Hepatocytes respond differently to major dietary \textit{trans} fatty acid isomers, elaidic acid and \textit{trans}-vaccenic acid

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Abstract

\textbf{Background:} It has been discussed if the adverse health effect associated with the ingestion of \textit{trans} fatty acids correlates with the food source, as the composition of the isomers varies in different foods. We have investigated the hepatocellular responses to the predominant \textit{trans} fatty acid isomers in industrially produced partially hydrogenated vegetable oils (elaidic acid) and products of ruminant origin (\textit{trans}-vaccenic acid).

\textbf{Results:} The responses of HepG2-SF cells exposed to 100 \textmuM fatty acids during 7 days were examined. Elaidic acid decreased the cellular proliferation rate while \textit{trans}-vaccenic acid had no effect. Analysis of cellular triacylglycerol fractions showed, that both \textit{trans} fatty acids were metabolized by HepG2-SF cells, although elaidic acid, to a higher degree than \textit{trans}-vaccenic, accumulated in the triacylglycerol fraction. Proteome analysis revealed that the overlap of differentially regulated proteins only contained four proteins, suggesting that the two \textit{trans} fatty acid isomers affect the cells in different ways. The data are available via ProteomeXchange with identifier PXD000760.

\textbf{Conclusions:} Our investigations revealed that the hepatocellular response to the two most abundant dietary positional C18:1 \textit{trans} fatty acid isomers differ substantially. In addition, the results suggest that \textit{trans}-vaccenic acid does not affect cholesterol metabolism adversely compared to elaidic acid.

\textbf{Keywords:} Cardiovascular disease, Lipid metabolism, Trans fatty acid, Proteomics, SILAC

Background

\textit{Trans} fatty acids (TFA) in the human diet originate from industrial partial hydrogenation of vegetable oils (iTFA) and the naturally occurring TFA in milk and body fat of ruminants (rTFA). The intake of iTFA can reach 9 % of the total energy intake [1] whereas the intake of rTFA rarely exceeds 0.5 % [2]. Dietary iTFA causes increased ratios in plasma of total cholesterol and LDL-cholesterol to HDL-cholesterol and of apoB to apoA1, therefore, increasing the risk of cardiovascular disease (CVD) (reviewed in [3]). The impact of rTFA on human health has also been investigated, but the results have been inconclusive (reviewed in [4]). Most studies do not show a relationship between rTFA and CVD [5–7] although a few have indicated an inverse association [8, 9]. In contrast to this potential positive effect of rTFA in relation to CVD, a high concentration of rTFA in serum and erythrocytes results in an increased risk of breast cancer [10, 11].

The main iTFA isomer is the \textit{trans}9-C18:1 named elaidic acid (EA), whereas the main isomer of rTFA is the \textit{trans}11-C18:1 named \textit{trans}-vaccenic acid (\textit{trans}VA) (40–60 % of rTFA [12]). The \textit{trans}VA originates from incomplete biohydrogenation of polyunsaturated fatty acids by microorganisms in the rumen of cows, goats and sheep [13]. Besides from being the major rTFA, \textit{trans}VA is also a constituent of iTFA contributing up to 5 % of the fat in margarine and shortening. Additionally, EA is present in rTFA, where it may constitute up to 10 % of the total rTFA [12]. Consequently, both EA and \textit{trans}VA intake may originate from several sources, complicating the evaluation of the impact of the individual isomer on human health. It is possible to enrich ruminant products for \textit{trans}VA through feeding strategies, by increasing time

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on pasture and decreasing silage in cattle feed, but the effect on EA content has not been evaluated [14]. TFA isomer distribution and content in rTFA varies depending on starting oil, degree of hydrogenation, temperature and catalyst [15, 16]. Knowledge about responses to individual TFA isomers such as EA and transVA can contribute in developing guidelines for the process of partial hydrogenation of vegetable oils and food formulations by the industry, but also in the context of feeding strategies for milk and meat production.

Despite indications that differential responses to positional isomers exist [17, 18], to our knowledge the TFA isomer specific effects in human have not been directly investigated through dietary intervention studies. Human clinical studies comparing health impact of diets enriched in dairy products with diets rich in hydrogenated vegetable oils exist [19–21]. However, the effect caused by the particular TFAs was difficult to ascertain because both diets contained TFA mixtures with isomers in different amount. In other studies, TFA replaced saturated fatty acids or polyunsaturated fatty acids to keep calorie-intake constant between experimental diets [22, 23]. The result of these studies is likely a mixed effect of excluding some fatty acids and including others, and it is difficult to determine the effects of particular dietary TFA isomers [4]. Only few studies have been conducted on cultures of human cells to assess isomer specific responses in regards to CVD risk. The studies have focused on inflammatory markers in endothelial cells [24] and monocytes [25], and the results indicate, that EA is the most detrimental of the fatty acids, while transVA may even be anti-inflammatory.

Besides inflammatory conditions, a significant factor in development of CVD is the dysregulation of cholesterol and lipoprotein levels. The liver is responsible for the secretion and regulation of plasma proteins. In addition, ingested fatty acids are metabolized by the liver and together with cholesterol redistributed to other organs through lipoproteins. Thus, the hepatocyte responses to individual TFAs are essential for understanding the physiological effect of the intake of different TFAs.

Through proteomic, we have evaluated if human hepatocellular cell line, HepG2-SF, responses to supplemented EA and transVA are comparable by reference to their cis-isomers, oleic acid (OA) and cis-vaccenic acid (cisVA). The cellular response to the TFAs EA and transVA were compared and investigated by reference to their naturally occurring cis-isomers OA and cisVA. Due to cellular desaturation of transVA into conjugated linoleic acid (CLA) this fatty acid was also included. The concentration of total free fatty acids in human blood has been reported to be as high as 435 ± 11 μM and that of a single free fatty acid, such as oleic acid, can be as high as 158 ± 5 μM [26]. Previous studies of HepG2 cells showed physiological relevant responses to free fatty acids (ranging from 50 to 1000 μM) and further HepG2 cells expressed and secreted a broad range of known plasma proteins important to lipid metabolism [27–33]. The lower concentration range of free fatty acids (50–200 μM) only had a small effect on the cells [29, 31], whereas the free fatty acids were cytotoxic at higher concentrations [28]. These previous studies using HepG2 cells mainly investigated OA, EA and palmitic acid in relation to changes in cholesterol and lipoproteins levels. We used 100 μM free fatty acid in complex with human serum albumin, which mimics the natural transportation of free fatty acids in the blood when fatty acids are not transported as lipoproteins [32]. Further, we used the commercial available HepG2-SF cell line adapted to growth in serum free media (commercial available as SynQ), which contains a minimal fatty acid formulation (see Methods), this gives us crucial control of the presence of fatty acids in the cell culture media supplemented with the experimental fatty acids.

We used Stable Isotope Labeling by Amino acids in Cell culture (SILAC) to investigate both cellular and secreted proteins from HepG2-SF cells in two separate triplex setups where cells were supplemented with transVA, cisVA or EA and transVA, OA or CLA. This was chosen because of the limitation of multiplexing more than three groups with SILAC. Further, Difference in gel electrophoresis (DIGE) was used to investigate the secretome from HepG2-SF cells supplemented with EA, OA or cisVA. Special emphasis has been on analyzing the secretome of EA and transVA supplemented cells, as changes in particular plasma protein levels are essential in the progression of CVD.

**Results**

**Viability of HepG2-SF cells in fatty acid supplemented medium**

HepG2-SF cell viability in 100 μM fatty acid supplemented medium was analyzed using CyQuant NF proliferation assay, as some C18 unsaturated fatty acids may be cytotoxic for HepG2 cells at or above 100 μM [34]. During 7 days exposure, HepG2-SF cells were found viable in the presence of 100 μM of all the fatty acids tested (Fig. 1). Proliferation did not significantly differ between cisVA and transVA and these did not differ markedly from Control (no fatty acid supplementation) either. Cells supplemented with CLA and OA did not significantly differ from Control in cell numbers on day 7, however, CLA and OA supplemented cells showed lower proliferation rates at the beginning of the experimental period. Supplementation with EA causes a considerable decrease in proliferation rate as compared to the other experimental groups, which suggests that EA directly or indirectly inhibit proliferation.
Fatty acid esterification into TG of HepG2-SF cells
To test if the supplemented fatty acids were taken up by the HepG2-SF and incorporated into TG, the fatty acid composition of FAME derived from the HepG2-SF TG fraction was analyzed by gas–liquid chromatography (Fig. 2 and Additional file 1: Data S1). The result showed that HepG2-SF cells take up the different supplemented fatty acids and esterify them into their TG. Eukaryotes are not able to make the fatty acid double bond of the trans geometry, and so Control and cells supplemented with cisVA do not contain TFA. Upon cisVA supplementation the content of this fatty acid in cell TG increase from (8.3 ± 4.2) % to (24.1 ± 7.4) % (i.e. the supplemented cisVA corresponds to 15.8 % ± 8.5 of total TG derived FAME). Supplementing HepG2-SF cells for 7 days with transVA, this fatty acid is present at 9.5 % ± 1.1 of the TG (Fig. 2a). The relative amounts of the supplemented geometrical isomers transVA and cisVA added to HepG2-SF TG are thus comparable (9.5 % ± 1.1 and 15.8 % ± 8.5). This is in contrast to the relative amounts of the positional trans-isomers EA and transVA, which are very different, with EA present at approximately three times the concentration of transVA (28.1 % ± 3.6 versus 9.5 % ± 1.1). In humans, transVA is desaturated into CLA with a conversion rate estimated to be 19 % [35]. We observed 2.47 % ± 0.16 CLA, when supplementing with transVA, corresponding to 21 % of the combined transVA and CLA (Fig. 2b), thus reflecting the estimated human conversion rate. Palmitic acid is the measured non-supplemented fatty acid that differed the most in amount between experimental groups, and is lower when cells were supplemented with EA and OA than compared to both supplementation with transVA or cisVA (p < 0.05) and the largest difference is observed between transVA and EA supplementation. In conclusion, HepG2-SF cells take up and metabolize all the supplemented fatty acids. However, after 7 days of supplementation, the measured amounts of EA and transVA isomers in TG differed between experimental groups, indicating a differential lipid metabolic response to the TFA isomers.

Identification and quantitation of proteins differentially regulated by transVA, EA and cisVA using SILAC
SILAC was applied to investigate the cellular proteomic response of the predominant rTFA and iTFA, transVA and EA respectively, and compare their response to the cisVA. Both the secreted and the intracellular proteins were investigated. A total of 3074 proteins were identified and of these 538 were quantifiable across all three groups. In the trans-trans comparison EA/VA 25 proteins were significantly regulated with a fold > 1.3. In the trans-cis comparison transVA/cisVA 20 proteins were significantly regulated with a fold > 1.3, and in EA/cisVA 24 proteins were significantly regulated with fold > 1.3. A total of 47 differentially regulated proteins were found (p < 0.01, fold > 1.3, Table 1 and Additional file 2: Data S2). Functional clustering analysis showed that the most significant functions represented by the regulated proteins were lipid and cholesterol metabolic processes including specifically cholesterol biosynthetic process. Except for apoA1, the regulated proteins involved in cholesterol synthesis are specifically up-regulated by EA and not transVA, demonstrating that this response is isomer specific. Eight of the 17 proteins categorized in lipid metabolism process are up-regulated by EA when compared to both transVA and cisVA and only 3 non-lipid metabolism proteins (tricarboxylate transport protein–mitochondrial, membrane-associated progesterone receptor component 1, and filamin-B) are regulated in these comparisons.
Fig. 2 The fatty acid composition of HepG2-SF TG after fatty acid supplementation. HepG2-SF TG composition was analyzed by gas liquid chromatography after 7 days incubation in fatty acid supplemented medium (100 μM). Panel a and b are high and low abundant fatty acids present in the TG, respectively. Content of individual fatty acids (x-axis), analyzed as extracted FAME, are averaged over three biological replicas and displayed as % of total TG derived FAME measured (y-axis). The supplemented TFAs esterify at different levels into HepG2-SF TG and EA is found at approximately three times the level of trans VA. The analysis shows that the supplemented fatty acids are taken up and metabolized by HepG2-SF cells. In addition, it demonstrates that EA is accumulating in TG when compared to the other experimental groups. *significantly different from Control at p < 0.05. Fatty acids significantly differing between experimental groups at p < 0.05 are marked with: a) different from OA, b) different from cis VA and c) different from trans VA.
Table 1 Differentially regulated proteins between trans-Vaccenic, cis-Vaccenic and Elaidic revealed by SILAC

| Uniprot accession no. | Name | Extracellular | EA/ transVA | EA/ cisVA | transVA/cisVA | Fold | Geo. SD. | Fold | Geo. SD. | Fold | Geo. SD. | selected GO-terms |
|-----------------------|------|---------------|-------------|-----------|--------------|------|---------|------|---------|------|---------|------------------|
| P02457                | Apolipoprotein A-4 | 1.20 | 1.10 | -1.08 | 1.06 | -1.31 | 1.11 | a,b,c |
| P06772                | Apolipoprotein A-IV | 1.49 | 1.09 | 1.44 | 1.09 | -1.05 | 1.04 | b,c |
| Q8NBP7                | Proprotein convertase subtilisin/kexin type 9 | 2.01 | 1.14 | 1.73 | 1.11 | -1.18 | 1.07 | b,c |
| P10909                | Chitinase | -1.46 | 1.17 | 1.42 | 1.09 | 1.27 | 1.20 | c |
| P08185                | Corticosteroid-binding globulin | 1.71 | 1.14 | -1.16 | 1.16 | -1.53 | 1.09 | c |
| Q99690                | Retinoid acid receptor responder protein 2 | 1.51 | 1.11 | -1.08 | 1.03 | -1.25 | 1.14 | c |
| P02753                | Retinol-binding protein 4 | 1.69 | 1.13 | -1.13 | 1.11 | -1.34 | 1.06 | c |
| P25311                | Zinc-alpha-2-glycoprotein | -1.53 | 1.13 | 1.93 | 1.04 | c |
| P02765                | Alpha-2-HS-glycoprotein | 1.55 | 1.17 | -3.32 | 1.17 | -1.38 | 1.07 | c |
| P27487                | Diacylglycerol-phosphatidase | 1.53 | 1.09 | 1.53 | 1.16 | 1.30 | 1.19 | c |
| P02671                | Fibronectin alpha chain | 1.55 | 1.10 | -1.08 | 1.08 | -1.73 | 1.14 | c |
| P51654                | Glycine-3 | 1.25 | 1.16 | 1.01 | 1.20 | -1.35 | 1.15 | c |
| P10554                | Heparin cofactor 2 | 1.59 | 1.08 | -1.05 | 1.11 | -1.57 | 1.16 | c |
| P08833                | Insulin-like growth factors-binding protein 1 | -1.53 | 1.06 | -2.21 | 1.08 | -1.48 | 1.07 | c |
| P61620                | Lysozyme C | 1.33 | 1.03 | 1.26 | 1.08 | c |
| Q16819                | Mammalian alpha | 1.67 | 1.06 | -1.23 | 1.07 | -1.07 | 1.06 | c |
| P01033                | Metalloproteinase inhibitor 1 | -1.53 | 1.09 | -1.14 | 1.08 | -1.27 | 1.07 | c |
| P36955                | Pigment epithelium-derived factor | 1.23 | 1.03 | 1.09 | 1.09 | -1.30 | 1.07 | c |
| P05154                | Plasma serine protease inhibitor | 1.17 | 1.13 | -2.14 | 1.16 | -1.38 | 1.08 | c |
| P05121                | Plasminogen activator inhibitor 1 | -1.08 | 1.12 | -1.39 | 1.08 | -1.35 | 1.11 | c |
| P02787                | Serotransferrin | -2.98 | 1.00 | 3.11 | 1.11 | -1.22 | 1.46 | c |
| P55542                | Serum amyloid A-4 protein | 1.35 | 1.14 | -3.07 | 0.99 | -1.27 | 1.13 | c |
| Q76061                | Stomatin-like-2 | -1.24 | 1.23 | -1.45 | 1.10 | -1.20 | 1.16 | c |
| P06400                | Tissue factor pathway inhibitor | 1.15 | 1.10 | -1.13 | 1.13 | -1.33 | 1.13 | c |
| P02766                | Transthyretin | -1.91 | 1.39 | 1.97 | 1.06 | 3.47 | 1.33 | c |
| Intracellular | | | | | | | |
| Q9UBM7                | 7-dehydrocholesterol reductase | 1.63 | 1.07 | 1.73 | 1.02 | a,b,c |
| P13424                | Farnesyl pyrophosphatase synthase | 1.47 | 1.05 | 1.60 | 1.10 | a,b,c |
| Q13907                | Isopentenyl-diphosphate Delta-isomerase 1 | 1.51 | 1.04 | 1.40 | 1.06 | a,b,c |
| P37268                | Squalene synthase | 1.81 | 1.05 | 1.86 | 1.09 | a,b,c |
| Q15738                | Sterol-3-α-carboxylic acid dehydrogenase, deoxycholate | 1.55 | 1.08 | 1.37 | 1.10 | a,b,c |
| Q9WB1D1               | Acyl-CoA acyltransferase, cytosolic | 1.46 | 1.14 | 1.51 | 1.09 | a,b,c |
| Q86TX2                | Acyl-CoA synthase I like | 1.52 | 1.06 | 1.56 | 1.16 | a,b,c |
| P33396                | ATP-ase synthase | 1.69 | 1.09 | 1.86 | 1.08 | a,b,c |
| Q9169K3               | Acyl-CoA synthase short-chain family member 3, mitochondrial | 1.38 | 1.01 | 1.38 | 1.03 | c |
| P07785                | Catepsin B | 1.23 | 1.03 | 1.29 | 1.03 | -1.36 | 1.09 | c |
| P07292                | Ferritin light chain | -1.57 | 1.18 | 1.30 | 1.13 | 2.06 | 1.22 | c |
| Q75169                | Fermitin-B | 1.34 | 1.08 | 1.42 | 1.10 | 1.35 | 1.05 | c |
| P06479                | Heat shock protein beta-1 | 1.15 | 0.81 | 1.39 | 0.88 | 1.19 | 1.07 | c |
| P10619                | Lysosomal protective protein | -1.25 | 1.07 | 1.28 | 1.02 | 1.52 | 1.05 | c |
| Q000264               | Membrane-associated progesterone receptor component 1 | 1.45 | 1.10 | 1.62 | 1.08 | 1.00 | 1.07 | c |
| P17987                | T-complex protein 1 subunit alpha | -1.30 | 1.18 | -1.23 | 1.06 | -1.36 | 1.08 | c |
| Q16881                | Thiorodoxin reductase 1, cytoplasmic | -1.28 | 1.06 | -1.01 | 1.04 | 1.31 | 1.10 | c |
| P35007                | Tricarboxylic transport protein, mitochondrial | 1.52 | 1.15 | 1.50 | 1.13 | -1.03 | 1.03 | c |
| P06753                | Tropomyosin alpha-3 chain | 1.02 | 1.07 | 1.33 | 1.03 | -1.25 | 1.08 | c |
| P67936                | Tropomyosin alpha-4 chain | 1.04 | 1.03 | 1.35 | 1.03 | c |
| P23381                | Trypsinogen-tRNA ligase, cytoplasmic | -1.55 | 1.03 | -1.32 | 1.12 | -1.16 | 1.09 | c |
| P62979                | Ubiquitin-40S ribosomal protein S27a | -1.30 | 1.08 | -1.23 | 1.24 | -1.05 | 1.39 | c |

Proteins are reported with Uniprot accession number, Name, Fold regulation in individual comparisons, the geometric standard deviation (Geo.SD.) and selected GO-terms a) black bold: significant regulatory data ($p < 0.01$) with fold $> 1.3$, black italic: significant regulatory data ($p < 0.01$) with fold $< 1.3$, grey italic: not significant regulatory data ($p > 0.01$). b) a: cholesterol biosynthetic process (GO:0006695), b: cholesterol metabolic process (GO:0008203), and c: lipid metabolic process (GO:0006629).
Corticosteroid binding protein, classified in lipid metabolism, was down-regulated upon transVA-supplementation in both transVA/EA and transVA/cisVA. Heparin cofactor 2 and fibrinogen alpha chain are non-lipid metabolism proteins regulated by transVA-supplementation. They were both down-regulated. Three proteins were down-regulated by both TFA isomers when compared to cisVA, these were plasminogen activator inhibitor 1 (PAI1), alpha-2-HS-glycoprotein (AHSG) and insulin-like growth factor-binding protein 1 (IGFBP1). Transferrin (TTR) is up-regulated in both transVA/cisVA and EA/cisVA.

The SILAC analysis showed that the overlap of differentially regulated proteins between the comparisons EA/cisVA and transVA/cisVA is rather small, comprising 16% and 20%, respectively, of regulated proteins at fold > 1.3, p < 0.01. This suggests that cellular responses to the two major dietary TFAs are rather different. However, a shared TFA-induced response was observed for PAI1, AHSG, IGFBP1, and TTR, as mentioned above.

Identification and quantitation of secreted proteins differentially regulated by transVA, EA and cisVA using DIGE

To further investigate the effects of TFA isomers on plasma protein levels and to gain more information on differentially regulated secreted proteins, the experimental medium from fatty acid supplementations of HepG2-SF cells were analyzed by DIGE. Approximately 2000 spots were detected per gel and 1000 aligned across all analytical gels. Using the criteria of 1.3 fold regulation at p-value < 0.05 the numbers of differentially regulated spots were 44 in EA/transVA and 5 in transVA/cisVA. From the 44 differentially regulated spots in the trans-trans comparison EA/transVA, 12 unique proteins (excl. human serum albumin) were identified, and from the 5 differentially regulated spots in the trans-cis comparison transVA/cisVA, 3 unique proteins were identified (Table 2 and Additional file 3: Data S3). EA, compared to transVA, induced up-regulation of apoA1 and apoE, which are apolipoproteins of HDL and VLDL respectively, and down-regulation of AHSG, which inversely correlate with CVD risk [36] and is suggested as a biomarker for TFA intake [37]. ApoA4 was up-regulated in both EA/transVA and transVA/cisVA. Four spots, upregulated by transVA when compared to EA, were identified as complement C3 with migration in the 2D gel estimated at a pl a little below 5 and a mass of approximately 40 kDa (Table 2, Additional file 3: data S3 and Additional file 4: Figure S1). The peptides, derived from the spots and identifying the proteins as complement C3, all locate in the c-terminal part of C3 corresponding to the C3c alpha chain fragment 2. This also fits the mass and pl observed, thus indicates increased complement C3 activation by transVA. The DIGE results thus support and supplement the SILAC results, as will be discussed, which further underline the indication that the major iTFA (EA) and the major rTFA (transVA) promote relatively different hepatocellular responses.

Identification and quantitation of proteins differentially regulated by transVA, OA and CLA using SILAC

OA is the most abundant of naturally occurring C18:1 cis fatty acids in food stuff, and it was included in a separate SILAC analysis comparing OA-supplemented cells to transVA, to test if the transVA induced response, observed in the transVA-cisVA-EA comparison described above, could also be confirmed with reference to OA. CLA was included in this second SILAC setup, as mammalian cells are able to further desaturate transVA to yield CLA. The cellular response to 100 μM CLA was investigated to assess if the transVA to CLA conversion contributes to the observed transVA response.

A total of 3191 proteins were identified in this second SILAC triplex setup and of these 15 were differentially regulated in the transVA/OA comparison within the fold 1.3 criteria set, p < 0.01. For CLA/transVA and CLA/OA the numbers were 26 and 39, respectively (Additional file 5: Data S4). Five of the 15 proteins significantly regulated in transVA/OA were also regulated in the first SILAC transVA-cisVA-EA triplex. The down-regulation of AHSG, fibrinogen alpha chain and corticosteroid binding globulin was confirmed in transVA/OA together with down-regulation of glypican 3 and stanniocalcin 2.

If the cellular response to transVA was in part mediated by its conversion to CLA, supplementation with 100 μM CLA would be expected to enhance the response observed during supplementation with transVA. Ten of the 26 proteins significantly regulated in the CLA/transVA comparison were also regulated in the first SILAC transVA-cisVA-EA triplex. Eight of these were in CLA/VA regulated in the opposite direction as in the transVA/cisVA comparison. This demonstrates that the transVA induced response is not significantly contributed by the presence of CLA, and furthermore the data suggests that the cis-desaturation on the Δ9 position in CLA may neutralize some of the effects of the Δ11 trans-double bond.

Discussion

Investigations of adverse health effects related to TFA intake have not focused on the contribution of the individual positional TFA isomers. We have in earlier studies investigated the differential response of human hepatocytes, HepG2-SF, to the two geometric Δ9C18:1 isomers OA (cisΔ9C18:1) and EA (transΔ9C18:1). The studies revealed substantial differences in abundance of proteins involved in cholesterol synthesis and lipid metabolism in general and furthermore indicated remodeling of the phospholipids caused by the EA supplementation of the
In the present study, we investigate if the hepatocellular responses to the two most abundant positional C18:1 TFA isomers in the human diet, EA and trans VA, are comparable. The cis VA was included to test if also the Δ11 C18:1 geometrical isomers showed the same affect on HepG2-SF cells, as previously shown for the Δ9 C18:1 geometrical isomers.

Supplemented trans fatty acids esterify at different amounts into the TG of liver cells

To our knowledge, TFA induced hepatic responses have not earlier been investigated in human cell cultures, although a study using rat hepatocytes showed that short-term (2 h) exposure to EA or trans VA causes equal incorporation of the TFAs into TG [39]. Contrary to the results in rats, we showed, by the analyses of FAME derived from HepG2-SF TG, that after 7 days supplementation with EA or trans VA that the TG fraction contained more than double the amount of EA to that of trans VA. Upon supplementation, trans VA and cis VA esterified to similar extend into the TG fraction. TG acts as a cellular storage site for fatty acids, which can be used for energy production through β-oxidation in mitochondria and peroxisomes. In rat the β-oxidation rate of EA has been shown to be 30 % lower than for both trans VA and cis VA [40] because a β-oxidation intermediate of EA slows β-oxidation progress [41]. If this occurs in human cells, EA may thus be accumulating in TG as β-oxidation slows down and this may be the reason for our observation of different relative amounts of TFAs in HepG2-SF TG. However, the total amounts of TG per cell in each experimental group were not determined in the present study. Consequently, it cannot be concluded if EA-containing TGs are added to the TG pool or if EA substitutes for other fatty acids, for example palmitic acid, keeping total TG at a constant amount.

Table 2 Differentially regulated secreted proteins revealed by DIGE and identified by mass spectrometry

| Protein name | Uniprot name | spot no | Av. Ratio | p-value | Av. Ratio | p-value |
|--------------|--------------|---------|-----------|---------|-----------|---------|
| 14-3-3 protein zeta/delta | 1433Z/P63104 | 2180 | 1.43 | 3.0E-02 | -1.43 | 3.0E-02 |
| Aldose 1-epimerase | GALM/Q9623 | 1832 | 2 | 2.5E-03 | 2 | 2.5E-03 |
| Alpha-1-antitrypsin | A1AT/P01009 | 1330 | 1.79 | 3.6E-04 | 1.79 | 3.6E-04 |
| Alpha-1-antitrypsin | - | 1342 | 1.96 | 3.9E-03 | 1.96 | 3.9E-03 |
| Alpha-2-HS-glycoprotein | FETUA/P02765 | 1400 | -1.51 | 3.4E-02 | -1.51 | 3.4E-02 |
| Apolipoprotein A1 | APOA1/P02647 | 2333 | 1.57 | 2.0E-04 | 1.57 | 2.0E-04 |
| Apolipoprotein A4 | APOA4/P06727 | 1737 | 1.57 | 7.3E-03 | 1.57 | 7.3E-03 |
| Apolipoprotein A4 | - | 1741 | 1.7 | 1.7E-04 | 1.7 | 1.7E-04 |
| Apolipoprotein A4 | - | 1743 | 1.43 | 2.3E-02 | 1.43 | 2.3E-02 |
| Carboxypeptidase E | CBPE/P16870 | 1417 | -1.78 | 1.3E-03 | -1.78 | 1.3E-03 |
| Chloride intracellular channel protein1 | CLIC1/O00299 | 2106 | 1.75 | 1.9E-03 | 1.75 | 1.9E-03 |
| Complement C3 | CO3/P01024 | 1675 | -1.41 | 3.1E-02 | -1.41 | 3.1E-02 |
| Complement C3 | - | 1706 | -1.51 | 4.6E-03 | -1.51 | 4.6E-03 |
| Complement C3 | - | 1708 | -1.51 | 4.6E-03 | -1.51 | 4.6E-03 |
| Complement C3 | - | 1710 | -1.51 | 8.8E-04 | -1.51 | 8.8E-04 |
| Complement factor B | CFAB/P00751 | 726 | -1.32 | 2.5E-03 | -1.32 | 2.5E-03 |
| Endolysin | ENPL/P14625 | 737 | -1.51 | 4.8E-02 | -1.51 | 4.8E-02 |
| Proteasome subunit alpha type-5 | PSAS/P28066 | 2203 | -1.37 | 4.7E-02 | -1.37 | 4.7E-02 |
| Protein AMBP | AMBP/P02760 | 2042 | 1.62 | 2.2E-03 | 1.62 | 2.2E-03 |
| Serotransferrin | TRFE/P02787 | 1005 | 1.56 | 8.0E-04 | 1.56 | 8.0E-04 |
| Serotransferrin | 1007 | 2.02 | 2.7E-04 | 2.02 | 2.7E-04 |
| Serotransferrin | 1033 | 1.65 | 1.5E-04 | 1.65 | 1.5E-04 |
| Serotransferrin | 1765 | 1.43 | 1.1E-02 | 1.43 | 1.1E-02 |

The secretome of hepatocytes supplemented with trans VA, EA, or cis VA were analyzed by DIGE and subsequently the regulated proteins were identified by LC-MS/MS analyses. In the table proteins are reported with protein name, Uniprot name/accession number, 2D gel spot number (spot no), Average ratio (Av. ratio), and p-value for individual comparisons.
EA and transVA affect lipid metabolism-related proteins differently

The cellular proteomic responses to EA, transVA and cisVA were analyzed using SILAC and DIGE. The SILAC analysis identified 47 differentially regulated proteins in EA/transVA, transVA/cisVA and/or EA/cisVA (Table 1) and functional cluster analysis showed that the main perturbed cellular functions are lipid and cholesterol metabolism. In a previous study, comparing the HepG2-SF responses to EA and OA, we found that the major part of proteins associated with cholesterol synthesis were upregulated in the TFA treated cells [38]. Moreover, in rat hepatocytes EA supplementation caused an increased cholesteryl efflux as compared to both transVA and cisVA [39]. In the present study, we find several of the proteins involved in lipid metabolism, and especially cholesterol synthesis, to be up-regulated by EA, when compared to both transVA and cisVA. It supports the findings in rats and suggests that the increased cholesteryl efflux is in part a result of increased synthesis. Also, Proprotein convertase subtilisin/kexin type 9 (PCSK9) was up-regulated in both EA/transVA and EA/cisVA but unchanged in transVA/cisVA. This protein can cause degradation of LDL receptor [42] and thereby possibly decrease the LDL-cholesterol uptake by the cell contributing to extracellular cholesterol accumulation. DIGE was performed on the secreted proteins from HepG2-SF cells, because it has been suggested that TFA may influence CVD risk by altering secretion of plasma proteins from the liver. Our DIGE data shows that apoA1, which is the major apolipoprotein of HDL, is up-regulated by EA when compared to transVA and unchanged in transVA/cisVA (Table 2). In SILAC the regulatory data for apoA1 is 1.2 fold for EA/VA and −1.3 fold for transVA/cisVA. Cholesteryl ester transfer protein is responsible for cholesteryl ester transfer from HDL to LDL and its activity is increased by TFA [43]. If ApoA1 is up-regulated by EA, this may cause an increased extracellular capacity for cholesterol, and with increased CETP activity this may aid cholesterol accumulation in LDL.

Trans fatty acid specific responses

The SILAC analysis revealed four proteins that were significantly regulated in the same direction, but not necessarily to the same extent, by both TFAs when compared to cisVA. These included TTR, which is up-regulated, and PAI1, IGFBP1 and AHSG, which are down-regulated. We have in a previous biomarker study compared EA with OA and among other candidates found TTR, PAI1, IGFBP1 and AHSG as potential biomarkers for TFA-intake [37]. The present study supports that the regulation of these proteins are TFA specific. Decreased level of metalloproteinase inhibitor 1 (TIMP1) was also suggested as a potential biomarker for EA intake [37], and this protein was down-regulated in EA/cisVA (−1.42) and just below the fold cut-off in transVA/cisVA (−1.27), also indicating this to be a TFA-specific response.

In the previous biomarker study PCSK9, ApoA4, tissue factor pathway inhibitor (TFPI), AHSG, and serotransferrin were also shown to be potential biomarkers for EA supplementation, when compared to OA [37]. PCSK9, ApoA4 and serotransferrin were up-regulated, TFPI and AHSG down-regulated. In the present investigations, SILAC analysis confirmed that PCSK9 and ApoA4 were up-regulated and potential EA biomarkers. TFPI was down-regulated by transVA when compared to cis-VA (−1.33), and the fold down-regulation in EA/cisVA was 1.15. Serotransferrin was up-regulated in EA/cisVA (1.31), but lacked significance in regulation in the other comparisons. In the DIGE characterization of the proteins secreted by HepG2-SF cells, ApoA4 and serotransferrin were up-regulated (on average 1.57 and 1.67, respectively) and AHSG down-regulated (−1.51) in the EA/VA comparison (Table 2), confirming the observed changes in the EA/OA comparison [37] underlining that these are potential biomarkers for EA intake. However, ApoA4 was also up-regulated in the DIGE transVA/cisVA comparison (1.4), which shows that this response is not EA-specific though more pronounced by EA than by transVA supplementation. Noteworthy, all the regulated and discussed proteins (PCSK9, AHSG, ApoA4, TIMP1, TFPI, PAI1 and IGFBP1) have previously been mentioned in the context of CVD risk [36, 37, 44–47].

However, 18 of the 26 proteins differentially regulated in CLA/transVA categorize with the GO-term: Response to stress, all up-regulated by CLA, and seven of these are part of the acute inflammatory response. This may indicate that the high concentration of CLA is stressful or even toxic for HepG2-SF cells, though not decreasing viability after a seven days period. A blood concentration of 100 μM CLA is not easily reached through diet, and though interesting it is beyond the scope of this paper to further discuss the cellular response to CLA.

The complement system is affected by TFA

Atherosclerosis is considered an inflammatory condition [48]. It has been suggested that dietary TFA induces vascular inflammation and effects on several markers of endothelial cell dysfunction and inflammatory responses have been documented [24, 49, 50]. Though a TFA, transVA did not induce this effect in endothelial cells and may even protect against vascular inflammation measured as decreased T-helper cell cytokine production [24, 25]. Part of the vascular inflammatory response is the activation of the complement system, and a key protein is complement factor C3 (CO3). Blood CO3 is a predictor of myocardial infarction [51] and correlates positively with obesity and coronary artery disease [52]. In the DIGE
study we identify CO3 in 4 spots, up-regulated by transVA when compared to EA and unchanged when compared to cisVA (Table 2, Additional file 3: Data S3 and Additional file 4: Figure S1). These spots correspond to the most C-terminal part of CO3, the C3c alpha chain fragment 2. By SILAC it is observed that the overall CO3 level is significantly increased by EA compared to transVA (1.27 fold), and up-regulation in cisVA/VA is also indicated (Additional file 2: Data S2). The regulatory data for CO3 suggests that supplementation with transVA gives the lowest overall level of CO3, but the highest level of CO3 activation relative to total amount. Complement factor B is also an important protein in the complement system, which upon binding to CO3 b fragment interacts with and activates complement factor D, which in turn cleaves factor B [53]. The cleaved form of factor B forms a complex with the CO3 b fragment in the formation of C3 convertase, which further accelerate the cleavage of CO3. Complement factor B was significantly up-regulated 1.2 fold in SILAC in both EA/VA and cisVA/transVA i.e. down-regulated by transVA (Additional file 2: Data S2). In DIGE judging by mass, pl, spot pattern, and identification by MS/MS, the full-length complement factor B was observed as a train of spots, with one spot up-regulated 1.42 fold and another down-regulated 1.32 fold in EA/VA. This observation in DIGE suggests a change in the glycosylation pattern, with an EA induced deglycosylation of factor B. Deglycosylation at Asn260 of factor B causes increased affinity for the CO3 b fragment [54] and deglycosylation at Asn353 is suggested to increase cleavage by factor D and thus deglycosylation at both sites can increase formation of the C3 convertase [55]. Whether the observed change in spot pattern on our 2D gels is due to deglycosylation at either of the mentioned sites, with implication for complement activation due to EA, requires further investigations.

Some proteins categorized in the acute inflammatory/ acute phase response are also regulated by the TFAs investigated, though not clearly confirming or rejecting an acute phase response. These include the positive acute phase proteins: fibrinogen alpha chain (SILAC: EA/VA 1.55 fold, transVA/cisVA −1.73 fold), PAI1 (SILAC: EA/cisVA −1.39, transVA/cisVA −1.35), serum amyloid A-4 (SILAC: EA/transVA 1.33, transVA/cisVA −1.27) and ferritin light chain (SILAC: EA/transVA −1.57, transVA/cisVA 2.06), and the negative acute phase proteins: AHSG (SILAC: EA/cisVA −1.32, transVA/cisVA −1.38; DIGE EA/VA −1.51), serotransferrin (SILAC: EA/cisVA 1.31; DIGE EA/transVA 1.67) and TTR (SILAC: EA/cisVA 1.97, transVA/cisVA 3.47).

**Regulators of blood coagulation are affected by TFA**

By SILAC fibrinogen alpha chain was the only protein to be significantly regulated specifically by transVA when compared to both EA and cisVA (−1.55 and −1.73 fold regulation, respectively). Though not significant at p < 0.01 both fibrinogen beta and gamma chain also showed approximately 1.6 fold down-regulation by transVA supplementation (Additional file 2: Data S2). PAI1 was decreased by both EA and transVA. Increased plasma fibrinogen and PAI1 levels correlates with increased thrombosis risk [44, 56], and thus transVA may have a potential beneficial influence on the level of these proteins in regards of CVD risk. However, the observed decrease in level of fibrinogen could also indicate a transVA-induced shift towards coagulation of the equilibrium between coagulation and fibrinolysis, resulting in consumption of fibrinogen. The possibility of increased coagulation is supported by the observation that both TFPI and heparin cofactor 2 are also decreased by transVA-supplementation. TFPI is a major inhibitor of the extrinsic coagulation pathway through its interaction with Factor Xa and tissue factor-factor VIIa complex [57, 58], and heparin cofactor 2 inhibits thrombin action [59], thus low level of both may promote thrombophilia. Whether transVA promotes or protects against thrombophilia is thus difficult to conclude from the present experiments, however it clearly affects levels of regulators of blood coagulation.

**Conclusion**

It has previously been established that iTFAs correlate with CVD risk [3]. However, a diet very high in rTFA also negatively affects CVD risk factors [20]. It has therefore previously been discussed if transVA and EA in food have different effects on human health, or whether it is merely a matter of the total dietary TFA amount consumed [60]. Our study shows that partially differential hepatocellular responses to the two C18:1 TFA isomers transVA and EA occurs. EA specifically induces an increase in proteins involved in cholesterol synthesis and cholesterol transport, a response not shared with transVA. The results of this investigation suggest that EA has an adverse effect on cholesterol metabolism that could be unique to the main TFA isomers found in foodstuff and explain its stronger correlation with CVD risk than for transVA, but this requires more investigations in animal models and ultimately in clinical intervention studies.

**Methods**

**Materials**

Cis-vaccenic acid (> 97 % purity) and Conjugated linoleic acid (> 96 % purity) were purchased from Cayman Chemicals. $^{13}$C<sub>6</sub>Arginine and $^{13}$C<sub>6</sub>N<sub>4</sub>Arginine (both 99 % purity) were from Cambridge isotope laboratories (Andover, MA, USA). Elaidic acid, Oleic acid, trans-vaccenic acid , L-Lysine and Arginine (all 99 % purity), Anhydrous dimethyl formamid (99.5 % purity), iodoacetamide, tetra-methyl-
ethylene-diamine, L-Glutamine, Human serum albumin and RPMI-1640 without Arginine, Lysine and Leucine were purchased from Sigma. Regular SynQ serum substitute and SynQ without Arginine and Lysine was from Cell Culture Service, Hamburg Germany. All reagents for DIGE were PlusOne quality from GE Healthcare. Also CyDye DIGE flours, IPG running buffers and PD10 columns were purchased from GE Healthcare. Penicillin, Streptomycin, regular RPMI-1640 and CyQuant NF proliferation assay kit were from Invitrogen.

Cell culture
HepG2-SF cells (HepG2 cells optimized for serum free growth, Cell Culture Service, Hamburg, Germany) were propagated in 75 cm² Nunc culture flasks in serum free medium composed of RPMI-1640, 10 % SynQ Serum substitute (Contains: Oleic acid, 3.5 μM; Linoleic acid, 500 ng/mL; Thiolic acid, 250 ng/mL), 4 mM L-glutamine, 20 U/mL penicillin and 20 μg/mL streptomycin, which was changed every third day. Cells were maintained at 37 °C in a 5 % CO₂ humidified incubator and subcultured every two weeks. For preparation of fatty acid supplemented medium, fatty acid in a 2:1 molar complex with human serum albumin [61] was adjusted to a final concentration of 100 μM in serum free medium.

Assessment of HepG2-SF growth in fatty acid supplemented medium by CyQuant proliferation assay
In a 96 well (low fluorescence) microtiter-plate 5,500 cells were seeded per well and allowed to attach for 24 h before 300 μL 100 μM fatty acid supplemented regular medium was added (4 replicas for each experimental group). On day 0, 3, 5 and 7 after addition of supplemented media proliferation was assessed by adding 100 μL CyQuant dye solution per well, and after 30 min incubation at 37 °C fluorescence was measured using a FLUOstar Omega (BMG Labtech) until a plateau was reached (excitation 480 nm, 520 nm emission, 3 h). For comparison of experimental groups with Control equality of variance were tested (F-test) and appropriate two-tailed t-tests were performed.

Measurement of fatty acids esterified into triacylglycerols (TG) by gas–liquid chromatography
For each experimental group 3 replicas of three million cells each, incubated 7 days in fatty acid supplemented serum free medium as described above, were washed 2 times in sucrose buffer (250 mM sucrose, 50 mM KCl, 2 mM MgCl₂, 20 mM Tris–HCl, pH 7.6) and scraped off the petridish in 1 ml sucrose buffer. Cells were centrifuged for 3 min at 180 g and the supernatant was discarded. Pellets were resuspended in methanol, vortexed and transferred to glass tubes with Teflon lined caps. Lipids were extracted with methyl-tert-butyl-ether according to [62] and subjected to thin layer chromatography on 20 × 20 cm silica gel 60 plates with glass backing (Merck) using a two-buffer system (buffer 1, 30 min: chloroform:methanol:acetic acid:water-50:30:8:3, buffer 2, 45 min: heptane-diethyl ether:acetic acid – 70:30:2). Plates were dried after the development and lipids were visualized by placing the plates in an iodine chamber, the TG bands were marked and scraped off the plate when iodine was evaporated. TG were extracted from silica with chloroform:methanol (2:1) and dried. Fatty acid methyl esters (FAME) were generated from the TG using BF₃. First samples were mixed with 1 ml methanolic NaOH (0.5 M) and heated for 5 min at 80 °C. After cooling to room temperature 1 ml of BF₃-methanol (13–15 %) was added and again samples were heated at 80 °C for 2 min and cooled rapidly to room temperature. To improve FAME extraction to the organic phase, 0.2 ml of a salt solution containing NaCl (370 mg/ml) and K₂CO₃ (1.5 mg/ml) in water was added followed by 1 ml of heptane. After 5 min centrifugation at 4000 rpm the upper organic phase was isolated and dried over anhydrous Na₂SO₄ before application to gas–liquid chromatography. The FAME were analyzed on a Thermo Trace GC Ultra (Thermo Scientific, USA) equipped with auto-sampler, flame ionization detector and a Supelco SLB-IL100 column (60 m x 0.25 mm x 0.2 μm film thickness, Sigma-Aldrich). Helium was used as a carrier gas with a flow rate of 1 ml/min. An isothermal program at 180 °C for 25 min was applied. The injector and detector temperatures were set at 200 °C and 220 °C, respectively. Five μl samples with a split flow of 10:1 were injected. Using Xcalibur software fatty acids were identified by comparing their retention times to standard mixtures and those eluting between 11.5 min and 23.5 min are expressed as mol % based on an internal standard (methyl heptadecanoate).

Incubation of HepG2-SF cells in fatty acid supplemented media for Stable Isotope Labeling by Amino acids in Cell culture (SILAC)
Serum free medium was prepared from RPMI-1640 without Arginine, Lysine and Leucine and SynQ without Arginine and Lysine. The medium was then supplemented with Leucine and Lysine to a final concentration of 0.38 mM and 1.02 mM, respectively. From this medium three different SILAC growth media were prepared containing 1.15 mM Arginine, 1³C₆Arginine or 1³C₆-1⁵N₄Arginine. To facilitate full incorporation of 1³C₆Arginine and 1³C₆-1⁵N₄Arginine the cells were cultivated as described above for 5 population doublings with medium change every third day. The level of incorporation was tested by MALDI-MS prior to incubations with fatty acids in SILAC media. For preparation of fatty acid supplemented medium fatty acid in a 2:1 complex with human serum albumin [61] was adjusted to a final concentration of
100 μM in SILAC serum free medium. Two SILAC triplex setups were made one using transVA (Arginine), EA ($^{13}$C$_6$Arginine) and cisVA ($^{15}$C$_6$$^{15}$N$_4$Arginine), the other using transVA (Arginine), OA ($^{13}$C$_6$Arginine) and CLA ($^{13}$C$_6$$^{15}$N$_4$Arginine).

For each experimental group 75 cm$^2$ culture flasks containing 90 % confluent cells were trypsinized and seeded into four 6 cm petri dishes (= four replicas/experimental group). Cells were allowed to attach for 24 h before 3 mL fatty acid supplemented medium was added. The medium was changed on day two, four and six. On day 7 medium was aspirated and filtered (Sarstedt syringe filters, 0.22 μm) and kept on ice until further processing as described below. Cells were trypsinized, washed three times in cold PBS and lysed in lysis buffer (1 % NP-40, 150 mM NaCl, 50 mM Tris, 0.1 % SDS, 1 mM PMSF, 25 μM E64, 2 mM 1.10 Phenanthroline, pH 7.4) by sonication on ice. Lysate was centrifuged (16000 g, 4 °C) and supernatant collected and kept on ice.

**SILAC sample preparation**

Cell medium was depleted for albumin by affinity chromatography on a column with a recombinant albumin binding domain from Streptococcal protein G [63]. The flow through was collected and the column regenerated using 20 mM Na-citrate, 150 mM NaCl, pH 2.5 before application of next sample. Between experimental groups the column was cleaned using 30 % isopropanol, 2 M NaCl. Protein concentration in depleted samples were determined using Bradford assay kit [64]. The 4 biological replicates in each group were pooled based on protein concentration and then the pools from different fatty acid incubations were mixed in a 1:1:1 ratio. The protein concentration in cell lysates were determined by Bradford assay kit [64]. The 4 biological replicates in each group were pooled based on protein concentration and then the pools from different fatty acid incubations were mixed in a 1:1:1 ratio. The protein concentration in cell lysates were determined by Bradford assay kit [64]. The 4 biological replicates in each group were pooled based on protein concentration and then the pools from different fatty acid incubations were mixed in a 1:1:1 ratio. The protein concentration in cell lysates were determined by Bradford assay kit [64]. The 4 biological replicates in each group were pooled based on protein concentration and then the pools from different fatty acid incubations were mixed in a 1:1:1 ratio. The protein concentration in cell lysates were determined by Bradford assay kit [64]. The 4 biological replicates in each group were pooled based on protein concentration and then the pools from different fatty acid incubations were mixed in a 1:1:1 ratio. The protein concentration in cell lysates were determined by Bradford assay kit [64].

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**LC-MS/MS of SILAC samples**

The peptides were separated on an Easy-nLC II HPLC system (Thermo Scientific) equipped with a trap column (ReproSil-Pur C18-AQ (5 μm, 2 cm x 100 μm I.D., Thermo Scientific) and an analytical column (ReproSil-Pur C18-AQ column, 3 μm, 10 cm x 75 μm I.D., Thermo Scientific) in-line to a NanoSpray III source (AB Sciei) connected to a TripleTOF 5600 mass spectrometer (AB Sciei) operated under Analyst TF 1.5.1 control. Peptides were eluted at a constant flow of 250 nl/min with a 50 min gradient from 5 to 35 % solvent B (90 % ACN, 0.1 % formic acid) followed by re-equilibration for 10 min back to the starting conditions. Information dependent acquisition was employed acquiring up to 25 MS/MS spectra per cycle using 1.6 s cycle time with an exclusion window of 6 s.

**SILAC data analysis**

All raw MS files were processed using Mascot Distiller 2.5.0 (Matrix Science). The MS data obtained by the analysis of gel lanes were merged into a multi-file-project using the default settings from the ABDsciex_5600.opt file except that the MS/MS Peak Picking “Same as MS Peak Picking” was deselected and “Fit method” was set to “Single Peak”. After peak picking all scans, the data were searched against Swiss-Prot Homo Sapiens database (v. 2013_11/12) using Mascot v. 2.3.02 (Matrix Science) [67]. The Search parameters allowed one missed trypsin cleavage site, propionamide as a fixed modification, and oxidation of methionine as a variable modification. The mass accuracy of the precursor and product ions were set to 10 ppm and 0.2 Da, respectively, the instrument setting was specified as ESI-QUAD-TOF, and a significance threshold of 0.01 was used. The default SILAC R + 6 R + 10 [MD] quantitation protocol was selected using a significance threshold at 0.01, matched rho was 0.7, XIC threshold was 0.1, isolated precursor threshold was set at 0.5 and normalization set to median. Mascot Distiller results were exported to xml files, imported into MS Data Miner v.1.2.0 (http://sourceforge.net/p/msdataminer [68]) and exported into Excel reports. Keratin was excluded as a general contaminant and a fold regulation threshold of 1.3 was selected for significant differentially regulated proteins. Spectra for differentially regulated proteins were manually inspected and proteins were excluded if not at least two unique peptides showed peaks for both light, medium and heavy label significantly above background (ion counts > 50). The data are available via ProteomeXchange with identifier PXD000760 (http://proteomecentral.proteomexchange.org). Clustering analysis was done using the Functional Annotation tools of DAVID Bioinformatics Resources 6.7 [69, 70].

**Incubation of HepG2-SF cells in fatty acid supplemented media for DIGE**

Four 75 cm$^2$ culture flasks containing 90 % confluent cells were trypsinized and seeded into 16 new flasks (4 replicas/group), and allowed to attach for two days before addition of fatty acid supplemented medium (10 ml/flask). Medium was changed on day 3 and 6. On day 6 only 5 ml fatty acid supplemented medium was added. The 5 ml conditioned medium was collected on day 7, filtered, and stored on ice. Samples for DIGE was depleted for albumin as described for SILAC above and desalted using a PD10 column into 2.5 mM Tris, pH 7.4. Aliquots of 50 μg protein were dried in a SpeedVac and stored at −20 °C until use.
DIGE analysis of proteins secreted by HepG2-SF cells

A total of 50 μg of protein was labeled using GE Healthcare CyDye DIGE Flours (minimal dyes) according to the manufacturers instructions (GE Healthcare). The samples were labeled using 400 pmol Cy3 or Cy5 and as an internal standard, a pool of 25 μg of each sample to be analyzed was labeled by 400 pmol Cy2 per 50 μg protein. Labeling was conducted in 7 M Urea, 2 M Thiourea, 4 % w/v CHAPS, 10 mM Tris, pH 8.5. After 30 min incubation the labeling reaction was quenched by adding 1 μl 10 mM lysine. Four experimental groups with 4 replicates were analyzed (two replicates in each group labeled with Cy3 and two with Cy5) resulting in a total of 8 analytical DIGE gels. The combination of samples differed in all gels and no gel contained two replicates from the same experimental group. Two samples labeled with Cy3 and Cy5 respectively were mixed with 50 μg Cy2-labeled internal standard and volume adjusted to 130 μl in rehydration solution (7 M Urea, 2 M Thiourea, 4 % w/v CHAPS, 0.5 % v/v pH4-7 IPG carrier ampholytes, 40 mM DTT, 10 mM Tris, pH 8.5). The mixed samples were incubated in the dark, rotating at room temperature for 20 min and centrifuged 30 min at 16000 g. Supernatants were subjected to isoelectric focusing by cup-loading on 24 cm pH 4–7 Immobiline Drystrips (GE Healthcare) rehydrated overnight in rehydration solution. Isoelectric focusing was performed in an EttaN IPGphor II (GE Healthcare) at 25 °C with maximum 50 μAmp/strip using the following program: step 1 h at 150 V, gradient 1 h to 500 V, step 2 h at 500 V, gradient 7 h to 1000 V, gradient 4 h to 8000 V, step 5 h 8000 V.

After focusing, proteins in IPG-strips were reduced with DTT (10 mg/ml in equilibration buffer composed of 50 mM Tris, 6 M Urea, 30 % glycerol, 2 % SDS, pH 8.8) for 15 min followed by alkylation 15 min with iodoacetamide (25 mg/ml in equilibration buffer). The strips were equilibrated in upper reservoir Laemmli buffer (50 mM Tris, 384 mM Glycine, 0.2 % w/v SDS) and placed on top of an acrylamide/bis (10 % T, 0.87 % C) Tris–HCl, pH 8.8, gel in low fluorescent glass plates. Second dimension electrophoresis was conducted in the EttaN Dalt six gel system (GE Healthcare) program as follows: 2 h at 0.3 W/gel, 10 h at 1 W/gel, 4 h at 2 W/gel.

DIGE-gels were scanned on Typhoon 8600 scanner at a pixel resolution of 100 μm, the photomultiplier tubes at 600 V and laser and emission filters set at standard DIGE wavelengths. DeCyder software v. 6.0 (GE Healthcare) was used for image analysis. Spots more than 1.3 fold regulated were mixed with 500 μl 5 % acetonitrile and no gels contained two replicas from the same experimental group. Two samples labeled with Cy3 and Cy5 respectively were mixed with 50 μg Cy2-labeled internal standard and volume adjusted to 130 μl in rehydration solution (7 M Urea, 2 M Thiourea, 4 % w/v CHAPS, 0.5 % v/v pH4-7 IPG carrier ampholytes, 40 mM DTT, 10 mM Tris, pH 8.5). The mixed samples were incubated in the dark, rotating at room temperature for 20 min and centrifuged 30 min at 16000 g. Supernatants were subjected to isoelectric focusing by cup-loading on 24 cm pH 4–7 Immobiline Drystrips (GE Healthcare) rehydrated overnight in rehydration solution. Isoelectric focusing was performed in an EttaN IPGphor II (GE Healthcare) at 25 °C with maximum 50 μAmp/strip using the following program: step 1 h at 150 V, gradient 1 h to 500 V, step 2 h at 500 V, gradient 7 h to 1000 V, gradient 4 h to 8000 V, step 5 h 8000 V.

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Identification of proteins of interest using LC MS/MS

Picked gel plugs were dehydrated and destained using acetonitrile and NH₄HCO₃ and the gel-plugs were dried before reswelling in 50 mM NH₄HCO₃ with 100 ng trypsin (Promega) pr plug and left over night at 37 °C for in-gel digestion [66]. At the end of digestion, formic acid was added. Peptides were purified on C18 reverse phase material using Stage-tips and eluted with 2 μl 70 % acetonitrile into 18 μl 0.1 % formic acid. The samples were subjected to LC MS/MS using an Easy nLC (Proxen) (50 min gradient of 5–40 % acetonitrile in 0.1 % formic acid) coupled in-line with a Q-TOF Ultima API mass-spectrometer (Micromass/Waters). Data were processed using MassLynx 4.0 software (Waters). MS/MS data are searched against SwissProt (v. 2014_02) limited to human taxonomy using Mascot v. 2.3.02 (Matrix Sciences). Trypsin was selected and a maximum of one missed cleavage was allowed. The following variable modifications were accepted: Carbamidomethyl, propionamide, and oxidation on methionine. Peptide tolerance was set at 1.2 Da and MS/MS tolerance at 0.6 Da. Instrument setting was specified as ESI-QUAD-TOF. Protein identifications were based on significant MOWSE-score, peptide scores > 30, low e-values, and visual inspection of MS/MS spectra. Spots were excluded from further analysis if identified as albumin, due to the use of albumin as delivery vehicle for fatty acids.

Additional files

Additional file 1: Data S1. G.LC. Results from the gas-liquid chromatography analysis. On the left are in bold averages of mol% for three biological replicates and on the right their respective standard deviations. Raw data is provided for each replicate in separate sheets. (XLSX 48 kb)

Additional file 2: Data S2. SILAC transvA-EA- cisvA. Data from the SILAC investigation of the cellular response to transvA, EA and cisvA. (XLSX 867 kb)

Additional file 3: Data S3. DIGE identifications. Protein identifications by LC-MS/MS from DIGE analysis of the secretomes from cells incubated with transvA, EA and cisvA. Spots with significantly different intensities between samples in the DIGE analysis were excised, and the proteins were identified by LC-MS/MS and Mascot searches. Proteins identified by 2 or more peptides with ion scores ≥ 30 are highlighted. (XLSX 23 kb)

Additional file 4: Figure S1. DIGE 2D-gel with indications of the significant differentially regulated spots in EA/transvA (panel A) and transvA/ cisvA (panel B). The numbers correspond to Table 2 and supplemental data S3. (DOCX 1198 kb)

Additional file 5: Data S4. SILAC transvA-OA-CLA. Data from the SILAC investigation of the cellular response to transvA, OA and CLA. In sheet 1, “full data”, unfiltered data from this second SILAC triplex is displayed. In sheet 2, “signif. 1.3 fold”, proteins significantly regulated ≥ 1.3 fold in ≥ one comparison are displayed. Proteins are reported with Uniprot accession number, Name, Fold regulation in individual comparisons, the geometric standard deviation (Geo.SD), and selected GO-terms. GO00006950 response
to stress (x) and GO:0002526 acute inflammatory response (y). Black bold: significant regulatory data (p < 0.01) with fold > 1.3, black italic: significant regulatory data (p < 0.01) with fold < 1.3, grey italic: not significant regulatory data (p > 0.01). DILXS 925 kb

Abbreviations

CLA: Conjugated linoleic acid, (trans,cis)Δ9,11-C18:2, cis14; cis-15: cis-vaccenic acid (cis11,17-C18:2); CVD: Cardiovascular disease; DGE: Difference in in-gel electrophoresis; EA: Elaidic acid (transΔ9C18:1); FAME: Fatty acid methyl esters; ITFA: Industrially produced TFA; OA: Oleic acid (cisΔ9C18:1); ITTA: Ruminant TFA; SILAC: Stable isotope labeling by amino acids in cell culture; TFA: Trans fatty acid; TG: triacylglycerol; transVA: trans-vaccenic acid (transΔ11C18:1); TIMP1: metalloproteinase inhibitor 1; TTR: transthyretin.

Competing interests

The author(s) declare that they have no competing interests.

Authors’ contributions

JJE, LVN and TPK were responsible for project conception; LVN, TPK, DK, CS, and TFD performed the experiments and analyzed the data; KWS contributed to the interpretation of the data and edits of the final manuscript; TPK, LVN and JJE wrote the manuscript; and JJE had primary responsibility for the final content. All authors read and approved the final manuscript.

Acknowledgements

The Danish council for Strategic Research – Health, Food and Welfare financially supported the work. Xuebing Xu and Zheng Guo from Lipid Lab at the Department of Molecular Biology and Genetics at Aarhus University, Denmark, are acknowledged for valuable discussion on the interpretation of GC data. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [71] with the dataset identifier PXD000760 and DOI:10.6019/PXD000760.

Received: 5 May 2015 Accepted: 22 November 2015

Published online: 01 December 2015

References

1. Stender S, Dyerberg J, Astrup A. High levels of industrially produced trans fat in popular fast foods. N Engl J Med. 2006;354:1650–2.
2. Micha R, Mozaffarian D. Trans fatty acids: effects on metabolic syndrome, heart disease and diabetes. Nat Rev Endocrinol. 2009;5:335–44.
3. Mozaffarian D, Clarke R. Quantitative effects on cardiovascular risk factors and coronary heart disease risk of replacing partially hydrogenated vegetable oils with other fats and oils. Eur J Clin Nutr. 2009;63 Suppl 2:S22–33.
4. Gebauer SK, Chardigny JIM, Jakobsen MU, Lamarche B, Lock AL, Proctor SD, et al. Effects of ruminant trans fatty acids on cardiovascular disease and cancer: a comprehensive review of epidemiological, clinical, and mechanistic studies. Adv Nutr. 2012;3:322–54.
5. Willett WC, Stampfer MJ, Manson JE, Colditz GA, Speizer FE, Rosner BA, et al. Intake of trans fatty acids and risk of coronary heart disease among women. Lancet. 1993;341:581–5.
6. Dreon DM, Orce MC, Feskens EJ, van Erp-Baart MA, Kok FJ, Kromhout D. Association between trans fatty acid intake and 10-year risk of coronary heart disease in the Zutphen Elderly Study: a prospective population-based study. Lancet. 2001;357:746–51.
7. Jakobsen MU, Overvad K, Dyerberg J, Heitmann BL. Intake of ruminant trans fatty acids and risk of coronary heart disease. Int J Epidemiol. 2008;37:173–82.
8. Pietinen P, Ascherio A, Korhonen P, Hartman AM, Willett WC, Albanes D, et al. Intake of fatty acids and risk of coronary heart disease in a cohort of Finnish men. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Am J Epidemiol. 1997;145:876–87.
9. Bolton-Smith C, Woodward M, Fenton S, Brown CA. Does dietary trans fatty acid intake relate to the prevalence of coronary heart disease in Scotland? Eur Heart J. 1996;17:837–45.
10. Rissanen H, Knelt P, Janvins R, Salminen I, Hakulinen T. Serum fatty acids and breast cancer incidence. Nutr Cancer. 2003;45:168–75.
11. Shannon J, King IB, Moschosky R, Lampe JW, Gao DL, Ray RM, et al. Erythrocyte fatty acids and breast cancer risk: a case–control study in Shanghai, China. Am J Clin Nutr. 2007;85:1900–7.
12. Precht D, Molkentin J. Trans fatty acids: implications for health, analytical methods, incidence in edible fats and intake (a review). Nahrung. 1995;39:343–74.
13. Kepler CR, Hiromi KP, McNeill JJ, Tove SB. Intermediates and products of the biobiohydrogenation of fatty acid by Butyrobivrio fibrisolvens. J Biol Chem. 1966;241:1550–4.
14. Slots T, Butler G, Leifert C, Kristensen T, Skibsted LH, Nielsen JH. Potential to differentiate milk composition by different feeding strategies. J Dairy Sci. 2009;92:2057–66.
15. Sebedio JL, Ratnayake WMN. Analysis of trans mono- and polysaturated fatty acids. In: Dijkstra AJ, Hamilton RJ, Hamw w, editors. Trans fatty acids. Oxford; Ames: Iowa: Blackwell Pub; 2008. p. 102–28.
16. Beers A, Ariaans R, Okneke D. Trans isomer control in hydrogenation of edible oils. In: Dijkstra AJ, Hamilton RJ, Hamw w, editors. Trans fatty acids. Oxford; Ames: Iowa: Blackwell Pub; 2008. p. 147–80.
17. Clifton PM, Keogh JB, Noakes M. Trans fatty acids in adipose tissue and the food supply are associated with myocardial infarction. J Nutr. 2004;134:874–9.
18. Hodgson JM, Wahlsqvist ML, Boxall JA, Balazs ND. Platelet trans fatty acids in relation to angiographically assessed coronary artery disease. Atherosclerosis. 1996;120:47–54.
19. Lintzemuth AH, Erkllä AT, Lamarche B, Schweib US, Jalsberg SM, Ausman LM. Influence of hydrogenated fat and butter on CVD risk factors: remnant-like particles, glucose and insulin, blood pressure and C-reactive protein. Atherosclerosis. 2003;171:97–107.
20. Motard-Belanger A, Charest A, Grenier G, Paquin P, Chouinard Y, Lemieux S, et al. Study of the effect of trans fatty acids from ruminants on blood lipids and other risk factors for cardiovascular disease. Am J Clin Nutr. 2008;87:593–9.
21. Aro A, Jauhainen M, Partaran M, Salminen I, Mutanen M. Searic acid, trans fatty acids, and dairy fat: effects on serum and lipoprotein lipids, apolipoproteins, lipoprotein(a), and lipid transfer proteins in healthy subjects. Am J Clin Nutr. 1997;65:1419–26.
22. de Roos NW, Bots ML, Kanant MB. Replacement of dietary saturated fatty acids by trans fatty acids lowers serum HDL cholesterol and impairs endothelial function in healthy men and women. Arterioscler Thromb Vasc Biol. 2001;21:1233–7.
23. Chardigny JIM, Destaillats F, Malpuech-Brugere C, Moulin J, Bauman DE, Lock AL, et al. Do trans fatty acids from industrially produced sources and from natural sources have the same effect on cardiovascular disease risk factors in healthy subjects? Results of the trans Fatty Acids Collaboration (TRANSFACT) study. Am J Clin Nutr. 2008;87:593–9.
24. Iwata NG, Pham M, Rizzo NO, Cheng AM, Maloney E, Kim F. Trans fatty acids induce vascular inflammation and reduce vascular nitric oxide production in endothelial cells. Plos One. 2011;6:e29600.
25. Jauhais A, Jähres H, Schörmann W, Fischer J, Kramer R, Degen C, et al. Vaccenic acid-mediated reduction in cytokine production is independent of c9,11-CLA in human peripheral blood mononuclear cells. Biochim Biophys Acta. 2012;1821:1316–22.
26. Lopes SM, Tribble SL, Mascioli EA, Blackburn GL. Human plasma fatty acid variations and how they are related to dietary intake. Am J Clin Nutr. 1991;53:362–7.
27. Swagell CD, Henley DC, Morris CP. Regulation of human hepatocyte gene expression by fatty acids. Biochem Biophys Res Commun. 2007;352:374–80.
28. Kondo Y, Kawada T, Urade R. Activation of caspase 3 in HepG2 cells by elaidic acid (18:1). Biochim Biophys Acta. 2007;1771:500–5.
29. Vock C, Gleisner M, Klapper M, Doring F. Identification of palmitate-regulated genes in HepG2 cells by applying microarray analysis. Biochim Biophys Acta. 2007;1770:1283–9.
30. Möberly JB, Cole TG, Alpers DH, Schonfeld G. Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco-2 cells occurs post-transcriptionally. Biochim Biophys Acta. 1990;1042:70–80.
31. Vock C, Gleisner M, Klapper M, Doring F. Oleate regulates genes controlled by signaling pathways of mitogen-activated protein kinase, insulin, and hypoxia. Nutr Res. 2008;28:681–9.
and secretion of apolipoprotein A-I and apolipoprotein B-containing lipoproteins in HepG2 cells. J Lipid Res. 2000;41:1980–90.

33. Arrol S, Mackness MJ, Durrington PN. The effects of fatty acids on apolipoprotein B secretion by human hepatoma cells (HEP G2). Atherosclerosis. 2000;150:255–64.

34. Vinciguerra M, Carozzino F, Peycuo M, Carbone S, Montesano R, Benelli R, et al. Unsaturated fatty acids promote hepatoma proliferation and progression through downregulation of the tumor suppressor PTEN. J Hepatol. 2009;50:1132–41.

35. Turpeinen AM, Mutanen M, Aro A, Salminen L, Basu S, Palmquist DL, et al. Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am J Clin Nutr. 2002;76:504–10.

36. Ix JH, Chertow GM, Shlipak MG, Brandenburg VM, Kettelhut M, Whooley MA. Association of retinol-α with mitral annular calcification and aortic stenosis among persons with coronary heart disease: data from the Heart and Soul Study. Circulation. 2007;115:2533–9.

37. Krogager TP, Nielsen LV, Bak S, Young C, Ferre C, Jensen ON, et al. Identification of a potential biomarker panel for the intake of the common trans fatty acid elaidic (trans-C18:1). J Proteomics. 2012;75:2685–96.

38. Vendel Nielsen L, Krogager TP, Young C, Ferre C, Chatgilialoglu C, Norrhaardt Jensen O, et al. Effects of elaidic acid on lipid metabolism in HepG2 cells, investigated by an integrated approach of lipidomics, transcriptomics and proteomics. PloS One. 2013;8:e74283.

39. Du ZY, Degrace P, Gresti J, Lareae O, Clouet P. Vaccenic and elaidic acids equally esterify into triacylglycerols, but differently into phospholipids of fed rat liver cells. Lipids. 2011;46:567–77.

40. Du ZY, Degrace P, Gresti J, Lareae O, Clouet P. Dissimilar properties of vaccenic versus elaidic acid in beta-oxidation activities and gene regulation in rat liver cells. Lipids. 2010;45:581–91.

41. Yu W, Liang X, Ensenauer RE, Vockley J, Sweetman L, Schulz H. Leaky C3 convertase formation. Science. 2010;330:1816–20.

42. Ix JH, Chertow GM, Shlipak MG, Brandenburg VM, Vekhoff M, Whooley MA. Postprandial increase of complement component 3 of C3b in complex with factors B and D give insight into complement factor xa formation by tissue factor pathway inhibitor. J Biol Chem. 2006;281:20290–9.

43. Lagrost L. Differential effects of cis and trans fatty acid isomers, oleic and vaccenic acids, on the cholesterol ester transfer protein activity. Biochim Biophys Acta. 1992;1124:159–62.

44. Kohler HP, Grant PJ. Plasminogen-activator inhibitor type 1 and coronary artery disease. N Engl J Med. 2000;342:2906–8.

45. Heald AH, Cruickshank JK, Riste LK, Cade JE, Anderson S, Greenhalgh A, et al. Close relation of fasting insulin-like growth factor binding protein-1 (IGFBP-1) with glucose tolerance and cardiovascular risk in two populations. Diabetologia. 2001;44:1333–9.

46. Laughlin GA, Barret-Connon E, Ciriq MH, Kirtz-Silverstein D. The prospective association of serum insulin-like growth factor binding protein-1 (IGFBP-1) with glucose tolerance and cardiovascular risk in two populations. Diabetes. 2001;44:1333–9.

47. Kronenberg F, Stuhlinger M, Trenkwalder E, Geethanjali FS, Pachinger O, von Eckardstein A, et al. Low apolipoprotein A-I plasma concentrations in men with coronary artery disease. J Am Coll Cardiol. 2000;36:751–7.

48. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med. 1999;340:115–26.

49. Baer DJ, Judd JT, Clevendence BA, Tracy RP. Dietary fatty acids affect plasma markers of inflammation in healthy men fed controlled diets: a randomized, crossover study. Am J Clin Nutr. 2004;79:969–73.

50. Lopez-Garcia E, Schulze MB, Meigs JB, Manson JAE, Rifai N, Stampfer MJ, et al. Consumption of Trans fatty acids is related to plasma biomarkers of inflammation and endothelial dysfunction. J Nutr. 2005;135:562–6.

51. Muscaria A, Massarelli G, Bastagi L, Poggiopollini G, Tornessi V, Drago G, et al. Relationship of serum C3 to fasting insulin, risk factors and previous ischaemic events in middle-aged men. Eur Heart J. 2000;21:1081–90.

52. Halles CJ, van Dijk H, de Laeijere RP, Plokker HW, van der Helm Y, Erkelens DW, et al. Postprandial increase of complement component 3 in normolipidemic patients with coronary artery disease: effects of expanded-dose simvastatin. Atherosclerosis. 2001;152:26–36.

53. Formens F, Ricklin D, Wu J, Tzekou A, Wallace RS, Lambris JD, et al. Structures of C3b in complex with factors B and D give insight into complement conversion formation. Science. 2010;330:1816–20.

54. Hourcade DE, Mitchell LM, Ogleby TJ. Mutations of the type A domain of complement factor B that promote high-affinity C3b-binding. J Immunol. 1999;162:2906–11.

55. Ritchie GE, Moffatt BE, Sim RB, Morgan EP, Dwek RA, Rudd PM. Glycosylation and the complement system. Chem Rev. 2002;102:305–320. 319.

56. Sec J, Silberhart T, Toller GH, Matheney TH, Sutherland P, Lipinetska L, et al. Association of fibrinogen with cardiovascular risk factors and cardiovascular disease in the Framingham Offspring Population. Circulation. 2000;102:1634–8.

57. Baugh RJ, Broze Jr GJ, Krishnawamy S. Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor. J Biol Chem. 1998;273:4788–86.

58. Griend TJ, Warren IA, Norovoy WF, Lillik KM, Brown SG, Mleitch JP, et al. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature. 1989;338:18–20.

59. Ahara K, Azuma H, Takamori N, Kanagawa Y, Akaite M, Fujimura M, et al. Heparin cofactor II is a novel protective factor against carotid atherosclerosis in elderly individuals. Circulation. 2004;109:2761–5.

60. Weggeman RM, Rudmin M, Trautwein EA. Intake of ruminant versus industrial trans fatty acids and risk of coronary heart disease - what is the evidence? Eur J Lipid Sci Technol. 2004;106:390–7.

61. Coulouba S, Delomene C, Rousseau D, Paul JL, Leynberg A, Pourci ML. Fatty acid incorporation in endothelial cells and effects on endothelial nitric oxide synthase. Eur J Clin Invest. 2007;37:692–9.

62. Mayrath V, Liebisch G, Kurzchala TF, Shevchenko A, Schwudke D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res. 2008;49:1137–46.

63. Kromvall G, Simmons A, Myhre EB, Jonsson S. Specific absorption of human serum albumin, immunoglobulin A, and immunoglobulin G with selected strains of group A and G streptococci. Infect Immun. 1979:251–1. 10–

64. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.

65. Bury AF. Analysis of protein and peptide mixtures : Evaluation of three sodium dodecyl sulphate-polyacylamide gel electrophoresis buffer systems. J Chromatogr. 1981;162:491–500.

66. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2006;1:2856–60.

67. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis. 1999;20:3551–67.

68. Dytldn TF, Poulsen ET, Scavenger C, Sangeda KW, Enghild JJ. MS Data Miner: a web-based software tool to analyze, compare, and share mass spectrometry protein identifications. Proteomics. 2012;12:2792–6.

69. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4: 44–57.

70. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37:1–13.

71. Vazcaino JA, Cote RG, Cozad A, Dianes JA, Fabregat A, Foster JM, et al. The Proteomics IDentifications (PRIDE) database and associated tools: status in 2013. Nucleic Acids Res. 2013;41:D1063–9.