Molecular effects of the consumption of margarine and butter varying in trans fat composition: a parallel human intervention study

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Research Article

Keywords: Butter, margarine, trans fatty acids, circulating lipids, metabolome, transcriptome

Posted Date: February 16th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1358254/v1

Abstract

Background: Dietary intake of industrial *trans* fatty acids (iTFA) is associated with inflammation, cardiovascular disease and type 2 diabetes but it is unclear whether naturally occurring *trans* fatty acids (TFA) exert these effects.

Methods: In a parallel study, the molecular effects of consuming dairy fat containing ruminant TFA (rTFA) or margarine containing iTFA were investigated. After a run-in period of margarine without TFA (wTFA), 42 healthy volunteers (45 to 69 y), were randomly assigned to diets enriched with margarine wTFA (control), margarine with iTFA, or alpine butter (rTFA) for 4 weeks. Changes in fasting blood values of lipid profiles (GC with flame-ionization detection), metabolome profiles (LC-MS, GC-MS) and gene expression (microarray) were measured.

Results: Eighteen fatty acids, as well as 242 additional features measured by LC-MS (185) and GC-MS (54) showed significantly different responses to the diets ($P_{FDR\text{-}adjusted} < 0.05$), mainly distinguishing butter from the margarines while gene expression was not differentially affected. The most abundant TFA in the dietary fats (elaidic and vaccenic acids) were reflected in the significantly different serum TFAs after the TFA interventions.

Conclusions: The TFA interventions did not appear to regulate a common molecular pathway. This could be attributable to the different TFAs present in rTFA and iTFA, which could differentially affect cholesterol and lipid metabolism.

Trial registration: ClinicalTrials.gov NCT00933322

Introduction

Trans fatty acids (TFA) are fatty acids (FA) that have at least one double bond in *trans* configuration [1]. Historically, the major dietary source of TFA were partially hydrogenated fats, which were widely promoted to address obesity and cardiovascular disease (CVD) [2]. However, emergence of evidence that causally linked these fats with an increased risk of CVD [3-5], has led to widespread implementation of recommendations to limit consumption of partially hydrogenated oils, the majority stipulating that dietary TFA should be kept as low as possible [6, 7].

TFAs also occur naturally, in small quantities, in meat and dairy products, due to the action of bacterial enzymes that act in the rumen to bio-hydrogenate unsaturated FA. The TFA isomers from industrial (iTFA) and ruminant (rTFA) fats are chemically similar, although they vary in their relative concentrations [8]. Given the decline in iTFA intake, the relative importance of rTFA in the diet has increased, with recent estimates showing that rTFA was the major dietary source of TFA (0.1 to 0.7% energy intake) for more than two-thirds of the countries surveyed [9].

The impact of rTFA on risk of CVD is still under debate [10]. Many epidemiological studies have demonstrated deleterious effects of iTFA but not rTFA on CVD risk [11-14], but others have reported equivalent effects of all TFA isomers on CVD risk factors [15, 16]. Furthermore, in a review of intervention studies, a positive relationship between all FA with a double bond in *trans* configuration and plasma LDL to HDL cholesterol ratio was described [8]. Some inconsistencies in these findings can be attributed to the different quantities of TFA consumed; indeed while positive associations between circulating lipoproteins and high intakes of rTFA or iTFA have been reported, these associations were lost at lower intakes of rTFA, as generally consumed in the diet [17, 18]. Nevertheless, a systematic review of randomised clinical trials found no evidence for an association between CVD risk factors and rTFA intakes of up to 4.2% energy intake [19].

In contrast to the wealth of observational data associating TFA with cardiovascular health, the understanding of molecular mechanisms mediating the effects of TFA on CVD risk is more limited [20]. However, there is evidence that TFA influences the regulation of multiple physiological processes [13], including hyperlipidemia [21], promotion of inflammation and cell death [22], reduced transforming growth factor-β (TGF-β) responsiveness [23], and endothelial dysfunction [13, 24]. Interestingly, preclinical studies comparing the major isoforms of rTFA and iTFA suggest that while these fats show some common behaviour, they can also act differentially depending on the molecular pathway assessed; for example rTFA may mediate its effects on cholesterol and FA synthesis via PPARs, while iTFA seems to specifically stimulate cholesterol synthesis via the activation of SREBP2-mediated gene regulation [20]. Thus, evaluation of the CVD risk of rTFA may require an understanding of the molecular effects of the different fats present in the food matrix. Importantly, dietary rTFA are not consumed in isolation but with a complex mixture of associated FAs. Few human studies have compared the effects of rTFA and iTFA on non-targeted circulating lipid profiles [25, 26]. However, lipid profiling together with omics technologies (transcriptomics and metabolomics) could be a powerful approach for demonstrating the effects of TFA on molecular and metabolic functions and pathways.

We previously reported the primary objectives of a parallel intervention study in which healthy subjects showed a small increase in total cholesterol and LDL-cholesterol after butter intake compared to margarines with or without TFA [27]. In the present work, we explore the molecular changes associated with lipid metabolism induced by the diets using a combination of ‘omics’ approaches.

Methods

Subjects

Participant recruitment, inclusion and exclusion criteria, randomization and compliance have been described in detail previously [27]. Briefly, a total of 142 healthy men and women, between 45 and 69 years were enrolled in the study. Of these, 125 participants were included in the per protocol analysis. Written informed consent for participation in the study was obtained from all participants and all procedures were conducted in accordance with the Declaration of Helsinki.

For the present analysis, we report on a predefined subgroup of 42 subjects selected from the main cohort using stratified sampling with respect to sex and treatment group (fourteen subjects per group). High-resolution lipid-analysis (HR-lipids), gas chromatography (GC)- and liquid chromatography (LC) mass spectrometry (MS) analysis was completed for the entire subset while gene expression was assessed in whole blood of 21 randomly selected subjects from the same subset (seven subjects per intervention group).

Study design and interventions

The study was a randomized, controlled, double-blind, three-arm, parallel-group intervention, designed to investigate the health effects of TFA intake from industrial and ruminant sources [27]. The study was approved by the cantonal ethical committee of Bern, Switzerland and registered at www.clinicaltrials.gov (NCT00933322). Block randomization with stratification for gender and age was used to assign subjects to one of three diets: a diet with alpine butter rich in ruminant TFA (rTFA group), a diet with margarine rich in industrial TFA (iTFA group) and a diet with margarine without TFA (wTFA group, control).

The study protocol began with a run-in period of two weeks, during which all study participants followed the wTFA diet, and was followed by a 4-week intervention period with the assigned diets (rTFA, iTFA, wTFA) according to the randomization. All dietary fats for the study were provided to the subjects (33-36% of individual energy requirements) in the form of the designated study products plus 15-25 g/day rapeseed oil, as described in detail previously [27]. The dietary fat supplements resulted in comparable levels of 2% energy intake from TFA for iTFA and rTFA diets. During all study phases, subjects adhered to a prescribed diet, defined by a dietitian to exclude all other dietary sources of TFA and meet individually calculated daily energy requirements. Details of the dietary restrictions and evaluation of dietary adherence are published elsewhere [27]. At each visit, weight was assessed and BMI calculated.

Blood sampling

Blood samples were collected from the antecubital vein at baseline (after the 2-week run-in period) and at the end of the intervention period (week 6) after an overnight fast. The preparation and processing of blood samples to assess clinical biochemistry and biomarkers of inflammation, coagulation and endothelium function have been described previously [27]. For GC-MS and LC-MS analyses, serum was separated and stored in microtubes at -80°C until assayed. Whole blood was collected for microarray analysis in PAXgene blood RNA tubes and frozen at -20°C for 24 h before transfer to -80°C until further treatment.

High-resolution lipid analysis with GC-FID

Serum samples were prepared for analysis by addition of 15 µL of internal standard (C13:0, 7.5 µg/15 µL) to 100 µL of serum, followed by methylation of free FA with MeOH/HCl (25°C for 45 min). A post-reaction treatment for neutralization was applied with Na_2CO_3 and extraction was performed with 300 µL hexane. Samples were measured by gas chromatography with flame ionization detector (GC-FID) (6890, Agilent Technologies, Santa Clara, CA) with injection volumes of 0.5 µL as described previously [28]. Butter and margarine samples were assayed previously by Radtke *et al.* [27], using the same GC-FID technique as for serum samples with sample preparation according to the method of Collomb and Bühler [28]. The data was reprocessed and reintegrated here with some additional reference lipids included. Eighty-five features (66 single FA and 19 sum parameters) were quantitatively analysed in the serum and dietary fats.

Transcriptomic analysis and data preprocessing

Total RNA was extracted from whole blood samples and treated to deplete for globin mRNA according to the protocol described by Gille *et al.* [29]. Whole genome transcript profiling was then performed with HG-U219 oligonucleotide expression probe arrays (Affymetrix, USA). Preparation of samples for profiling including reverse transcription, amplification, amplified RNA labelling, purification, fragmentation and hybridization steps were performed as defined previously [29]. Arrays were measured in accordance with manufacturer's recommendations using Affymetrix GeneAtlas™System. Raw array data was imported to the R environment (version 4.1.2) [30] and corrected for background noise, log2 transformed, normalized for inter-array variation (quantile normalization) [31] and summarized using the *rma* (Robust Multichip Average) function from affy (version 1.72.0) [32]. In addition, probe sets were filtered to keep only: the most variable probe set per gene (based on standard deviation), probe sets assigned to gene symbols (hgu219.db, version 3.2.3) [33] and probe sets with average expression (log2) > 5. Of the 49'386 probe sets, 6'128 probe sets were retained for further analysis.

Untargeted GC-MS analysis and data pre-processing

Sample preparation for the untargeted analysis of the serum samples by GC-MS was based on the method published by Trimigno *et al.* [34] using a GC-MS 7890B/MS5977A (Agilent Technologies, Santa Clara, CA, USA) with a CombiPAL autosampler (CTC-Analytics AG, Zwingen, Switzerland). After deconvolution, features from subjects that only appeared in less than three samples were eliminated (n = 4828 remaining features). A second manual integration was conducted on 54 selected features that showed significant differences between treatment group responses to the interventions (see *Statistical Analyses*). The areas were normalized with the isotopically-labelled fructose. The features demonstrating a significant treatment effect were searched in EI-Mass Spectral Library (NIST 2017, Gaithersburg, MD 20899-6410, USA), masslib-library (www.masslib.com). The molecules demonstrating sufficient potential for identification were acquired from Sigma-Aldrich (Buchs, Switzerland) and analyzed by GC-MS.

Untargeted LC-MS analysis and data pre-processing

Serum samples were thawed, treated and analyzed according to the protocol described by Pimentel *et al.* [35], using a maXis 4G+ (Bruker, Bremen, Germany) coupled to a 3000 RS UHPLC (Thermo, Basel, Switzerland). Raw data (m/z values, retention times and intensities) were imported into Progenesis QI (V2.4) (Waters, Switzerland) for retention time alignment, peak detection, sample loading normalization, deconvolution of masses (including detection of adducts) and final export of peak volumes for further statistical treatment. The pre-processed dataset (~85'000 features) was imported into R (version 3.5.2) [36] and filtered in two steps. All features that showed a mean in QC samples (n = 21) that were < 4 times higher than the mean for the final three blanks were excluded. In addition, features with a relative standard deviation > 30% in the QC samples were excluded to leave a reduced dataset of 11'616 features. Identification was carried out by a mass search in the Human Metabolome Database (HMDB v4.0 2018 [37]) and NIST (National Institute of Standards and Technology Database, V14). Successive identification was then confirmed by LC-MS where exact mass and retention time of the metabolites were compared with standards. The identification was differentiated at four levels [38-40]. We report here in detail on metabolites identified at the highest level (level 1), *i.e.* compound identified with accurate retention time (+/- 10%) and mass (+/- 10 ppm) when confirmed with a chemical reference standard.

Statistical Analyses

Data exploration

All statistical analyses were performed in the R environment (version 3.5.2) [36]. The normality of baseline clinical characteristics and metabolomics datasets was explored through Shapiro-Wilk tests of normality and visually by quantile-quantile plots. Principal components analysis (PCA) was used with all datasets to visually inspect sources of variation, in particular to exclude the presence of batch effects, outliers or potential confounders (e.g. sex) (lipids and metabolomics datasets: ropls package, v1.4.4, 2016, [41]; transcriptomics dataset: stat function 'prcomp' visualized with ggplot2 (version 3.3.5) [42].

Clinical data

Non-parametric univariate statistics were used for the current analysis in view of the reduced study population size and multiple features that were not distributed normally. Baseline clinical characteristics, blood biochemistry, inflammation and endothelium biomarkers of the study population were compared by treatment group using the Kruskal-Wallis test (P < 0.05).

GC-FID Lipid analysis

The Kruskal Wallis test was used to compare the changes in lipid profiles in response to the three interventions. For significant results, a *post hoc* Wilcoxon signed-rank test was completed to compare each with adjustment of P-values for multiple testing using the Benjamini-Hochberg FDR method [43] (significance where $P_{FDR\text{-}adjusted} < 0.05$). The sum parameters for the classes of fatty acids were integrated into this analysis. The response to the intervention for these analyses was defined for each participant as the delta change between measures at endpoint (week 6) and baseline (week 2). Features showing a significant difference between the three treatments were presented in heatmaps using the "Metabolomics" package (v0.1.4) [44] with distance calculated by "*Canberra*" method and clustering using the "*ward.D2*" method [45].

Gene and pathway analysis

The molecular effects of the dietary interventions were investigated by assessing the change in gene expression in whole blood (*i.e.* delta change between baseline and post-treatment), with delta values calculated using log_2-transformed data, thus interpretable as log_2-fold expression changes (log_2FC). Differentially expressed genes for each group and between groups were identified by performing a paired moderated t-test on the responses to the intervention using R package Limma (Linear Models for Microarray Data) (version 3.50.0) ($P_{FDR\text{-}adjusted} < 0.05$) [43].

Gene set enrichment analysis (GSEA) [46] was used to explore the results of the differential analyses. GSEA determines whether a predefined set of genes show statistically significant, consistent differences between two biological states by calculation of an enrichment score (ES) for each geneset. A 'normalized' ES (NES) is calculated to account for differences in number of genes in each geneset. GSEA was carried out for a selection of genesets (n = 20) from the Hallmark human reference geneset collection (mSigDB, Broad Institute, version 7.5.1) that describe pathways that are regulated by TFA, including lipid and cholesterol metabolism, inflammation, apoptosis, autophagy, coagulation, adipose tissue regulation and oxidative stress [20]. Significance of enrichments were confirmed by comparing the ES to those obtained by random permutations (n = 1000 iterations) of the ranked gene with FDR correction for multiple testing (significance where $P_{FDR\text{-}adjusted} < 0.05$) [43].

Untargeted metabolomics analysis

The same statistical workflow as described above for the GC-FID lipid analysis was applied for the untargeted LC-MS metabolomics dataset to identify molecular biomarkers that were differentially modulated by the interventions, (comparing delta change between baseline and post-treatment).For the untargeted GC-MS dataset, a two-step statistical analysis was applied to select putative compounds that could discriminate the different effects of the interventions (delta change between baseline and post-treatment) for the reintegration step of data processing. In the first step, univariate analyses were applied to identify metabolites showing a different response between the dietary treatments using the Kruskal-

Wallis test ($P < 0.05$). This was complemented with the identification of further discriminating metabolites from orthogonal partial least squares - discriminant analysis (OPLS-DA) models using the Ropls package (v1.4.4, 2016) [41] to compare the effects of each pair of treatments (for valid models, the top 100 metabolites were selected for reintegration). After reintegration, the second step of analysis applied a Wilcoxon signed-rank test to compare the responses of the reintegrated metabolites to the treatment in pairwise assessments ($P_{FDR\text{-}adjusted} < 0.05$).

Correlation analyses

Correlation analyses were conducted to assess the relationships between biomarkers measured previously [27], including blood biochemistry (glycemia, insulinemia, blood lipids) and biomarkers of inflammation and endothelium function and the FAs measured by targeted GC-FID analysis. Spearman's correlation test was used to associate the biomarker and FA changes in response to the dietary interventions (delta change between baseline and post-treatment) with visualization by corrplot (v0.84) [47] with significance where $P_{FDR\text{-}adjusted} < 0.05$ [43]. This analysis was restricted to the fourteen clinical parameters that showed no significant difference between groups at baseline. The effect of the different dietary intervention on these associations was considered by repeating the analysis with Spearman's partial correlation test to control for the effect of diet (ppcor Package, v 1.1) [48].

Results

Subject characteristics and clinical chemistry

The 42 participants selected for the nutrigenomic analyses showed similar baseline clinical characteristics to the main cohort [27] (Table 1). There were significant differences between the treatment groups for baseline levels of insulin, interleukin-6 (IL-6), tumor necrosis factor (TNF) receptors (1 and 2) and endothelial leucocyte adhesion molecule (ELAM-1). Therefore, these markers were not considered further.

Altered lipid profiles in serum reflect dietary fat composition

The total fat content of the three test products were very similar but differences were present for specific FAs (Table 2). By design, the butter contained comparable levels of total TFA to the margarine with iTFA, and it was confirmed that the margarine without TFA (wTFA) contained only traces of TFA. However, the relative composition of the most abundant TFAs differed between the iTFA margarine and the butter; the TFA in the iTFA margarine contained both elaidic- (1.8 %) and vaccenic acid (1.4 %), whereas the TFA in rTFA in butter was mainly vaccenic acid (3.5 %). In addition, the butter contained higher levels of SFA (in particular palmitic acid, C16:0 and stearic acid, C18:0) than the margarines despite lower levels of lauric

acid (C12:0). Total MUFA and total PUFAs were present at higher levels in both margarines than the butter due to higher levels of 9-oleic acid and linoleic acid, respectively.

Out of 85 features (i.e. 66 FA and 19 sum parameters), a total of 18 FA (four long-chain SFA, three branched-chain FA, three PUFAs and all TFAs) as well as six sum parameters showed significantly different responses for at least one intervention ($P_{FDR\text{-}adjusted} < 0.05$) (Table 3). A heatmap of the 24 FA's (inclusive of six sum parameters) that showed significantly different responses to the treatment is presented in Figure 1, showing clustering of the 42 subjects into the three treatment groups (iTFA, wTFA and rTFA) with six exceptions.

Elaidic acid (C18:1 t10-11) and vaccenic acid (C18:1t12) were the major TFA isomers found in the test products. The quantified levels of these two lipids in the test products are shown in Figure 2a, together with their relative changes in serum after the dietary interventions (Figure 2b). Whereas serum levels of elaidic and vaccenic acid were both increased in the iTFA group (elaidic acid being significantly higher than vaccenic acid), only vaccenic acid was increased in the rTFA group. Neither TFA was significantly modified in the wTFA group.

Associations between circulating lipids and clinical markers

An overview of the significant correlations found between changes in plasma free FAs and clinical biomarkers after the dietary interventions is presented in Figure 3. The strongest correlations were observed between FAs. Most of the significant associations were between lipids or between the clinical biomarkers as these two categories of parameters were ranked separately with the execution of C12:0. Also, the FAs had stronger correlations coefficients than the clinical biomarkers. Finally, most of the correlations were positive, in particular for the FAs. Nevertheless, some significant associations between targeted FAs and clinical biomarkers were identified, as detailed in Supplement Table 1. Multiple positive correlations were observed for the FAs and LDL-cholesterol including dairy-associated biomarkers, pentadecanoic acid (C15:0) and heptadecanoic (margaric) acid (C17:0), several PUFAs, and individual TFAs, vaccenic acid (C18:1t10-11) and 9-*trans*, 12-*cis*-octadecadienoic acid (C18:2t9+c12). All of these correlations remained significant after adjusting for the treatment effect. Several FAs that were associated with LDL-cholesterol were also positively associated with changes in total cholesterol. Conversely, HDL-cholesterol was not significantly associated with any lipids. Some FAs were positively associated with blood glycemia, hsCRP, intercellular adhesion molecule and lipoprotein a, but these associations were generally not significant after controlling for the effect of treatment with the exception of heptadecanoic (margaric) acid (C17:0) that was inversely associated with inter cellular adhesion molecule ICAM. No significant associations were observed between the selected lipids and triglycerides, apolipoprotein B, endothelin, TNF-a, and vascular cellular adhesion molecule (VCAM). In addition, no significant associations with clinical biomarkers were observed for the dominant TFA species in iTFA, elaidic acid (C18:1t6-9).

Limited effects of dietary fats on gene expression in blood

Overall, 496 genes were differentially expressed between baseline (week 2) and endpoint (week 6) in at least one of the three intervention groups ($P < 0.05$) but the responses were not statistically significant nor did they differ significantly between the intervention groups ($P_{FDR\text{-}adjusted} > 0.05$). GSEA pathway enrichment did not reveal any pathway that was significantly differentially enriched whether for individual treatments or when comparing treatments.

Biomarkers of butter revealed in untargeted metabolomics analyses of serum

LC-MS untargeted metabolomics

A total of 185 features measured in blood by LC-MS showed significantly different responses to the three interventions ($P_{FDR\text{-}adjusted} < 0.05$). These metabolites are presented in a heatmap (Figure 4) which shows almost exclusively positive values, i.e. increased concentrations at the end of the 4-week intervention, as well as a separation of the butter group from the margarine groups while the margarine groups (wTFA and iTFA) were not differentiated. Of the 185 significant features, two were identified at level 1 and two tentatively identified (level: 2-3) (Figure 5, Supplement Table S2). Of the identified metabolites (Figure 6), three showed a relative decrease in the rTFA group, relative to the wTFA and/or iTFA groups, including the fat-soluble vitamin, retinol. An inspection of the levels of these metabolites at the end of the run-in phase showed they were present at similar levels between the three treatment groups while after the butter treatment the levels of retinol and prostaglandins decreased (Supplement Figure S1). 4-isopropylbenzoic acid showed an increase after the rTFA intervention relative to the wTFA and iTFA groups.

GC-MS untargeted metabolomics

The GC-MS analysis revealed three identified metabolites that showed significantly different responses to the treatments after manual integration of the signals ($P_{FDR\text{-}adjusted} < 0.05$) (Figure 6). Phytanic acid was clearly increased in butter treatment (P=<0.001) compared to wTFA and iTFA. An isomer of octadecadienonic acid (C18:2), was also significantly increased in the rTFA group, though it was present both in butter and margarine (P=0.007). This molecule was classified at the level 3 of identification because of its unknown isomeric structure. Finally, glycolic acid, an a-hydroxy acid, was significantly decreased in rTFA (P=0.037), relative to the wTFA group.

Discussion

The present study investigated the molecular and metabolic impact of four weeks butter compared to margarine intake (with and without TFA) on fasting blood using a targeted analysis of free FA (GC-FID), transcriptomic analysis of whole blood gene expression and two untargeted metabolomic approaches (LC-MS and GC-MS).

Lipid composition of dietary interventions is reflected in serum of healthy adults

The profiling of the lipids present in the intervention diets in serum before and after dietary interventions revealed a distinct lipid signature of the intervention diets in serum. For those lipids that responded differently to the interventions, remarkable consensus could be observed between the relative change in serum and the different quantity of lipids in the products. Indeed, with the exception of total TFA that included CLA, the relative changes in serum lipids directly reflected the differences in the product composition, even for those lipids that were present in relatively low levels such as the PUFAs. We are able to demonstrate this close relationship between composition of TFA in different dietary fats and circulating levels of TFA after exposure to diets with differing but still relatively low TFA content.

Among the SFA that discriminated between rTFA and iTFA diets, pentadecanoic acid (C15:0) and margaric acid (C17:0) were both specifically increased after rTFA, confirming their previously described role as dietary biomarkers of dairy intake [49, 50]. Several other odd-chain methylated FA were also raised specifically after dairy intake including methyl-palmitate (15-methyl C17:0), which has previously been associated with butter intake [51].

The PUFAs arachidonic acid, EPA and DPA were significantly increased after the rTFA diet, despite their presence at very low levels in butter (albeit higher than the margarines). Linoleic acid, a dietary precursor of eicosanoids, was actually more abundant in the margarine diets although no significant differences in this lipid was observed in serum. The serum differences in eicosanoids therefore do not seem to be explained by the dietary composition of lipids but may rather reflect differences in metabolism of linolenic acid and linoleic acids. Indeed, there is some evidence to suggest that certain TFA can disrupt the metabolism of essential PUFAs [52]. A change in the metabolism of PUFAs could not be confirmed in our transcriptomic analysis of blood cells. As the synthesis of PUFAs is localised in the liver, blood cell transcription might not have captured such tissue-specific regulation, particularly in the fasting state. Another limitation in using fasting samples is that changes in FA that are rapidly metabolised may not be visible. Indeed, the absence of diet-specific differences for some short-chain SFA and MUFAs despite differences in their presence in the composition of the diets may be attributable to rapid metabolism of these fats.

Different TFAs in serum distinguish diets rich in rTFA compared to iTFA

Both diets containing TFA (iTFA and rTFA) induced similar increases in total TFA in serum that were significantly higher than the control diet without TFA (wTFA). Conversely, several TFAs showed high specificity to the source of TFA both in the product content and the serum after exposure to the diet. As expected, CLA was specifically associated with the rTFA diet due to the well-known production of CLA from linoleic acid by rumen bacteria [53]. Interestingly, diet-related changes in TFAs were found for FAs present in relatively low quantities in the diet such as C16:1t and C18:2t isomers. The most abundant TFAs in the rTFA and iTFA diets, respectively vaccenic and elaidic acid, were both specifically elevated in serum after the corresponding diet. Elaidic acid has already been identified as a putative dietary intake biomarker of margarine and hardened vegetable fats, as like other TFAs it cannot be synthesized by the body [54]. Conversely, the major isomer of rTFA, *trans*-11-vaccenic acid [55], is also present in dietary sources of iTFA, though at lower levels, as reflected by lower but significant increases in serum levels for the iTFA group.

Elaidic acid and vaccenic acid: associations to cholesterol metabolism

In our study we also explored the relationship between circulating lipids following the intervention diet and various biomarkers of molecular pathways that can be influenced by TFAs, including inflammation, lipid/cholesterol metabolism and coagulation. Our correlation data confirmed a positive association between various TFAs that were specifically elevated after the rTFA intervention (including vaccenic acid) and the small but significant increase in LDL-cholesterol after rTFA, which was described previously for the full dataset [27]. It is however important to note the similar associations observed for other FAs that were specifically elevated after rTFA including the dairy biomarkers C15:0 and C17:0. Thus the correlations might be interpreted as a collective effect of the dairy matrix on the LDL-cholesterol rather than a specific TFA-modulation of cholesterol [56]. No associations were found between elaidic acid and the circulating biomarkers of inflammation and lipid metabolism. While serum elaidic acid has been associated with CVD outcomes Oshita *et al.* [57], the iTFA contained relatively low levels of TFA and serum levels of the FA in this short-term intervention remained consistently low, which could explain the lack of effect of the intervention on surrogate markers of cholesterol metabolism.

Transcriptomics and metabolomics approaches are methods sensitive to detect early biological responses and were selected for this study to help assess the effects of the relatively low levels of trans fats in the intervention diets. However, our transcriptomics data showed limited associations with the dietary interventions, which concurred with the previously reported absence of effects of the high intake of iTFA and rTFA for three weeks on the plasma proteome of healthy men [58]. Although blood transcriptomics can reflect systemic transcription changes in response to dietary interventions with fats, in particular under postprandial conditions [59], in this healthy population and the relatively low level of TFA used in the diets, it was unfortunately not possible to confirm a distinct effect of the diets on the fasting transcriptome. Furthermore, only one type of metabolite was shown to distinguish between the three diets, a prostaglandin (A2/B2/J2), which was decreased after both TFA-containing diets but to a

greater extent after rTFA, while prostaglandin D3 was only decreased after butter. Prostaglandins are regulatory compounds derived from dietary fatty acids, in particular arachidonic acid, that play important roles in many physiologic processes on many human organ systems, including inflammation [60]. Interestingly elaidic acid and vaccenic acid differently impact prostaglandins production in endothelial cells [61]. Although the prostaglandins tentatively identified in this report have hardly been reported in humans, our results suggest that plasma prostaglandins might be sensitive markers to assess the impact of dietary fats on inflammation in humans. However in the absence of responses in other metabolites or inflammatory biomarkers, this result should be interpreted with caution.

In previous studies, TFA intake has been associated with inflammatory markers CRP and IL6, particularly in populations predisposed to metabolic illness [62, 63]. In our study with healthy subjects, no correlation between elaidic acid or vaccenic acid and the two inflammation markers were found. Similarly, Motard-Belanger *et al.* found no increase of CRP at the end of four weeks of rTFA or iTFA in a nutritional intervention, compared to the control [16]. It is noteworthy that both diets comprise relatively low but comparable levels of TFA (2% total energy intake), a little over the WHO public health guidelines which could explain the limited clinical effects of the diet previously reported by Radtke *et al.* [27].

Biomarkers of dietary intake

The majority of metabolites that responded differently to the dietary interventions differentiated the butter effect from the similar effects of the two margarines. Several of the identified metabolites were increased in the rTFA group and might be considered as biomarkers of ruminant animal fat intake. These included phenol lipid 4-isopropyl-benzoic acid and fatty acids, octadecadienoic acid and phytanic acid. The branched-chain fatty acid phytanic acid is a degradation product from chlorophyll that is found in ruminant animal fat and has been proposed a putative biomarker of dairy fat [49, 64]. Some health benefits from phytanic acid intake have been described [65], such as prevention of metabolic syndrome or type 2 diabetes although dietary reduction of phytanic acid was also recommended for the management of infantile Refsum disease, one of the less severe of Zellweger spectrum disorders [66]. 4-isopropyl-benzoic acid (cuminic acid) is present in the seeds of *Cuminum cyminum* (cumin) (https://foodb.ca/compounds/FDB013929). Although its presence in foods, in particular dairy products, has not been reported it is interesting to note that *Cuminum cyminum* is used as a feed additive in livestock. The untargeted GC-MS methodology used to characterize octadecadienoic acid is not specific enough to differentiate the various C18:2 molecules that can possibly be present. The reader is thus referred to the GC-FID results for further details.

Dietary biomarkers of margarine could also be inferred by considering metabolites showing a relative reduction in the rTFA group relative to the baseline, which was the end of the run-in with the wTFA margarine. These included retinol which although present in butter is supplemented, often at high levels, in margarines. Of note, dihydrophylloquinone, a hydrogenated form of vitamin K1 not naturally present in

vegetable oils, has previously been reported as a candidate biomarker of TFA-intake from partially hydrogenated fat [67] but was not found with our untargeted LC-MS method.

Strengths and limitations of study

A strength of this study was the use of different techniques to investigate the molecular effects of the dietary interventions under study. In particular, by using a targeted lipid panel, we were able to discriminate between the circulating changes in specific FA and TFAs that were present in the dietary sources of TFA. This is important in the context of the distinct biological roles of these lipids. Using different metabolomics approaches in this study, we were able to consider the wider impact of the diets on metabolites other than lipids but, although the clustering of LC-MS data was more efficient than the targeted FA analytics to separate the intake of butter from the intake of margarine, we were limited by the challenge of identifying discriminating metabolites. Finally, our study was limited by the restricted number of samples evaluated for whole blood transcriptomics which did not capture the expected changes in lipid metabolism following the change in dietary fats.

Conclusion

The application of a combination of targeted and untargeted metabolomics to serum samples of humans supported the identification of distinct chemical signatures associated with the different dietary fats. We confirm that the different types and abundance of TFA isomers in rTFA and iTFA diets results in distinct changes in serum TFA after 4 weeks of these diets. In particular, elaidic and vaccenic acids are differentially modulated by the TFA diets in concordance with their relative presence in the dietary fats. The associations of the different TFAs with clinical biomarkers did not seem specific to the TFAs but was rather related to the overall lipid composition of the dietary fats. Beyond the lipid analysis, we did not find clear biomarkers that distinguished the rTFA from iTFA perhaps reflecting the relatively low dose of TFA used that did not result in clinically different phenotypes in the larger cohort of this study [27]. However, we did identify biomarkers that distinguished butter from the margarines including some putative exogenous markers, suggesting that this approach is useful for discriminating metabolic effects of similar foods.

Abbreviations

CLA: conjugated linoleic acid; CRP: C-reactive protein; GC: gas-chromatography; GC-FID: GC with flame ionization detector; GSEA: geneset enrichment analyses; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high-sensitivity C-reactive protein; ICAM: intercellular adhesion molecule; iTFA: industrial trans fatty acids; IL-6: interleukin-6; LC: liquid-chromatography; LDL-C: low-density lipoprotein cholesterol MS: mass spectrometry; MUFA: monounsaturated fatty acids; OPLS-DA: orthogonal partial least squares – discriminant analysis; PUFA: polyunsaturated fatty acids; rTFA: ruminant trans fatty acids; SFA: saturated

fatty acids; TFA: trans fatty acids; TNF: tumor necrosis factor; VCAM: vascular cellular adhesion molecule; wTFA: without trans fatty acids

Declarations

Acknowledgements

The authors thank Monika Spahni for measuring the fatty acids in blood.

Authors' contributions

K.J.B.P., G.V. and B.W. designed the omics profiling of the study; T.R., H.S. and A.S. recruited and conducted the clinical trial on which the omics profiling is based; D.S., K.J.B.P., R.B., C.B., R.P., N.Z. and U.B. analysed the data; D.G. and K.J.B.P. prepared the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by Agroscope.

Availability of data and materials

The transcriptome and metabolome datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the cantonal ethical committee of Bern, Switzerland (KEK 124/09) and the study was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent for participation in the study. The study was registered at www.clinicaltrials.gov (NCT00933322).

Consent for publication

All authors consent to publish the present results.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1

Baseline characteristics of subjects. Assessment after the run-in phase (week 2).

	wTFA group	iTFA group	rTFA group	P
	(*n* = 14)	(*n* = 14)	(*n* = 14)	
Age (years)	56.5 (52.3, 58.8)	52.0 (46.8, 58)	51.0 (47, 56.5)	0.373
Sex (% male)	50	50	50	
BMI (kg/m^2)	25.20 (21.0, 26.8)	24.60 (22.5, 28.2)	25.35 (23.8, 27.4)	0.778
Glucose (mmol/L)	4.68 (4.5, 5.0)	5.10 (4.7, 5.4)	4.73 (4.4, 5.0)	0.260
Insulin (mU/L)	5.00 (3.3, 6.3)	5.75 (3.5, 7.9)	3.65 (1.9, 4.4)	0.040*
Total cholesterol (mmol/L)	5.035 (4.6, 5.3)	4.895 (4.0, 5.6)	4.465 (4.3, 5.0)	0.338
HDL-C (mmol/L)	1.49 (1.3, 1.8)	1.36 (1.1, 1.5)	1.33 (1.2, 1.7)	0.401
LDL-C (mmol/L)	3.22 (2.8, 3.7)	3.06 (2.6, 3.8)	2.67 (2.5, 3.3)	0.193
Ox-LDL Ab (mU/ml)	254 (162, 476)	483 (347, 1394)	811 (411, 1167)	0.092
Triglycerides (mmol/L)	0.87 (0.75, 1.0)	0.89 (0.62, 1.4)	0.66 (0.58, 0.92)	0.314
Lp-a (mg/L)	0.13 (0.11, 0.35)	0.20 (0.08, 0.47)	0.12 (0.04, 0.8)	0.933
Apo A1 (g/L)	2.61 (1.3, 3.4)	2.05 (1.5, 2.4)	2.14 (1.7, 2.8)	0.457
Apo B (g/L)	0.87 (0.29, 1.23)	0.78 (0.41, 1.02)	1.05 (0.86, 1.39)	0.156
hs-CRP (mg/L)	0.6 (0.24, 1.49)	0.96 (0.7, 1.98)	0.82 (0.5, 2.09)	0.371
IL-6 (pg/ml)	0.74 (0.65, 0.98)	0.7 (0.6, 0.96)	1.09 (0.97, 1.38)	0.027*
TNFa (pg/ml)	2.40 (2, 5)	3.37 (1, 5.2)	3.81 (1.8, 7.2)	0.701
TNF-Receptor 1 (pg/ml)	798 (705, 974)	995 (941, 1103)	1197 (997, 1395)	0.007**
TNF-Receptor 2 (pg/ml)	2636 (2290, 4089)	2664 (2457, 3406)	1748 (1461, 2098)	0.033*
ELAM-1 (ng/ml)	2.5 (1.6, 3.4)	2.6 (1.2, 4.8)	5.5 (3.9, 8.2)	0.007**
Endothelin (pg/ml)	1.9 (1.6, 2.7)	2 (1.7, 2.3)	1.7 (1.3)	0.638
ICAM (ng/ml)	17.5 (10, 32.4)	19.1 (10.9, 32.2)	25.8 (20.7, 29.7)	0.274
VCAM (ng/ml)	212 (124, 320)	150 (113, 241)	286 (183, 415)	0.099

Legend: Data are presented as medians and IQ1, IQ3 between brackets. Kruskall-Wallis test was used to determine significant difference between subjects in the three groups. P < 0.05: *, P < 0.01: **, P < 0.001: ***. Abbreviations: Apo A1, apolipoprotein A1; Apo B, apolipoprotein B; hs-CRP, high-sensitivity C reactive protein; ELAM-1, endothelial leucocyte adhesion molecule; HDL-C, high-density lipoprotein cholesterol; ICAM, intercellular adhesion molecule; iTFA, diet enriched with industrial trans fatty acids; IL-6, interleukin-

6; LDL-C, low-density lipoprotein cholesterol; Lp-a, lipoprotein a; ox-LDL Ab, antibodies of oxidized low-density lipoprotein; rTFA, diet enriched with ruminant trans fatty acids; TNF, tumor necrosis factor; VCAM, vascular cellular adhesion molecule; wTFA: diet without trans fatty acids.

Table 2

Composition of the most important fatty acids of the butter and the two margarines with and without TFA (g 100 g^{-1} product), used in this study.

Experimental fat (g/100g product)	Margarine without TFA (wTFA)	Margarine with TFA (iTFA)	Butter (rTFA)
Total fat content	83.4	84.6	85.1
SFA			
C4:0 (butyric acid)	-	-	2.6
C6:0 (caproic acid)	0.2	0.2	1.5
C8:0 (caprylic acid)	2.1	2.1	0.8
C10:0 (capric acid)	1.5	1.6	1.7
C12:0 (lauric acid)	11.4	11.9	1.9
C14:0 (myristic acid)	4.8	4.9	7.3
C15:0 (pentadecanoic acid)	-	-	1
C16:0 (palmitic acid)	18.1	16.2	19.6
C17:0 (heptadecanoic/margaric acid)	-	-	0.4
C18:0 (stearic acid)	3.4	4.2	8.5
C20:0 (arachidonic acid)	0.2	0.2	0.1
C 22:0 (behenic acid)	-	-	0.1
13-Methyl C15:0 iso (13-methylmyristic acid)	-	-	0.3
12-Methyl C15:0 aiso (12-methylmyristic acid)	-	-	0.5
14-Methyl C16:0 iso (14-methylpentadecanoic acid)	-	-	0.3
15-Methyl C17:0 iso (15-methylpalmitic acid)	-	-	0.3
14-Methyl C17:0 aiso (14-methylpalmitic acid)	-	-	0.3
16-Methyl C18:0 iso (16-methylmargaric acid)	-	-	0.1
Total SFA	41.7	41.5	47
MUFA *cis*			
C14:1c (myristoleic acid)	-	-	0.6
C16:1c9 (ω7) (palmitoleic acid)	0.1	0.1	1.1
C18:1c9 (ω9) (9-oleic acid)	24.4	23.9	15.2

C18:1c11 (ω7) (cis vaccenic acid)	0.8	0.9	0.7
C18:1c12 (ω6) (12-oleic acid)	-	0.2	0.2
C18:1c13 (ω5) (13-oleic acid)	-	0.1	0.1
C20:1c8+c9 (gadoleic acid)	-	-	0.1
C20:1c11 (ω9) (gondoic acid)	0.1	0.1	-
Total MUFA *cis*	25.4	25.3	18
PUFA *cis*			
C18:2c9c12 (linoleic acid)	5	4.6	1.2
C18:3c9c12c15 (linolenic acid)	1.1	1.2	1
C20:4c (arachidonic acid)	-	-	0.1
C20:5c (EPA)	-	-	0.1
C22:5c (DPA)	-	-	0.1
Total PUFA *cis*	6.1	5.8	2.5
TFA			
C16:1t9 (ω7) (palmitelaidic acid)	-	-	0.2
C18:1t6-9 (petroselaidic, elaidic acid)	0.1	1.8	0.2
C18:1t10-11 (trans vaccenic acid)	0.1	1.4	3.5
C18:1t12 (12-elaidic acid)	-	0.3	0.3
C18:1t13+c6+c7 (13-trans-octadecenoic acid)	-	0.4	0.5
C18:1u	-	0.1	0.3
C18:2u	0.1	0.1	0.2
C18:2t9c12 (9-trans, 12-cis-octadecadienoic acid)	0.1	-	0.5
C18:2c9t11 (ω7) (9-cis,11-*trans*-Octadecadienoic acid)	-	-	1.5
Total trans (without CLA)	0.4	4.1	6.3
Sum parameters (cumulative values)			
Total C18:1t	0.2	4	5
Total C18:2t (with CLA)	0.1	0.1	2.7
Total C18:2t (without CLA)	0.1	0.1	1.1

Total CLA	-	-	1.6
Total TFA (without CLA)	0.4	4.1	6.3
Total TFA (with CLA)	0.4	4.1	7.9
Total ω-3 FA	1.2	1.2	1.7
Total ω-6 FA	5	5.3	2

Legend: - = concentration < 0.01 g. Abbreviations: u, unknown; CLA, conjugated fatty acids; DPA, Clupanodonic acid; EPA, Timnodonic acid; iTFA, industrial *trans* fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; rTFA, ruminant *trans* fatty acids; SFA, saturated fatty acids; TFA, *trans* fatty acids; wTFA, without *trans* fatty acids.

Table 3

Delta change in serum levels of lipids [µg mL^{-1} serum] after 4 week treatments. Only lipids that change significantly differently between treatments are shown. The Spearman's correlation between median serum change in lipid per treatment group and content of lipids in the corresponding food products is represented by a letter code: +: weak positive (rho < 0.6); ++ moderate positive (rho 0.7-0.9); +++: strong positive (rho > 0.9). All correlations are positive.

Fatty Acids	Median wTFA	IQR wTFA (Q1, Q3)	Median iTFA	IQR iTFA (Q1, Q3)	Median rTFA	IQR rTFA (Q1, Q3)	$P_{FDR\text{-}adjusted}$ #	sp
[µg mL^{-1}]	(n=14)		(n=14)		(n=14)			
SFA								
C12:0	10[b]	-15, 45	0[b]	-10, 15	-60[a]	-70, -17.5	**	+
C15:0	0[a]	-10, 10	-5[a]	-17.5, 7.5	30[b]	20, 40	***	++
C17:0	5[a]	-10, 17.5	-5[a]	-17.5, 10	35[b]	30, 82.5	**	++
C18:0	30[a]	-368, 583	-125[a]	-320, 300	660[b]	190, 1783	*	+
12-Methyl C15:0 aiso	0[a]	10, 0	0[a]	-10, 0	10[b]	10, 20	**	++
14-Methyl C16:0 iso	0[a]	-7.5, 10	0[a]	0, 10	10[b]	10, 20	**	++
15-Methyl C17:0 iso	0[a]	-7.5, 10	5[a]	-10, 10	30[b]	2.5, 50	*	++
PUFA cis								
C20:4c	0[a]	0, 10	-5[a]	-10, 0	20[b]	10, 35	**	++
C20:5c	15[a]	0, 42.5	-10[a]	-5, 37.5	65[b]	30, 153	**	++
C22:5c	10[a]	-10, 27.5	-10[a]	-20, 15	60[b]	32.5, 128	***	++
TFA								
C16:1t9	0[a]	0, 10	0[a]	-10, 10	25[b]	10, 47.5	**	++
C18:1t6-9	10[a]	-17.5, 10	115[b]	92.5, 185	10[a]	-7.5, 20	**	+++
C18:1t10-11	0[a]	-10, 10	75[b]	27.5, 90	115[c]	95, 153	***	+++
C18:1t12	0[a]	0, 0	25[b]	12.5, 30	10[b]	0, 17.5	**	++
C18:1t13+c6+c7	0[a]	0, 10	30[b]	12.5, 38	20[b]	12.5, 30	**	+
C18:2u	0[a]	-10, 7.5	0[a]	-10, 0	10[b]	10, 17.5	**	++

C18:2t9+c12	0^{b}	0, 7.5	-5^{a}	-10, 0	10^{b}	0, 10	**	+++
C18:2c9+t11	0^{a}	-10, 7.5	0^{a}	-17.5, 7.5	80^{b}	52.5, 105	**	++
Sum parameters								
Total C18:1t	10^{a}	-7.5, 27.5	285^{b}	163, 335	155^{b}	103, 198	***	+
Total C18:2t (with CLA)	-5^{a}	-17.5, 0	0^{a}	-27.5, 10	80^{b}	40, 123	***	++
Total CLA	-10^{a}	-10, 10	0^{a}	-17.5, 10	75^{b}	45, 115	***	++
Total TFA (without CLA)	20^{a}	-7.5, 47.5	275^{b}	115, 328	165^{b}	125, 225	***	+
Total TFA (with CLA)	20^{a}	-10, 62.5	290^{b}	77.5, 338	225^{b}	172.5, 337.5	***	+
Total ω-3 FA	90^{ab}	-40, 228	-15^{a}	-200, 135	260^{b}	102.5, 908	*	++

Legend: #Kruskal wallis test results ($P_{FDR\text{-}adjusted}$ <0.05: *, $P_{FDR\text{-}adjusted}$ <0.01: **, $P_{FDR\text{-}adjusted}$ <0.001: ***). Different letters (a, b, c) denote significant differences between treatment based on paired Wilcoxon signed-rank test ($P_{FDR\text{-}adjusted}$ <0.05). Abbreviation: sp: Spearman correlation; IQR, interquartile range.

Figures

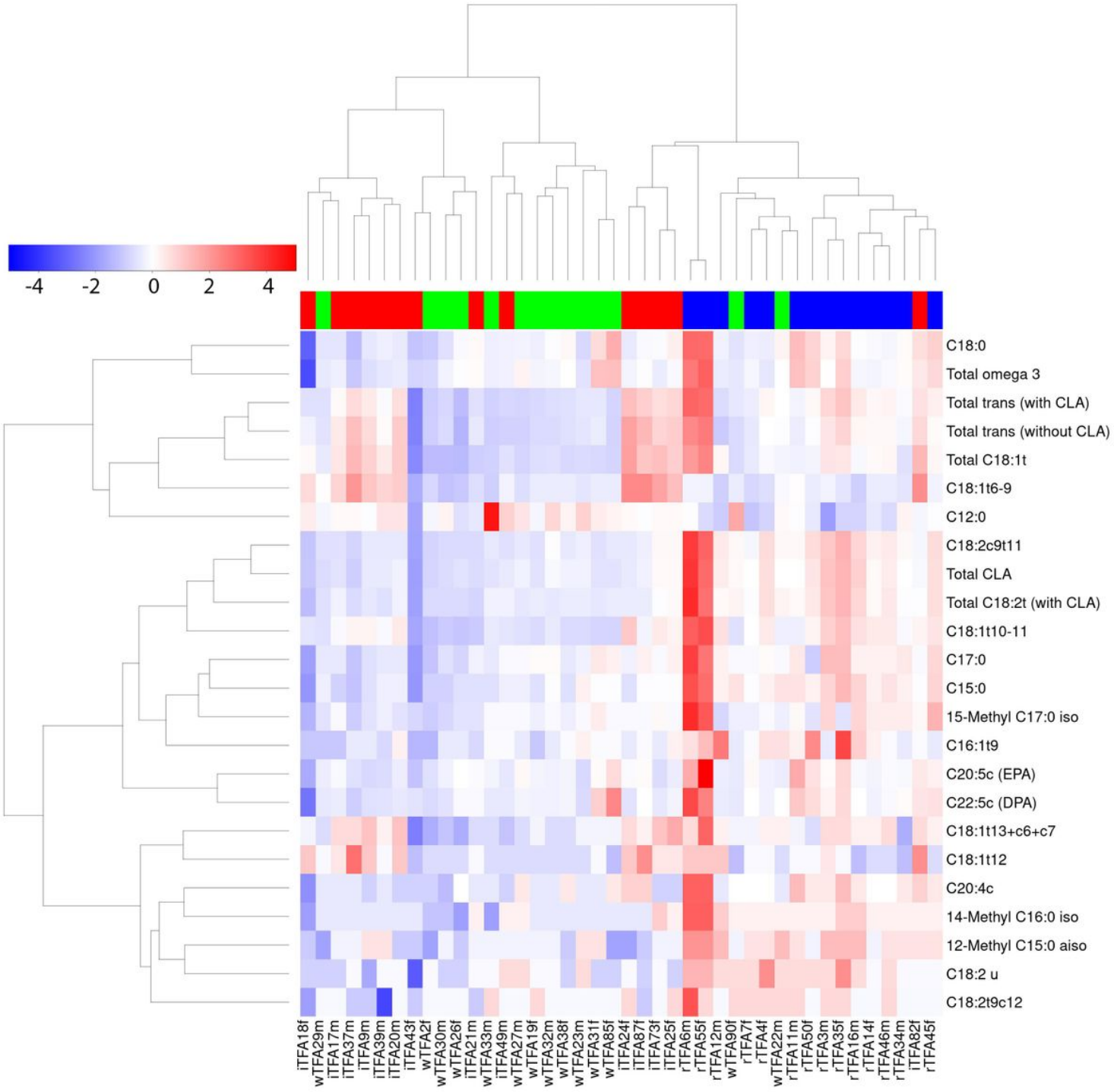

Figure 1

Heatmap of 24 lipid parameters (18 lipids and 6 sum parameters) measured by high-resolution GC-FID that were selected from 85 features (66 single lipids and 19 sum parameters) by a Kruskal-Wallis test ($P_{adjusted\ FDR} < 0.05$). The response of each subject (delta change between baseline and post-treatment) is denoted in columns with annotation for treatment groups, rTFA group (blue), margarine wTFA (green) and margarine with iTFA (red), study number and sex (m=male, f=female).

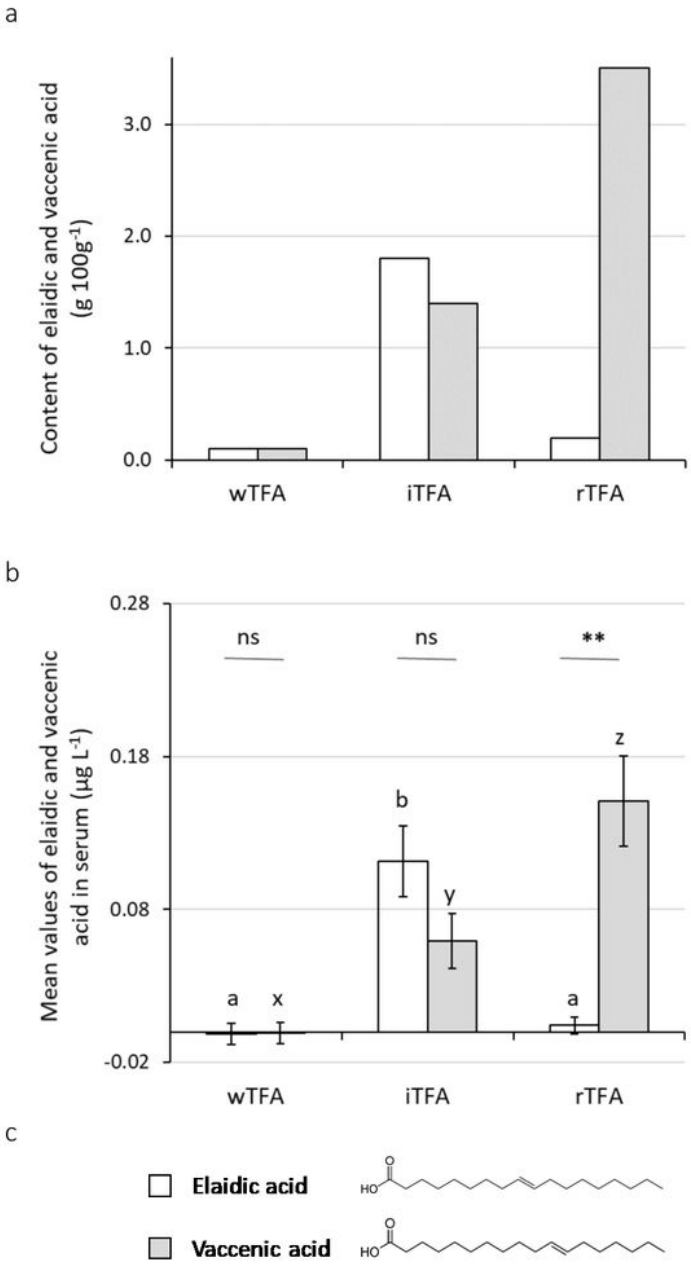

Figure 2

Amounts of elaidic and vaccenic acid (a) in the three different test products and (b) blood (delta change between baseline and post-treatment). Elaidic and vaccenic acids were measured in the serum of subjects after the treatment with butter (rTFA), margarine with TFA (iTFA), margarine without TFA (wTFA) (Mean and standard error of the mean) Different superscript letters for the same acid indicate significant

differences between the treatments (P < 0.05); ns, not significant; ** = P < 0.01. **c.** Chemical structure of elaidic and vaccenic acid.

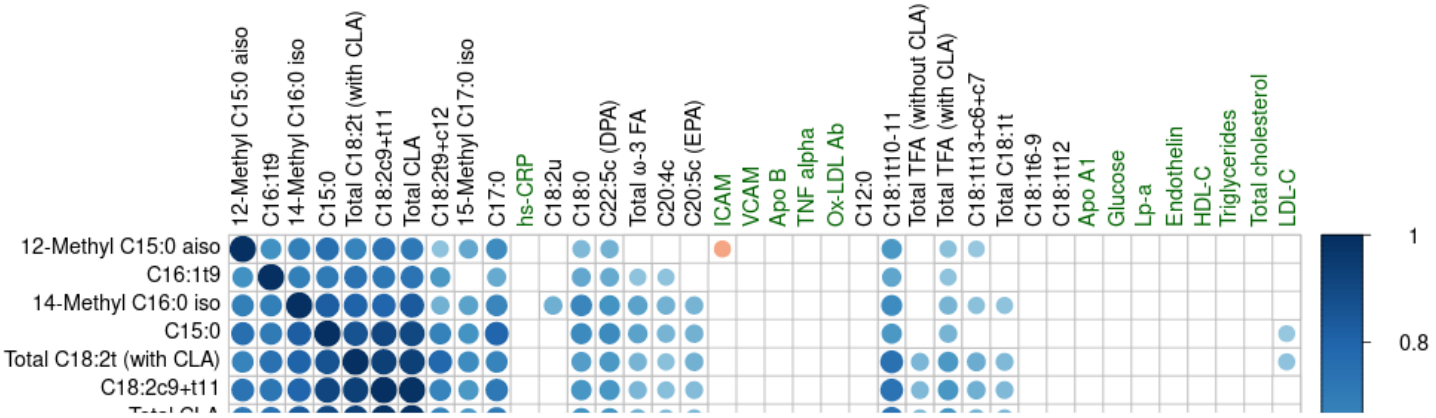

Figure 3

Spearman correlation analysis in the whole study-population identified some positive (blue) and negative (red) significant associations between blood lipids showing a significant response to the interventions (*n* = 24) and clinical parameters (*n* = 14) after the four week treatments (delta change between baseline and

post-treatment). Significance was considered where $P_{adjusted\ FDR} < 0.05$. Non-significant correlations are left blank. The clustering method was Ward D2. The size of the coloured symbols and the coloured scale to the right indicate the rho correlation value. Clinical parameters highlighted in green.

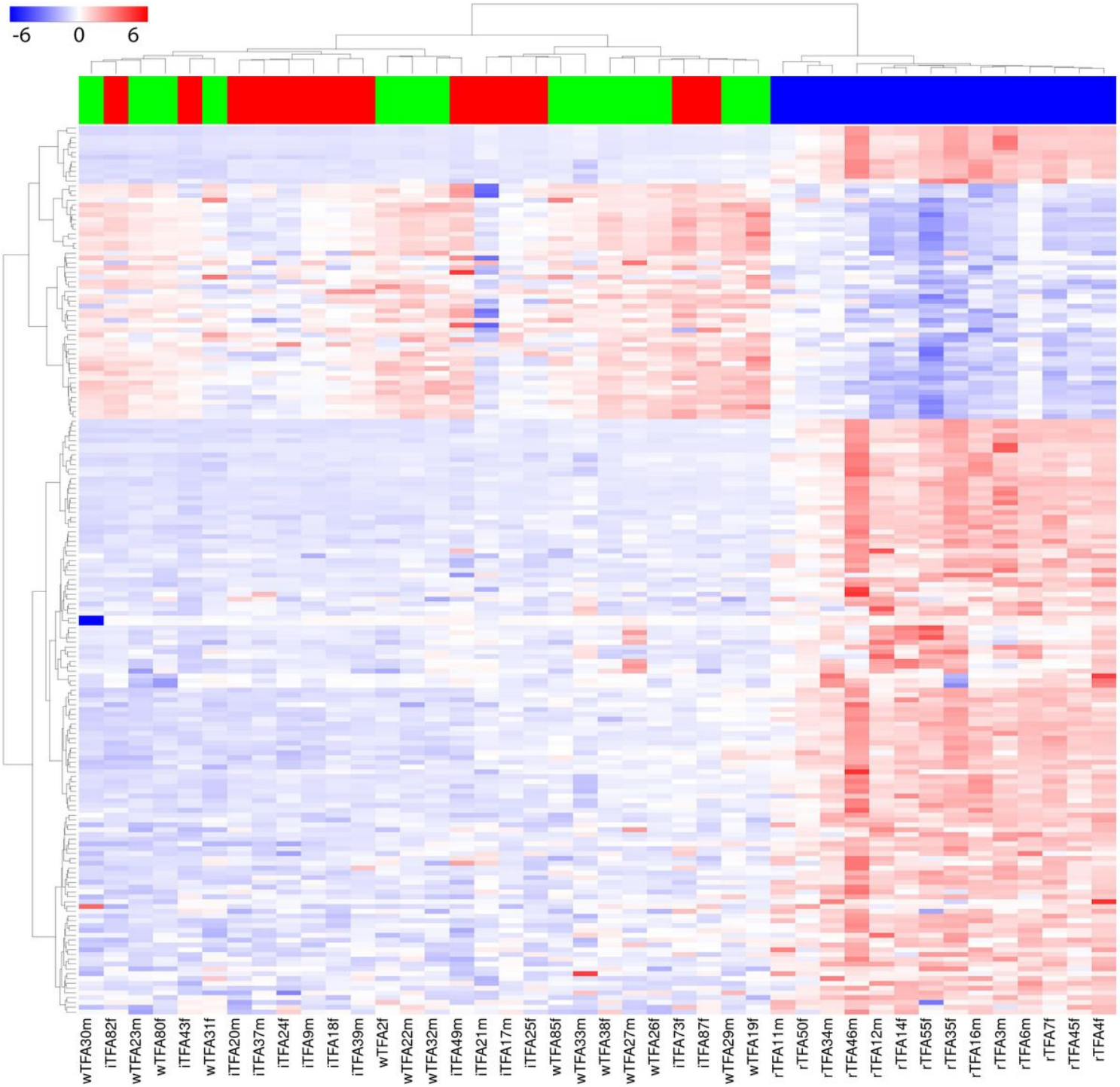

Figure 4

Heatmap of 185 LC-MS features (rows) that were selected from 11’616 (normalised) LC-MS features by a Kruskal-Wallis test ($P_{adjusted\ FDR} < 0.05$). The response of each subject (delta change between baseline

and post-treatment) is denoted in columns with annotation for treatment groups, rTFA group (blue), margarine wTFA (green) and margarine with iTFA (red), study number and sex.

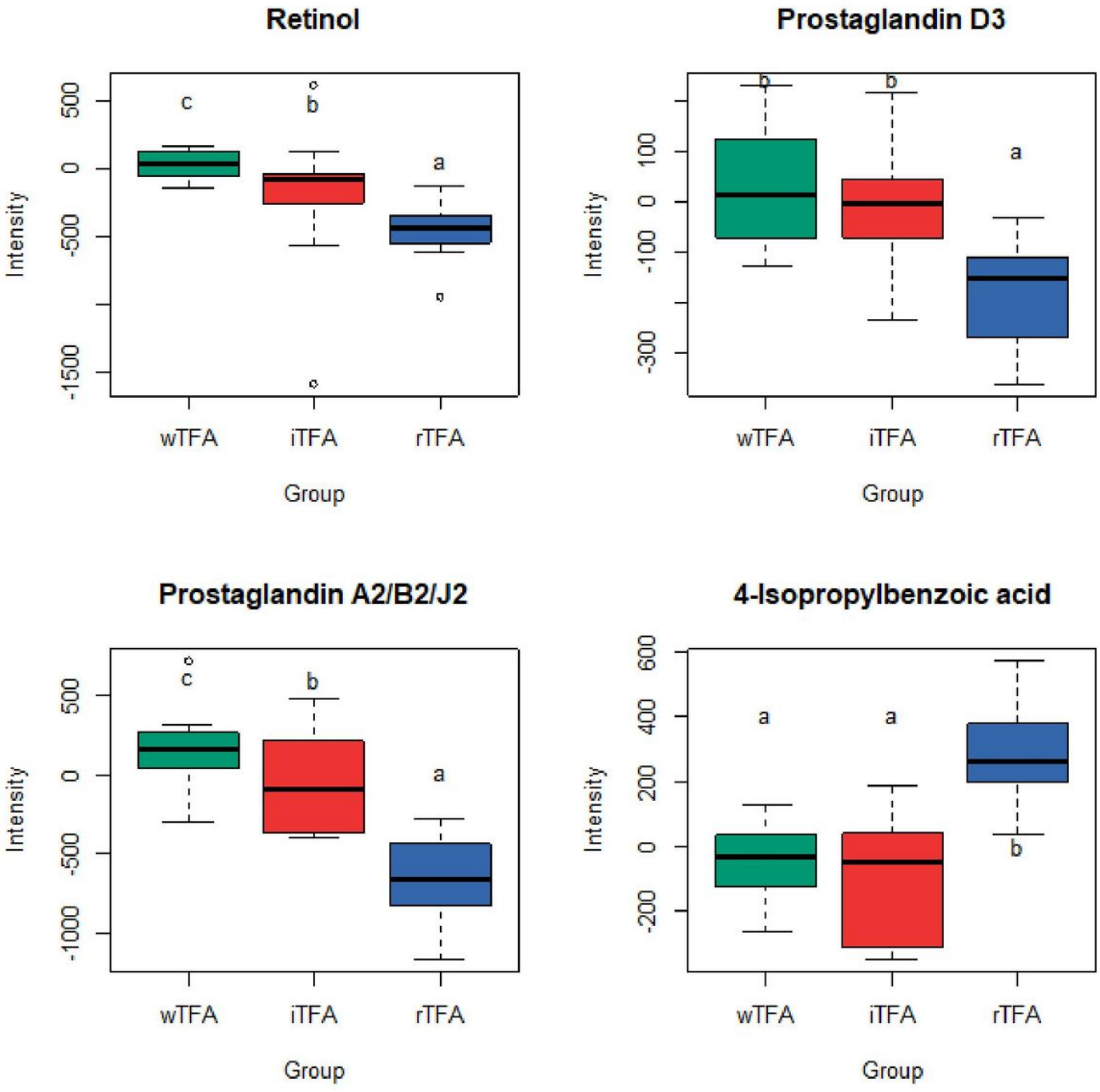

Figure 5

Boxplots of identified LC-MS metabolites that show a significant response (delta change between baseline and post-treatment) to the diets grouped by those that are either increased or decreased in the rTFA group. Different letters indicate significantly different values (paired Wilcoxon signed-rank Test,

$P_{FDR\text{-}adjusted} < 0.05$) paired. Plots show the IQR (box), the median dividing the IQR (—), with dashed line whiskers that extend to the last point included in the 1.5 x IQR range and outliers outside this range identified (o). One outlier in wTFA boxplot for 4-isopropylbenzoic acid was eliminated (baseline: 475.6, endpoint: 6464.7). This subject was however not removed from the robust statistical analysis.

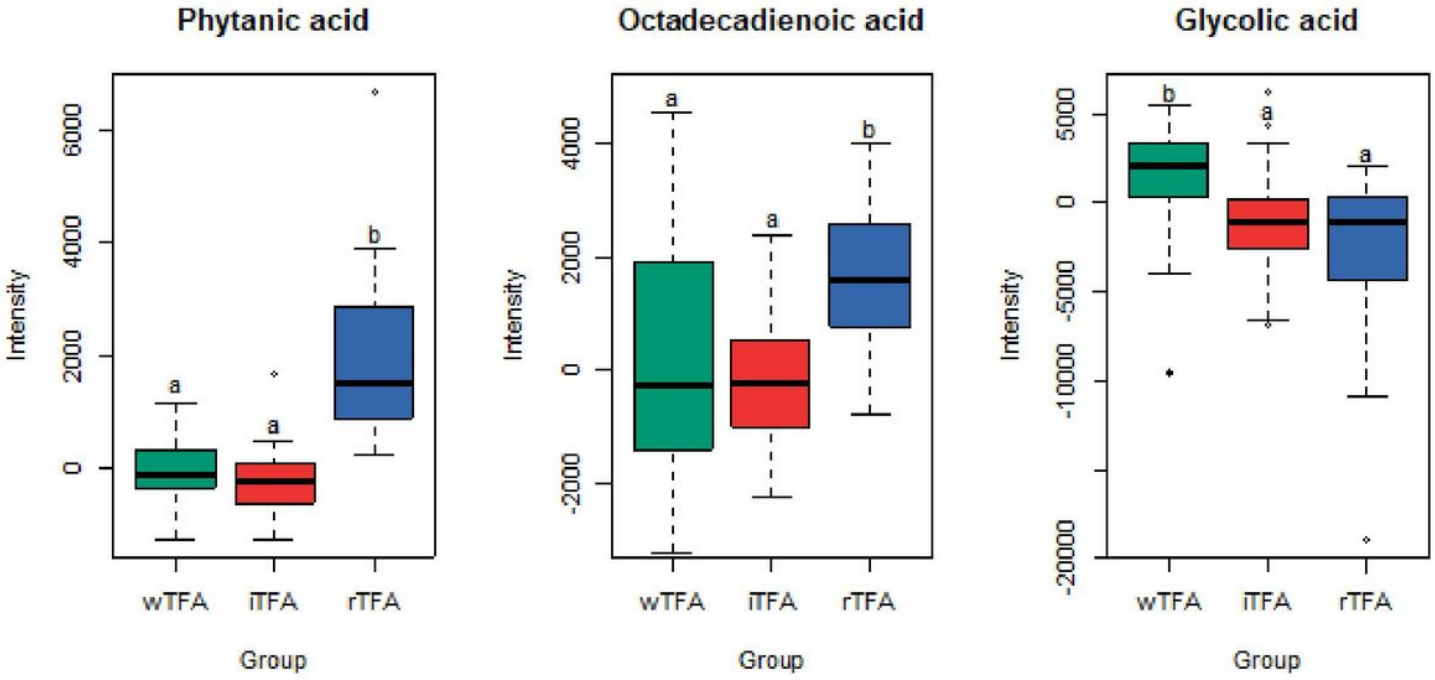

Figure 6

Boxplots of identified GC-MS metabolites that show different responses (delta change between baseline and post-treatment) to the diets. Different letters indicate significantly different values (paired Wilcoxon signed-rank Test, $P_{FDR\text{-}adjusted} < 0.05$) paired. Plots show the IQR (box), the median dividing the IQR (—), with dashed line whiskers that extend to the last point included in the 1.5 x IQR range and outliers outside this range identified (◊).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TFASupplementFile20220214.docx