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Prolonged monitoring of postprandial lipid metabolism after a western meal rich in linoleic acid (LA) and carbohydrates

Engy Shokry, Roxana Raab, Franca F. Kirchberg, Christian Hellmuth, Mario Klingler, Hans Demmelmaier, Berthold Koletzko*, Olaf Uhl

LMU - Ludwig-Maximilians-Universität München, Division of Metabolic and Nutritional Medicine, Dr. von Hauner Children’s Hospital, University of Munich Medical Centre, Munich, Germany

Co-authors’ email addresses:
Engy Shokry: Engy.Shokry@med.uni-muenchen.de
Roxana Raab: roxana.raab@tum.de
Franca F. Kirchberg: Franca.Kirchberg@med.uni-muenchen.de
Christian Hellmuth: Christian.Hellmuth@med.uni-muenchen.de
Mario Klingler: marioklingler@hotmail.com
Hans Demmelmaier: Hans.Demmelmaier@med.uni-muenchen.de
Berthold Koletzko: Berthold.Koletzko@med.uni-muenchen.de
Olaf Uhl: Olaf.Uhl@med.uni-muenchen.de

* Correspondence: Dr. Berthold Koletzko, Prof. Of Paediatrics, LMU - Ludwig-Maximilians-Universität München, Dr. von Hauner Children’s Hospital, LMU Medical Center, Campus Innenstadt, Lindwurmstr. 4, D-80337 Munich, Germany.
Tel: +49 89 44005 2826, Fax: +49 89 44005 7742, E-mail: Berthold.Koletzko@med.uni-muenchen.de; office.koletzko@med.lmu.de
Abstract: Today, increased awareness has been raised regarding high consumption of n-6 polyunsaturated fatty acids (n-6 PUFA) in western diets. A comprehensive analysis of total and individual postprandial fatty acid (FA) profiles would provide insights into metabolic turnover and related health effects. After an overnight fast, nine healthy adults consumed a mixed meal composed of 97 g carbohydrate and 45 g fat of which 26.4 g was linoleic acid (LA). Non-esterified fatty acids (NEFA), phospholipid fatty acids (PL-FA) and triacylglycerol fatty acids (TG-FA) were monitored in plasma samples, at baseline and hourly over 7 h postprandial. Total TG-FA concentration peaked at 2h postprandial followed by a constant decline. LA from TG18:2n-6 and behenic acid from TG22:0 showed the highest response among TG-FA, with a biphasic response detected for the former. PL-FA exhibited no change. Total NEFA initially decreased reaching nadir at 1h, then increased reaching its maximum value at 7h. The individual NEFA showed the same response curve except LA and some very long chain saturated fatty acids (VLCSFA, ≥ 20 carbon chain length) that markedly increased shortly after the meal intake. The similarities and dissimilarities in lipid profiles between study subjects at different time points were visualized using non-metric multidimensional scaling (NMDS). Overall, the results indicate that postprandial levels of LA and VLCSFA either as NEFA or TG were most affected by the test meal, which might provide an explanation for the health effects of this dietary lifestyle characterized by high intake of mixed meals rich in n-6 PUFA.

Key words: meal challenge, metabolism, linoleic acid, postprandial, very long-chain saturated fatty acids (VLCSFA), polyunsaturated fatty acids (PUFA).
Introduction

Fatty acid (FA) trafficking across the tissues is highly dependent on the nutritional state (Hodson and Fielding 2010). In the fasted state, activated lipolysis leads to a net flow of non-esterified FAs (NEFA) from the adipose tissue to peripheral tissues to serve as an energy source (Hodson and Fielding 2010). On the other hand, after a meal, especially following a high-fat meal, major changes occur in FA trafficking as a response to the large influx of meal fat. Dietary fat is mainly composed of triacylglycerols (TG), which after digestion and absorption appear in the circulation as chylomicron-TG (CM-TG) (Snook et al. 1996). The adipose tissue plays a key role in buffering postprandial flux of dietary fat into the circulation as it suppresses the release of NEFA into the circulation, increases plasma TG clearance, and increases fat reuptake (Frayn 2002). This clearance is largely mediated by the action of lipoprotein lipase (LPL), which hydrolyzes CM-TG, releasing FA which are subsequently taken-up primarily by the adipose tissue. Therefore, the net trafficking of FA in the postprandial state is directed towards storage rather than release. This is one of the metabolic pathways used to limit the increase in plasma lipids, thus protecting other tissues from exposure to excessive lipid levels (Hodson and Fielding 2010). The FA composition of the meal is an important factor affecting the absorption, transport, and uptake by adipose tissue of dietary fat, thus it can influence the duration, magnitude, and composition of postprandial lipemia, both qualitatively and quantitatively (Tumova et al. 2016). This is important to note, as sequential meal intake (< 5 h) is common among western communities, and means that most individuals spend most of the day in the postprandial state (Leech et al. 2015).

In the majority of the previously conducted postprandial studies, large, calorie dense and high-fat test meals (∼1300 kcal and 60% fat) were used for monitoring postprandial lipid
levels (Brandauer et al. 2013; Harrison et al. 2009; Katsanos et al. 2004; Koutsari et al. 2001a, 2001b; Peddie et al. 2012; Peluso et al. 2012; Schwander et al. 2014; Tsetsonis et al. 1997). The problem with large high-fat test meals is, that at very high doses of dietary lipids, there is no clear dose dependence of postprandial hypertriglyceridemia on the fat load. However, a dose-dependent increase has been reported for mixed meals (650-950 kcal) with moderate intake of fat (30-60g, 50-65% of total energy content) and a significant proportion of carbohydrates (50-100g, 25-40% of total energy content) to ensure the effective release of insulin (Zhang et al. 1998). These parameters were used to design the test diet in the current study. The composition of macronutrients in the meal used is typical for a meal consumed by many individuals in western communities on a daily basis, according to reports released by UN Food and Agriculture Organization (FAO) (Roser and Ritchie 2018). Specifically, the test diet was rich in linoleic acid (LA, NEFA18:2n-6), which is the most frequently consumed polyunsaturated fatty acid (PUFA) in the western diet and is found in virtually all commonly consumed foods. LA is an essential fatty acid (EFA), which cannot be synthesized de novo by humans. Therefore, it can be used as a reliable biomarker of dietary intake by tracking its movement within the endogenous plasma pool. According to the existing literature, adipose tissue and plasma PUFA, specifically LA and alpha-linolenic acids (ALA) were found to be the best indicators of dietary intake (Baylin et al. 2002; Hedrick et al. 2012). On these bases, the objective of the present work was to introduce a hypothesis-free explorative study addressing the question of real-life postprandial individual FA response following a typical western meal, containing moderately high fat (45g, ≈45% of total energy content) and energy contents (≈905kcal). The present study also monitored other
PUFA including arachidonic acid, the precursor of leukotrienes and prostaglandins which play a role in inflammation.

Materials and methods

Participants and sample collection

Briefly, nine adults (3 males and 6 females) initially participated in the study. The study participants did not suffer from any endocrine, gastroenterological or glucose metabolic disorders, renal or liver failure, and had not undergone any bariatric surgeries or procedures. Medical history of the study participants was obtained based on self-reported data before inclusion in the study. The study was approved by the Ethics Committee of the Medical Faculty, Ludwig-Maximilians-Universität München (LMU) (251-10) and written informed consent was obtained from all participants. Later, one female participant was excluded from further investigation because of missing blood sampling at three time points of the study. Six of the remaining 8 study participants were normal-weight (NW) (18.50≥BMI≤24.99) and 2 were overweight (OW) or preobese (25.00≥BMI≤29.99) (WHO 2000) with an overall mean body mass index (BMI; in kg/m²) of 21.0 ± 3.0. All participants showed normal baseline concentrations of HDL-cholesterol (HDL-c) with a median of 1.8 mol/m³. Three of the 8 participants showed borderline high baseline LDL-cholesterol (LDL-c) but normal LDL-c/HDL-c ratios with overall medians of 3.1 mol/m³ and 1.7, respectively. Only one participant (#1) presented borderline high values of TG, LDL-c and LDL-c/HDL-c ratios with values of 1.8 mol/m³, 3.5 mol/m³, and 3.9, respectively.

The subjects were asked to avoid alcohol consumption and strenuous physical activity for 24 h preceding the study, to minimize environmental influences on metabolism. Weight was assessed in a standardized manner wearing light clothes and no shoes. After an
overnight fast of 12 h, participants consumed a standardized breakfast meal consisting of a muffin, a chicken sandwich and a glass of orange juice. The energy content of the meal was 3785 KJ (≈905 kcal), provided as 26 g, 45 g, and 97 g of protein, fat, and carbohydrates, respectively. A detailed overview of the meal composition is given in Table 1. Most of the total fat content in the meal (42 g, 93.3%) was provided by sunflower oil. The total FA composition of sunflower oil was determined as described by Drzymała-Czyż et al. (2017) using a 25 μl oil sample and the results are presented in Table 2. Both the chicken sandwich and the muffin were rich in PUFA and MUFA, represented principally by LA and OA. The meal was consumed within 20 minutes. Fasting blood samples were collected right before the ingestion of the meal and blood samples were taken hourly over 7 h postprandial. Blood was collected via venipuncture from antecubital vein in 7.7 ml ethylenediamine tetraacetic acid (EDTA) monovettes (Sarstedt, Nümbrecht, Germany). Collected blood samples were immediately centrifuged (1000xg, 10 min, 4°C), or kept on ice and centrifuged within 2 h. Samples were stored at -80 °C until analysis.

**Non-esterified fatty acid (NEFA) analysis**

NEFA in plasma were analyzed by liquid chromatography (1200 Agilent, Santa Clara, USA), coupled to triple quadrupole mass spectrometry (4000 QTRAP, Sciex, Framingham, USA) as described previously (Hellmuth et al. 2012). In short, proteins were precipitated by adding isopropanol (200 μl) to 20 μl of serum in a 96-deep-well plate. After centrifugation, 10 μl of the supernatant were injected with an eluent flow rate of 700 μl/min. Gradient elution was performed with eluent A (5 mM ammonium acetate and 2.1 mM acetic acid) and eluent B (acetonitrile with 20% isopropanol) on Pursuit UPS Diphenyl column (1.9 μm, 100 x 3.0 mm; Varian, Darmstadt, Germany) at 40°C. All
samples were measured within one batch, and the quality of the measurements was assessed with quality control (QC) samples. Three QC samples were analyzed with the study samples. NEFA with a coefficient of variation (CV %) > 10% in the QC samples were excluded from analysis. Absolute baseline and postprandial concentrations were calculated in µmol/L then converted to mol/m³ (standard international (SI) unit).

**Fatty acid (FA) analysis of phospholipids (PL) and triacylglycerols (TG)**

PL and TG were extracted from plasma according to a modified Folch procedure with chloroform/methanol (2:1, v/v). Lipid fractions were separated by thin layer chromatography (TLC) and fatty acid methyl esters (FAME) were synthesized at room temperature by adding 50 µl of sodium methoxide (25% weight in methanol, Sigma Aldrich). The transesterification reaction was stopped after 3 minutes by adding 150 µl of 3 M methanolic HCl. FAME were extracted twice in 600 µl of hexane. Then, the extracts were combined, evaporated under nitrogen, and re-dissolved in 40 µl hexane. Individual FAME were quantified by gas chromatography with flame ionization detection (GC-FID) as µmol/L (Agilent 5890 series II, Waldbronn, Germany) using a BPX 70 column (25 m × 0.22 mm, 0.25 µm film, SGE, Weiterstadt, Germany).

**Statistical analyses**

Data were analyzed using Excel 2010 and R version 3.3.1. Concentration data for the individual FA were provided as absolute postprandial concentrations. To calculate the relative concentrations, postprandial concentrations were divided by the respective baseline values. Areas under the curves (AUC) were calculated using the relative values. Concentrations and AUC are provided in Supplementary Table 1 (Apnm-2018-0798suppla). Non-metric multidimensional scaling (NMDS) using Bray–Curtis dissimilarity index was performed in the R vegan package to visualize the clustering of
samples, based on concentration data of the measured FA species at the different time points (0-7 h postprandial) (Oksanen 2018). A NMDS plot was performed to show the spatial distribution of samples collected from participants at different time points in the study settings or “scores plot” using the measured species (both at baseline & postprandial) (Fig. 1) and significant differences were tested by PERMANOVA, using Adonis methodology. Adonis function in R vegan package implements a multivariate analysis of variances using distance matrices. It partitions dissimilarities for the sources of variation and uses permutation tests to inspect the significances of those partitions. In Adonis, a R2 value (effect size) is computed showing the percentage of variation explained by the supplied category, as well as a p-value indicating the statistical significance (Oksanen 2018).

Time versus concentration data were plotted collectively for the three main lipid classes (TG, NEFA, PL) (Fig. 2) as well as for their individual subclasses (saturated fatty acids (SFA), very long chain saturated fatty acids (VLCSFA), PUFA, and MUFA) (Fig. 3-5). Error bars were not included in Fig. 3-5 due to the high overlap between the curves.

**Results**

To examine the effect of a mixed meal rich in LA on a broad spectrum of FA, plasma concentrations were assessed at baseline (before the meal intake) and hourly over a period of 7 h in healthy volunteers. Detailed demographic characteristics and baseline metabolic data of the study participants are provided in Supplementary Table 2 (Apnm-2018-0798supplb). Each subject provided 8 blood samples (from 8 time points). In total, 44 NEFA, 27 TG-FA and 26 PL-FA were quantified.

**Non-multidimensional scaling (NMDS) plot**
NMDS ordination plots of Bray-Curtis dissimilarities between samples taken from 8 participants at 8 time points demonstrated significant clustering (p=0.001, R²=0.285). Eight Clusters of samples corresponding to the 8 time points of the experiment were identified in the NMDS plot. Clusters corresponding to baseline, 5, 6, and to a lesser extent 7 h postprandial were ordinated close to one another (Group A). The same applied to those at 1, 3 and to a lesser extent 2 h postprandial (Group B). A single cluster corresponding to 4 h postprandial showed almost a similar spatial distance to groups A and B, thus ordinated midway between the 2 groups (Fig. 1).

**Total lipid response**

A concentration-time course plot for the medians of the total FA of the investigated lipid species (TG, PL, and NEFA) is presented in Fig. 2. The plot shows similar profiles of the lipid species at baseline and 5 h postprandial. The total PL-FA did not respond to the meal challenge in contrast to the TG-FA which peaked at 2 h postprandial, then steadily decreased reaching baseline levels by 6 h and nadir by 7 h after ingestion of the test meal. Total NEFA followed the expected postprandial fall and rise pattern. The median total NEFA concentration decreased to nadir by 1 h following the meal challenge, then steadily increased reaching baseline and highest values at 5 and 7 h postprandial, respectively. Total fasting NEFA levels ranged from 0.17 to 0.79 mol/m³ with a median of 0.33 mol/m³.

**Triacylglycerols (TG) response**

The majority of individual TG-FA followed the total TG concentration-time course pattern with different magnitudes. LA from TG18:2n-6 and behenic acid from TG22:0 showed the highest response to the meal challenge with 0-7 h AUC values of 13.22 and 21.27, respectively. Additionally, a biphasic response was detected for LA from
TG18:2n-6 at 2 and 6 h (Fig. 3). AUCs (0-7 h) of all other TG-FA ranged between -0.76 (TG16:1n-7) and 4.78 (TG24:0).

**Non-esterified fatty acids (NEFA) response**

By examining the concentrations of individual NEFA, the highest baseline levels were detected for NEFA18:1 with a median of 0.14 mol/m$^3$. NEFA18:2, NEFA16:0, and NEFA18:0 also showed high baseline concentrations. After meal ingestion, the majority of individual NEFA showed the same concentration-time curve observed for total NEFA with initial suppression followed by a continuous rise to highest levels, reached by the end of the study. Characteristically, LA did not show the initial decrease suffered by the majority of other NEFA ($\leq 1$ h). It directly started to increase till 4 h, decreased at 5 h concomitant with the increase in the other NEFA, then continuously increased reaching the highest value at 7 h after the meal intake. Other NEFA, including NEFA22:0, NEFA24:0, and NEFA26:0 also did not decrease shortly after meal ingestion, but markedly increased reaching the highest values at 2-3 h. This was followed by a slow decreasing trend and a kink by 5 h, after which the concentrations rather stagnated (Fig. 4). The observations seen from the curves are also reflected in the 0-7 h AUCs with values of 30.31, 19.72, 10.04, and 6.83 obtained for NEFA22:0, NEFA24:0, NEFA26:0, and NEFA18:2, respectively.

**Phospholipids (PL) response**

No substantial changes from baseline levels were seen for individual PL-FA concentrations in response to the test meal over a period of 7 h (Fig. 5). AUC were in the range of -0.87 (PL18:3n-3) to 0.65 (PL22:2n-6).
Discussion

The present study investigated the effect of the composition of a typical western meal on levels of different circulating FA in TG, PL, and NEFA fractions, unlike the majority of the previously conducted postprandial studies, in which often only the total NEFA concentration has been determined (Jackson et al. 2005). Using a test meal rich in LA helped in interpreting the findings since it is a reliable biomarker useful to track the ingested lipid within the endogenous plasma pool. This was reflected in the study results which demonstrated that LA in addition to VLCSFA either as NEFA or TG were the most affected by the test meal, where they not only showed the highest response but also a different behaviour from the other individual species. The study also allowed monitoring of the residual lipid profile usually subjected to the second meal effect by using a time course of 7h postprandial in the study design. The residual lipid profile is the amount of fat left-over after the assimilation of nutrients of the previous meal usually taking place within 5-6 h after the meal consumption, thus produces an additive effect with fat from the next meal consumed (Woerle et al. 2003). Elevation of the residual lipid profile might indicate potential health risks, since it is associated with accumulation of lipids in plasma and extended hyperlipidemia, triggering coronary artery diseases (CAD) and cardiovascular incidents (Bell et al. 2008; Tushuizen et al. 2005; Garber 2012). At 7h postprandial, LA and behenic acid as TG18:2n-6 and TG22:0, respectively were the only TG-FA that did not return to their baseline values and the same applied to the total NEFA, especially LA which reached their highest response at 7h. Interestingly, the study was also able to detect the interindividual differences in postprandial lipid responses which were related to the corresponding baseline levels according to results of NMDS analysis.
In detail, the NMDS plot showed a similarity in the lipid responses at baseline with the late postprandial period (5-7 h postprandial), where samples at these time points were ordinated close to each other (Fig. 1). Additionally, it was observed, that samples obtained from participant #5, especially at time point 4, 5, 6, 7 h, and to a lesser extent at 1 h, 3 h, and baseline, were ordinated further away from the rest of the study participants’ samples, indicating a different profile. The same applied to participant #1 and #7, especially among samples collected in the late postprandial period (6 h and 7 h). Inspection of the raw data available in Supplementary Table 2 (Apnm-2018-0798supplb) showed that participant #5 and #1 have the lowest and highest baseline TG concentrations, respectively (especially TG18:2n-6, TG18:1n-7, TG18:1n-9, and total TG concentrations). They also maintained similar behaviour of these TG species postprandially at 1, 4, 6 and 7 h in comparison to corresponding samples from other study participants. Samples from participant #7 at time points 0, 4, 6 and 7 h were also relatively distant from the samples of the rest of the study participants. This participant showed higher baseline and postprandial TG concentrations relative to participant #5, however lower than the rest of the study participants. These observations provide information on the correlations between baseline and postprandial TG concentrations, especially at the lowest and highest ranges. Previous studies have demonstrated that fasting hypertriglyceridemia displayed exaggerated and prolonged triglyceride responses and vice versa, which indicates a close correlation between postprandial and baseline blood TG concentrations (Tiihonen et al. 2015). This elevated TG response, especially in the late postprandial phase (5–8 h) was linked to increased risk of CVD (Patsch et al. 1992). Interestingly, according to the present findings, participants #5 & 7 with the lowest TG values (baseline & postprandial) showed the lowest LDL-c/HDL-c ratios while participant #1 with the highest TG values showed the highest LDL-
c/HDL-c ratios. LDL-c/ HDL-c ratio was reported as a risk indicator for CVD (Kunutsor et al. 2017).

Regarding the individual concentration-time course curves of the three lipid classes involved, they showed a decrease in NEFA in the immediate postprandial period (≤ 1h), with a corresponding increase in TG, which typically reached its peak at 2 h and no significant change in PL (Fig. 2). This is considered a normal response, since during fasting; adipose tissue tends to release FA into the systemic circulation in order to supply tissues with a high requirement for FA, thus causing high fasting plasma NEFA concentrations. Shortly after meals, the adipose tissue metabolism starts to deal with the increased concentration of the plasma TG resulting from entry of chylomicron-TG (CM-TG) into the circulation (Hunter et al. 2001). However, it should not be excluded that the hepatic very low-density lipoproteins (VLDL) might also partially explain the increase in TG in the postprandial period, although CM are better substrates for LPL than VLDL (Xiang et al. 1999, Heller et al. 1993). The total plasma TG typically increased to reach a broad peak at 2 h before decreasing gradually due to clearance from the circulation (Fig. 2). This was found in agreement with reports on the monophasic response of the mean concentrations of plasma TG postprandially, but in contrast with other studies demonstrating biphasic response in the first 6 h period after a high fat meal (Cohn et al. 1988; Heller et al. 1993; Kashyap et al. 1983; Olefsky et al. 1976; Peel et al. 1993; Williams et al. 1992). Interestingly, in this study, a biphasic response was detected in TG18:2n-6 comprising LA, the most abundant FA in the test meal (Fig. 3b). TG18:2n-6 showed 2 peaks at 2 h and 6 h postprandial. The early sharp peak at 2 h corresponded to the early rise in plasma TG concentrations due to the first entry of CM-TG containing LA into the circulation (Bysted et al. 2005; Hodson et al. 2009; Yli-Jokipii et al. 2003).
Ockner et al. (1969) demonstrated that the increase in exogenous postprandial TG-LA levels is entirely a result of the increase in CM-TG with no increase in VLDL-TG. In other reports, the TG fatty acid composition of CM in the systemic circulation was consistently found to resemble that of the dietary fat consumed (Hodson and Fielding 2010). The delayed peak at 6 h might be attributed to the carry-over effect, suggesting the release of preformed CM retained either in enterocytes (primary site of synthesis) or lymph. (Zhu et al. 2009). This hypothesis is supported by the fact that LA was discovered to form large sized bulky CM, which partially reside in the enterocytes before being released by lymphatic drainage. In the literature, the size of CM produced by LA was compared to palmitic acid (NEFA16:0), a SFA. It was found that both FA formed CM of significantly different sizes corresponding to >800 angstroms (Å) in diameter for TG18:2n-6 versus 300 to 800 Å for TG16:0, thus showing that the latter resulted in particles similar to the smaller VLDL (Ockner et al. 1971, 1972). Carlier et al. (1991) reported that LA produced an increase in both the size and proportion of CM, which may cause a stepwise release. Thus, this could possibly provide an explanation for the delayed peak detected for TG18:2n-6 at 6 h. Negative health impacts were related to the formation of large CM particles (800-3600 Å) because of their role as the primary cause of formation of atherosclerotic lesions (Kocmur 2017). It could also be that the second peak was no longer confined to CM but might represent newly synthesized VLDL-TG.

This biphasic response of TG18:2n-6 could be also attributed to the consumption of a mixed meal rich in carbohydrates where a high carbohydrate content might have led to a rapid rate of gastric emptying and fat digestion which was absorbed in two waves (Zampelas et al. 1998). This hypothesis is further supported by a similar finding previously reported on another exogenous fat (alpha-linolenic acid, ALA), which showed
a biphasic pattern in both its TG and NEFA fractions after consuming a meal rich in carbohydrates (136g) with a fat intake of 0.5 g fat/kg body weight comprising ALA in rapeseed oil (12 g/100 g) (Shishehbor et al. 1998). Overall, these findings provide evidence that consuming a mixed meal of high carbohydrates with a fat meal could cause increased residence time and reduced clearance of postprandial TG in the late stage of postprandial phase, especially CM-TG which increase its atherogenicity (Bravo et al. 2010).

In the present study, TG18:2n-6 along with TG22:0 were the only two TG-FA that did not return to the baseline values at 7 h postprandial, unlike the other measured individual TG-FA, that returned back to their baseline values or below baseline at the end of the study (Fig. 3b & 3d). It is also worth mentioning, that at 2 h, the TG comprising VLCSFA (≥ 20 carbon chain length) showed a relatively high increase from their baseline values compared to the other individual TG species (Fig. 3d).

The plausible explanation is the relatively high concentration of these TG in the meal, which are absorbed and transported as CM to the lymphatic circulation. Some reports do not consider diet as a major source of these FA inside the body (USDA 2014). However, hydrogenated fats or partially hydrogenated vegetable oils, commonly found in processed food, could represent an unusual dietary source of VLCSFA (Ajmi 2005). These VLCSFA were suggested to be products of desaturation and elongation of trans fatty acids (TFA) from hydrogenated oils in the human body (Ajmi 2005). Hydrogenated fat has also been previously recognized as a source of VLCSFA based on studies performed on rats fed a diet with partially and totally hydrogenated fish oils (Granlund et al. 2000). Additionally, increased levels of serum TG of arachidic (20:0) and behenic (22:0) acids were found in rats fed a diet rich in VLCSFA (Ajmi 2005). Some human studies found
that the proportions of behenic acid (22:0) and lignoceric acid (24:0) in PL and cholesterol esters (CE) increased by n-6 PUFA consumption compared with SFA, while arachidic acid (20:0) exhibited no change (Lauritzen and Hellgren 2015).

In agreement with previous literature, the total NEFA concentration was initially suppressed after meal ingestion followed by a steady increase to values above baseline levels (Bickerton et al. 2007; Dubois et al. 1998; Griffiths et al. 1994; Jackson et al. 2005). The initial decrease was attributed to insulin driven inhibition of lipolysis in adipose tissue and subsequent suppression of NEFA release into plasma (Fielding 2011). The following rising part of the NEFA curve likely represented an interplay between (1) spill-over of FA into plasma caused by the ineffective uptake of FA released from circulating TG (especially after a fat-rich meal) by the adipose tissue (Evans et al. 2002; Fielding et al. 1996), (2) FA release from adipose tissue by lipolysis, and (3) uptake of NEFAs into peripheral tissues (Fielding 2011).

In contrast to the general behaviour observed in the majority of NEFA, LA showed a characteristic profile, since its concentration did not decrease initially unlike the majority of NEFA (≤ 1 h). Several hypotheses could be suggested to provide an explanation for this finding. One of which is the spill-over effect, which accounts for 40-50% of the total plasma NEFA pool in the postprandial period. NEFA18:2n-6 might be affected to a large extent by this effect, as it represented the most abundant FA in the test meal (64.59% in sunflower oil) (Fielding 1996). It could also be that the high baseline concentration of this NEFA dampened the effect on its plasma levels. Another hypothesis is, that LA has the ability to bypass the lymphatic pathway and went directly to the portal circulation. Previous reports demonstrated that an important proportion of PUFA, especially LA and
ALA might pass through the enterocyte cytosol and go directly to the portal venous flow (McDonald and Weidman 1987; Ramírez et al. 2001; Thomson et al. 1989).

LA is assumed to be preferentially taken-up by the adipose tissue because it is the principal FA in the test meal and postprandial TG. Therefore, the unexpected increase in its plasma levels in the early postprandial period would imply a decreased capacity of its entrapment within the adipose tissue. This hypothesis was generated based on earlier reports on the close correlation between FA composition of the adipose tissue TG and diet in the early postprandial period (2-4 h) (Hynes et al. 2003; Summers et al. 2000). This means that FA derived from LPL-mediated CM-TG hydrolysis are more likely to be taken-up by the adipose tissue than FA from the circulating NEFA pool. This assumption is further supported by the sudden decrease of LA at 5 h concomitant with the increase in the other NEFA because, at this time point, lipolysis reached its maximum and the uptake load of the adipose tissue was spared. It should not be disregarded that this pattern was also obtained in a previous study where participants consumed a meal high in carbohydrates and fat in the form of rapeseed oil, as mentioned earlier. ALA, similarly, showed a characteristic “surge-null-surge” response for both plasma TG and NEFA fractions with no clear explanation (Shishehbor et al. 1998).

Another class of FA that stood out regarding their postprandial response were VLCSFA, namely: NEFA22:0, NEFA24:0, and NEFA26:0), one very long chain monounsaturated fatty acid (VLCMUFA, NEFA26:1), and two very long chain polyunsaturated fatty acids VLCPUFA (NEFA26:2 and NEFA26:3) (Fig. 4). Even though baseline concentrations of these FA were very low, the extent to which these FA responded was not expected and did not seem to be proportional to their initial concentrations. VLCSFA (NEFA22:0 and NEFA24:0) showed a biphasic pattern where they showed two maxima and two minima
at 3 h and 5 h and similar to NEFA18:2, they showed initial increase in their levels shortly after meal intake and did not return to the baseline at any time point during the experiment (Fig. 4b & 4d). Moreover, the magnitude of their response was very high relative to NEFA18:2n-6, in spite that the latter is the major component of the meal. This could suggest different magnitudes of spill-over effect, resulting from selective uptake of PUFA relative to VLCSFA, either by the liver or the adipose tissue or both. In fact, this finding is difficult to be directly interpreted since the plasma levels of NEFA are governed by different factors, including passive diffusion along a concentration gradient and facilitated diffusion by transfer proteins. These factors may be affected by concentration, chain length, saturation/unsaturation, as well as competition between different FA, particularly in case of saturation. Previous studies have demonstrated that in case of the competition of chain lengths, the shorter chain length is preferred and in case of equal chain length, the more unsaturated FA are preferred. Based on that, in the current situation of a high PUFA meal especially LA, when saturation of adipose tissue occurs, spill-over will show much stronger influence on the less polar VLCSFA (longer chain length and with no double bonds) than the more polar PUFA (Abumrad et al. 1998). Furthermore, it should not be neglected that the higher magnitude of response in VLCSFA relative to LA, could be due to their very low baseline values in comparison to high baseline values for LA, as shown in Supplementary Table 1 (Apnm-2018-0798suppla). In spite of the novelty of these results, it is difficult to relate them to potential health effects. In the literature, VLCSFA in plasma were associated with a favourable metabolic pattern and a reduced risk for diabetes and coronary heart diseases (Forouhi et al. 2014; Lemaitre et al. 2015; Malik et al. 2015). Conversely, excess serum VLCSFA were
identified as hepatotoxic substances which induce lipoapoptosis and liver damage (Malhi et al. 2006).

In general, it was monitored that the plasma NEFA concentrations were higher at the end of the study period than at baseline which could also be explained by the so-called “rebound” effect of spill-over FA, which were less easily up-taken by the adipose tissue against a concentration gradient, i.e. when the adipose tissue lipolysis is higher at the end of the postprandial state (Evans et al. 2008; Miles and Nelson 2007). It could also be speculated, that physical activity during the study period resulted in these elevated NEFA levels since the majority of energy used by muscles is derived by NEFA from the adipose tissue during moderate physical activity (Frayn 2010). The subjects were advised to stay in the examination room, but they were allowed to move and work in a sitting position (e.g. on computers). This activity consumes obviously more energy than overnight sleeping, thus the higher NEFA levels might have resulted from the increased energy demand. Overall, both prolonged postprandial hypertriglyceridemia and elevated NEFA concentrations (caused by less stimulated tissue uptake leading to augmented spill over) have been previously linked to obesity and diabetes (Lairon 1996).

**Limitations**

The study presented limitations that deserve to be mentioned. The study design did not involve measurement of the partitioning or contribution of the FA within the individual lipoprotein subclasses (CM- or VLDL-TG) using tracer technology. This makes interpretation of the findings somewhat speculative and provides limited information about the associated health risks of the ingested lipids, which is highly dependent on the dynamic metabolic processes of the different major lipoprotein fractions. However, tracer technology might not be appropriate in studies involving highly non-steady metabolic
states such as the postprandial state (i.e. kinetic studies) which requires an ‘instantaneous’ replacement of carbon source with the labeled nutrient, followed by rapid quenching and extraction of metabolites at defined time points (Magkos and Mittendorfer 2009). This goes along with relatively high costs and a substantial burden added to subjects as they have to avoid special foods naturally enriched in 13C if 13C-tracer method is used (Lambert and Parks 2012).

Conclusion

In this work, the behaviour of a broad spectrum of plasma FA species was investigated over an extended postprandial period of 7 h, in response to a typical western meal rich in LA. The study results demonstrated that, postprandially, LA and VLCSFA either as NEFA or TG were most affected by the test meal. Among the TG-FA, LA from TG18:2n-6 and behenic acid from TG22:0 showed the highest response and were the only two TG-FA that did not return to their baseline values at the end of the study in addition to a characteristic postprandial biphasic response observed for the former. As NEFA, LA and some VLCSFA showed a different behaviour from the other individual NEFA, since they markedly increased shortly after the meal intake. The study also demonstrated that postprandial TG responses were elevated in subjects with the highest baseline TG concentrations indicating a high correlation between postprandial and baseline TG levels. Concomitantly, the results pose new questions to be further investigated, especially regarding potential health implications of western dietary lifestyle characterized by high intake of mixed meals rich in n-6 PUFA, especially in high metabolic risk subjects (obese and diabetic).
Abbreviations:
Å (angstrom), ALA (alpha linolenic acid), AUC (Areas under the curves), BMI (body mass index), CV% (coefficient of variation), FA (fatty acid), FABP (fatty acid binding protein), FAME (fatty acid methyl esters), GC-FID (gas chromatography with flame ionization detection), HDL-c (high-density lipoprotein cholesterol), IQR (interquartile ranges), LA (linoleic acid), LC-MUFA (long chain monounsaturated fatty acid), LC-PUFA (long chain polyunsaturated fatty acid), LC-SFA (long chain saturated fatty acid), LDL-c (low-density lipoprotein cholesterol), LPL (lipoprotein lipase), MUFA (monounsaturated fatty acid), NEFA (non-esterified fatty acid), OA (oleic acid), PL (phospholipids), PUFA (polyunsaturated fatty acid), QC (quality control), SFA (saturated fatty acid), TFA (trans fatty acids), triacylglycerols (TG), TLC (thin layer chromatography), VLDL (very low-density lipoprotein), VLCMUFA (very long chain monounsaturated fatty acid), VLCPUFA (very long chain polyunsaturated fatty acids), VLCSFA (very long chain saturated fatty acid).

Acknowledgment

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Conflict of interest statement

The authors have no conflicts of interest to report.
References


Table 1. Nutritional composition of the test meal

<table>
<thead>
<tr>
<th></th>
<th>Muffin</th>
<th>Sandwich</th>
<th>Juice</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (KJ)</td>
<td>1735</td>
<td>1764</td>
<td>286</td>
<td>3785</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>6</td>
<td>19</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>47</td>
<td>35</td>
<td>15</td>
<td>97</td>
</tr>
<tr>
<td>Fat* (g)</td>
<td>22</td>
<td>23</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>C 18:2 n-6 (g)</td>
<td>13.2</td>
<td>13.1</td>
<td>-</td>
<td>26.4</td>
</tr>
<tr>
<td>C 18:1 n-9 (g)</td>
<td>5.7</td>
<td>5.4</td>
<td>-</td>
<td>11.1</td>
</tr>
<tr>
<td>C 16:0 (g)</td>
<td>1.8</td>
<td>1.6</td>
<td>-</td>
<td>3.4</td>
</tr>
<tr>
<td>C 18:0 (g)</td>
<td>0.7</td>
<td>0.7</td>
<td>-</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* 42g of total fat in the meal is provided by sunflower oil
Table 2. Fatty acid composition of the sunflower oil

<table>
<thead>
<tr>
<th>FA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:2n-6</td>
<td>64.59</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>23.73</td>
</tr>
<tr>
<td>C16:0</td>
<td>6.49</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.99</td>
</tr>
<tr>
<td>C18:1n-7</td>
<td>0.78</td>
</tr>
<tr>
<td>C22:0</td>
<td>0.55</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.21</td>
</tr>
<tr>
<td>C24:1n-9</td>
<td>0.19</td>
</tr>
<tr>
<td>C20:1n-9</td>
<td>0.14</td>
</tr>
<tr>
<td>C16:1n-7</td>
<td>0.11</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.07</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>0.05</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.03</td>
</tr>
<tr>
<td>C22:2n-6</td>
<td>0.03</td>
</tr>
<tr>
<td>C16:1 t</td>
<td>0.01</td>
</tr>
<tr>
<td>C20:2n-6</td>
<td>0.01</td>
</tr>
<tr>
<td>C20:3n-9</td>
<td>0.01</td>
</tr>
<tr>
<td>n-6 PUFA</td>
<td>64.63</td>
</tr>
</tbody>
</table>

Data are expressed as weight percentage of total fatty acids.
Figure legends

**Fig. 1.** NMDS ordinations based on Bray-Curtis distances between all samples displaying the distance between samples collected from the study participants \((n = 8)\) at different time points after the standardized meal intake. The numbers (0-7) refer to the time points starting from baseline till 7h postprandial in 1h intervals. The samples are identified by a combination of 2 numbers, the participant ID (number) and the timepoint at which the sample was collected, separated by an underscore (_).

**Fig. 2.** Median of the sums of triacylglycerol, phospholipid, and non-esterified fatty acid levels of study participants \((n = 8)\) at baseline and after a standard breakfast meal. Values are given in relation to baseline values.

**Fig. 3.** Curves depicting postprandial relative concentrations of plasma triglycerides (TG) of study participants \((n = 8)\), comprising: (a) saturated fatty acids (SFA) (b) polyunsaturated fatty acids (PUFA) (c) monounsaturated fatty acids (d) Very long chain saturated fatty acids (VLCSFA) versus time (h) after the standardized meal intake. Results are given in relation to baseline values.

**Fig. 4.** Curves depicting postprandial relative concentrations of plasma non-esterified fatty acids (NEFA) of study participants \((n = 8)\), comprising: (a) saturated fatty acids (SFA) (b) polyunsaturated fatty acids (PUFA) (c) monounsaturated fatty acids (d) Very long chain saturated fatty acids (VLCSFA) versus time (h) after the standardized meal intake. Results are given in relation to baseline values.

**Fig. 5.** Curves depicting postprandial relative concentrations of plasma phospholipids (PL) of study participants \((n = 8)\), comprising: (a) saturated fatty acids (SFA) (b) polyunsaturated fatty acids (PUFA) (c) monounsaturated fatty acids (d) Very long chain saturated fatty acids (VLCSFA) versus time (h) after the standardized meal intake. Results are given in relation to baseline values.
Fig. 1. NMDS ordinations based on Bray-Curtis distances between all samples displaying the distance between samples collected from the study participants (n = 8) at different time points after the standardized meal intake. The numbers (0-7) refer to the time points starting from baseline till 7h postprandial in 1h intervals. The samples are identified by a combination of 2 numbers, the participant ID (number) and the timepoint at which the sample was collected, separated by an underscore (_).
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