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Lipidomic profiling of chylomicron triacylglycerols in response to high fat meals

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Short title Lipidomic analysis of chylomicrons

CM chylomicron-rich
FAME fatty acid methyl esters
MetS metabolic syndrome
MRM multiple-reaction monitoring
TAG triacylglycerol
AUC area under the curve
MS mass spectrometry

CM TAG chylomicron triacylglycerol
PUFA polyunsaturated fatty acid
FA fatty acid
FAME fatty acid methyl ester
UPLC ultra high pressure liquid chromatography
ESI electrospray ionization
APCI atmospheric pressure chemical ionization

Abstract

Using lipidomic methodologies the impact that meal lipid composition and metabolic syndrome (MetS) exerts on the postprandial chylomicron triacylglycerol (TAG) response was examined. Males (9 control; 11 MetS) participated in a randomised crossover trial ingesting two high fat breakfast meals composed of either dairy-based foods or vegetable oil-based foods. The postprandial lipidomic molecular composition of the TAG in the chylomicron-rich (CM) fraction was analysed with tandem mass spectrometry coupled with liquid chromatography to profile CM TAG species and targeted TAG regioisomers. Postprandial CM TAG concentrations were significantly lower after the dairy-based foods compared with the vegetable oil-based foods for both control and MetS subjects. The CM TAG response to the ingested meals involved both significant and differential depletion of TAG species containing shorter- and medium-chain fatty acids (FA) and enrichment of TAG molecular species containing C16 and C18 saturated, monounsaturated and diunsaturated FA. Furthermore, there were significant changes in the TAG species between the food TAG and CM TAG and between the 3- and 5- hour postprandial samples for the CM TAG regioisomers. Unexpectedly, the postprandial CM TAG concentration and CM TAG lipidomic responses did not differ between the control and MetS subjects. Lipidomic analysing of CM TAG molecular species revealed dynamic changes in the molecular species of CM TAG during the postprandial phase suggesting either preferential CM TAG species formation and/or clearance.

Key words

Chylomicron, lipidomics, postprandial, lipid metabolism, metabolic syndrome

Introduction

The postprandial plasma or chylomicron (CM) triacylglycerol (TAG) response is a combination of genetic and environmental factors including, but not limited to meal, diet, genotype, gender and visceral fat deposition (1-3). People consuming a typical Western eating pattern spend the majority of their daytime in a postprandial state with a continual fluctuation in the degree of lipemia throughout the day. Studies in the postprandial state are important as hypertriglyceridaemia, or the accumulation of TAG rich lipoprotein fractions, is a major risk factor for cardiovascular disease (4, 5).

Metabolic syndrome (MetS) is a cluster of metabolic abnormalities characterised by obesity, dyslipidemia and high blood pressure. MetS is associated with high fasting TAG concentrations, low HDL cholesterol concentrations and impaired fasting glycaemia as well as an exaggerated postprandial plasma TAG response following dietary intervention (6). In particular, intakes of dietary saturated fat have been observed to result in an exaggerated postprandial lipaemia in men with MetS in an acute intervention (7). Conversely, epidemiological evidence supports an inverse correlation between dairy intake and development of MetS (8, 9) but the mechanism is not clear.

Dairy fat contains short and medium chain fatty acids (FA) (about 11 % C10 or shorter by weight) (10), and is one of the most complex dietary fat sources. Unlike long chain FA, which are transported in chylomicrons, short and medium chain FA are transported predominantly in the portal blood and are preferentially oxidized in the liver (11). Ultimately, replacement of long chain FA with short and medium chain FA may increase lipid oxidation and result in enhanced satiety (12).

Corroboration for the loss of short or medium chain FA to the portal system is supported by Mekki et al (13) who observed a lower postprandial response (plasma and CM TAG concentrations) in healthy men fed 40 g of butterfat compared with olive or sunflower oil. Conversely, Thomsen et al (14) found higher plasma and CM TAG concentrations after a meal containing 100 g butter compared with a meal containing 80 g olive oil. In addition to FA composition, the location of the FA in dietary TAG molecules influences the metabolic fate of fatty acids. This is especially seen for saturated FA and medium chain FA, with increased transportation in CM from the *sn*-2 position of dietary fat (15-17). Thus, the health implications of dairy fat are being continuously re-evaluated (18-21).

Postprandial chylomicronaemia is a dynamic, non-steady state condition and a function of several mechanisms including TAG hydrolysis in the gut, transfer of hydrolysis products to mucosal cells, TAG reassembly, TAG clearance from blood, and remnant uptake by the liver. These steps may be affected by differences in the molecular species of TAG ingested. Novel molecular level LC-MS/MS analysis methods are starting to reveal the selective formation and/or clearance of individual TAG in humans (22-24).

The aim of this investigation was to examine the postprandial CM lipidomic TAG profiles in response to high fat breakfast meals differing significantly in TAG composition (dairy- and vegetable oil-based) from men identified with MetS compared with control subjects. The effect of consumption of dairy-based foods on postprandial CM TAG at the molecular level has not previously been studied. It was hypothesised that lipidomic analysis of the CM TAG would reveal more detailed changes in the composition of the CM TAG molecular species than standard fatty acid analysis of CM TAG, and this would help in understanding what kind of fats influence the formation and the clearance of CM TAG.

Materials and Methods

Subjects

A total of 21 male subjects (aged 40 – 60 y) were recruited by newspaper, poster and flyer advertisements. Subjects were screened prior to initiating the study for risk factors associated with the MetS. Subjects were classified as being MetS (n=11) based on the International Diabetes Federation criteria (25) meaning they had abdominal obesity (waist circumference ≥ 94 cm) and two of the following factors – raised serum TAG (≥ 1.7 mmol/l), reduced serum HDL cholesterol (< 1.03 mmol/l), impaired fasting glycaemia (fasting plasma glucose ≥ 5.6 mmol/l) or raised blood pressure (systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg). A cohort of age and height matched subjects, were also recruited to act as a control group (n=10). All subjects were non-smokers and not taking any supplements known to interfere with lipid metabolism. Subjects with fasting glucose concentrations indicative of type 2 diabetes mellitus were excluded along with people on diabetic medication. All experimental procedures were carried out in accordance with the declaration of Helsinki (2000) of the World Medical Association and were formally approved by the Deakin University Human Research Ethics Committee (DHNC EC 120-

2008). Informed written consent was obtained from each subject before participation in the study and after the nature, purpose and risks of the study were explained. Subjects were informed of their right to withdraw from the trial at any stage of the investigation. Two subjects were excluded from the statistical analysis owing to exceptionally high postprandial TAG responses (mean concentrations were greater than 3 x standard deviations from the other subjects). The trial was registered with the Australian New Zealand Clinical Trial Registry (ACTRN12610000562077).

Study Design

According to a crossover design, all subjects were studied on 2 separate occasions at intervals of 4-6 weeks apart. To prevent possible differences between subjects at baseline from their previous meal, subjects were provided with a dinner to be consumed the night before the investigation. The meal consisted of a pre-packaged lasagne and fruit yoghurt providing a total of 2502 kJ as 20% fat, 19% protein and 61% carbohydrates. Subjects were asked to eat the provided food only. In the period between the study days, subjects followed their habitual diet. On the day before each study, subjects were instructed to refrain from alcohol and vigorous physical activity. Subjects were randomly assigned to the test meals, and arrived at the clinical laboratory after a 12 h fast. On arrival subjects had their weight, height and waist circumference as well as blood pressure measurements recorded following standardised procedures. Subjects were fitted with a canula in the antecubital vein and fasting blood samples were collected immediately before (0 h) and at every hour thereafter up to 5 h following the test meal. Subjects remained in a semi-recumbent position for the duration of the study and provided with unlimited water. At each time point, 14 mL of blood was collected for subsequent analysis.

Dietary recording

Dietary information was collected using a 24 h recall by a dietitian *via* a face to face interview with each participant on two occasions. Food and drink consumed by the participants was entered into FoodWorks (FoodWorks®version 2009, Xyris, Brisbane) and an estimation of energy and macronutrient consumption, based on the average of these two records, was recorded (Table 1).

Test meals

Subjects were randomly assigned to either receive first a breakfast meal high in dairy-based foods or high in vegetable oil-based foods that mainly contained soy protein, soy bean oil and sunflower oil. Both meals contained 54 g fat and were matched on protein content. The meal high in dairy foods consisted of cheddar cheese (60 g), butter (20 g) and extra creamy full milk (300 mL) with

toast (50 g) (3120 kJ, 54 g fat, 29 g protein and 37 g carbohydrate) and the meal high in vegetable oil-based foods contained cheddar flavoured soy cheese (100 g), soy beverage (300 mL), a non-dairy spread (20g) and toast (50g) (3290 kJ, 54 g fat 29 g protein and 47 g carbohydrate). The calcium content of the two meals was similar (672 mg in the dairy-based meal of which 89% was derived from dairy foods) and 737 mg in the vegetable oil-based meal. Subjects consumed the meals within 15 min.

Isolation of plasma and a chylomicron rich fraction

EDTA blood samples were centrifuged (591 x g for 15 min) to separate plasma. A chylomicron-rich fraction (Svedberg flotation unit (Sf) > 400, later abbreviated as CM) was isolated from plasma by ultracentrifugation using a Beckman ultracentrifuge and TLA 100.4 rotor (Beckman instruments, Palo Alto, CA) as previously described (26). Briefly, 1.8 mL of EDTA plasma was overlaid with saline solution (density = 1.006 kg/l) in ultracentrifuge tubes and centrifuged at 35039 x g for 30 min at 4°C. The top 1 millimetre was aspirated to remove the CM. All samples were frozen at -80°C prior analysis.

TAG concentrations

TAG concentrations in plasma and CM as well as plasma cholesterol and glucose concentrations were measured on a Roche Cobas Integra 400 plus autoanalyser (Roche, Laval, Quebec, Canada) by enzymatic colorimetric methods using commercially available kits (CHOL2, TRIGL and Glucose HK Gen 3) as per the manufacturer's instructions (Roche, Laval, Quebec, Canada).

Lipid extraction

For the FA composition and regioisomerism of CM and food lipids, the extraction was performed using a modification of the method by Folch et al (27). Briefly, an internal standard mixture of triheptadecanoin (Sigma-Aldrich, St.Louis,MO), dinonadecanoylphosphatidylcholine (Sigma-Aldrich, St.Louis, MO) and cholesteryl pentadecanoate (Nu-Chek Prep. Inc., Elysian, MN) was added to the isolated chylomicrons. Then 1.5 mL methanol, 3 mL chloroform and 0.8 mL 0.88 % potassium chloride in water were added and the blend was thoroughly vortexed after each addition. The tubes were centrifuged 2000 x g for 3 minutes to separate the layers, and the chloroform rich layer was removed and evaporated to dryness.

For the molecular species analysis, the lipids of the CM as well as lipids of the food were extracted with chloroform:methanol (2:1, 20 volumes), mixed, sonicated for 30 minutes to assist the

liberation of lipids from the proteins and allowed to stand for 20 minutes. Samples were centrifuged (16 000 x g, 10 min) and the supernatant transferred to a 96 well plate and dried under a stream of nitrogen at 40°C. Immediately before analysis, samples were resuspended in water saturated butanol and methanol containing 10mM ammonium formate.

TAG isolation and fatty acid composition analysis

The TAG were isolated with solid phase extraction based on silica columns (28). TAG from the CM fraction from the 3 and 5 h time points were chosen for FA composition analysis. Fatty acid methyl esters (FAME) were prepared from the isolated TAG using the boron trifluoride method. In short, the lipids were evaporated to dryness under a stream of nitrogen. 0.1 mL toluene and 0.5 mL boron trifluoride in methanol ($\approx 1.3M$, Sigma-Aldrich, St Louis, MO) were added to each tube. The tubes were tightly capped and placed into a block heater (90°C) for 60 min. After cooling, 0.8 mL distilled water and 0.8 mL hexane were added and the tubes were shaken. Hexane containing FAME were directly taken for gas chromatographic analysis (29) or stored at -20°C prior to analysis. The FAME were analysed in duplicate with gas chromatography (Shimadzu GC-2010 equipped with AOC-20i Auto Injector, Flame ionization detector (Shimadzu corporation, Kyoto, Japan) and wall coated open tubular column DB-23 (60 m x 0.25 mm i.d., liquid film 0.25 μm , Agilent technologies, J.W. Scientific, Santa Clara, CA). Supelco 37 Component FAME Mix (Supelco, St. Louis, MO) and 68D (Nu-Chek-Prep, Elysian, MN) were used as external standards and triheptadecanoin (Sigma-Aldrich, St.Louis, MO) as an internal standard.

Lipidomic analysis

(a) Molecular species analysis of CM TAG: TAG analysis of the CM fraction was performed by liquid chromatography, electrospray ionisation-tandem mass spectrometry using an Applied Biosystems 4000 QTRAP mass spectrometer running Analyst 1.5 software. Liquid chromatography was performed on a Zorbax C18, 1.8 μm , 50 x 2.1 mm column (Agilent technologies, Santa Clara CA). Samples were resuspended in 50 μl saturated butanol and 50 μl MeOH containing 10 mM ammonium formate (NH_4COOH). The mobile phase was tetrahydrofuran:methanol:water in a 30:20:50 ratio (A) and 75:20:5 (B) both containing 10 mM NH_4COOH . TAG were separated with an isocratic flow (100 $\mu L/min$) of 85% mobile phase B. Quantification of individual TAG species was performed using scheduled multiple-reaction monitoring (MRM) in positive ion mode (30). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the internal standard (using Multiquant 1.2 software). Total TAG was calculated by summing the individual species. This analysis resulted in TAG FA combinations where the location of the FA in

different *sn* position was not determined. These TAG are marked as A-B-C. As there were no standards available for each TAG species, no adjustment was made for different response factors and the relative proportions of different species presented in Figure 3 should be taken as semi-quantitative.

(b) Regioisomer analysis of CM TAG: The CM TAG of 3 and 5 hours were chosen for regioisomer analysis. The TAG were separated using Acquity UPLC™ (Waters, Milford, MA) with Kinetex™ C18 reversed phase column (100 x 2.1 mm, i.d. 1.7 µm, Phenomenex, Torrance, CA). The solvent gradient program was as follows: initial acetonitrile; linear from 0 to 22 min to acetone/acetonitrile (70:30). The flow rate was 0.4 mL/min. MS/MS analyses were performed with a Quattro Premier tandem quadrupole mass spectrometer (Waters, Milford, MA), using positive ESI. Ammonium gas (purity 5.0; Linde AG, Munich, Germany) was introduced to the nebulizer gas flow to produce ammonium adducts of TAG. The daughter ion spectra of precursor ions of TAG were collected in the analyses. Analyses of reference TAG 1-oleoyl-2-linoleoyl-3-oleoyl-*sn*-glycerol, 1(3)-linoleoyl-2-oleoyl-3(1)-oleoyl-*sn*-glycerol, 1-linoleoyl-2-oleoyl-3-linoleoyl-*sn*-glycerol, 1(3)-linoleoyl-2-linoleoyl-3(1)-oleoyl-*sn*-glycerol, 1-linoleoyl-2-linolenoyl-3-linoleoyl-*sn*-glycerol, 1(3)-linoleoyl-2-linoleoyl-3(1)-linolenoyl-*sn*-glycerol, 1-oleoyl-2-palmitoyl-3-oleoyl-*sn*-glycerol, 1(3)-palmitoyl-2-oleoyl-3(1)-oleoyl-*sn*-glycerol, 1-palmitoyl-2-oleoyl-3-palmitoyl-*sn*-glycerol, 1(3)-palmitoyl-2-palmitoyl-3(1)-oleoyl-*sn*-glycerol, 1-oleoyl-2-stearoyl-3-oleoyl-*sn*-glycerol, 1(3)-oleoyl-2-oleoyl-3(1)-stearoyl-*sn*-glycerol, 1-oleoyl-2-myristoyl-3-oleoyl-*sn*-glycerol and 1(3)-myristoyl-2-oleoyl-3(1)-oleoyl-*sn*-glycerol (Larodan Fine Chemicals, Malmö, Sweden) were conducted in quadruplicate. The ratio of DAG ion intensities in ESI-MS/MS analyses was determined for each standard regioisomer and regioisomer mixture, and calibration curves were made as described previously (31). The relative proportions of regioisomers of the TAG in CM samples were calculated on the basis of the corresponding ratios of the DAG ion intensities in the calibration curves. For example, to measure the relative proportions of POO/OOP and OPO (1(3)-palmitoyl-2-oleoyl-3(1)-oleoyl-*sn*-glycerol and 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol) the following mixtures were prepared from commercial standards (100% POO/OOP, 75% POO/OOP + 25% OPO, 50% POO/OOP + 50% OPO, 25 % POO/OOP + 75% OPO, 100% OPO. The calibration curve was then obtained by plotting the OPO content on the X axis and the amount of DAG $[OP]^+ / ([OP]^+ + [OO]^+)$ formed from the standard mixtures on the Y -axis. The responses were linear and the correlation coefficients (R^2) were above 0.95. The relative proportions of POO/OOP and OPO in the sample were then read from the X axis of the calibration curve based on the proportions of $[OP]^+$ and $[OO]^+$ in the sample. Each sample was analysed in triplicate. To calculate the molar

proportions of the TAG regioisomer pairs in the chylomicron rich fraction the proportions of 17 most abundant molecular weight species were analysed by UPLC/APCI-MS analysis with Quattro Premier tandem quadrupole mass spectrometer (Waters, Milford, MA) with positive ion mode. Acquity UPLC™ (Waters, Milford, MA) with Kinetex™ C18 reversed phase column (100 x 2,1 mm, i.d. 1.7µm, Phenomenex, Torrance, CA) was used to separate TAG prior to MS. The binary solvent gradient program was as follows: initial acetonitrile; linear from 0 to 31 min to acetone. The flow rate was 0.4 mL/min. Single ion recording was used. Triheptadecanoin (Sigma-Aldrich, St.Louis, MO) was used as an internal standard and linoleoyldioleoylglycerol, dilinoleoylloleoylglycerol, dioleoylpalmitoylglycerol, oleoyldipalmitoylglycerol, dioleoylstearyl glycerol, myristoyldioleoylglycerol, linolenoyldilinoleoylglycerol, dilinoleoylpalmitoylglycerol, linoleoylloleoylpalmitoylglycerol, triolein, oleoyldistearoylglycerol and oleoyldipalmitoleoylglycerol (Larodan Fine Chemicals, Malmö, Sweden) as external standards. Molecular weight species concentrations were calculated by using QuanLynx software (Waters, Milford, MA), which uses internal standard and calibration curves of external standards for quantification.

Statistical analysis

The number of subjects included was estimated to allow an 80% power to detect a difference in plasma TAG concentrations of 0.16 mmol/L between the dairy-based breakfast and vegetable oil-based breakfast in the control group at three hours postprandial at a significance level of $p < 0.05$. The statistical analyses were carried out using SPSS statistical package (version 17, SPSS, Chicago, IL). Distributions of data were checked for normality. Independent t-tests were used at baseline to identify differences between subjects with MetS and controls in anthropometric, biochemical, fatty acid, molecular weight species and regioisomerism measures. Postprandial changes were assessed using two-way ANOVA for repeated measures with meal and time as within-subject factors and group (MetS or control) as a between subject factor. Where significant differences were observed Bonferroni adjustment was used to correct for multiple testing. The 0 to 5 h incremental area under the curve (AUC) was calculated using the trapezoid rule and comparison between meal AUC was performed using one-way ANOVA for repeated measures and the Tukey test for post-hoc comparison. All TAG lipidomic analysis showed very little between subject group differences (control vs MetS), and none that seemed of physiological importance. For example, there were only three significant between group (control and MetS) differences in the 44 comparisons made of TAG FA composition (Table 2). To strengthen between meal differences, the lipidomic results were pooled in further statistical analyses. A value of $p < 0.05$ was taken as significant.

Results

Subject characteristics

The control and MetS group did not differ significantly in age and height. All biomarkers used as diagnostic criteria for MetS were significantly higher in MetS subjects compared with control subjects with the exception of HDL cholesterol which was, as expected, significantly lower. Although subjects with MetS were on average 23 kg heavier than their control counterparts, estimated energy intakes measured by 24 h recall were not different between the two groups. The baseline characteristics of subjects are presented in Table 1.

All subjects completed the study and there were no difficulties associated with consumption of the test meals. No significant changes in the baseline circulating levels of plasma cholesterol, glucose and TAG or in body weight were observed in subjects between test days (data not shown).

Plasma and CM TAG responses

Postprandial plasma concentrations of TAG (Figure 1a) and AUC analyses (data not shown) were not influenced by type of meal ($p = 0.731$) or whether subjects were classified as MetS or not ($p=0.911$).

Postprandial concentrations of the TAG in the CM fraction were not significantly different between subjects presenting with MetS and control subjects. An effect of meal, however, was observed in the CM TAG concentration with a significantly lower postprandial response observed after the dairy-based meal ($p = 0.023$) compared with the vegetable oil-based meal (Figure 1b). Paired t-tests indicated that at all timepoints (1-5 hr inclusive), except baseline, postprandial concentrations of TAG in the CM were significant lower for the subjects (MetS and control subjects) consuming the dairy-based meal compared with the vegetable oil-based meal. These findings are supported by AUC analyses (data not shown) which also revealed no effect of MetS on postprandial CM TAG response ($p = 0.489$) but a significant effect of meal ($p= <0.001$).

The analysis of the TAG in the CM fraction

The pooled results of the analysis of CM TAG in both control and MetS subjects have been divided into (1) FA composition analysis (Figure 2), (2) molecular species data in which the three FA in each TAG are identified but the distribution of each of these FA in the three different *sn*-position is

not determined (Figure 3), and (3) regioisomerism data in which the three different FA in each TAG are determined and the prevalence of the FA in the *sn*-2 position is distinguished from the prevalence of FA in the *sn*-1/3 positions (Table 3).

Fatty acid composition of the meal TAG and CM TAG

There were significantly higher proportions of the saturated FA 10:0, 12:0, 14:0 and 18:0 in the dairy-based meal TAG than in the CM TAG at 3h and 5h postprandially. The FA 16:0, 16:1(n-7), 18:1(n-9), 18:1(n-7) and 18:2(n-6) were more abundant in the CM TAG than in the dairy-based breakfast TAG.

The FA composition of the vegetable oil-based meal TAG and the CM TAG after the meal resembled each other, although some significant differences such as the increased presence of 16:1(n-7) in the CM TAG compared with the meal TAG were seen (Figure 2).

There were significant differences between the 3h and 5h CM TAG samples in terms of FA composition (Figure 2). After the dairy-based meal, the proportions of 10:0, 12:0, 14:0, 16:0 and 18:0 were significantly lower and the proportions of 16:1(n-7) and 18:1(n-9) significantly higher 5 h postprandially compared with 3 h postprandially. After the vegetable oil-based meal, the proportion of 16:0 was higher and the proportion of 18:1(n-9) lower at 5 h postprandially than 3 h postprandially.

Molecular species analysis of the TAG in the meals

A total of 80 different molecular species of TAG were detected in the dairy-based meal (concentration $\geq 0.1\%$ total TAG), compared with the 31 different molecular species of TAG detected in the vegetable oil-based meal. Another major difference between the meals was in the greater proportion of TAG with short and medium chain FA (8:0 up to 12:0) observed in the dairy-based meal TAG, compared with the vegetable oil-based meal which consisted of higher molecular weight TAG species consisting primarily of TAG molecules with 16:0, 18:0, 18:1 and 18:2 (see Figure 3 for major species).

Molecular species analysis of the TAG in the CM

Many of the lower molecular weight trisaturated TAG detected in the dairy meal were found in significantly lower proportions in the CM TAG (Figure 3). Typical TAG species which increased in

proportion from the dairy meal TAG to the CM TAG were those containing 16:0, 16:1, 18:1 and 18:2. For example, TAG molecular species 16:0-18:1-18:1 increased from 2.1% in food TAG to 13.0% in the CM TAG, at 3 h postprandially.

In addition, after the vegetable oil-based meal, the highest increases from meal TAG to CM TAG occurred in molecular species containing 16:0, 16:1, 18:1 and 18:2. For example, TAG molecular species 16:0-18:1-18:2 increased from 4.1% in food to 10.8% in the CM TAG fraction.

Significant changes in the composition of the molecular species of TAG in the CM were seen after both meals with time. The only CM TAG that did not have significant differences in proportions between one and five hours were 16:0-16:0-18:2 and 16:0-16:1-18:1 after the dairy meal. CM molecular species showing major increasing trends with time were 14:0-16:0-18:2 and 18:1-14:0-16:0 for the dairy meal CM TAG and 16:0-16:0-18:2 and 16:0-18:1-18:2 for the vegetable oil-based CM TAG. CM molecular species showing major decreasing trends with time were 16:0-18:1-18:2 and 16:0-18:1-18:1 for the dairy-based meal CM TAG and 18:1-18:2-18:2 and 18:1-18:1-18:1 for the vegetable oil-based CM TAG.

Regioisomers of the TAG from the meal and CM

Significant differences were found in the proportions of regioisomers between the meal and the CM TAG as well as between the CM TAG at 3 and 5 h postprandially (Table 3).

After the dairy meal, the proportions of *sn*-18:1-16:0-18:1 and *sn*-18:1-18:2-18:1 decreased significantly and the proportions of *sn*-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0 and *sn*-18:2-18:1-18:1 + *sn*-18:1-18:1-18:2 increased significantly from meal to 3 h CM TAG and further to 5 h CM TAG. In addition to the above, the proportion of *sn*-18:1-18:1-18:0 + *sn*-18:0-18:1-18:1 was significantly higher at the 5 h time point than at the 3 h time point and that of *sn*-18:1-18:0-18:1 was significantly lower at the 5 h time point than at the 3 h time point.

After the vegetable oil-based meal, the proportion of *sn*-18:2-18:2-18:1 + *sn*-18:1-18:2-18:2 decreased significantly and the proportion of *sn*-18:2-18:1-18:2 increased significantly from meal to 3 h and further to 5 h CM TAG. *Sn*-16:0-16:0-18:1 + *sn*-18:1-16:0-16:0 was detected in the vegetable oil-based meal TAG, and it was also detected together with *sn*-16:0-18:1-16:0 in the CM TAG (in approximately equal proportions).

Sn-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0 was the most prevalent CM TAG species analysed, comprising of 11.4 +/- 2.2 (mean +/- sd) and 14.7 +/- 3.4 molar percentages of all CM TAG at 3 h and 5 h, respectively, after the dairy meal and 11.9 +/-1.7 and 14.1 +/- 2.7 molar percentages of all CM TAG at 3 h and 5 h, respectively, after the vegetable oil-based meal.

The only regioisomers whose molar proportions in CM TAG did not differ after the dairy-based and vegetable oil-based meals were *sn*-18:1-18:1-18:0 + *sn*-18:0-18:1-18:1 at 3 h and *sn*-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0 at both 3 and 5 h.

Discussion

The present study examined the effect of high fat meals from dairy foods or from vegetable oil-based foods on postprandial plasma TAG and CM TAG concentrations and CM TAG molecular composition in men with or without MetS. It was hypothesised that lipidomic analysis of the molecular species of CM TAG would reveal more detail of complexity of the changes in CM TAG molecular species in the postprandial period than standard FA analysis of CM TAG. This could potentially help us understand what kind of TAG molecules are formed and/or cleared in favour of others, i.e. what kind of molecules prolong postprandial lipemia, as has been reported in type II diabetic subjects (32).

There were three major findings, [a] consistent with the hypothesis, the lipidomic analysis of the CM TAG revealed more detail than the simple measurement of CM TAG FA composition, in terms of the dynamic changes in the CM TAG molecular compositions during the postprandial phase, [b] the postprandial CM responses in the MetS subjects to the high fat meals were the same as the control subjects, and [c] the postprandial CM TAG concentrations were significantly lower after the dairy-based meal relative to the vegetable oil-based meal in both control and MetS participants.

The first major finding in this study was the extent to which the chylomicrons undergo dynamic changes in TAG molecular species composition during the absorptive and postprandial phases. While significant changes of CM TAG during the postprandial period was evident from the CM TAG FA proportions (Figure 2), a clearer picture of these complex changes was revealed by the use of the different LC-MS/MS techniques to examine the individual TAG species in the food lipids and CM. For example, in both dairy and vegetable oil-based CM, there was a loss of trisaturated- and disaturated-TAG species and an increase in some mono- and di-unsaturated TAG species (at 3 h postprandially) compared with the food TAG. For both meals, there were four molecular species which increased substantially from low proportions in the food TAG to high proportions in the CM TAG: 16:0-18:1-18:1, 16:0-18:1-18:2, 16:0-16:1-18:1 and 16:0-18:2-18:2. Significant changes of CM TAG molecular species composition have been reported previously in studies with fats/oils such as palm oil (33) and olive oil (22). In the palm oil study, significant alterations in the composition of CM were reported relative to the TAG molecular species in the palm oil i.e. a decreased proportion of *sn*-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0. In the olive oil study, the proportions of tri-18:1 decreased from 62% in the ingested oil to 42% in the CM TAG (6 h post ingestion), while at the same time the proportion of 18:1-18:1-18:2 more than doubled in the CM.

Not only were there substantial changes of CM TAG molecular species composition during the absorptive phase, there was evidence of significant changes in composition between 3 and 5 h postprandially. After the dairy meal, the proportions of all saturated FA (10:0, 12:0, 14:0, 16:0 and 18:0) were lower at 5 h than at 3 h suggesting altered digestion and/or clearance of these species (based on the FA composition analysis, Figure 2). The non-steady state of CM TAG molecular species composition was more evident in the regioisomers between 3- and 5-hours (Table 3). This was dramatically revealed in the dairy meal TAG and dairy CM TAG where there was a doubling/halving of regioisomers from food in comparison with the CM (*sn*-18:2-18:1-18:1 + *sn*-18:1-18:1-18:2 proportions doubled, while *sn*-18:1-18:2-18:1 proportions were approximately halved), with clear evidence of change in the proportions between 3 and 5 hours. This suggests that the different regioisomers are not equivalent in terms of substrates for the lipoprotein lipase and therefore the regioisomer composition could influence the rate of metabolism of CM to CM remnants. It was also evident that the use of the lipidomic techniques enabled a better understanding of the dynamics of CM TAG molecular species observed from diet TAG to CM TAG, and between 3 and 5 h postprandially, compared with FA compositional analysis alone. For example, the proportions of palmitic, oleic and linoleic acids alone did not reveal the extent of difference between the meal and CM in TAG 16:0-18:1-18:1 nor the large change between the 3 h and 5 h CM composition seen in *sn*-18:2-18:1-18:1 + *sn*-18:1-18:1-18:2.

The second major finding was the failure to detect a difference between the control subjects and the MetS in the plasma TAG and CM TAG responses for both meals. A possible explanation may reside in the fact that both the control and MetS subjects were quite heterogeneous in their characteristics. For example, those defined as MetS did not have excessively high plasma TAG levels at baseline. Reports in the literature have observed an increased postprandial plasma TAG levels in men presenting with MetS relative to age matched controls (6) and in men with mild to moderate cholesterolaemia (34). Recently, Adiels et al (35) did not find a difference in postprandial CM response between subjects with and without type II diabetes, which they attributed to a lack of difference in fasting CM clearance rates between the two groups.

The third major finding was the reduced CM TAG concentrations following the dairy-based meal relative to the vegetable oil-based meal. There are a number of possible explanations for the lowered postprandial CM TAG response to a high fat dairy-based meal. Firstly, the higher concentrations of short and medium chain FA present in dairy products may have resulted in absorption of these FA into the portal system rather than incorporation into CM (16, 36) owing to

the preferential positioning of the shorter chain FA in the *sn*-1 and *sn*-3 positions in the TAG molecule, and to the water solubility of the shorter chain FA. Swift et al (37) fed subjects different liquid formulas (40% energy from fat) containing either medium or long chain FA. The CM TAG concentration after consuming the medium chain FA was only 20% of that after the long chain FA infusion despite there being the same amount of fat in the infusions. After the medium chain FA infusion, only 8-13% of CM TAG could be accounted for from medium chain FA compared with a 90% contribution of long chain FA to CM TAG after a long chain FA infusion. In addition, the 8:0 content of the CM was significantly lower than the 10:0 content even though the medium chain FA infusion contained more than twice as much 8:0 compared with 10:0. Our study demonstrated that medium chain FA were incorporated into CM TAG, but at a much reduced level compared with long chain FA. As seen from the FA composition and TAG molecular species data, 8:0 and 10:0 are probably transported mainly *via* the portal venous route, as noted previously (17). It is possible that analysis of the plasma free FA composition during the postprandial phase may have detected these medium chain FA after the dairy meal.

Secondly, it is possible that the higher dairy calcium intake in the dairy-based meal could have contributed to the lowered postprandial chylomicronaemia, as reported by Lorenzen et al (38) and Lorenzen and Astrup (39). These authors ascribed the effect to increased fat excretion, presumably by the formation of insoluble calcium FA soaps. In the present study, faecal fat levels were not measured. It is noted that while the estimated total calcium content of both meals was similar, the dairy meal contained 595 mg of dairy-derived calcium compared with no dairy derived calcium in the vegetable oil-based meal. Soy milk in Australia is fortified with calcium up to the levels naturally occurring in milk. Studies have shown that dairy calcium results in higher fat excretion than an equivalent dose of calcium given as a supplement (38) and also a study by Heaney et al (40) observed that calcium fortified soy-beverages do not constitute a comparable source of calcium when compared with cow's milk. Taking these findings into consideration, we believe there is justification for proposing that the form of calcium i.e. dairy calcium may have contributed to the lower postprandial CM TAG response observed after the high fat dairy meal. Based on the CM FA composition it can be seen (Figure 2) that the proportions of 14:0 and 18:0 were significantly lower in the CM TAG compared with the dairy meal, while the proportion of 16:0 was significantly raised. These data do not support a uniform potential loss of saturated FA as excreted complexes, following the dairy meal. The nature of the excreted fats should be investigated in future studies with dairy foods.

To conclude, in the most frequently applied analytical procedures for characterization of fats, the individual TAG molecular species, are largely overlooked. In this study, GC and HPLC-MS/MS methods were used to reveal the dynamic or non-steady state of postprandial TAG in CM; this dynamic state is likely to be a reflection of differential rates of CM secretion and clearance which Adiels et al (35) regard as a significant determinant of postprandial CM accumulation. Such detailed information on human CM TAG is still scarce in the published literature. We cannot conclude from the present data, whether the molecular species of dietary fats differentially influence the rates of secretion of CM TAG or rates of clearance of the CM TAG by lipoprotein lipase, however this is a topic that should be investigated in future studies.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Table 1 Baseline characteristics of participants

	Participants with metabolic syndrome (n = 10)	Participants without metabolic syndrome (n = 9)	P value
Age (years)	53.7 (1.6)	51.4 (1.5)	0.54
Height (m)	1.75 (0.0)	1.78 (0.1)	0.32
Weight (kg)	106.6 (5.5)	83.2 (2.1)	0.00*
Body Mass Index (kg/m ²)	34.9 (1.9)	26.3 (0.8)	0.00*
Waist circumference (cm)	115.0 (3.5)	92.2 (2.0)	0.00*
Plasma Glucose (mmol/L)	6.5 (0.8)	5.4 (0.2)	0.02*
Plasma Triacylglycerols (mmol/L)	1.9 (0.2)	1.3 (0.1)	0.00*
Plasma HDL cholesterol (mmol/L)	1.1 (0.1)	1.3 (0.1)	0.02*
Energy intake (kj)	10178 (704)	10693 (560)	0.58

Values are mean $\pm$ SE

*Subjects with MetS were significantly different at baseline ($p < 0.05$) compared to controls (analysed by independent t-test)

Table 2: Fatty acid composition of chylomicron TAG three hours (180 minutes) and five hours (300 minutes) postprandially as molar percentages after the dairy-based breakfast and the vegetable oil-based breakfast. In the 44 comparisons made, the only significant differences between the groups (control vs MetS) were 16:0 180 min after the dairy meal ($p=0.037$), 18:3(n-3) 180 min after the vegetable oil based meal ($p=0.047$), and other fatty acids 300 min after the vegetable oil based meal ($p=0.022$). The lack of physiologically important differences between the two volunteer groups enabled pooling of the results in the manuscript to strengthen between-meal comparison.

FA	Dairy based breakfast meal				Vegetable based breakfast meal			
	180 minutes		300 minutes		180 minutes		300 minutes	
	MetS Mean (SD)	Control Mean (SD)	MetS Mean (SD)	Control Mean (SD)	MetS Mean (SD)	Control Mean (SD)	MetS Mean (SD)	Control Mean (SD)
10:0	1.3 (0.6)	2.0 (1.1)	0.7 (0.0)		0.8 (0.0)	0.8 (0.2)		
12:0	2.2 (0.7)	2.1 (0.9)	1.3 (0.6)	1.4 (0.6)	0.2 (0.1)	0.2 (0.1)	0.5 (0.3)	0.3 (0.1)
14:0	8.5 (2.0)	8.6 (1.9)	6.2 (1.8)	7.4 (2.0)	1.6 (0.3)	1.7 (0.4)	1.7 (0.3)	1.8 (0.3)
16:0	35.9 (2.2)	33.8 (1.9)	32.9 (2.6)	33.5 (1.9)	32.6 (2.3)	30.9 (2.7)	33.6 (1.0)	33.0 (2.9)
16:1(n-7)	3.9 (0.9)	3.8 (1.2)	4.3 (1.3)	4.2 (1.1)	2.3 (0.8)	1.6 (0.9)	2.0 (0.8)	1.9 (1.0)
18:0	5.8 (0.9)	5.5 (0.9)	5.1 (1.1)	4.3 (1.7)	4.1 (0.4)	4.4 (0.7)	4.2 (0.3)	4.1 (0.3)
18:1(n-9)	28.5 (2.9)	28.8 (3.0)	31.9 (3.2)	31.3 (2.3)	37.7 (2.1)	37.5 (1.9)	35.7 (1.2)	36.6 (2.6)
18:1(n-7)	1.6 (0.5)	1.6 (0.3)	2.0 (0.5)	1.8 (0.4)	1.8 (0.5)	1.8 (0.3)	1.7 (0.3)	1.7 (0.4)
18:2(n-6)	7.5 (2.0)	8.2 (1.6)	7.9 (1.9)	9.1 (1.9)	16.1 (3.0)	17.2 (1.6)	15.9 (2.5)	16.8 (2.7)
18:3(n-3)	1.0 (0.3)	1.1 (0.3)	1.0 (0.2)	1.1 (0.3)	2.1 (0.5)	2.6 (0.5)	1.6 (0.3)	1.9 (0.4)
Others	7.3	7.7	10.7	9.4	3.0	3.6	5.2	3.8

Table 3 Proportions of selected regioisomer pairs of dairy-based and vegetable oil-based breakfast TAG and the chylomicron TAG at three and five hours postprandially. The molar percentages of the isomers are presented in parenthesis.

	Dairy breakfast*	Chylomicrons 3h	Chylomicrons 5h
<i>sn</i> -18:1-18:1-18:0 + <i>sn</i> -18:0-18:1-18:1	73.4 ^a	75.2 ± 11.8 ^a (5.0)	83.1 ± 14.1 ^b (2.2)
<i>sn</i> -18:1-18:0-18:1	26.6 ^a	24.8 ± 11.8 ^a (1.8)	16.9 ± 14.1 ^b (0.6)
<i>sn</i> -18:2-18:1-18:1 + <i>sn</i> -18:1-18:1-18:2	31.3 ^a	47.7 ± 14.1 ^b (2.6)	62.6 ± 16.2 ^c (3.1)
<i>sn</i> -18:1-18:2-18:1	68.7 ^a	52.3 ± 14.1 ^{a,b} (2.9)	37.4 ± 16.2 ^b (1.9)
<i>sn</i> -18:2-18:2-18:1 + <i>sn</i> -18:1-18:2-18:2	100	n.d.**	n.d.**
<i>sn</i> -18:2-18:1-18:2	0	n.d.**	n.d.**
<i>sn</i> -16:0-18:1-18:1 + <i>sn</i> -18:1-18:1-16:0	58.0 ^a	80.2 ± 9.1 ^b (11.4)	87.6 ± 10.1 ^c (14.7)
<i>sn</i> -18:1-16:0-18:1	42.0 ^a	19.8 ± 9.1 ^b (2.7)	12.4 ± 10.1 ^c (1.9)
<i>sn</i> -16:0-16:0-18:1 + <i>sn</i> -18:1-16:0-16:0	n.a.	84.1 ± 10.9 (9.2)	87.4 ± 12.9 (8.9)
<i>sn</i> -16:0-18:1-16:0	n.a.	15.9 ± 10.9 (1.9)	12.6 ± 12.9 (1.4)
	Vegetable oil -based breakfast*	Chylomicrons 3h	Chylomicrons 5h
<i>sn</i> -18:1-18:1-18:0 + <i>sn</i> -18:0-18:1-18:1	100 ^a	88.7 ± 10.4 ^b (4.9)	95.7 ± 6.1 ^{a,b} (2.6)
<i>sn</i> -18:1-18:0-18:1	0 ^a	11.3 ± 10.4 ^b (0.7)	4.3 ± 6.1 ^{a,b} (0.1)
<i>sn</i> -18:2-18:1-18:1 + <i>sn</i> -18:1-18:1-18:2	50.9 ^a	44.8 ± 10.3 ^b (5.0)	58.1 ± 11.7 ^c (5.1)
<i>sn</i> -18:1-18:2-18:1	49.1 ^a	55.2 ± 10.3 ^b (6.2)	41.9 ± 11.7 ^c (3.7)
<i>sn</i> -18:2-18:2-18:1 + <i>sn</i> -18:1-18:2-18:2	100 ^a	92.9 ± 9.2 ^b (5.3)	81.1 ± 15.7 ^c (3.6)
<i>sn</i> -18:2-18:1-18:2	0 ^a	7.1 ± 9.2 ^b (0.4)	18.9 ± 15.7 ^c (0.9)
<i>sn</i> -16:0-18:1-18:1 + <i>sn</i> -18:1-18:1-16:0	100 ^a	92.4 ± 7.7 ^b (11.9)	96.4 ± 6.1 ^{a,b} (14.1)
<i>sn</i> -18:1-16:0-18:1	0 ^a	7.6 ± 7.7 ^b (0.9)	3.6 ± 6.1 ^{a,b} (0.5)
<i>sn</i> -16:0-16:0-18:1 + <i>sn</i> -18:1-16:0-16:0	99.7 ^a	42.9 ± 13.8 ^b (3.6)	44.8 ± 16.0 ^b (4.3)
<i>sn</i> -16:0-18:1-16:0	0.3 ^a	57.1 ± 13.8 ^b (4.9)	55.2 ± 16.0 ^b (5.5)

* The non-availability of standards of the numerous TAG in the dairy fat including the low molecular weight TAG prevented the calculation of the meal TAG into molar percentages

** The regioisomer was detected in 4 out of 19 subjects at the 3 h time point and one out of 19 subjects at the 5 h samples. In the samples that contained this regioisomer pair, it was >94% in the form of *sn*-18:2-18:2-18:1 + *sn*-18:1-18:2-18:2

n.a. not available

Data marked with different letters differ significantly ($p < 0.05$) in each row.

.

Figure legends

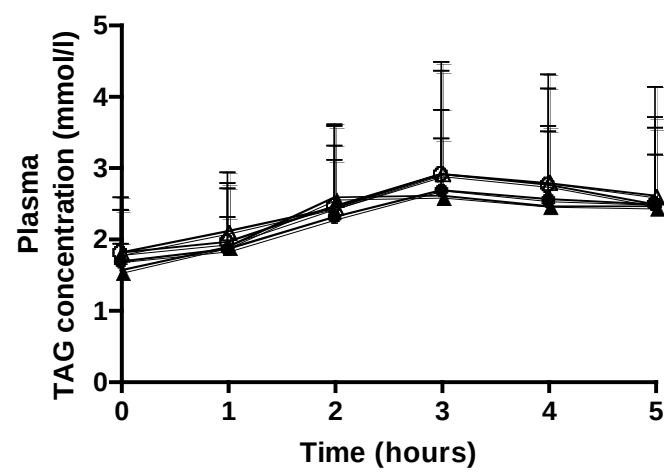
Figure 1 Concentration of plasma and chylomicron TAG after consuming high fat dairy-based and vegetable oil-based breakfasts in subjects with MetS (n = 9) and control subjects (n = 10). A: no effect on diet or MetS on postprandial plasma TAG concentrations; B: CM TAG concentrations were significantly higher after a vegetable oil-based oil breakfast compared with the a dairy breakfast in MetS and control subjects at all timepoints except baseline *(significant at 1 h, 2 h, 3 h, 4 h and 5 h (p<0.05)). Figures are mean $\pm$ SD; ○ = MetS dairy; ● = control dairy; △ = MetS vegetable; ▲ = control vegetable

Figure 2 Fatty acid composition of meals (white bars) and chylomicron TAG three hours (light gray bars) and five hours (dark gray bars) postprandially (mean and sd, n=19) as molar percentages after the dairy-based breakfast (A) and the vegetable oil-based breakfast (B). Data of subjects with and without metabolic syndrome are pooled, as there were no differences between the groups. Data marked with different letters differ significantly (p<0.05) in each fatty acid

Figure 3 Major (molar prevalence of more than 2% of all measured TAG in the TAG of the breakfast or in chylomicron TAG) chylomicron TAG species after the dairy-based breakfast (A) and the vegetable oil-based breakfast (B) (mean and standard deviation). Black bars represent the meal, and the following bars chylomicrons at the times of blood draw: 1h, 2h, 3h, 4h and 5h. Between time (one to five hours) differences (ANOVA for repeated measurements) are significant (p < 0.05) excluding the following TAG: 16:0/16:0/18:2 and 16:0/16:1/18:1 after the dairy meal. Data of subjects with and without metabolic syndrome are pooled, as there were no differences between the groups. Values are molar percentages, but between TAG differences should be considered semi-quantitative due to lack of individual standards (see Methods section).

Figure 1

A



B

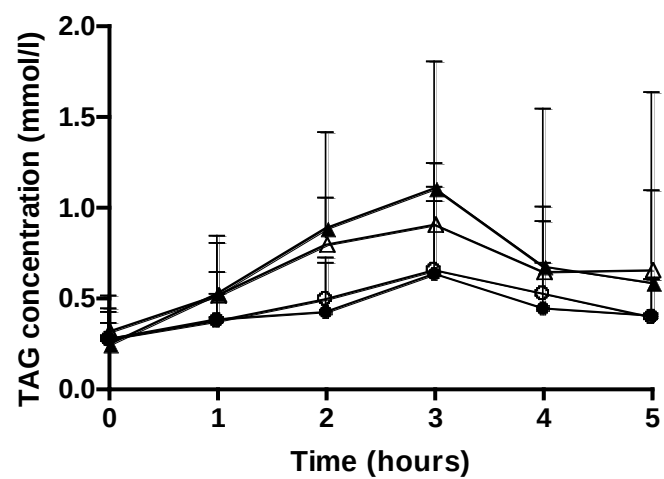


Figure 2

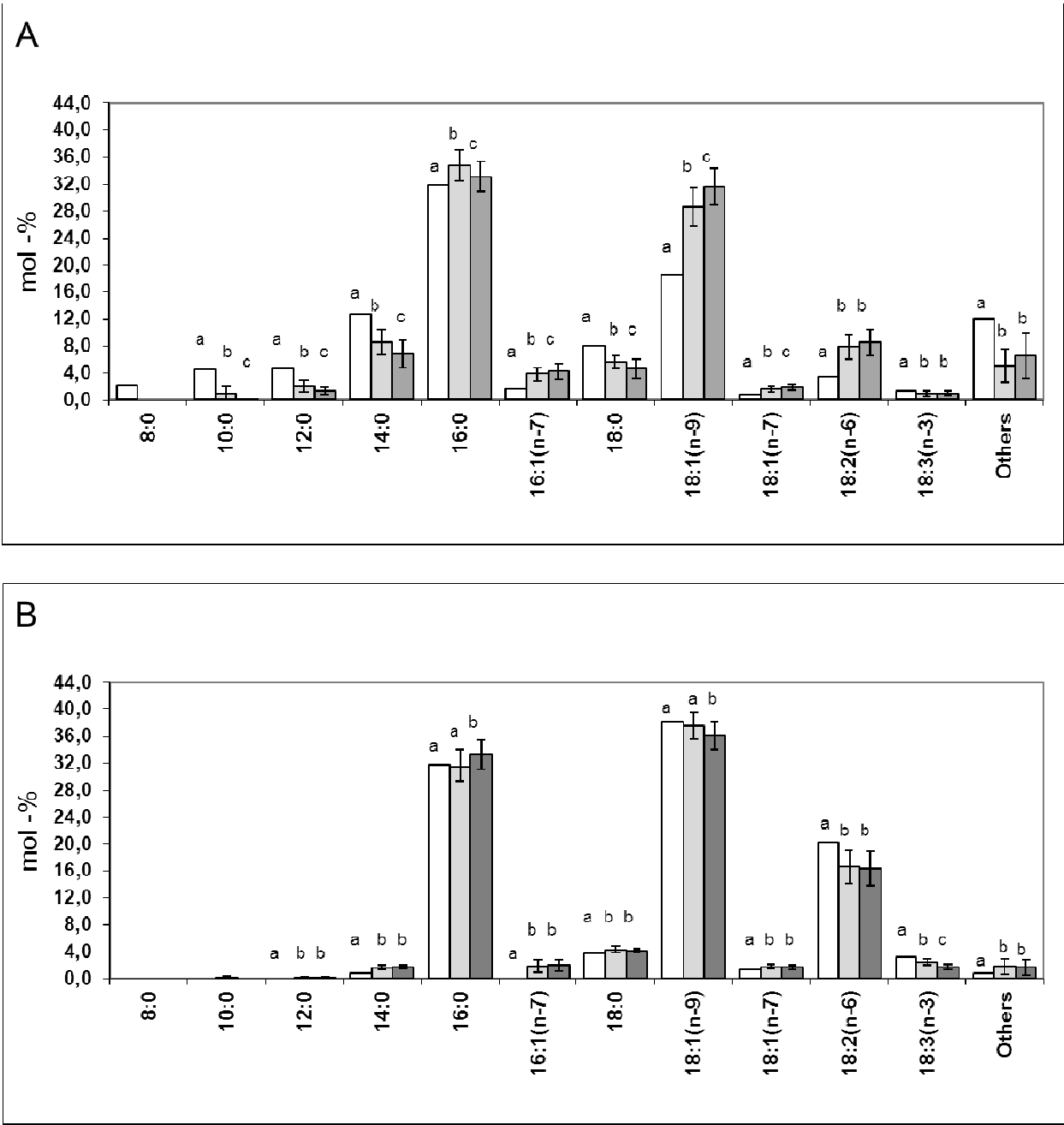


Figure 3

