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A meal rich in palm oil or butter modifies the sphingolipid profile of postprandial triglyceride-rich lipoproteins from type 2 diabetic women

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Abbreviations: CE, cholesteryl esters ; Cer, ceramides ; CM, chylomicrons; T2D, type 2 diabetes ; GM3, ganglioside GM3 ; Palm Nut, hazelnut-cocoa palm oil spread ; PL, phospholipids ; Spha, sphinganine ; SP, sphingolipids ; SM, sphingomyelins ; Spho, sphingosine ; S1P, sphingosine-1-phosphate ; SPC, sphingosylphosphorylcholine; TG, triglycerides ; TGRL, triglyceride-rich lipoproteins.

Keywords: ceramides, chylomicrons, dietary lipids, tandem mass spectrometry, type 2 diabetes.

Highlights

- Elevated postprandial triglyceride-rich lipoproteins (TGRL) and triglycerides are risk factors for cardiovascular disease.
- Some plasma sphingolipids are bioactive lipid mediators in type 2 diabetes (T2D).
- Higher concentrations of ceramides (Cer) and ganglioside GM3 in fasting T2D women.
- A fat-rich meal increased Cer concentrations in postprandial TGRL from T2D women compared to fasting ones.
- A fat-rich meal particularly increased C16:0, C24:0 and C24:1 Cer in postprandial T2D TGRL.

Abstract

Elevated concentrations of triglyceride-rich lipoproteins (TGRL) in the fasting and postprandial states are risk factors for cardiovascular events, especially in type 2 diabetes (T2D). T2D modifies the lipid composition of plasma and lipoproteins and some sphingolipids (SP) have been validated as potent predictive biomarkers of cardiovascular disease occurrence. The main objectives of the present study were to characterize the plasma SP profile in fasting T2D patients and to determine whether SP are modified in postprandial TGRL from these patients compared to fasting TGRL. In a randomized parallel-group study, 30 T2D women ingested a breakfast including 20g lipids from either hazelnut cocoa palm oil-rich spread (Palm Nut) or Butter. Plasma was collected and TGRL were isolated by ultracentrifugation at fasting and 4h after the meal. Fasting samples of 6 control subjects from another cohort were analyzed for comparison. SP were analyzed by tandem mass spectrometry. Plasma from fasting T2D patients had higher ceramide (Cer) and ganglioside GM3 concentrations, and lower concentrations of sphingosylphosphorylcholine vs healthy subjects. In postprandial TGRL from T2D patients compared to those in the fasting state, Cer concentrations and especially C16:0, C24:1 and C24:0 molecular species, increased after the Palm Nut or Butter breakfast. A positive correlation was observed in the Palm Nut group between changes (Δ 4h-fasting) of summed C16:0+C22:0+C24:1+C24:0 Cer concentrations in TGRL, and changes in plasma TG, TGRL-TG and TGRL-C16:0 concentrations. Altogether in T2D, the altered profile of plasma SP and the increased Cer concentrations in postprandial TGRL could contribute to the increased atherogenicity of TGRL.

1. Introduction

It is now well established that the plasma lipidome is modified in metabolic diseases, such as type 2 diabetes (T2D). Besides changes in major lipid classes (triglycerides (TG), cholesteryl esters (CE), non-esterified cholesterol and phospholipids (PL)), plasma concentrations of some sphingolipid (SP) classes, such as ceramides (Cer), sphingomyelins (SM), sphingosine-1-phosphate (S1P) and GM3 ganglioside (GM3), among others, are altered in the plasma of T2D patients compared to healthy control subjects [1-4] and recent studies validated plasma SP as candidate biomarkers of cardiovascular disease [5]. There is also growing interest in the lipidomic analysis of plasma lipoproteins, since the distribution and the concentrations of lipids differ across lipoprotein classes [6]. Dyslipidemia in T2D results in significant alterations of lipoprotein lipids which have a contributing impact on the functional properties of lipoproteins leading to increased cardiovascular risk in metabolic diseases. However, most studies have investigated modifications of major core lipids in cholesterol-rich lipoproteins (LDL and HDL) compared to studies focusing on minor surface lipids such as SP. Regarding T2D, a few studies reported that minor lipid composition of HDL and LDL is modified [7,8], causing alteration in their functionality [9]. For example, compared to particles from control subjects, HDL from diabetic patients are depleted in S1P [10,11], and this could alter their cardioprotective function [10]. It was also shown that modification in phosphatidylcholine (PC) and SM contents of HDL2 and HDL3 could affect their capacity to manage cholesterol trafficking in cells expressing SR-B1 [12]. LDL from dyslipidemic T2D patients contained more diacylglycerol and less CE in their lipid core as well as a reduction in SM, Cer and lysophosphatidylcholine [13].

Besides LDL and HDL, elevated concentrations of triglyceride-rich lipoproteins (TGRL) are recognized as a risk factor for ischaemic cardiovascular diseases [14]. TGRL are constituted of chylomicrons (CM), VLDL as well as their remnants, whose composition varies according to the amount and type of dietary fats [15]. Many epidemiologic studies have well established that an increase of postprandial TG is an independent risk factor for cardiovascular events incidence, especially in diabetic women compared to men [16-18]. Among postprandial lipids, polar lipids such as SP are present in various foods, particularly

dairy products mainly in the form of SM [19]. Recent studies from our laboratory showed that, compared to soybean polar lipids, the ingestion of milk polar lipids in mice led to an early increase in postprandial lipemia, a faster clearance of CM from plasma as well as increased concentrations of SM and C24:1 Cer in CM [20]. In post-menopausal overweight women, a 4-week milk polar lipid supplementation lowered total-, LDL-cholesterol and TG at fasting and postprandially [21] and decreased some SM and Cer species both in plasma and CM [22]. Specific nutritional interventions, such as the PREDIMED trial, showed positive associations between specific Cer species (C16:0, C22:0, C24:1 and C24:0) and the incidence of cardiovascular events [23]. Thus, there is growing interest towards the modulation of the SP composition of the diet to prevent metabolic disorders and/or improve/restore metabolic status [24]. Our objectives were to characterize the SP profile of plasma and TGRL from T2D patients and more specifically, to study the SP composition of plasma and TGRL isolated before and after a mixed breakfast meal including a realistic amount of lipids (20g) from a vegetable palm oil source or from butter.

2. Material and Methods

2.1. Study participants

Thirty women with T2D were recruited for a parallel-group clinical trial described in a previous publication (COMPLETE, clinical trial registration number at ClinicalTrials.gov: NCT03561571) [15]. The primary outcome of the COMPLETE study was to determine the effect of fasting and postprandial TGRL on thromboxane B₂, a marker of platelet activation [15]. Analysis of the sphingolipidome of plasma and TGRL was one of the predefined secondary outcomes. After a 12 hour overnight fast, blood was drawn and the patients ingested a breakfast containing 20g lipids from either hazelnut-cocoa spread (Palm Nut) rich in palm oil (n=15) or Butter (n=15). A second blood sample was collected 4 hours (T240) after the ingestion of the breakfast.

In order to validate the fasting SP phenotyping of COMPLETE T2D volunteers with that of healthy subjects, fasting plasma from six healthy women (control, CTL) who were part of a previous trial (FiTaBlé, clinical trial registration number at ClinicalTrials.gov: NCT03717311) were used. CTL women had normal fasting glycemia, triglyceridemia and cholesterol concentrations. Both trials were

approved by a national Scientific Ethics Committee (COMPLETE ID-RCB: 2018-A01414-51; FiTaBlé ID-RCB : 2018-A00949-46) and conducted in accordance with the principles of the Helsinki declaration. Informed written consent was obtained from all subjects.

Blood was collected into EDTA tubes and plasma was immediately isolated by centrifugation ($1500 \times g$, 10 min, at 4°C) and stored in aliquots at -20°C . The clinical and metabolic characteristics of the participants are shown in Table 1. T2D patients were obese, had a long history of diabetes, mild dyslipidemia with mild hypertriglyceridemia, normal HDL cholesterol and moderately high LDL cholesterol. Regarding the anti-diabetic and lipid lowering medication, 28 of 30 patients had metformin, 18 had sulfonylurea, 11 had glucagon-like peptide 1 analog, 8 had gliptin, and 1 had acarbose. In addition, 17 of 30 had statins and 1 had a combined therapy of statins and ezetimibe. None was treated with fibrates or insulin. Healthy CTL subjects had all the parameters mentioned above in the expected normal range.

2.2 Test Breakfasts Composition

Both experimental breakfasts contained 20g lipids and were similar in carbohydrates, proteins, and caloric contents (supplemental Table S1). The Palm Nut breakfast provided 439 calories with 51.1%, 40.9%, and 6.5% of energy as carbohydrates, lipids including a commercial hazelnut-cocoa refined palm oil spread (60 g), and proteins, respectively. The Butter breakfast was composed of 51.7% carbohydrates, 40.9% lipids including commercial butter (22.5 g, 82% fat) and 4.6% proteins for a total of 440 calories. Regarding the total fatty acid (FA) composition of both fats, Palm Nut mostly contained palmitic (C16:0, 33.2%) and oleic (C18:1 n-9, 45.2%) acids. Butter contained 78.8% saturated fatty acids, including 42.2% palmitic acid [15]. Regarding SP, Palm Nut in the breakfast contained 0.03 mg Cer, 1.03 mg SM and 0.03 mg GM3 while 22.5g butter contained 0.38 mg Cer, 6.70 mg SM and 0.28 mg GM3.

2.3. Isolation of TGRL fractions and determination of protein concentration

TGRL fractions were isolated from the plasma by ultracentrifugation at $9777 \times g$ for 1 hour at 12°C using a Beckman Coulter Optima TLX ultracentrifuge and a Beckman TLA 100.2 rotor. Top 100 μL of

the floating layer corresponding to TGRL with density <1.000 g/ml was collected and immediately stored at -20°C. Protein concentration of the fractions was measured using a modified Lowry method [25] prior to storage. The hydrodynamic diameter of TGRL was measured by dynamic light scattering at 25 °C with a Zeta-Sizer NanoS (Malvern, UK) [15].

2.4. Determination of plasma and TGRL sphingolipid compositions

2.4.1. Lipid extraction

Lipids from plasma (10 µL) and TGRL fractions (100 µL) were extracted in a single phase chloroform:methanol (1:2) procedure after addition of a mix of internal standards in methanol (details in supplemental Table S2). The lipid containing phase was evaporated with nitrogen and dried lipid extracts were stored at -20°C.

2.4.2. Saponification and purification

Dried lipid extracts were resuspended in 1.5 ml of chloroform:methanol (1:2) and saponified with 150µl of 1M KOH solution. Samples were incubated for two hours at 37°C and pH was adjusted to neutrality with acetic acid. Purification was performed on a solid phase extraction (SPE) C18 column by a double extraction using methanol:H₂O (1:1). Elution was successively done with methanol:H₂O (12:1) for GM3 and chloroform:methanol (1:2) for Cer and SM. Samples were evaporated with nitrogen and homogenized with chloroform:methanol (2:1) before direct injection in the mass spectrometer.

2.4.3. Tandem mass spectrometry analysis

Samples were homogenised in chloroform:methanol (1:2 v/v) and analysed by direct flow injection on a triple-quadrupole mass spectrometer (API 4500 QTRAP MS/MS; Sciex Applied Biosystems, Toronto, Canada) in the positive ionization mode for Cer and SM and in the negative ionization mode for GM3 (supplemental Table S2). These three classes of SP were quantified separately with the Multi Reaction Monitoring (MRM) method with a flow rate of 200 µl/min (analysis time of 3 minutes). The concentration of each molecular species (12 species for each class) was calculated from the ratio of its signal to that of the corresponding internal standard. Total Cer, SM and GM3, corresponded to the sum of the various species. Data were expressed either as µmol/L plasma or µmol/g TGRL protein. Results

are presented based on the assumption of sphingosine d18:1 as the major sphingoid base for determined GM3, SM and Cer species.

2.5. Determination of plasma and TGRL sphingoid bases (sphingosine-1-phosphate, sphingosine, sphinganine) and sphingosylphosphorylcholine compositions

2.5.1. Lipid extraction

Extraction of sphingoid bases and sphingosylphosphorylcholine (SPC) was based on the method of Pettazzoni et al. [26]. Plasma and TGRL fractions (200 μ L) were precipitated with 500 μ L of a mix of internal standards in methanol and 500 μ L of 2% H_3PO_4 (details in supplemental Table S2). Samples were centrifuged and the supernatant was directly transferred in SPE columns Oasis cartridge (MCX, Waters Corp) previously conditioned with 2% H_3PO_4 and methanol. Columns were washed with 2% formic acid and elution was performed successively with 0.2% formic acid in methanol and 2% NH_4OH in methanol. Lipid extracts were evaporated with nitrogen and redissolved with 500 μ L of 0.2 % formic acid in 37.5% acetonitrile.

2.5.2. Liquid chromatography and tandem mass spectrometry analysis

Liquid chromatography (LC) - tandem mass spectrometry (MS/MS) analysis was performed using two LC pumps (LC20AD) and an autosampler (SIL20AC) (Prominence Liquid Chromatograph, Shimadzu©, Kyoto, Japan) coupled to an API 4500 QTrap (Sciex Applied Biosystems, Toronto, Canada) operated in the MRM mode. MS/MS parameters and retention time (RT) for each molecule are described in supplemental Table S2. Positive ionization mode was used for sphinganine (Spha), sphingosine (Spho) and SPC; negative ionization mode for sphingosine-1-phosphate (S1P). Reverse phase LC was performed on a C_8 column (Uptisphere® 120 Å, 3 μ m, 2.1 mm x 50 mm, Interchim©) maintained at 25°C (phase A: formic acid 0.2% in H_2O , phase B: formic acid 0.2% in acetonitrile). The following gradient was run at a flow rate of 0.4 mL/min. The gradient was initiated at 37.5% of phase A, 0 to 4 min : 37.5 to 50 % B, 4 to 8 min : 50% B, 8 to 8.5 min : 50 to 75% B, 8.5 to 9.5 min : 75% B, 9.5 to 10 min : 75 to 37.5% B, 10 to 13 min : 37.5 % B). Fifty microliters were injected and the total run time was 13 minutes. Quantification was performed with the Analyst software version 1.6.2, using IS

and a nine point external calibration curve (linear regression). Data were expressed in nmol/L or nmol/g TGRL protein.

2.6. Statistical analysis

Data were expressed as means \pm SEM. Normality of data distribution was assessed using d'Agostino-Pearson normality test. Differences between T240 and T0 were assessed using paired Student's *t*-test or Wilcoxon test. Differences were considered significant at $P < 0.05$ (2-sided test). All statistical analyses were performed using GraphPad Prism 8.0 (Graphpad Software, San Diego, CA).

3. Results

3.1. The sphingolipid concentrations of plasma and TGRL are modified in fasting type 2 diabetic patients compared to fasting control subjects

3.1.1. Plasma sphingolipids

Characterization of T2D volunteers of COMPLETE study at fasting shows that total Cer and GM3 concentrations were higher by 34% and 28% respectively in plasma from these T2D women compared to plasma from CTL subjects (T2D Cer: $15.5 \pm 0.6 \mu\text{M}$ vs CTL Cer: $11.0 \pm 0.7 \mu\text{M}$; T2D GM3: $12.6 \pm 0.7 \mu\text{M}$ vs CTL GM3: $10.2 \pm 0.3 \mu\text{M}$) (Fig. 1A). No differences in plasma SM concentrations were observed between T2D patients and CTL subjects. Regarding sphingoid bases and lysosphingolipids, S1P, Spho and Spha concentrations were similar between T2D patients and CTL subjects whereas decreased concentrations of SPC were found in T2D patients (T2D SPC: $3.5 \pm 0.5 \text{ nmol/L}$ vs CTL SPC: $10.2 \pm 2.1 \text{ nmol/L}$) (Fig. 1B). Quantitative analyses of 12 molecular species within each class of SP showed differences between T2D patients and CTL subjects (Fig. 2). In Cer, most species were higher in plasma from T2D patients, especially the most abundant very long chain ones C22:0 (+55%), C24:1 (+34%) and C24:0 Cer (+65%) (Fig. 2A). Most molecular species of SM were similar between T2D and CTL, excepted for a slight increase in minor C16:1 and C18:1 SM species (Fig. 2B). In GM3, increased concentrations of GM3 containing saturated fatty acids, i.e. C18:0 (+54%), C20:0 (+75%), C22:0 (+78%) and C24:0 (+31%), as well as C22:1 (+22%) were found in plasma from T2D patients (Fig. 2C).

Total plasma T2D Cer concentrations were positively correlated with plasma TG ($r=0.42$, $P=0.021$), total cholesterol ($r=0.61$, $P=0.0004$), LDL-cholesterol ($r=0.43$, $P=0.018$) and apoB concentrations ($r=0.46$, $P=0.014$). Amongst molecular species of Cer, C16:0 Cer was also positively correlated with plasma TG ($r=0.46$, $P=0.021$), total cholesterol ($r=0.52$, $P=0.021$), LDL-cholesterol ($r=0.40$, $P=0.021$) and apoB levels ($r=0.38$, $P=0.021$). Plasma most abundant very long chain C22:0 Cer and C24:0 Cer species were correlated with TG ($r=0.48$, $P=0.007$ and $r=0.47$, $P=0.008$, respectively), and C22:0+C24:0+C24:1 Cer species were correlated with total cholesterol ($r=0.61$, $P=0.0004$, $r=0.56$, $P=0.001$ and $r=0.49$, $P=0.006$, respectively). No correlation was found between SP and glycemic parameters (either glycemia, HbA1C, HOMA-IR).

3.1.2. TGRL sphingolipids

No significant differences were observed between total Cer, SM and GM3 concentrations in T2D patients vs CTL subjects when concentrations were expressed per mg of protein (Fig. 1C). Regarding sphingoid bases and SPC, only S1P concentrations, expressed per mg protein, was increased in T2D TGRL (Fig. 1D). Of note, when results were expressed per L of plasma, SM, GM3, S1P and SPC concentrations were higher in TGRL from T2D patients compared to those from CTL subjects, whereas no change in Cer concentrations was observed (data not shown).

3.2. The sphingolipid concentrations of TGRL are modified in T2D patients after the ingestion of a single meal

COMPLETE study in T2D women shows that the ingestion of 20 g lipids in a single meal led to modifications of SP concentrations in postprandial diabetic TGRL compared to fasting T2D TGRL (Fig. 3) without any change in plasma SP (supplemental Table S3). In particular, total TGRL Cer concentrations increased by 45% at T240 after the intake of the Palm Nut meal and by 40% following the Butter breakfast compared to fasting TGRL Cer concentrations (0.51 ± 0.01 vs 0.35 ± 0.04 $\mu\text{mol/g}$ protein in the Palm Nut group, 0.83 ± 0.09 vs 0.60 ± 0.09 $\mu\text{mol/g}$ protein) (Fig. 3A). SM concentrations tended to increase in postprandial TGRL from Palm Nut group (7.14 ± 0.53 vs 6.56 ± 1.47 $\mu\text{mol/g}$

protein, $P=0.09$) but no change was noted in postprandial TGRL SM from the Butter group. Neither meal modified the concentrations of postprandial GM3, sphingoid bases and SPC (Fig. 3B). Amongst the molecular species of TGRL Cer, C16:0, C22:0, C24:1 and C24:0 increased postprandially after the Palm Nut or Butter breakfast ($P=0.07$ for C22:0 Cer in the Butter group) (Fig. 4A). Accordingly, as an overall marker of atherogenic species, the sum of C16:0+C22:0+C24:1+C24:0 Cer was different between fasting TGRL and postprandial TGRL both in the Palm Nut group ($0.17 \pm 0.03 \mu\text{mol/g protein}$ vs $0.30 \pm 0.04 \mu\text{mol/g protein}$, $P=0.0006$, +78%) and in the Butter group ($0.35 \pm 0.07 \mu\text{mol/g protein}$ vs $0.53 \pm 0.07 \mu\text{mol/g protein}$, $P=0.014$, +52%). There was no effect of either meal on the molecular species concentrations of TGRL SM and GM3 (Fig. 4B and C).

3.3. Postprandial sphingolipids are correlated with triglycerides in TGRL from T2D patients

A positive correlation was observed in the Palm Nut group between postprandial changes (Δ T240-T0) of TGRL C22:0 Cer, C24:0 Cer, summed C16:0+C22:0+C24:1+C24:0 Cer concentrations and postprandial changes in plasma TG, TGRL TG as well as Δ TGRL total C16:0 concentrations (Figure 5). No significant correlation in the Butter group was noted regarding these changes. Total Δ TGRL Cer concentrations ($0.16 \pm 0.05 \mu\text{mol/g protein}$) tended to be correlated with Δ TGRL total C16:0 concentrations ($118.3 \pm 35.6 \text{ nmol/mg protein}$ i.e. C16:0 concentration in postprandial TGRL total lipids: $231.5 \pm 42.3 \text{ nmol/mg protein}$ minus C16:0 concentration in fasting TGRL lipids: $113.2 \pm 19 \text{ nmol/mg protein}$) in the Palm Nut group ($r=0.49$, $P=0.06$) while no significant correlations were observed in the Butter group. In the Butter group, total Δ TGRL Cer concentrations tended to be associated with Δ TGRL mean diameters ($r=0.46$, $P=0.08$).

4. Discussion

In the present study, targeted lipidomic analyses of SP by MS/MS reveal detailed compositions of SP classes of TGRL from fasting T2D patients, expanding data on altered SP concentrations in T2D plasma. First, our data show that plasma Cer concentrations were increased (+34%) in fasting obese women with T2D from COMPLETE study compared to healthy women, with the greatest increase being observed for the most abundant molecular species, C24:0 Cer (+65%) followed by C22:0 Cer and C24:1 Cer. Our

data are consistent with reported elevated plasma Cer (+30%) in fasting obese T2D patients [1], with increased concentrations of C18:0, C20:0, C24:1 Cer quantified subspecies and C24:0 Cer being the most abundant plasma ceramide subspecies. We also observed increased concentrations of plasma total GM3 and more precisely of saturated fatty acids subspecies in T2D patients, in accordance with higher serum levels of GM3 in hypertriglyceridemic patients or T2D patients with severe obesity [4]. The positive correlations that we observed between plasma Cer, specific C16:0 Cer concentrations and total cholesterol, LDL-cholesterol as well as TG confirm that Cer and its transport into lipoproteins are altered by the dysregulated metabolism of lipids in diabetes [27,28]. Regarding SM, the most abundant SP class, no change of its concentration was evidenced in obese T2D patients, while plasma SM levels have been reported to be lower in diabetic patients than in non-diabetic patients before bariatric surgery [29]. By contrast, an increase of total SM levels was observed in TGRL from T2D patients. Such an increase could reflect higher cholesterol absorbers as shown in VLDL [30]. A novel observation of the present study is the detection of S1P in TGRL and their increased concentrations in T2D patients vs CTL subjects.

It is well established that in the postprandial state, CM and their remnants are the main carriers of dietary lipids, including TG, CE and polar lipids, and that the transient accumulation of TG in CM generally constitutes the first and main change in the circulation [31]. We previously showed in our COMPLETE randomized study that a 20g fat meal led to increased concentrations of TG in postprandial TGRL isolated from T2D obese women [15]. Besides TG, TGRL are constituted of a polar surface including SP that possesses biological functions of specific interest in T2D and cardiovascular risk. In this ancillary part of the COMPLETE study, we show that the intake of this single meal enriched in saturated fats increased total Cer concentrations in postprandial TGRL compared to fasting TGRL. Detailed analyses of TGRL molecular species revealed specific increase of C16:0 and very-long chain molecular species (C22:0, C24:0 and C24:1). It is known that following a meal, absorbed dietary lipids are taken up by enterocytes which incorporate most of them into CM for secretion. Amongst fatty acids, palmitic acid (C16:0) is the precursor of *de novo* Cer synthesis whom first step involves the condensation of palmitoyl-CoA and serine *via* serine palmitoyl transferase (SPT) [32]. In the breakfast given to our T2D patients, palmitic acid represented at least 33% of total lipids in Butter and Palm Nut. Therefore,

the observed increased Cer concentrations in postprandial TGRL are likely to result from a *de novo* synthesis of Cer in the intestine through the supply of dietary palmitic acid. These results support studies performed in mice showing that a palm oil bolus increased plasma total Cer 4 hours after the gavage and that pre-treatment of rodents by myriocin, an inhibitor of SPT, abolished the increase of total Cer, suggesting a *de novo* ceramide synthesis in the small intestine [33]. In human studies, lipid infusion increased plasma Cer levels [34] and saturated fatty acid overfeeding of overweight people for several weeks increased plasma Cer [35] including very long chain species, C24:0 [36] and C24:1 [35]. Regarding SM, we found here that total SM concentrations tended to increase in postprandial TGRL from the Palm Nut group compared to fasting concentrations. FA composition of plasma SM has been described to be rather stable [37] and no effect of a 5-week supplementation on SM molecular species distribution amongst lipoproteins classes has been evidenced in young adults [38]. Although SP are present in dairy-derived foods, it is very unlikely that the increased content of SP in TGRL is reflective of a dietary contribution of pre-formed SP rather than *de novo* synthesis, due to the very limited amount of SP in the Palm Nut (most likely traces via its skim milk powder content) and Butter breakfasts. We did not detect any difference of changes ($\Delta T_{240} - T_0$) of total TGRL Cer concentrations between the Palm Nut and Butter groups, despite differences in FA compositions of the meals (33% C16:0 and 45% C18:1 n-9 in the Palm Nut or 79% saturated fatty acids including 42% C16:0 in the butter). Therefore, our results indicate that the single intake of 20g lipids containing at least 33% C16:0 is sufficient to modify the SP profile of intestine-derived TGRL and the FA composition of Cer. The effects of other dietary fatty acids, including monounsaturated fatty acids and polyunsaturated fatty acids, on the sphingolipid profile of TGRL certainly deserve further investigation.

Many experimental *in vitro* studies showed the role of distinct ceramides as bioactive lipid mediators and biomarkers of insulin resistance, T2D, and coronary artery disease [39]. A recent meta-analysis highlighted the importance of studying the molecular species of Cer that are significantly associated with the risk of adverse cardiovascular events in patients [40]. Our results in type diabetic patients having ingested the palm oil-based breakfast showed positive correlations between C22:0 Cer, C24:0 Cer, summed C16:0+C22:0+C24:1+C24:0 Cer and TG in TGRL, suggesting a contributing (direct or indirect) effect of TGRL Cer in potential deleterious effects of increased postprandial TG levels in

metabolic diseases. Such a correlation was not found after butter consumption. In this regard, functional pro-aggregant effects of postprandial TGRL on platelets were evidenced in our COMPLETE study [15]. In the prospective case-cohort PREDIMED study enrolling participants with high cardiovascular risk [23], a positive association was documented between plasma C16:0+C22:0+C24:1+C24:0 Cer and the incidence of cardiovascular events. However, further studies are needed to test the potential biological effects of Cer with different acyl-chain lengths.

Although the small sample size is a limitation of our study, it is the first time to our knowledge that the distribution of SP classes and molecular species in fasting and postprandial TGRL from T2D patients is described. Our study only included women with T2D, because of their higher risk for cardiovascular morbidity and mortality compared to men with diabetes, so our results would need to be confirmed in T2D men and patients with obesity. In conclusion, the ingestion of a single 20g fat-rich meal from a vegetable or dairy source by T2D patients led to increased concentrations of deleterious molecular species of Cer in postprandial TGRL to a similar extent than the diabetes status *vs* healthy status in the fasting state. Ceramides in TGRL, although minor components of the lipidome, can be modulated by dietary lipids and could contribute to the increased atherogenicity of postprandial TGRL in metabolic diseases beyond the traditional risk factors for atherothrombosis.

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Author contribution

M.M.B. : conceptualization, methodology, validation, formal analysis, investigation, data curation, writing - original draft, visualization; **C.C.** : conceptualization, validation, investigation, data curation, writing - original draft, visualization, supervision; **M.P.** : methodology, validation, formal analysis; **T.L-B.** : investigation, resources; **C.B.** : investigation; **M.D.F.** : investigation; **A.D.** : investigation; **S. L-P.** : resources; **J-A.N.** : resources; **P.M.** : conceptualization, resources, funding acquisitions, review and

editing; **M-C.M.** : conceptualization, formal analysis, writing - review and editing, visualization, supervision, funding acquisitions; **D.C.** : conceptualization, methodology, validation, formal analysis, investigation, data curation, writing - review and editing, resources, supervision. All co-authors read, commented, and approved the manuscript.

Declaration of competing interests

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Table 1. Clinical and metabolic characteristics of women with type 2 diabetes in the COMPLETE study and comparison with healthy control women.

Characteristics	Control subjects (n=6)	COMPLETE study				P
		Type 2 diabetes (n=30)	P	Type 2 diabetes Palm Nut group (n=15)	Type 2 diabetes Butter group (n=15)	
Age (years)	29.7 ± 1.7	65.5 ± 1.4	<0.0001	62.9 ± 2.3	68.1 ± 1.4	NS
BMI (kg/m ²)	23.2 ± 0.9	34.1 ± 1.0	<0.0001	34.4 ± 1.7	33.9 ± 1.2	NS
Duration of diabetes (years)	N/A	12.9* ± 1.4	-	12.5 ± 1.7	13.2‡ ± 2.4	NS
Glucose homeostasis						
HbA _{1c} (%)	N/A	7.8 ± 0.2	-	7.7 ± 0.3	7.8 ± 0.3	NS
Glycemia (mmol/L)	4.6 ± 0.1	9.0 ± 0.7	<0.0001	8.8 ± 1.0	9.3 ± 0.9	NS
Insulin (mU/L)	N/A	12.3* ± 1.3	-	11.2 ± 1.4	13.4‡ ± 2.3	NS
HOMA-IR	N/A	5.0* ± 0.7	-	4.1 ± 0.4	5.9‡ ± 1.4	NS
Plasma lipids (mmol/L)						
Triglycerides	0.6 ± 0.1	1.69 ± 0.1	<0.0001	1.53 ± 0.1	1.85 ± 0.3	NS
Total cholesterol	3.45 ± 0.6	4.55 ± 0.2	NS	4.49 ± 0.2	4.62 ± 0.2	NS
HDL cholesterol	1.10 ± 0.3	1.24 ± 0.1	NS	1.27 ± 0.1	1.21 ± 0.1	NS
LDL cholesterol	1.40 ± 0.3	2.56 ± 0.1	0.003	2.53 ± 0.2	2.60 ± 0.2	NS
Plasma apolipoproteins B (g/L)						
Total ApoB		0.89† ± 0.04	-	0.86‡ ± 0.1	0.92‡ ± 0.1	NS
ApoB100	N/A	0.88† ± 0.04	-	0.85‡ ± 0.05	0.91‡ ± 0.1	NS
ApoB48		0.006† ± 0.001	-	0.005 ± 0.001	0.006§ ± 0.001	NS

*: n=29, †: n=28, ‡: n=14, §: n=13. Data are presented as means ± SEM. NA: not available, N/A: not applicable, NS: not significant.

Figure legends

Fig.1. Total sphingolipid concentrations in plasma and TGRL from fasting type 2 diabetic patients of COMPLETE study compared with control subjects.

Plasma concentrations of total ceramides (Cer), sphingomyelin (SM), ganglioside (GM3) (A), sphingosine-1-phosphate (S1P), sphingosine (Spho), sphinganine (Spha) and sphingosylphosphorylcholine (SPC) (B). Triglyceride-rich lipoproteins (TGRL) concentrations, expressed as $\mu\text{mol per g}$ of total proteins, of Cer, SM, GM3 (C), sphingoid bases and SPC (D). Mass spectrometric analyses were performed as described in Materials and Methods. The bars represent means \pm S.E.M. for control subjects (n=6) and type 2 diabetic (T2D) patients (n=30). * $P < 0.05$.

Fig. 2. Plasma sphingolipid molecular species in fasting type 2 diabetic patients of COMPLETE study compared with control subjects.

Molecular species concentrations of plasma ceramides (Cer) (A), sphingomyelins (SM) (B) and gangliosides (GM3). Cer and SM molecular species annotations were based on the assumption that sphingosine d18:1 is the major sphingoid base. Data are shown as means \pm S.E.M. for control subjects (n=6) and type 2 diabetic (T2D) patients (n=30). * $P < 0.05$.

Fig. 3. Sphingolipid concentrations in triglyceride-rich lipoproteins from type 2 diabetic patients before (T0) and after the breakfasts (T240) in COMPLETE study.

Concentrations of total ceramides (Cer), sphingomyelin (SM), ganglioside (GM3) (A), sphingosine-1-phosphate (S1P), sphingosine (Spho), sphinganine (Spha) and sphingosylphosphorylcholine (SPC) (B) in TGRL from type 2 diabetic patients at fasting (T0) and 240 min. after breakfast (T240). Bars, expressed as $\mu\text{mol per g}$ of total proteins, are means \pm S.E.M. of 15 type 2 diabetic patients in each group. Palm Nut: hazelnut-cocoa palm oil spread.

Fig. 4. Sphingolipid molecular species in TGRL from type 2 diabetic patients before (T0) and after the breakfasts (T240) in COMPLETE study.

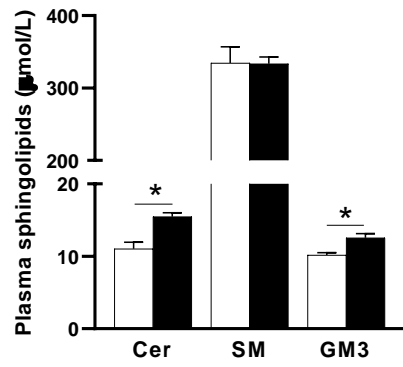
Molecular species concentrations of ceramides (Cer) (A), sphingomyelins (SM) (B) and gangliosides (GM3) (C) in TGRL from type 2 diabetic patients. Cer and SM molecular species annotations were based on the assumption that sphingosine d18:1 is the major sphingoid base. Data, expressed as μmol per g of total proteins, are means \pm S.E.M. of 15 type 2 diabetic patients in each group. Palm Nut: hazelnut-cocoa palm oil spread. $*P < 0.05$.

Fig. 5. Heat map of correlations between TGRL ceramide species and clinical parameters of type 2 diabetic patients before (T0) and after the breakfasts (T240) in COMPLETE study.

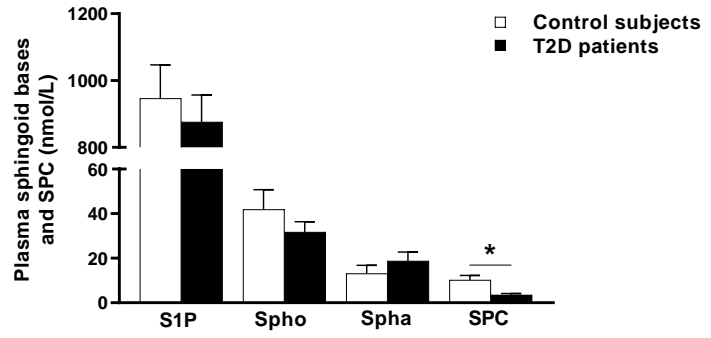
The (T240 - T0) differences (Δ) of selected parameters in type 2 diabetic patients having ingested the Palm Nut breakfast (n=15) or the Butter breakfast (n=15) were used to calculate Spearman's correlation coefficients between different ceramide (Cer) species and plasma triglycerides (TG), TGRL TG, C16:0 concentrations in TGRL total lipids, plasma apoB48 concentrations and hydrodynamic diameters of the TGRL. Asterisks show significant correlations. (*) $P < 0.10$; * $P < 0.05$; ** $P < 0.01$.

Figure 1

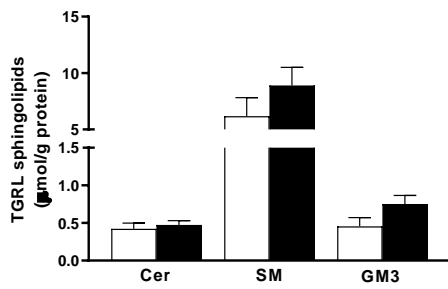
A



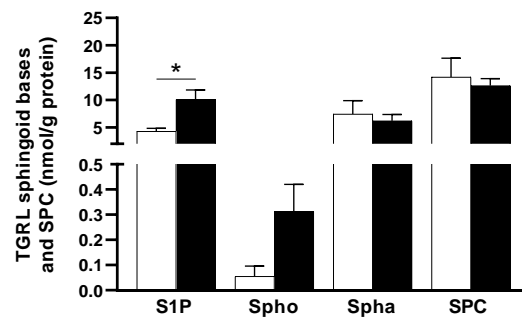
B



C



D



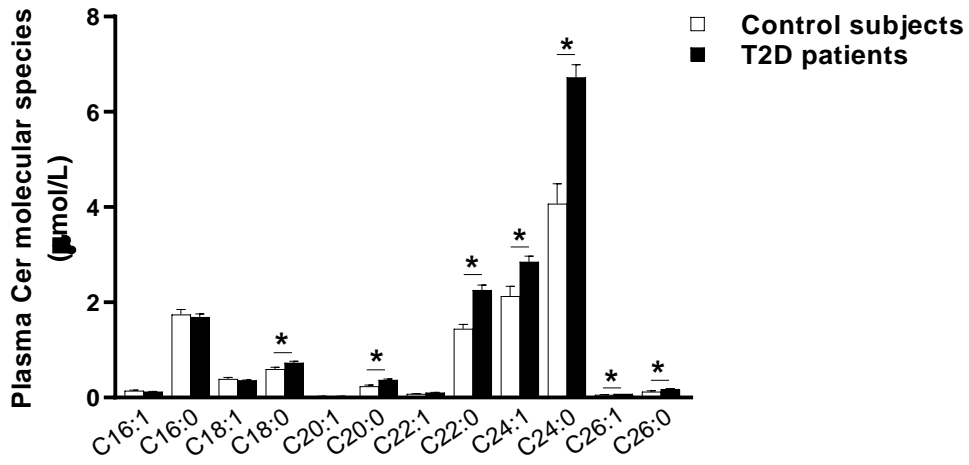
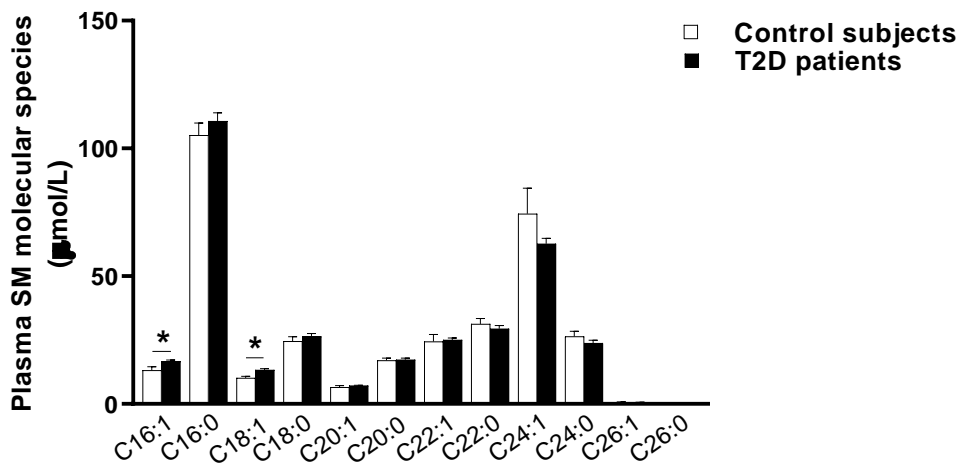
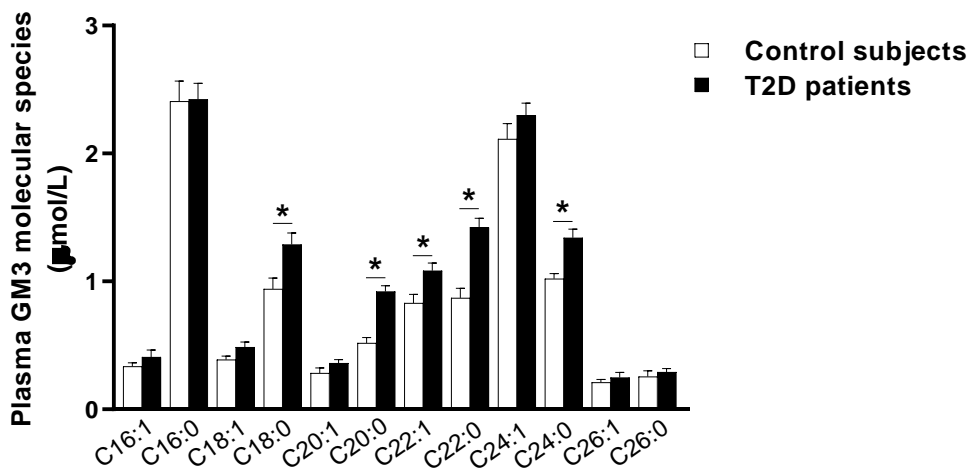
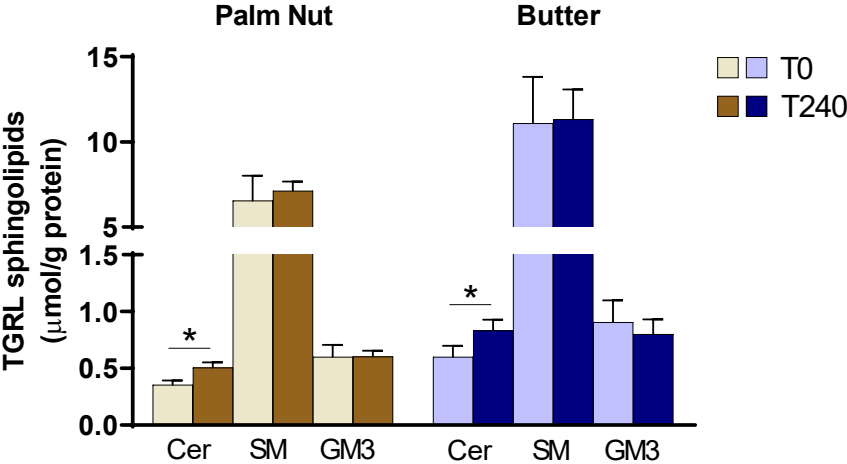
A**B****C**

Figure 3

A



B

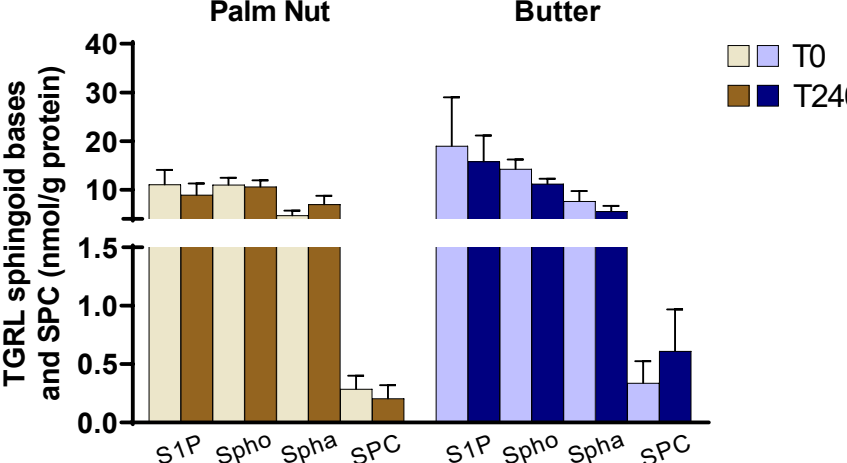
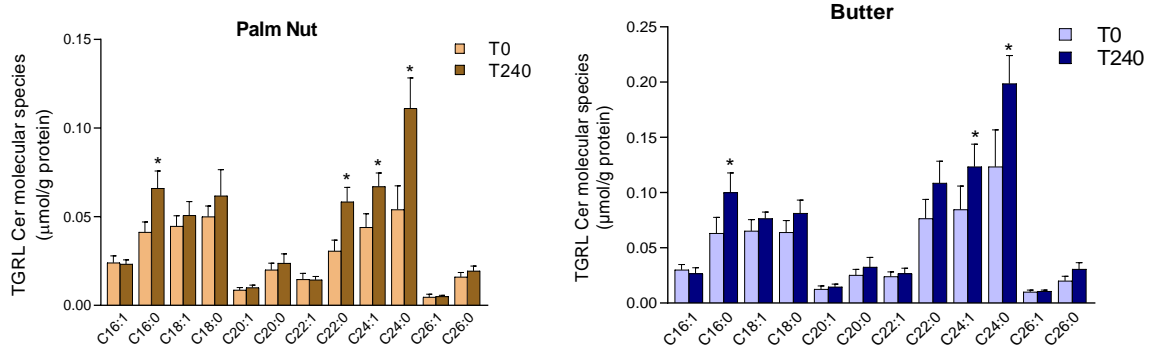
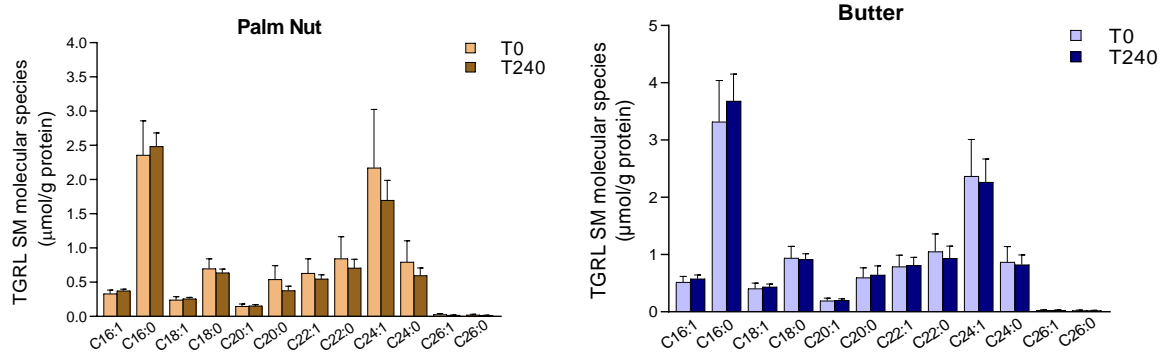


Figure 4

A



B



C

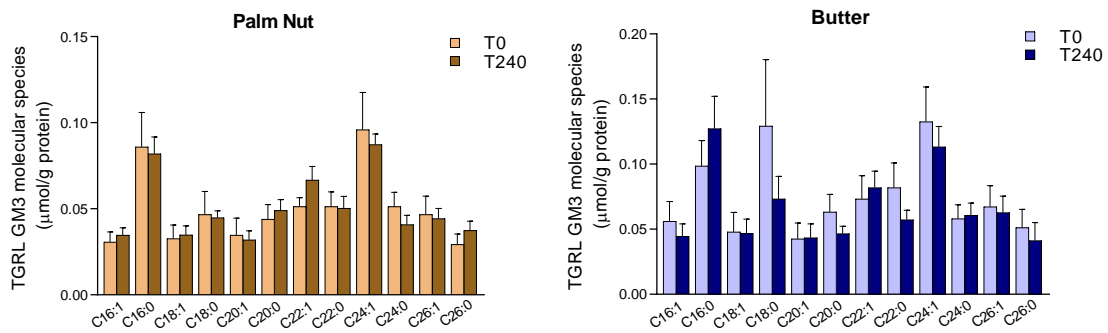


Figure 5

