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Marie Michèle Boulet, David Cheillan, Mathilde Di Filippo, Taïssia Lelekov-boissard, Charline Buisson, et al.. Postprandial Triglyceride-Rich Lipoproteins from Type 2 Diabetic Women Stimulate Platelet Activation Regardless of the Fat Source in the Meal. *Molecular Nutrition and Food Research*, 2020, 64 (19), pp.2000694. 10.1002/mnfr.202000694 . hal-03015175

HAL Id: hal-03015175

<https://hal.science/hal-03015175>

Submitted on 7 Dec 2020

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Postprandial triglyceride-rich lipoproteins from type 2 diabetic women stimulate platelet activation regardless of the fat source in the meal

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31 **Abbreviations list:** AA: arachidonic acid, CM: chylomicrons, HCS: hazelnut cocoa spread,

32 FA: fatty acids, LPL: lipoprotein lipase, PP: postprandial, PPP: platelet-poor plasma, PRP:

33 platelet-rich plasma, SFA: saturated fatty acid, TC: total cholesterol, TG: triglycerides,

34 TGRL: triglyceride-rich lipoproteins, TxB₂: thromboxane B₂

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36 **Keywords:** fatty acids, platelets, postprandial, triglyceride-rich lipoproteins, type 2 diabetes

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ABSTRACT

Scope. The aim of this study was to examine whether postprandial (PP) triglyceride-rich lipoproteins (TGRL) secreted after a moderate fat intake would activate platelets differently according to their fatty acid (FA) composition.

Methods and results. In a parallel single-blind randomized trial, 30 women with type 2 diabetes were assigned to a breakfast containing 20 g lipids from butter vs hazelnut cocoa spread rich in palm oil. Blood samples were collected at fasting and 4 h PP. FA composition of fasting and PP TGRL and their effects on the activation of platelets from healthy blood donors were assessed. Both breakfasts similarly increased plasma ApoB-48, plasma and TGRL triglycerides ($p < 0.05$). TGRL mean diameter increased after both breakfasts and was greater after the butter breakfast. Both breakfasts were rich in palmitic acid, and the HCS breakfast contained 45% oleic acid. TGRL FA composition reflected the dietary FA composition. Pre-incubation of platelets with fasting and PP TGRL increased collagen-stimulated aggregation ($p < 0.01$ vs control). Fasting and PP TGRL similarly increased agonist-induced thromboxane B_2 concentrations, and this effect was concentration-dependent for PP TGRL.

Conclusion. PP TGRL from type 2 diabetic women after a palm-oil spread vs butter-based mixed meal induce similar acute *in vitro* platelet activation.

1. INTRODUCTION

Type 2 diabetic (T2D) patients have a higher risk of cardiovascular diseases, including ischemic coronary artery events, atherosclerosis and atherothrombosis [1]. Epidemiologic studies also showed that women with T2D exhibit a worse cardiovascular risk profile [2] and have greater increase in cardiovascular morbidity and mortality compared to men [3]. Platelets from T2D individuals have been shown to be hyperactive, thus more easily prone to agonist-triggered aggregation in the event of plaque rupture [4]. Moreover, it is well documented that increased concentration of plasma triglycerides (TG), carried in the bloodstream by “triglyceride-rich lipoproteins” (TGRL) that are chylomicrons (CM), VLDL and their remnants, is an important risk factor in T2D [5]. Our group previously showed that large TGRL from fasting T2D patients enhanced *in vitro* platelet activation and aggregation [6].

It is now well acknowledged that postprandial (PP) TG concentrations are involved in cardiovascular risk. The concept of the atherogenicity of CM and VLDL was first introduced by Zilversmit 40 years ago [7]. Non-fasting TG levels were subsequently associated with the incidence of various cardiovascular events [8-12], independently of other risk factors [13, 14]. TGRL remnants are likely to be the main contributors of an enhanced atherosclerosis development [14, 15]. However, regarding atherothrombosis, the contribution of TGRL is not clearly established. The few studies on this topic were performed mostly in non-diabetic hyperlipidemic and normolipidemic individuals and had major differences in methodology. They showed that CM from both groups can have an inhibitory effect on platelet functions [16], while VLDL from healthy subjects [17] and remnants from healthy and T2D individuals as well as patients with lipoprotein lipase (LPL) deficiency [18] would have an enhancing effect. No study had been performed on the potential implication of PP TGRL from T2D

patients in atherothrombosis, and it is relevant to study, especially in this population at higher risk for cardiovascular complications.

Because the CM fraction of TGRL in the PP state is mainly constituted of dietary TG, the fatty acid (FA) composition of the ingested lipid influences their fatty acid composition in healthy individuals [19]. It is also known that there are exchanges of FA from plasma and lipoproteins to phosphatidylcholine in platelet membranes [20, 21]. Chain length of the saturated fatty acids (SFA) can also impact platelet aggregation. Studies in humans suggest deleterious stimulating effects of dietary long chain SFA on platelet function [22-24]. Also, it was shown that the PP state was associated with increased platelet activation in T2D patients following the consumption of a carbohydrate-rich meal and related to PP insulin [25, 26]. However the specific effect of TGRL secreted following the consumption of fats with different SFA composition on platelet aggregation has never been investigated in T2D women.

In this context, we hypothesized that PP TGRL from T2D women would have a modified FA composition following a realistic fat intake *via* a mixed meal from different food sources (dairy vs vegetal) and that this would change their effect on platelet activation and aggregation.

2. EXPERIMENTAL SECTION

Study participants

Thirty T2D women were recruited at the Department of Endocrinology and Metabolic Diseases, Cardiovascular Hospital, Lyon Bron (France) and randomized in one of the two groups. To be included, the subjects required to be women aged between 50 and 80 years old, with a post-menopausal status, a stable weight and a BMI ≥ 27 and ≤ 40 kg/m², an HbA_{1c} ≥ 6.5 and $\leq 10\%$ (≥ 48 and ≤ 86 mmol/mol) and no history of cardiovascular events or major hypertriglyceridemia. The anti-diabetic and lipid lowering medication was not discontinued for ethical reasons: 28 (93%) had metformin, 18 (60%) had sulfonylurea, 11 (37%) had glucagon-like peptide 1 analog, 8 (27%) had gliptin and 1 (3%) had Acarbose; none was treated with insulin injection. In addition, 17 (57%) had statins and 1 (0.03%) had a combined therapy of statins and ezetimibe. **Table 1** shows study participants characteristics. They were postmenopausal women with grade 1 obesity (mean BMI of 34.1 ± 1.0), and long duration of T2D (12.9 ± 1.4 years). They had low serum LDL cholesterol due to statin treatment and none was treated with fibrates. They had a mild increase in serum TG concentration (33.3% had plasma TG above 2 mmol/L).

Study design

The study is a randomized interventional parallel trial entitled *The Composition of Triglyceride Rich Lipoproteins and Platelet Activation in Type 2 Diabetes* (COMPLETE, clinical trial registration number at ClinicalTrials.gov: NCT03561571). The trial was approved by the Scientific Ethics Committee Ile de France VII and conducted in accordance with the principles of the Helsinki declaration. Informed written consent was obtained from all subjects. The subjects performed the trial from October 2018 to May 2019. A minimum sample size of 10 T2D women per group was necessary to detect a 30% difference in TxB₂

concentrations with an alpha level of 0.05 and 80% power, 15 women were recruited in each group to increase the statistical power. The primary outcome was the difference in TxB₂ concentrations in platelets incubated with fasting and PP TGRL. Secondary outcomes were the difference in the aggregation of platelets incubated with fasting and PP TGRL, the difference in TxB₂ concentrations in platelets incubated with TGRL collected after the consumption of a fat of different FA composition; and the difference in FA composition between fasting and PP TGRL from each group.

The interventions were randomized according to a block randomization performed by a biostatistician associated to the study using the R software (The R Foundation, Miami, FL). The block randomization (7 blocks of 4 and 1 block of 2 patients) was generated so that both treatments could be tested on platelets from the same donor in each block. The outcome assessors were blinded to the group assignment of the patients. After a 12 hour overnight fast, the first blood draw was made and the subjects were asked to eat the whole test breakfast according to their randomization group. A second blood sample was collected 4 hours after the ingestion of the breakfast. Blood sampling and test breakfast administration were done as part of the annual T2D follow-up at the outpatient clinic.

Test breakfasts composition

Both experimental breakfasts contained 20g of fats, one containing a majority of vegetal oil (mostly refined palm oil) within a commercial hazelnut-cocoa spread (HCS) and the other with butter. The FA composition of both fats was assessed by gas chromatography from 30 mg of butter and 70 mg of HCS with the same method as for the TGRL. Lipids were extracted with chloroform/methanol (2:1) according to the Folch method [27]. The HCS mostly contained palmitic (16:0) and oleic (18:1 n-9) acids. Butter contained a broader range of SFA

(Table 2). Both breakfasts were designed to be similar in carbohydrates, proteins and caloric contents. The butter breakfast was composed of plain crackers (30g), commercial butter (22.5g, 82% fat), jam (30g), orange juice (200 ml) and plain coffee or tea (200 ml) for a total of 440 calories, with 51.7%, 40.9% and 4.5% of energy as carbohydrates (56.9g), lipids (20g) and proteins (5g), respectively. The HCS breakfast contained plain crackers (30g), commercial HCS (60g) and plain coffee or tea (200 ml) providing 439 calories with 51.1%, 40.9% and 6.5% of energy as carbohydrates (56.1g), lipids (20g) and proteins (7.1g), respectively. All subjects were given a maximum of 20 minutes to eat the breakfast.

Plasma biochemistry

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. Measurement of ApoB-48 concentrations was performed by ELISA according to the manufacturer's instruction (SHIBAYAGI, Shibukawa, Gunma Prefecture, Japan). Total (TC), HDL cholesterol (HDL-c), TG, total ApoB and glucose were enzymatically determined using commercial kits (ABBOTT Diagnostics) on an Architect C16000 autoanalyser (ABBOTT Diagnostics, Illinois, USA). LDL cholesterol was calculated using Friedewald equation ($LDL-c = TC - HDL-c - 0.45 \times TG$). HbA_{1c} was determined by capillary electrophoresis using a Sebia Capillarys 3 (Sebia). Insulin was determined by immunoassay on an Architect I2000 autoanalyser. ApoB-100 was calculated by subtraction of ApoB-48 from total ApoB.

Isolation and characterization of TGRL fractions

In order to collect TGRL fractions, 250µL of plasma was carefully deposited under a layer of distilled water (850µL) followed 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 $<1.000 \text{ g/ml}$ was collected and stored at 4°C for no longer than 2 days or stored at -20°C for lipid analysis. Protein concentration of the fractions was measured using a modified Lowry method [28]. TG concentrations were obtained on an Architect C16000 autoanalyser. The hydrodynamic diameter of TGRL was measured by dynamic light scattering at 25°C with a ZetaSizer NanoS (Malvern, UK) using 1.0658 as viscosity and 1.445 as refractive index and 1.330 as refractive index of the aqueous phase [29].

TGRL fatty acid composition by gas chromatography

Total lipids were extracted twice from TGRL ($200\mu\text{L} + 300\mu\text{L H}_2\text{O}$) with ethanol:chloroform (1:3:6, respectively) after addition of 1,2,3-triheptadecanoyl-sn-glycerol as internal standard and BHT ($50\mu\text{M}$). FA were transmethylated with $\text{BF}_3/\text{methanol}$ (1.3 M, 10% in methanol) at 100°C for 1h30. FA methyl esters were extracted twice with isooctane and separated by gas chromatography using a gas chromatograph (HP6890 G1530) equipped with a SP 2380 capillary column ($0.25\mu\text{m}$, $30\text{m} \times 0.25\text{mm}$, Supelco, Bellefonte, PA, USA) and a flame ionization detector.

Platelet isolation

Venous blood was obtained from the local blood bank (Etablissement Français du Sang) on citrate-phosphate-dextrose anticoagulant (19.6 mmol/L citric acid, 89.4 mmol/L sodium citrate, $16.1 \text{ mmol/L NaH}_2\text{PO}_4$, 128.7 mmol/L dextrose; pH 5.6) from healthy volunteers who had not ingested any aspirin or anti-inflammatory drugs in the previous 10 days. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at $200 \times g$ for 17 minutes at 20°C .

PRP was collected, acidified to pH 6.4 with 0.15 M citric acid and centrifuged at $900 \times g$ for 12 minutes at 20°C. Platelet-poor plasma (PPP) was removed and platelets were suspended in a Tyrode-HEPES buffer (pH 7.35) [30]. The mean concentration for all platelet suspensions was 239 ± 24 G/L (min: 141 G/L, max: 354 G/L).

Platelet aggregation experiments

Aggregation was measured in isolated platelets in a Chrono-log dual-channel aggregometer (Coulter, Margency, France) according to the method of Born [31]. Platelet suspensions were preincubated for 5 minutes at 37°C in the presence or absence of different preparations of TGRL (25 µg proteins/mL) and then stimulated with subthreshold concentrations of collagen (0.13 ± 0.02 µg/mL) (type I from equine tendons, Diagnostica Stago, Asnières sur Seine, France) with continuous stirring at 1000 rpm. The subthreshold concentrations of collagen were defined as the concentrations of collagen that induced approximately less than a 10-20% increase in light transmission. The extent of platelet aggregation was expressed as percentage of change in light transmission 4 minutes after the addition of collagen [32].

Platelet thromboxane B₂ measurement

Freshly isolated platelet suspensions were incubated at 37°C for 30 minutes in the absence or presence of TGRL (25 µg proteins/mL) while being slowly agitated. Platelets were incubated at 37°C for an hour with non aggregating concentrations of collagen (0.5 µg/mL) or thrombin (0.05 U/mL) while being slowly agitated before being immediately stored at -80°C until TxB₂ measurement. Following 3 cycles of thawing and freezing of samples for platelets lysis, TxB₂ concentrations were determined by enzyme immunoassays according to the manufacturer's recommendations (Enzo Life Sciences Inc., Villeurbanne, France). Platelet proteins

concentrations were measured using the Bradford method [33] in order to express TxB₂ results.

Statistical analysis

Data were expressed as means \pm SEM. Normality of data distribution was assessed using d'Agostino-Pearson normality test. Paired Student's t-test or Wilcoxon test was used to assess differences between platelets incubated with TGRL and stimulated with an agonist compared to platelets stimulated with an agonist. Differences between platelets stimulated with an agonist compared to platelets alone were performed to ensure platelet functionality in response to agonists using Student's t-test or Wilcoxon test as well. Differences between T240 and T0 in each group were assessed using paired Student's *t*-test or Wilcoxon test and differences between the two groups for the means of variation deltas (T240-T0) were determined using unpaired Student's *t*-test or Mann-Whitney 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

Postprandial modification of plasma and triglyceride-rich lipoproteins

At the plasma level, consumption of both breakfasts increased ApoB-48 (HCS: +78%, Butter: +67%, $p < 0.001$ for both) and TG (HCS: +33%, Butter: +30%, $p \leq 0.0001$ for both) concentrations similarly (**Figure 1A and B**) at 4 hours after 20g fat meal. TGRL isolated from plasma of fasting T2D patients had a mean diameter of 146 ± 5 nm and a mean TG and total FA concentrations of 0.07 ± 0.01 mmol/L and 512 ± 6.5 nmol/mg protein, respectively. TGRL collected after the breakfasts had an increased diameter, TG and FA content 171 ± 4 nm, 0.12 ± 0.02 mmol/L and 907 ± 14 nmol/mg protein, respectively. TGRL mean concentration of TG was significantly increased in the PP state. As shown in **Figure 2A**, there was a 200% and 71% increase in TGRL-TG after the HCS and butter breakfasts, respectively ($p < 0.01$ for both groups). The type of fat does not impact TGRL-TG concentrations in PP plasma. Following the HCS breakfast, mean TGRL diameter was 10% higher, while it was increased by 23% after the butter breakfast (**Figure 2B**) ($p < 0.01$ for both groups). This increase in diameter after the butter breakfast was significantly higher than after the HCS breakfast (**Figure 2B**, $p < 0.01$).

As shown in **Table 3**, the main FA found in TGRL were palmitic (16:0), oleic (18:1 n-9) and linoleic (18:2 n-6) acids and their molar percents were not different between both groups in the fasting state. Ingestion of the HCS breakfast increased the proportion of oleic acid, the main MUFA, with decreased proportions of SFA and PUFA. After the butter breakfast, molar percentages of palmitic acid, the main SFA, and of medium chain SFA were significantly elevated, except for stearic acid (18:0), and proportions of PUFA were significantly decreased. As shown in **Figure 3**, there was a significant PP increase in TGRL SFA molar

percent (T240-T0) following the butter breakfast compared to the HCS one, while the MUFA molar percent increased in TGRL after the HCS breakfast compared to the butter breakfast.

Effect of fasting and postprandial TGRL on platelet aggregation

As shown in **Figure 4**, TGRL from fasting T2D patients increased collagen-stimulated platelet aggregation (HCS group: +62%, butter group: +86%, $p < 0.01$ for both) compared with collagen-stimulated platelets alone. When platelets were pre-incubated with TGRL isolated in the PP state, collagen-stimulated aggregation increased similarly in both groups (HCS group: +86%, butter group: +129%, $p < 0.01$ for both). When platelets pre-incubated with PP TGRL are compared to platelets pre-incubated with fasting TGRL, the difference was not significant.

Effect of fasting and postprandial TGRL on platelet thromboxane B_2 concentration

Concentrations of TxB_2 in collagen and thrombin-stimulated platelets alone or pre-incubated with TGRL from T2D patients before and after the test breakfasts are shown in **Figure 5**. When platelets were stimulated with non-aggregating concentrations of collagen, TGRL isolated in the fasting state in both groups significantly increased TxB_2 concentration similarly (HCS group: +28%, butter group: +31%, $p < 0.05$ for both) compared to collagen-stimulated platelets. TGRL isolated after the breakfasts did not further increase platelet TxB_2 concentration compared to TGRL collected in the fasting state (HCS group: +29%, $p = 0.07$, butter group: +23%, $p = \text{NS}$ vs control, **Figure 5A**). When platelets were pre-incubated with fasting and PP TGRL and stimulated with thrombin, there was an increase of TxB_2 platelet concentration (HCS group at T0: +54%, $p = 0.06$, HCS group at T240: +54%, $p = 0.02$, butter group at T0: +34%, $p = 0.007$, butter group at T240: +55%, $p = 0.001$ vs control) (**Figure 5B**).

We also tested the effect of a higher concentration of PP TGRL (50 µg/mL) on platelets, since TGRL concentration is elevated in the PP compared to the fasting state. The incubation of platelets with 50 µg/mL of PP TGRL significantly increased TxB₂ concentration compared to 25 µg/mL PP TGRL and fasting TGRL. This effect was not different between the HCS and butter group (**Figure 6**).

4. DISCUSSION

The association of non-fasting TG concentrations with the risk of ischemic cardiovascular events, independently of traditional risk factors, has highlighted the need of specific studies in order to understand the implication of PP TGRL on platelet function. Our randomized controlled study establishes that TGRL secreted in the PP state have a stimulatory effect on platelet aggregation. This finding extends our previous observation of the enhancing effect of large TGRL from fasting T2D patients on platelet aggregation [6]. This effect of PP TGRL was not dependent on their FA composition.

The concentrations of plasma TG and ApoB-48 increased in T2D patients after the ingestion of both realistic breakfasts in a similar way. This is in accordance with the observations of Sciarrillo *et al.* who showed that plasma TG levels in lean and obese subjects were increased in a similar manner after a meal containing 61% of calories (51-84g) from different fats (butter, coconut oil, olive oil and canola oil) [34]. Our results support the fact that PP TG metabolism is altered in the T2D population, because a similar lipemic response in healthy individuals would necessitate around 30-40g of lipids [35].

Residual fasting TGRL have a mean diameter of 146 nm, which is much larger than what is reported in healthy individuals. For example, Mekki *et al.* reported a mean diameter of ~91 nm for fasting TGRL in healthy individuals [36]. Interestingly, despite a similar amount of fat (20 g), we observed a greater diameter of PP TGRL 4 hours after the ingestion of the butter breakfast (183 nm) compared to the PP TGRL after the HCS breakfast (159 nm), and compared to the mean diameter reported for PP TGRL 4 hours after the consumption of a meal containing 40g of butter (~120 nm). In addition, our study shows that ingestion of a moderate amount of lipids (20g) leads to modification of the FA composition of TGRL 4 hours after the intake in T2D women. FA composition of PP TGRL reflects the main FA

composition of each fat consumed. TGRL secreted after the HCS breakfast, containing 45% of oleic acid, were enriched in MUFA (particularly oleic acid, 18:1 n-9). Those collected after the butter breakfast, which contained 79% of SFA (mainly palmitic acid, 16:0) and were accordingly rich in SFA (53% of total FA), especially palmitic acid (16:0).

The increased size of TGRL collected after the butter breakfast is intriguing and the difference in FA composition of TGRL after both breakfasts could be due to the positional distribution of fatty acids on the glycerol backbone of the TG molecules. In butter, palmitic acid (16:0) is mostly in the *sn*-2 position, while it is on the external *sn*-1 and 3 positions in TG from palm oil [37]. Our results are in line with studies on animal models demonstrating that the FA position at *sn*-2, particularly for palmitic acid (16:0), has a determining effect on its absorption and subsequent incorporation in TG [38, 39]. The FA in the *sn*-2 position of the ingested TG is favorably incorporated in the CM [40] and the presence of palmitic acid in the *sn*-2 position is likely to increase TG absorption [41]. However, in our case the palmitic acid content of both fats was similar and this may have minimized the effect of stereospecificity on TG absorption. Also, although the PP TGRL is significantly higher by 24 nm after the butter breakfast, it may not be enough of an increase to have a physiological impact on TGRL lipolysis.

Our results on the effect of TGRL from T2D patients on platelets show that PP TGRL significantly stimulate *in vitro* platelet aggregation. To the best of our knowledge, no study has already investigated the specific effect of PP TGRL from T2D patients on isolated platelets. For example, Aviram *et al.* tested the effect of different isolated lipoproteins fractions from healthy or hyperlipoproteinemic men after two meals containing 50g of fat either rich in SFA (25g) or PUFA (38g) on platelet aggregation. They observed that 3h after both meals, CM decreased collagen-stimulated aggregation while VLDL enhanced it in both groups [42]. An original aspect of our study is that we isolated and tested both fractions from

the fasting and PP state, which are different in composition. Indeed, the presence of large TG-rich particles in the basal state suggest that these are remnants of an inadequate CM and VLDL lipolysis [43], while the largest TGRL fractions isolated in the PP state contain CM and are representative of the heterogeneous mix of particles following a meal. We also show for the first time *in vitro* that an increase in TGRL, in physiological concentration, in the PP state has an enhancing effect on platelets TxB₂ concentration after a meal containing a realistic amount of fat rich in SFA. The increase in platelet activation and aggregation was similar after both fats, even though the TGRL FA composition was modified in PP. It is possible that an acute intervention of one breakfast containing a moderate quantity of lipids is not enough to cause a sufficient change in TGRL composition leading to a significant effect of TGRL FA on TGRL-induced platelet activation. A longer dietary intervention with different lipid amount and sources could induce more pronounced effects. Delgado-Lista *et al* studied in 20 healthy young men the effects of a chronic 28 days intake of a controlled diet followed by 3 tests meals containing 1g/kg of lipids of different FA composition [44]. A lipid-rich meal caused an increase of plasma tissue-type plasminogen activator, d-dimers and TxB₂ in the PP irrespective of the type of fat consumed, but not in the fasting state.

Our results show that fasting and PP TGRL also increase platelet TxB₂ concentration with differences in the extent of TGRL-induced stimulation according to the agonist used. We aimed to use physiological agonists that would not interfere with the lipid composition of TGRL and platelets (eg: arachidonic acid), and that would activate platelets by different signalling pathways. Tested collagen concentrations induce a moderate stimulation while used thrombin concentrations provoke strong platelet activation. The platelets used are from healthy donors, so it is possible that the effect of fasting and PP TGRL would be greater on platelets from T2D patients, since they are already hyperactive in the basal state.

Overall, we acknowledge some limitations to our study. The test fats were comprised in mixed meals so we cannot exclude that other components of the meals could have had an effect on TGRL composition and their effect on platelets, for example compounds from coffee or tea. Also, we could not obtain dietary data from the patients, which would have been useful to evaluate the usual fat intake, but randomization reduces the risk of bias in this case. Finally, our trial included only women, because of their higher risk for cardiovascular morbidity and mortality compared to diabetic men, so our results would need to be confirmed in T2D men.

In conclusion, this trial shows that 20g of lipids is sufficient to modify the FA composition of TGRL 4 hours after the ingestion in T2D patients. We confirm that fasting TGRL from T2D patients activate platelets and further demonstrate that PP TGRL have a similar enhancing effect on platelet aggregation and TxB₂ formation, independently of their FA composition. This study also highlights the importance of TG management in the diabetic population for prevention of cardiovascular complications, as well as dietary counseling regarding fat intake. Our randomized trial shows that a realistic acute intake of either a commercial hazelnut cocoa spread or butter has a similar detrimental effect on TGRL-induced platelet activation compared with TGRL from fasting patients with T2D.

ACKNOWLEDGEMENTS

We thank the nurses and the medical team of the department of Endocrinology, Metabolic Disease, Diabetes and Nutrition at Louis Pradel hospital for their essential collaboration and the dieticians at *Centre de Recherche en Nutrition Humaine Rhône-Alpes* for designing the test breakfasts.

FUNDING

This work was supported by Inserm and promoted by *Hospices Civils de Lyon*. MMB received a doctoral contract from the *Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation* and a grant from the *Nouvelle Société Francophone d'Athérosclérose*. CC thanks the CNRS.

CONFLICT OF INTEREST STATEMENT

MCM received other research funding on other topics from Sodial-Candia R&D, the *National Interprofessionnel de l'Economie Laitière* (CNIEL, French Dairy Interbranch Organization) and Nutricia Research and has consultancy activities for food & dairy companies. These activities had no link with the present study. MCM was the coordinator of a research project funded by the National Research Agency on the valuing and health properties of buttermilk and milk polar lipids (VALOBAB, ANR-2011-0007), in which DC participated; the present study is not part of this project. PM received research grants from Akcea, honoraria for clinical trials or talks paid to his university or to him from Akcea, Amgen, Boehringer, MSD, Novo Nordisk and Sanofi. Other authors have no conflict of interest to declare.

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AUTHOR CONTRIBUTIONS

MMB, CC, DC, MCM and PM designed the study. PM was the principal investigator of the clinical trial. TLB was the clinical research associate assigned to the trial. SLP, JAN and PM collaborated to the writing of the Scientific Ethics Committee application. MMB, CB, CC, and MDF performed the experiments and collected the data. MMB and CC analyzed the data. MMB, CC, DC, MCM and PM interpreted the data. MMB and CC wrote the manuscript. DC, MCM and PM critically revised the manuscript. All co-authors read, commented and approved the manuscript.

541 **TABLE 1.** Clinical and biological parameters of the study participants

Characteristics	Type 2 diabetes (n=30)	Type 2 diabetes HCS group (n=15)	Type 2 diabetes Butter group (n=15)	p
Age (years)	65.5 ± 1.4	62.9 ± 2.3	68.1 ± 1.4	NS
BMI (kg/m ²)	34.1 ± 1.0	34.4 ± 1.7	33.9 ± 1.2	NS
Duration of diabetes (years)	12.9* ± 1.4	12.5 ± 1.7	13.2‡ ± 2.4	NS
<i>Glucose homeostasis</i>				
HbA _{1c} (%)	7.8 ± 0.2	7.7 ± 0.3	7.8 ± 0.3	NS
Glycemia (mmol/L)	9.0 ± 0.7	8.8 ± 1.0	9.3 ± 0.9	NS
Insulin (mU/L)	12.3* ± 1.3	11.2 ± 1.4	13.4‡ ± 2.3	NS
HOMA-IR	5.0* ± 0.7	4.1 ± 0.4	5.9‡ ± 1.4	NS
<i>Plasma lipids (mmol/L)</i>				
Triglycerides	1.69 ± 0.1	1.53 ± 0.1	1.85 ± 0.3	NS
Total cholesterol	4.55 ± 0.2	4.49 ± 0.2	4.62 ± 0.2	NS
HDL cholesterol	1.24 ± 0.1	1.27 ± 0.1	1.21 ± 0.1	NS
LDL cholesterol	2.56 ± 0.1	2.53 ± 0.2	2.60 ± 0.2	NS
<i>Plasma apolipoproteins B (g/L)</i>				
Total ApoB	0.89† ± 0.04	0.86‡ ± 0.1	0.92‡ ± 0.1	NS
ApoB-100	0.88† ± 0.04	0.85‡ ± 0.05	0.91‡ ± 0.1	NS
ApoB-48	0.006† ± 0.001	0.005 ± 0.001	0.006§ ± 0.001	NS
<i>Antidiabetic and lipid lowering-medication (n(%))</i>				
Metformin	28 (93)	14 (93)	14 (93)	-
Sulfonylurea	18 (60)	8 (53)	10 (67)	-

Gliptin	8 (27)	4 (27)	4 (27)	-
GLP-1 analogs	11 (37)	5 (33)	6 (40)	-
Acarbose	1 (3)	0	1 (7)	
Statins	18 (60)	8 (53)	10 (67)	-
Ezetimibe	1 (3)	1 (7)	0	-

542 *: n=29, †: n=28, ‡: n=14, §: n=13. Data are presented as means ± SEM. HCS: hazelnut-cocoa spread, NS: not
543 significant.

544 **TABLE 2.** Fatty acid composition of the fats in the test breakfasts

FA (mol %)	HCS	Butter
12:0	0.1	4.9
14:0	0.9	16.1
15:0	0	1.6
16:0	33.2	42.2
18:0	6.5	10.6
18:1 n-9	45.2	15.1
trans 18:1 n-9	4.3	1.7
18:2 n-6	8.3	1.0
18:3 n-3	0.2	0.2
20:0	0.3	0.1
22:0	0	0.1
Total		
SFA	41.0	78.8
MUFA	50.5	19.9
PUFA	8.5	1.3

545 Minor FA are not listed. HCS: hazelnut cocoa spread, FA: fatty acids, SFA: saturated fatty acids

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549 **TABLE 3.** Fatty acid composition of TGRL total lipids

Fatty acids (mol %)	HCS T0	HCS T240	p	Butter T0	Butter T240	p
12:0	0.07±0.07	0.03±0.03	NS	0.09±0.06	0.38±0.10	0.002
14:0	2.22±0.28	1.39±0.20	0.0009	1.81±0.26	4.35±0.52	<0.0001
15:0	0.20±0.08	0.12±0.04	NS	0.35±0.10	0.54±0.11	0.02
16:0	29.12±0.81	27.57±0.46	0.004	28.56±0.79	32.71±0.49	0.0002
16:1 n-7	2.00±0.30	1.58±0.22	0.007	1.95±0.38	2.23±0.26	NS
18:0	16.54±2.60	12.61±1.95	0.02	16.07±2.59	14.32±2.15	NS
18:1 n-9	22.66±1.23	32.35±1.34	0.0001	24.65±1.88	25.49±1.33	NS
trans 18:1 n-9	0.33±0.12	0.59±0.19	NS	0.30±0.11	0.33±0.09	NS
18:2 n-6	17.48±1.76	16.07±1.16	NS	16.75±1.31	12.27±0.63	<0.0001
18:3 n-3	0.24±0.08	0.22±0.07	NS	0.37±0.12	0.35±0.08	NS
20:0	0.24±0.11	0.19±0.05	NS	0.26±0.10	0.13±0.03	NS
20:3 n-6	0.55±0.11	0.44±0.08	NS	0.58±0.12	0.52±0.07	NS
20:4 n-6	2.90±0.41	2.48±0.33	0.02	3.18±0.22	2.41±0.12	0.007
20:5 n-3	0.21±0.10	0.17±0.06	NS	0.29±0.10	0.22±0.06	NS
22:0	0.5±0.48	0.42±0.37	NS	0.06±0.02	0.08±0.02	NS
22:6 n-3	0.63±0.11	0.49±0.08	0.007	0.63±0.12	0.58±0.08	NS
24:0	0.51±0.13	0.32±0.08	0.05	0.62±0.15	0.36±0.06	0.01
24:1 n-9	0.20±0.08	0.13±0.05	NS	0.27±0.07	0.15±0.04	0.04
Total						
SFA	49.40±2.45	42.88±1.85	0.005	47.91±2.63	53.05±1.95	0.02
MUFA	28.31±1.90	36.98±1.82	0.0001	29.83±2.39	30.28±1.84	NS
PUFA	22.31±1.68	20.17±1.02	0.04	22.26±1.49	16.72±0.69	0.0002

550 Data are presented as means \pm SEM. Other minor FA are not listed. HCS: hazelnut cocoa spread, NS: not
551 significant, SFA: saturated fatty acids.

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FIGURE LEGENDS

FIGURE 1. Increase in plasma ApoB-48 and triglyceride concentrations following the test breakfasts

(A) Plasma ApoB-48. (B) Plasma triglycerides. Results are expressed as mean \pm SEM of 15 patients in each group: $*p<0.05$ for both parameters. HCS: hazelnut cocoa spread.

FIGURE 2. Increase of TGRL triglyceride concentration and diameter following the test breakfasts

(A) TGRL triglyceride concentration in plasma. (B) TGRL hydrodynamic diameter. Results are expressed as mean \pm SEM of 15 patients in each group: $*p<0.01$. HCS: hazelnut cocoa spread.

FIGURE 3. Modification of the fatty acid profile of TGRL total lipids following the test breakfasts

Results are expressed as mean of the delta of (FA mol% at T240-FA mol% at T0) \pm SEM of 15 patients in each group: $*p<0.05$. HCS: hazelnut cocoa spread, SFA: saturated fatty acids.

FIGURE 4. Collagen-stimulated platelet aggregation is increased by fasting and postprandial TGRL from type 2 diabetic patients

Experiences were performed with platelets from 8 different donors. Results are expressed as mean \pm SEM of aggregation percentage of collagen-stimulated platelets without TGRL (black bars) or pre-incubated with fasting (white bars) or PP TGRL (brown and light blue bars) of 15 patients in each group $*p<0.01$. HCS: hazelnut chocolate spread.

FIGURE 5. Effects of fasting and postprandial TGRL on agonist-stimulated platelet thromboxane B₂

(A) Collagen stimulation. Results are expressed as mean platelet TxB₂ concentrations in pmol/mg of total proteins \pm SEM of 15 patients in each group* $p<0.05$. [#] $p=0.07$. HCS: hazelnut cocoa spread.

(B) Thrombin stimulation. Results are expressed as mean platelet TxB₂ concentrations in pmol/mg of total proteins \pm SEM of 15 patients in each group* $p<0.05$. [#] $p=0.06$. HCS: hazelnut cocoa spread.

Experiments were performed with platelets from 8 different donors.

FIGURE 6. Platelet activation by postprandial TGRL is concentration dependant, but does not differ according to the fat consumed

Results are expressed as mean platelet TxB₂ concentrations in pmol/mg of total proteins \pm SEM. $n=9$, * $p<0.05$. [#] $p=0.06$. HCS: hazelnut cocoa spread, control: platelets + thrombin, TGRLT0_25: platelets + 25 μ g/mL TGRL T0 + thrombin, TGRL T240_25: platelets + 25 μ g/mL TGRL T240 + thrombin, TGRL T240_50: platelets + 50 μ g/mL TGRL T240 + thrombin. Experiment was performed with platelets from 1 donor.