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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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1     **Postprandial triglyceride-rich lipoproteins from type 2 diabetic**  
2     **women stimulate platelet activation regardless of the fat source in**  
3                                     **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, TxB<sub>2</sub>: thromboxane B<sub>2</sub>

35

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

37

38

39 **ABSTRACT**

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

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

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

57

58        **1. INTRODUCTION**

59        Type 2 diabetic (T2D) patients have a higher risk of cardiovascular diseases, including  
60        ischemic coronary artery events, atherosclerosis and atherothrombosis [1]. Epidemiologic  
61        studies also showed that women with T2D exhibit a worse cardiovascular risk profile [2] and  
62        have greater increase in cardiovascular morbidity and mortality compared to men [3].  
63        Platelets from T2D individuals have been shown to be hyperactive, thus more easily prone to  
64        agonist-triggered aggregation in the event of plaque rupture [4]. Moreover, it is well  
65        documented that increased concentration of plasma triglycerides (TG), carried in the  
66        bloodstream by “triglyceride-rich lipoproteins” (TGRL) that are chylomicrons (CM), VLDL  
67        and their remnants, is an important risk factor in T2D [5]. Our group previously showed that  
68        large TGRL from fasting T2D patients enhanced *in vitro* platelet activation and aggregation  
69        [6].

70        It is now well acknowledged that postprandial (PP) TG concentrations are involved in  
71        cardiovascular risk. The concept of the atherogenicity of CM and VLDL was first introduced  
72        by Zilversmit 40 years ago [7]. Non-fasting TG levels were subsequently associated with the  
73        incidence of various cardiovascular events [8-12], independently of other risk factors [13, 14].  
74        TGRL remnants are likely to be the main contributors of an enhanced atherosclerosis  
75        development [14, 15]. However, regarding atherothrombosis, the contribution of TGRL is not  
76        clearly established. The few studies on this topic were performed mostly in non-diabetic  
77        hyperlipidemic and normolipidemic individuals and had major differences in methodology.  
78        They showed that CM from both groups can have an inhibitory effect on platelet functions  
79        [16], while VLDL from healthy subjects [17] and remnants from healthy and T2D individuals  
80        as well as patients with lipoprotein lipase (LPL) deficiency [18] would have an enhancing  
81        effect. No study had been performed on the potential implication of PP TGRL from T2D

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

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

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

99

## 2. EXPERIMENTAL SECTION

### 101 *Study participants*

102 Thirty T2D women were recruited at the Department of Endocrinology and Metabolic  
103 Diseases, Cardiovascular Hospital, Lyon Bron (France) and randomized in one of the two  
104 groups. To be included, the subjects required to be women aged between 50 and 80 years old,  
105 with a post-menopausal status, a stable weight and a BMI  $\geq 27$  and  $\leq 40$  kg/m<sup>2</sup>, an HbA<sub>1c</sub>  $\geq 6,5$   
106 and  $\leq 10\%$  ( $\geq 48$  and  $\leq 86$  mmol/mol) and no history of cardiovascular events or major  
107 hypertriglyceridemia. The anti-diabetic and lipid lowering medication was not discontinued  
108 for ethical reasons: 28 (93%) had metformin, 18 (60%) had sulfonylurea, 11 (37%) had  
109 glucagon-like peptide 1 analog, 8 (27%) had gliptin and 1 (3%) had Acarbose; none was  
110 treated with insulin injection. In addition, 17 (57%) had statins and 1 (0.03%) had a combined  
111 therapy of statins and ezetimibe. **Table 1** shows study participants characteristics. They were  
112 postmenopausal women with grade 1 obesity (mean BMI of  $34.1 \pm 1.0$ ), and long duration of  
113 T2D ( $12.9 \pm 1.4$  years). They had low serum LDL cholesterol due to statin treatment and none  
114 was treated with fibrates. They had a mild increase in serum TG concentration (33.3% had  
115 plasma TG above 2 mmol/L).

116

### 117 *Study design*

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

125 concentrations with an alpha level of 0.05 and 80% power, 15 women were recruited in each  
126 group to increase the statistical power. The primary outcome was the difference in TxB<sub>2</sub>  
127 concentrations in platelets incubated with fasting and PP TGRL. Secondary outcomes were  
128 the difference in the aggregation of platelets incubated with fasting and PP TGRL, the  
129 difference in TxB<sub>2</sub> concentrations in platelets incubated with TGRL collected after the  
130 consumption of a fat of different FA composition; and the difference in FA composition  
131 between fasting and PP TGRL from each group.

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

141

#### 142 *Test breakfasts composition*

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



149 **(Table 2)**. Both breakfasts were designed to be similar in carbohydrates, proteins and caloric  
150 contents. The butter breakfast was composed of plain crackers (30g), commercial butter  
151 (22.5g, 82% fat), jam (30g), orange juice (200 ml) and plain coffee or tea (200 ml) for a total  
152 of 440 calories, with 51.7%, 40.9% and 4.5% of energy as carbohydrates (56.9g), lipids (20g)  
153 and proteins (5g), respectively. The HCS breakfast contained plain crackers (30g),  
154 commercial HCS (60g) and plain coffee or tea (200 ml) providing 439 calories with 51.1%,  
155 40.9% and 6.5% of energy as carbohydrates (56.1g), lipids (20g) and proteins (7.1g),  
156 respectively. All subjects were given a maximum of 20 minutes to eat the breakfast.

157

#### 158 *Plasma biochemistry*

159 Blood was collected into EDTA tubes and plasma was immediately isolated by centrifugation  
160 ( $1500 \times g$ , 10 min, at 4 °C) and stored in aliquots at -20 °C. Measurement of ApoB-48  
161 concentrations was performed by ELISA according to the manufacturer's instruction  
162 (SHIBAYAGI, Shibukawa, Gunma Prefecture, Japan). Total (TC), HDL cholesterol (HDL-c),  
163 TG, total ApoB and glucose were enzymatically determined using commercial kits (ABBOTT  
164 Diagnostics) on an Architect C16000 autoanalyser (ABBOTT Diagnostics, Illinois, USA).  
165 LDL cholesterol was calculated using Friedewald equation ( $LDL-c = TC - HDL-c -$   
166  $0.45 * TG$ ). HbA<sub>1c</sub> was determined by capillary electrophoresis using a Sebia Capillarys 3  
167 (Sebia). Insulin was determined by immunoassay on an Architect I2000 autoanalyser. ApoB-  
168 100 was calculated by subtraction of ApoB-48 from total ApoB.

169

#### 170 *Isolation and characterization of TGRL fractions*

171 In order to collect TGRL fractions, 250µL of plasma was carefully deposited under a layer of  
172 distilled water (850µL) followed by ultracentrifugation at  $9777 \times g$  for 1 hour at 12°C using a

173 Beckman Coulter Optima TLX ultracentrifuge and a Beckman TLA 100.2 rotor. Top 100  $\mu\text{L}$   
174 of the floating layer corresponding to TGRL with density  $<1.000 \text{ g/ml}$  was collected and  
175 stored at  $4^\circ\text{C}$  for no longer than 2 days or stored at  $-20^\circ\text{C}$  for lipid analysis. Protein  
176 concentration of the fractions was measured using a modified Lowry method [28]. TG  
177 concentrations were obtained on an Architect C16000 autoanalyser. The hydrodynamic  
178 diameter of TGRL was measured by dynamic light scattering at  $25^\circ\text{C}$  with a ZetaSizer NanoS  
179 (Malvern, UK) using 1.0658 as viscosity and 1.445 as refractive index and 1.330 as refractive  
180 index of the aqueous phase [29].

181

#### 182 *TGRL fatty acid composition by gas chromatography*

183 Total lipids were extracted twice from TGRL ( $200\mu\text{L} + 300\mu\text{L H}_2\text{O}$ ) with ethanol:chloroform  
184 (1:3:6, respectively) after addition of 1,2,3-triheptadecanoyl-sn-glycerol as internal standard  
185 and BHT ( $50\mu\text{M}$ ). FA were transmethylated with  $\text{BF}_3/\text{methanol}$  (1.3 M, 10% in methanol) at  
186  $100^\circ\text{C}$  for 1h30. FA methyl esters were extracted twice with isooctane and separated by gas  
187 chromatography using a gas chromatograph (HP6890 G1530) equipped with a SP 2380  
188 capillary column ( $0.25\mu\text{m}$ ,  $30\text{m} \times 0.25\text{mm}$ , Supelco, Bellefonte, PA, USA) and a flame  
189 ionization detector.

190

#### 191 *Platelet isolation*

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

197 PRP was collected, acidified to pH 6.4 with 0.15 M citric acid and centrifuged at  $900 \times g$  for  
198 12 minutes at 20°C. Platelet-poor plasma (PPP) was removed and platelets were suspended in  
199 a Tyrode-HEPES buffer (pH 7.35) [30]. The mean concentration for all platelet suspensions  
200 was  $239 \pm 24$  G/L (min: 141 G/L, max: 354 G/L).

201

#### 202 *Platelet aggregation experiments*

203 Aggregation was measured in isolated platelets in a Chrono-log dual-channel aggregometer  
204 (Coulter, Margency, France) according to the method of Born [31]. Platelet suspensions were  
205 preincubated for 5 minutes at 37°C in the presence or absence of different preparations of  
206 TGRL (25 µg proteins/mL) and then stimulated with subthreshold concentrations of collagen  
207 ( $0.13 \pm 0.02$  µg/mL) (type I from equine tendons, Diagnostica Stago, Asnières sur Seine,  
208 France) with continuous stirring at 1000 rpm. The subthreshold concentrations of collagen  
209 were defined as the concentrations of collagen that induced approximately less than a 10-20%  
210 increase in light transmission. The extent of platelet aggregation was expressed as percentage  
211 of change in light transmission 4 minutes after the addition of collagen [32].

212

#### 213 *Platelet thromboxane B<sub>2</sub> measurement*

214 Freshly isolated platelet suspensions were incubated at 37°C for 30 minutes in the absence or  
215 presence of TGRL (25 µg proteins/mL) while being slowly agitated. Platelets were incubated  
216 at 37°C for an hour with non aggregating concentrations of collagen (0.5 µg/mL) or thrombin  
217 (0.05 U/mL) while being slowly agitated before being immediately stored at -80°C until TxB<sub>2</sub>  
218 measurement. Following 3 cycles of thawing and freezing of samples for platelets lysis, TxB<sub>2</sub>  
219 concentrations were determined by enzyme immunoassays according to the manufacturer's  
220 recommendations (Enzo Life Sciences Inc., Villeurbanne, France). Platelet proteins

221 concentrations were measured using the Bradford method [33] in order to express TxB<sub>2</sub>  
222 results.

223

224 *Statistical analysis*

225 Data were expressed as means  $\pm$  SEM. Normality of data distribution was assessed using  
226 d'Agostino-Pearson normality test. Paired Student's t-test or Wilcoxon test was used to assess  
227 differences between platelets incubated with TGRL and stimulated with an agonist compared  
228 to platelets stimulated with an agonist. Differences between platelets stimulated with an  
229 agonist compared to platelets alone were performed to ensure platelet functionality in  
230 response to agonists using Student's t-test or Wilcoxon test as well. Differences between  
231 T240 and T0 in each group were assessed using paired Student's *t*-test or Wilcoxon test and  
232 differences between the two groups for the means of variation deltas (T240-T0) were  
233 determined using unpaired Student's *t*-test or Mann-Whitney test. Differences were  
234 considered significant at  $p < 0.05$  (2 sided test). All statistical analyses were performed using  
235 GraphPad Prism 8.0 (Graphpad Software, San Diego, CA).

236

237

238 **3. RESULTS**

239

240 *Postprandial modification of plasma and triglyceride-rich lipoproteins*

241 At the plasma level, consumption of both breakfasts increased ApoB-48 (HCS: +78%, Butter:  
242 +67%,  $p < 0.001$  for both) and TG (HCS: +33%, Butter: +30%,  $p \leq 0.0001$  for both)  
243 concentrations similarly (**Figure 1A and B**) at 4 hours after 20g fat meal. TGRL isolated from  
244 plasma of fasting T2D patients had a mean diameter of  $146 \pm 5$  nm and a mean TG and total  
245 FA concentrations of  $0.07 \pm 0.01$  mmol/L and  $512 \pm 6.5$  nmol/mg protein, respectively. TGRL  
246 collected after the breakfasts had an increased diameter, TG and FA content  $171 \pm 4$  nm,  
247  $0.12 \pm 0.02$  mmol/L and  $907 \pm 14$  nmol/mg protein, respectively. TGRL mean concentration of  
248 TG was significantly increased in the PP state. As shown in **Figure 2A**, there was a 200% and  
249 71% increase in TGRL-TG after the HCS and butter breakfasts, respectively ( $p < 0.01$  for both  
250 groups). The type of fat does not impact TGRL-TG concentrations in PP plasma. Following  
251 the HCS breakfast, mean TGRL diameter was 10% higher, while it was increased by 23%  
252 after the butter breakfast (**Figure 2B**) ( $p < 0.01$  for both groups). This increase in diameter after  
253 the butter breakfast was significantly higher than after the HCS breakfast (**Figure 2B**,  
254  $p < 0.01$ ).

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

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

264

265 *Effect of fasting and postprandial TGRL on platelet aggregation*

266 As shown in **Figure 4**, TGRL from fasting T2D patients increased collagen-stimulated  
267 platelet aggregation (HCS group: +62%, butter group: +86%,  $p < 0.01$  for both) compared with  
268 collagen-stimulated platelets alone. When platelets were pre-incubated with TGRL isolated in  
269 the PP state, collagen-stimulated aggregation increased similarly in both groups (HCS group:  
270 +86%, butter group: +129%,  $p < 0.01$  for both). When platelets pre-incubated with PP TGRL  
271 are compared to platelets pre-incubated with fasting TGRL, the difference was not significant.

272

273 *Effect of fasting and postprandial TGRL on platelet thromboxane  $B_2$  concentration*

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

285 We also tested the effect of a higher concentration of PP TGRL (50 µg/mL) on platelets, since  
286 TGRL concentration is elevated in the PP compared to the fasting state. The incubation of  
287 platelets with 50 µg/mL of PP TGRL significantly increased TxB<sub>2</sub> concentration compared to  
288 25 µg/mL PP TGRL and fasting TGRL. This effect was not different between the HCS and  
289 butter group (**Figure 6**).

290

291 **4. DISCUSSION**

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

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

306 Residual fasting TGRL have a mean diameter of 146 nm, which is much larger than what is  
307 reported in healthy individuals. For example, Mekki *et al.* reported a mean diameter of ~91  
308 nm for fasting TGRL in healthy individuals [36]. Interestingly, despite a similar amount of fat  
309 (20 g), we observed a greater diameter of PP TGRL 4 hours after the ingestion of the butter  
310 breakfast (183 nm) compared to the PP TGRL after the HCS breakfast (159 nm), and  
311 compared to the mean diameter reported for PP TGRL 4 hours after the consumption of a  
312 meal containing 40g of butter (~120 nm). In addition, our study shows that ingestion of a  
313 moderate amount of lipids (20g) leads to modification of the FA composition of TGRL 4  
314 hours after the intake in T2D women. FA composition of PP TGRL reflects the main FA



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

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

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

340 the fasting and PP state, which are different in composition. Indeed, the presence of large TG-  
341 rich particles in the basal state suggest that these are remnants of an inadequate CM and  
342 VLDL lipolysis [43], while the largest TGRL fractions isolated in the PP state contain CM  
343 and are representative of the heterogeneous mix of particles following a meal. We also show  
344 for the first time *in vitro* that an increase in TGRL, in physiological concentration, in the PP  
345 state has an enhancing effect on platelets TxB<sub>2</sub> concentration after a meal containing a  
346 realistic amount of fat rich in SFA. The increase in platelet activation and aggregation was  
347 similar after both fats, even though the TGRL FA composition was modified in PP. It is  
348 possible that an acute intervention of one breakfast containing a moderate quantity of lipids is  
349 not enough to cause a sufficient change in TGRL composition leading to a significant effect  
350 of TGRL FA on TGRL-induced platelet activation. A longer dietary intervention with  
351 different lipid amount and sources could induce more pronounced effects. Delgado-Lista *et al*  
352 studied in 20 healthy young men the effects of a chronic 28 days intake of a controlled diet  
353 followed by 3 tests meals containing 1g/kg of lipids of different FA composition [44]. A lipid-  
354 rich meal caused an increase of plasma tissue-type plasminogen activator, d-dimers and TxB<sub>2</sub>  
355 in the PP irrespective of the type of fat consumed, but not in the fasting state.

356 Our results show that fasting and PP TGRL also increase platelet TxB<sub>2</sub> concentration with  
357 differences in the extent of TGRL-induced stimulation according to the agonist used. We  
358 aimed to use physiological agonists that would not interfere with the lipid composition of  
359 TGRL and platelets (eg: arachidonic acid), and that would activate platelets by different  
360 signalling pathways. Tested collagen concentrations induce a moderate stimulation while used  
361 thrombin concentrations provoke strong platelet activation. The platelets used are from  
362 healthy donors, so it is possible that the effect of fasting and PP TGRL would be greater on  
363 platelets from T2D patients, since they are already hyperactive in the basal state.

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

372 In conclusion, this trial shows that 20g of lipids is sufficient to modify the FA composition of  
373 TGRL 4 hours after the ingestion in T2D patients. We confirm that fasting TGRL from T2D  
374 patients activate platelets and further demonstrate that PP TGRL have a similar enhancing  
375 effect on platelet aggregation and TxB<sub>2</sub> formation, independently of their FA composition.  
376 This study also highlights the importance of TG management in the diabetic population for  
377 prevention of cardiovascular complications, as well as dietary counseling regarding fat intake.  
378 Our randomized trial shows that a realistic acute intake of either a commercial hazelnut cocoa  
379 spread or butter has a similar detrimental effect on TGRL-induced platelet activation  
380 compared with TGRL from fasting patients with T2D.

381

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393

394 **CONFLICT OF INTEREST STATEMENT**

395 MCM received other research funding on other topics from Sodiaal-Candia R&D, the *Centre*  
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404 declare.

405

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529

530

531 **AUTHOR CONTRIBUTIONS**

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

539

540

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

Characteristics	Type 2	Type 2 diabetes	Type 2 diabetes	p
	diabetes (n=30)	HCS group (n=15)	Butter group (n=15)	
Age (years)	65.5 ± 1.4	62.9 ± 2.3	68.1 ± 1.4	NS
BMI (kg/m <sup>2</sup> )	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
<b><i>Glucose homeostasis</i></b>				
HbA <sub>1c</sub> (%)	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
<b><i>Plasma lipids (mmol/L)</i></b>				
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
<b><i>Plasma apolipoproteins B (g/L)</i></b>				
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
<b><i>Antidiabetic and lipid lowering-medication (n(%))</i></b>				
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	-

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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

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

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

546

547

548

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

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

552

553



554 **FIGURE LEGENDS**

555

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

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

560

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

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

566

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

569 Results are expressed as mean of the delta of (FA mol% at T240-FA mol% at T0)  $\pm$  SEM of  
570 15 patients in each group: \* $p < 0.05$ . HCS: hazelnut cocoa spread, SFA: saturated fatty acids.

571

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

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

578

579 **FIGURE 5. Effects of fasting and postprandial TGRL on agonist-stimulated platelet**  
580 **thromboxane B<sub>2</sub>**

581 (A) Collagen stimulation. Results are expressed as mean platelet TxB<sub>2</sub> concentrations in  
582 pmol/mg of total proteins ± SEM of 15 patients in each group\* $p < 0.05$ . # $p = 0.07$ . HCS:  
583 hazelnut cocoa spread.

584 (B) Thrombin stimulation. Results are expressed as mean platelet TxB<sub>2</sub> concentrations in  
585 pmol/mg of total proteins ± SEM of 15 patients in each group\* $p < 0.05$ . # $p = 0.06$ . HCS:  
586 hazelnut cocoa spread.

587 Experiments were performed with platelets from 8 different donors.

588

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

591 Results are expressed as mean platelet TxB<sub>2</sub> concentrations in pmol/mg of total proteins ±  
592 SEM. n=9, \* $p < 0.05$ . # $p = 0.06$ . HCS: hazelnut cocoa spread, control: platelets + thrombin,  
593 TGRLT0\_25: platelets + 25 µg/mL TGRL T0 + thrombin, TGRL T240\_25: platelets + 25  
594 µg/mL TGRL T240 + thrombin, TGRL T240\_50: platelets + 50 µg/mL TGRL T240 +  
595 thrombin. Experiment was performed with platelets from 1 donor.

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