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Large Triglyceride-Rich Lipoproteins from fasting patients with Type 2 Diabetes activate platelets

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ABSTRACT

Aim. Type 2 diabetic (T2D) patients present risk factors for atherothrombosis such as fasting hypertriglyceridemia and platelet hyperactivity. Our objective was to determine the effect of large triglyceride-rich lipoproteins (large TGRL) from fasting T2D patients on platelet aggregation and, if any, to identify the signaling pathway involved. **Methods.** Large TGRL were isolated from the plasma of 25 T2D patients by ultra-centrifugation ($d < 1.000$). Platelets were isolated from healthy blood donors and suspended in buffer. Platelets were pre-incubated in the presence or absence of TGRL and stimulated with either collagen or thrombin. Platelet aggregation and the arachidonic acid (AA) signaling pathway were studied. **Results.** Fasting T2D large TGRL were mostly of hepatic origin (ApoB-100/ApoB-48 ratio of 42 ± 7) and were enriched in TG (TG/total ApoB ratio of 4.2 ± 0.5). They potentiated agonist-stimulated platelet aggregation (collagen: +68%, $p < 0.05$, thrombin: +771%, $p < 0.05$). It should be mentioned that TGRL from the plasma of healthy blood donors ($n=7$) had no effect on platelet aggregation. T2D large TGRL increased thromboxane B₂ (TxB₂) concentration in platelets stimulated with collagen (+34%, $p < 0.05$) or thrombin (+37%, $p < 0.05$) compared to platelets stimulated with one or the other agonists alone. Phosphorylation of p38 MAPK and cytosolic phospholipase A₂ (cPLA₂) was enhanced after incubation of platelets with T2D TGRL and thrombin (+87% and +32%, respectively, $p < 0.05$) compared to platelets incubated with thrombin. **Conclusion.** Large TGRL from fasting T2D patients may play a role in the development of atherothrombosis by increasing platelet aggregation and activating platelet AA signaling pathway.

Keywords: *Triglyceride rich lipoproteins, platelets, type 2 diabetes, cardiovascular risk*

1. INTRODUCTION

Type 2 diabetes (T2D) and ischemic cardiovascular risk are highly associated and diabetic patients have a risk of ischemic coronary artery event increased by two to four fold [1]. The presence of T2D is associated with increased atherosclerotic plaque formation and eventually atherothrombosis when platelets that are already hyperactive are triggered following plaque rupture, leading to stroke or myocardial infarction [2]. It is well documented that risk factors for atherothrombosis in T2D patients include elevation of fasting plasma triglycerides, which are mostly carried by triglyceride rich lipoproteins (TGRL) including remnants from chylomicrons (CM) and very large density lipoproteins (VLDL) [3]. There is growing evidence that the increased production of large triglyceride-enriched VLDL1 and remnants particles in insulin resistant states and their prolonged presence in circulation may play a significant role in the atherothrombosis process [4]. While there are many studies about functional effects of modifications of LDL and HDL particles size, concentration and composition in diabetic patients [5-7], a direct involvement of the large triglyceride-rich lipoproteins subfraction in T2D-associated atherothrombotic events remains unclear. A direct role of TGRL has already been demonstrated in the inflammatory response of endothelial cells to cytokine stimulation and in the recruitment of monocytes on inflamed endothelial cells under shear flow [8]. However, there is currently very little data about the functional properties of TGRL on platelets compared to the numerous data regarding atherogenic functions of other particles such as LDL.

Hyperglycemia, glycemic variability and insulin resistance are also associated with altered blood platelet activation, implying a change in platelet composition and function in diabetic

Abbreviations: AA: arachidonic acid, BMI: body mass index, CM: chylomicrons, cPLA₂: cytosolic phospholipase A₂, HBD: healthy blood donors, HDL: high density lipoproteins, HTG: hypertriglyceridemia, LDL: low density lipoproteins, PRP: platelet-rich plasma, VLDL: very low density lipoproteins, TGRL: triglyceride rich lipoproteins, TG: triglycerides, T2D: type 2 diabetes, TxA₂: thromboxane A₂, TxB₂: thromboxane B₂.

individuals. High plasma lipids or glucose concentrations often observed in T2D patients can cause modifications of the lipid composition of platelets, reduce their membrane fluidity and lead to protein glycation [9, 10]. Consequently, platelets from T2D patients exhibit an increased reactivity/activation [9, 11], and this even in the absence of previous vascular complications [12], as well as increased adhesion and aggregation [10].

Many studies focused on interactions between platelets and lipoproteins, especially LDL, HDL and their oxidized forms. Our group, as well as others, showed an inhibitory effect of HDL particles on platelet aggregation and a stimulating effect of oxidized LDL [13-16]. Binding of lipoproteins to the platelet receptors can have an impact on multiple agonist dependent signaling pathways essentials for platelet activation and aggregation. Among them, activation of p38 MAPK results in phosphorylation and activation of cytosolic phospholipase A₂ (cPLA₂) that catalyzes the release of arachidonic acid from membrane phospholipids. This leads to the formation of thromboxane A₂ (TxA₂) that is released and amplifies the activation of adjacent platelets promoting thrombus formation [16, 17]. To our knowledge, there are no studies on the potential effect of large TGRL from fasting T2D patients on platelet function and the mechanisms implied.

Thus, the aims of our study were to determine if large TGRL from fasting T2D patients have an effect on platelets and if so, to identify the platelet signaling pathway involved.

2. SUBJECTS AND METHODS

2.1. T2D patients characteristics

T2D patients were recruited at the Endocrinology, Metabolic Disease, Diabetes and Nutrition Department at the Louis Pradel Hospital (Hospices Civils de Lyon) in Bron, France and written informed consent was obtained for biobanking prior to their inclusion into the study.

Blood was collected after a **12 hours** overnight fast. Exclusion criteria were presence of any metabolic disease other than T2D and record of any type of cardiovascular complications.

Patients were treated with either one or a combination of oral anti-diabetic medication: 92% had metformin, 40% had sulfonylurea, 32% had gliptin and 24% had GLP-1 inhibitor) and 28% had insulin therapy. A total of 72% of the patients had lipid lowering therapy: 64% had statin therapy and 8% had a combined therapy of statin and ezetimibe. Subjects in the control group were non-diabetic blood donors for which general good health status was determined by a confidential medical assessment at the Etablissement Français du Sang prior to blood donation. The study was conducted in accordance with the principles of the Helsinki declaration and medication was not discontinued for ethical reasons in the T2D patients group prior to venipuncture. **Table 1** shows study participants characteristics (n=32). The mean age was of 59.8 ± 2 years old for T2D patients and 27 ± 4 years old for controls. T2D patients mean BMI was in the obese category (31.9 ± 1.4) and lipid profiles and blood glucose variables were in the normal range since patients had an ongoing follow-up.

2.2. Plasma biochemistry

Measurements of ApoB-100 and ApoB-48 were performed by ELISA specific for each apolipoprotein (MABTECH, Nacka Strand, Sweden and SHIBAYAGI, Shibukawa, Gunma Prefecture, Japan, respectively). Total cholesterol (TC) and triglycerides (TG) were enzymatically determined using commercial kits (ABBOTT Diagnostics) on an Architect C16000 autoanalyser (ABBOTT Diagnostics, Illinois, USA). HbA1c was determined by high-performance liquid chromatography using a Variant II Hemoglobin testing system (Bio-Rad).

2.3. Isolation and characterization of TGRL fractions

Blood was collected into EDTA tubes and plasma was immediately isolated by centrifugation ($1500 \times g$, 10 min, at 4°C). Plasma was stored in aliquots at -20°C . Collection of the TGRL fraction was done by careful deposition of plasma under a layer of distilled water followed by ultracentrifugation at $9777 \times g$ for 1 hour at 12°C using a Beckman Coulter Optima TLX ultracentrifuge. 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. Protein concentration of the fractions was measured using a modified Lowry method [18].

TGRL ApoB-48, ApoB-100, cholesterol and triglycerides concentrations were measured using the same methods as plasma samples. The hydrodynamic diameter of TGRL was measured by dynamic light scattering at 25°C with a ZetaSizer using 1.0658 as viscosity and 1.445 as refractive index and 1.330 as refractive index of the aqueous phase.

2.4. 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 mmol/L NaH_2PO_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).

144

145 **2.5. Platelet aggregation experiments**

146 Aggregation was measured in isolated platelets in a Chrono-log dual-channel aggregometer
147 (Coulter, Margency, France) according to the method of Born [19]. A volume of 400 μ L of
148 platelet suspensions was preincubated for 5 minutes at 37°C in the presence or absence of
149 different preparations of TGRL (25 μ g proteins/mL) and then stimulated with subthreshold
150 concentrations of collagen (Diagnostica Stago, Asnières sur Seine, France) or thrombin
151 (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) with continuous stirring at 1000 rpm. The
152 subthreshold concentrations of collagen or thrombin were defined as the concentrations of
153 agonists that induced approximately less than a 20-30% increase in light transmission. The
154 extent of platelet aggregation was expressed as percentage of change in light transmission 4
155 minutes after the addition of either agonist.

156

157 **2.6. Platelet thromboxane B_2 measurement**

158 Freshly isolated platelet suspensions were incubated at 37°C for 30 minutes in the absence or
159 presence of TGRL (25 μ g proteins/mL) while being slowly agitated. Platelets were then
160 incubated at 37°C for an hour with either collagen (0.5 μ g/mL) or thrombin (0.1 U/mL) while
161 being slowly agitated before being immediately stored at -80°C until measurement. Following
162 3 cycles of thawing and freezing of samples for platelets lysis, TxB₂ concentrations were
163 determined by enzyme immunoassays according to the manufacturer's recommendations
164 (Enzo Life Sciences Inc., Villeurbanne, France).

165

166 **2.7. Western blotting analysis**

Following platelet incubation as described above and platelet lysis in the presence of anti-phosphatases and anti-proteases, proteins were denatured, electrophoresed in 12% bis-Tris or 3-8% Tris-Acetate (Bio-Rad Laboratories, Marnes-la-Coquette, France) and transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk and incubated with either 1:2500 anti-phospho-p38 MAPK or 1:1000 anti-phospho-cPLA₂ polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA), washed, and incubated with 1:5000 or 1:2000 goat anti-rabbit horseradish peroxidase conjugate. Phospho-p38 MAPK (38 kDa) and phospho-cPLA₂ (85 kDa) were visualized by enhanced chemiluminescence (GE Healthcare, Little Chalfont Buckinghamshire, UK), and bands were quantified by densitometry using Quantity One (Bio-Rad). Results were normalized for β -actin protein levels.

2.8. Statistical analyses

Data were expressed as means \pm S.E.M. Normality of data distribution was assessed using d'Agostino-Pearson normality test. Paired Student's t-test was used to assess differences between platelets incubated with large 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 as well. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 7.0 (Graphpad Software, San Diego, CA).

3. RESULTS

3.1. TGRL fractions characterization

Lipoprotein particles recovered in the top aqueous fractions of ultracentrifuged plasma had a density lower than 1.000 g/ml. Both apoB-48 and apoB-100 were detected in the isolated fractions, indicative of the presence of CM and CM remnants containing apoB-48, as well as VLDL and their remnants containing apoB-100. Collected particles had a mean hydrodynamic diameter of 205 nm confirming that they were large. TG/total apoB and TG/total cholesterol ratios were 4-fold higher in TGRL than in plasma showing an enrichment of the particles in TG (**Table 2**).

3.2. Effect of TGRL isolated from T2D patients on collagen-stimulated platelet aggregation and TxB₂ concentration

As shown in **Figure 1A**, there was a significant increase of 68% ($168 \pm 22\%$) of collagen-stimulated platelet aggregation when platelets were preincubated with 25µg/mL T2D patients large TGRL compared with 100% for platelets incubated with subthreshold concentrations of collagen (n=25). We also determined the effect of large TGRL isolated from healthy blood donors (HBD) on platelets (n=7). There was no impact of the incubation of platelets with large TGRL from HBD prior to collagen stimulation: $102 \pm 31\%$ aggregation in platelets pre-incubated with 25µg/mL HBD TGRL and stimulated with 0.13 ± 0.02 µg/mL collagen vs 100% for platelets incubated with the same collagen concentration (**Figure 1A**). Addition of large TGRL from T2D patients or HBD without agonist stimulation did not trigger platelet aggregation on their own (not shown). To determine whether stimulating effects of large T2D TGRL on platelet aggregation were associated with an increased biosynthesis of TxA₂ produced from arachidonic acid by the cyclooxygenase pathway, concentrations of TxB₂, its stable catabolite, were measured in platelets pre-incubated with or without 25µg/mL T2D large TGRL and stimulated with 0.37 ± 0.04 µg/mL collagen (n=22). As expected, there was first a 55% increase in TxB₂ concentration when platelets were incubated with collagen

($p < 0.05$), and the increase was even greater (+108% vs control platelets) when platelets were pre-incubated with T2D large TGRL and with the same collagen concentration ($p < 0.05$) (**Figure 1B**).

3.3. Effect of large TGRL isolated from T2D patients on thrombin-stimulated platelet aggregation and thromboxane B_2 concentration

We also performed aggregation measurements on platelets pre-incubated with 25 $\mu\text{g/mL}$ large TGRL from T2D patients and subthreshold concentrations of thrombin compared with platelets incubated with thrombin alone. There was a 771% increase of aggregation when platelets were pre-incubated with T2D large TGRL compared to 100% for platelets stimulated with the same thrombin concentration as shown in **Figure 2A**. Platelets pre-incubated with 25 $\mu\text{g/mL}$ T2D large TGRL and further stimulated with thrombin (0.1 U/ml) showed higher concentrations of TxB_2 than platelets only stimulated with thrombin ($p < 0.05$) (**Figure 2B**).

3.4. Effect of T2D large TGRL on platelet p38MAPK and cPLA₂

Because TxA_2 biosynthesis requires p38 MAPK-dependent activation of cPLA₂, which hydrolyzes membrane phospholipids to release arachidonic acid, we measured the phosphorylation of p38 MAPK and cPLA₂ in platelets pre-incubated with or without 25 $\mu\text{g/mL}$ T2D large TGRL and thrombin (0.09 ± 0.01 U/ml). As shown in **Figure 2C** and **2D**, there was an increase of phosphorylated amounts of both enzymes with the addition of thrombin (phospho-p38MAPK: +48%, $p < 0.05$, phospho-cPLA₂: +110%, $p < 0.05$). An additional increase in phosphorylation of both enzymes was observed when platelets were pre-incubated with T2D large TGRL and thrombin compared to platelets stimulated with thrombin (phospho-p38MAPK: +87%, $p < 0.05$, phospho-cPLA₂: +32%, $p < 0.05$).

4. DISCUSSION

The presence of large TGRL in the fasting state is known as a major lipid abnormality in T2D patients; however, the potential implication of these specific particles in the development of atherothrombosis is unknown. The present study supports the presence of remaining large TGRL containing ApoB-48 and B-100 in the plasma of fasting T2D patients. Our data show for the first time that large TGRL from fasting T2D patients potentiate activation of isolated platelets when incubated with non-aggregating subthreshold concentrations of collagen or thrombin. This platelet activation is triggered through the activation of the AA signaling pathway.

Our lipoprotein fractions corresponded to ultracentrifuged fractions of the lightest density ($d < 1.000$) and were a mixture of large lipoproteins and their remnants enriched in triglycerides. They had a mean diameter of 205 nm, which was higher than the reported values in healthy individuals of 30-80 nm for VLDL and CM remnants [20]. We were able to detect and quantify ApoB-48 and ApoB-100 in plasma from fasting T2D patients, also indicative of the presence of large CM remnants and large light VLDL. These findings are in agreement with data showing that T2D patients present a higher production of large VLDL1 particles as well as a reduction of their hepatic catabolism [21, 22]. Since each triglyceride-rich particle contains only one apolipoprotein B, the ratio of TG/Total ApoB of 4.2 ± 0.5 shows the particles isolated from fasting T2D patients' plasma display an enrichment in TG, characteristic of large TGRL. Concentrations of ApoB-48 and ApoB-100 similar to ours have been reported in plasma, CM and large VLDL fractions of T2D patients [23]. The centrifugation method used for large TGRL isolation ensured that there was no contamination

with smaller particles like IDL, and most probably caused a loss of the small VLDL2 subclass, allowing the collection of the largest particles. Altogether, our results regarding the presence of large TG-rich particles in fasting T2D patients are in accordance with the literature [24-27].

In this study, we chose to focus on large TGRL interactions with platelets in T2D patients since this population presents an increased risk of developing cardiovascular complications compared to non-diabetic. Indeed, in fasting healthy individuals, CM and their remnants are rapidly cleared from circulation and negligible concentrations of ApoB-48 are reported in their plasma [28-30]. These particles are unlikely to play a role in atherothrombotic events in healthy subjects [31] therefore, the relevance of studying the effect of fasting large TGRL from healthy subjects on platelets is weak. By contrast, previous studies have shown that large TGRL particles from T2D and/or HTG patients display increased residence time in the circulation, which may increase their atherogenicity and contribute to cardiovascular complications [32]. We tested also the effect of large TGRL from fasting T2D patients on platelets isolated from healthy donors and not on platelets from T2D patients. Since it was already shown that platelets from T2D patients are prone to activation in a basal state, it would have been uncertain if the effects on activation and aggregation were really caused by large TGRL rather than by the dysfunction of the platelets itself.

Regarding the interaction of large TGRL with platelets, only a few studies have reported an effect of large TGRL on platelet function in various populations in the postprandial state [33-37]. It is difficult to compare these post-prandial results with ours from fasting T2D patients, since we cannot rule out that the large TGRL composition could be modified after a meal and therefore have a different impact on platelet aggregation. There are also many methodological differences between the studies that limit their comparison and the conclusions drawing.

Indeed, isolation method of the large TGRL, measurement of platelet aggregation performed in whole blood, platelet-rich plasma (PRP) or washed platelets as well as the concentrations of large TGRL and agonists used to assess platelet aggregation can all have a major impact on the results. Our experiments were carried out in plasma-free platelet suspensions, which avoid the interference of plasma proteins present in PRP (such as albumin and other lipoproteins), and of other blood cells (such as erythrocytes) present in whole blood. Indeed, Yamazaki *et al.* showed that post-prandial remnant-like particles in non-diabetic individuals enhanced shear induced platelet aggregation and P-selectin expression in whole blood and suggested that ADP released from erythrocytes might have activated platelets [38]. In addition, our large TGRL fractions were isolated from fasting type 2 diabetic patients, which excludes the effect of other fatty compounds released in the post-prandial state that could potentially stimulate platelets. The T2D large TGRL fractions we tested were a mixture of apoB-48 and apoB-100 containing particles, thus it is not possible to conclude which class of lipoproteins in particular drives the stimulating effect observed on platelet activation. However, our fractions are representative of those of the large TGRL found *in-vivo* in plasma from fasting T2D patients. Our results demonstrate for the first time that the aggregation response of platelets from healthy donors to physiological agonists is enhanced by large TGRL from fasting T2D patients.

This study also showed that the increased platelet activation caused by large TGRL from fasting T2D patients involved the activation of the platelet AA signaling pathway. Indeed, Tx_{B2} concentration as well as phosphorylated amounts of p38MAPK and cPLA₂ were higher following platelet incubation with the particles and stimulation by an agonist compared to agonist-stimulated platelets. Beitz *et al.* showed that VLDL from fasting patients with coronary heart disease increased platelet generation of Tx_{A2} in whole blood from healthy

volunteers [39]. Later, Englyst *et al.* showed that incubation of PRP with high concentrations of VLDL from healthy volunteers in a post-prandial state increased collagen-stimulated platelet TxB₂ concentration when compared with platelets stimulated with collagen [35].

In conclusion, we showed that large TGRL from fasting T2D patients, containing both apoB-48 and apoB-100, have an enhancing effect on platelet aggregation *via* activation of the AA signaling pathway and might contribute to the increased atherothrombotic risk of T2D patients. Further studies about these fasting lipoproteins specific to T2D patients would be required to have a better understanding of their implication in the development of cardiovascular complications in this population. It would also be relevant to determine whether postprandial large TGRL from T2D patients or from healthy subjects activate platelets to the same extent as large TGRL from fasting T2D patients.

CONFLICT OF INTEREST

The authors have no conflict of interest related to this work to disclose.

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AUTHOR CONTRIBUTIONS

MMB, MCM, DC and CC designed the study. MMB, MDF and CB performed the experiments and collected the data. MMB and CC analyzed the data. MMB, DC, MCM, PM and CC 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.

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

Characteristics	T2D	HBD
n	25	7
Age (years)	59.8 ± 2	27 ± 4 ^b
BMI (kg/m ²)	31.9 ± 1.4	ND
TG (mmol/L)	1.63 ± 0.20	0.79 ± 0.14 ^b
HDL-C (mmol/L)	1.06 ± 0.05	1.04 ± 0.07 ^b
LDL-C (mmol/L)	2.22 ± 0.20	2.25 ± 0.27 ^b
Glycemia (mmol/L)	9.0 ± 0.6	ND
HbA1c (%)	7.8 ± 0.3	ND
T2D duration (years)	13.8 ± 2.0^a	NA

467 a: n=24, b: n=6

468 Data are presented as means ± SEM

469

470 **TABLE 2.** Characteristics of plasma and corresponding large TGRL fractions from T2D
471 patients

Characteristics	Plasma	Large TGRL fractions
Hydrodynamic diameter (nm)	-	205 ± 15
ApoB-48 (mg/L)	15.2 ± 3.7 ^a	0.94 ± 0.40 ^b
ApoB-100 (mg/L)	1327.2 ± 126.8 ^a	28.1 ± 5.2 ^b
TG (mmol/L)	1.37 ± 0.16	0.16 ± 0.04
Total cholesterol (mmol/L)	3.89 ± 0.32	0.09 ± 0.01
Ratios (mg/L)		
ApoB-100/apoB-48	153.9 ± 42.0	42.4 ± 6.5
TG/total cholesterol	0.4 ± 0.04	4.0 ± 0.4
TG/total apoB	0.9 ± 0.1	4.2 ± 0.5

472 n=10, a: n=9, b: n=7

473 Data are presented as means ± SEM

FIGURE LEGENDS

FIGURE 1. Collagen-stimulated platelet aggregation and thromboxane B₂ concentration are increased by large TGRL from type 2 diabetic patients.

(A): (i) Platelets from healthy blood donors were incubated in the absence or presence of TGRL from healthy blood donors followed by stimulation with a concentration of collagen of $0.13 \mu\text{g/ml} \pm 0.01$ and agitated at 1000 rpm for 4 minutes at 37°C. Results are expressed as mean percentage of control determined by platelets stimulated with collagen and are means \pm SEM of 7 healthy blood donors: NS. **(ii)** Platelets from healthy donors were incubated in the absence or presence of T2D patients large TGRL followed by stimulation with a concentration of collagen of $0.17 \mu\text{g/ml} \pm 0.02$ and agitated at 1000 rpm for 4 minutes at 37°C. Results are expressed as mean percentage of control determined by platelets stimulated with collagen and are means \pm SEM of 25 patients: $*p < 0.05$.

(B): Platelets from healthy donors were incubated at 37°C for 30 minutes in the absence or presence of T2D large TGRL ($25 \mu\text{g prot/ml}$) and stimulated with collagen ($0.37 \pm 0.04 \mu\text{g/ml}$) for 1 hour at 37°C while undergoing slow agitation. Results are expressed as mean TxB₂ concentrations in $\text{pmol}/10^9$ platelets \pm SEM of 25 patients. Platelets + collagen vs platelet with vehicle: +36%, $*p < 0.05$. Platelets + T2D large TGRL + collagen vs platelets + collagen: +34%, $*p < 0.05$.

FIGURE 2. Large TGRL from type 2 diabetic patients increase thrombin-stimulated platelet aggregation and thromboxane B₂ concentration via activation of the arachidonic acid signaling pathway.

(A): Platelets from healthy donors were incubated in the absence or presence of T2D large TGRL ($25 \mu\text{g prot/ml}$) followed by stimulation with thrombin ($0.013 \text{ U/ml} \pm 0.001$) and agitated at 1000 rpm for 4 minutes Results are expressed as mean percentage of control

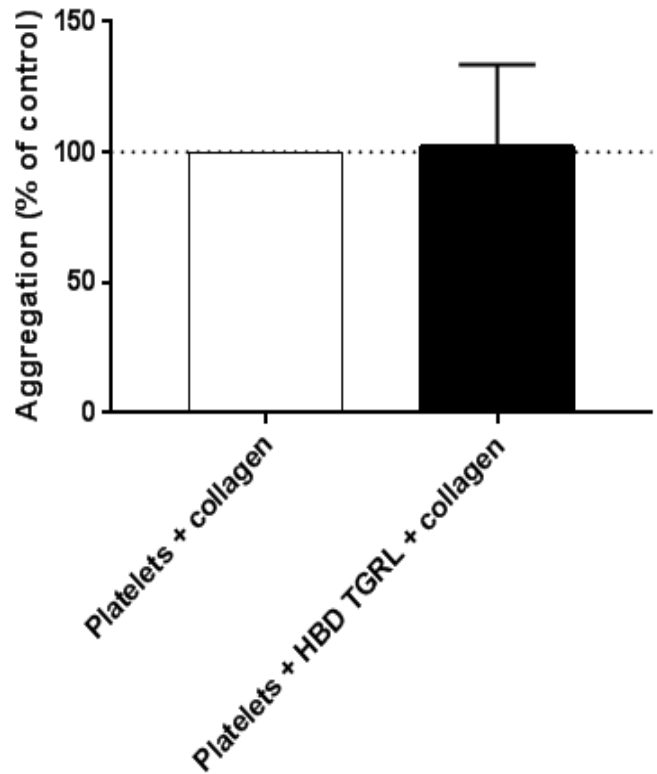
determined by platelets stimulated with thrombin and are means \pm SEM of 6 patients:
 $*p<0.05$.

(B): Platelets from healthy donors were incubated at 37°C for 30 minutes in the absence or presence of T2D large TGRL (25 μ g prot/ml) and stimulated with thrombin (0.1 U/ml) for 1 hour at 37°C while undergoing slow agitation (n=13). Results are expressed as mean TxB₂ concentrations in pmol/10⁹ platelets \pm SEM. Platelets + thrombin vs platelets alone: +1453%, $*p<0.05$. Platelets + T2D large TGRL + thrombin vs platelets + thrombin: +37%, $*p<0.05$.

(C and D): Platelets from healthy donors were incubated at 37°C with or without thrombin (0.09 \pm 0.01 U/ml) while undergoing slow agitation for controls. Platelets were incubated with 25 μ g prot/ml T2D large TGRL and thrombin under the same conditions for testing. Phosphorylation of p38MAPK and cPLA₂ was measured after SDS-PAGE separation and immunoblotting with anti-phospho p38 MAPK or anti-phospho cPLA₂ antibodies, followed by reprobing with beta-actin antibody. (n=6 and n=3, respectively). Results are expressed as mean percentage of control determined by platelets stimulated with thrombin **(C) phospho-p38MAPK:** Platelets + thrombin vs platelets alone: $*p<0.05$. Platelets + T2D large TGRL + thrombin vs platelets + thrombin: $*p<0.05$ and the representative immunoblots. **(D) phospho-cPLA₂:** Platelets + thrombin vs platelets alone: $*p<0.05$. Platelets + T2D large TGRL + thrombin vs platelets + thrombin: $*p<0.05$ and the representative immunoblots.

Figure 1A.

(i)



(ii)

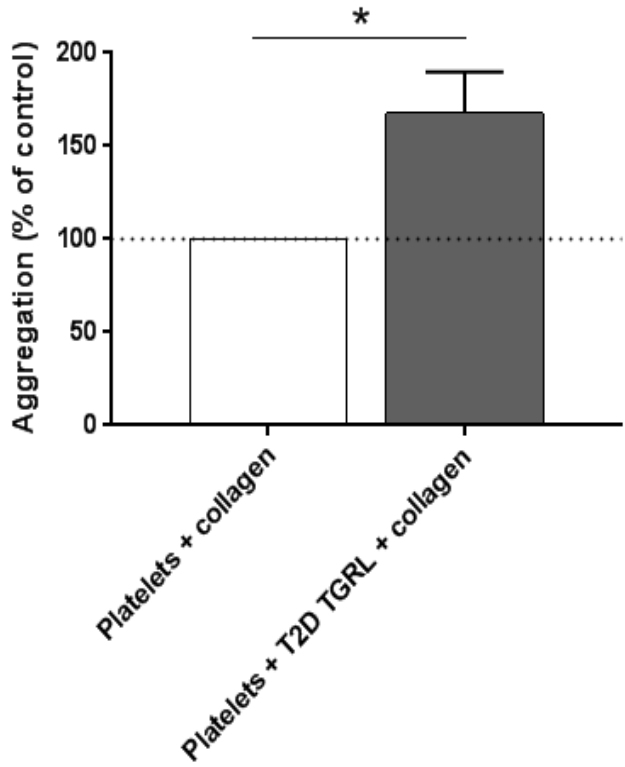


Figure 1B.

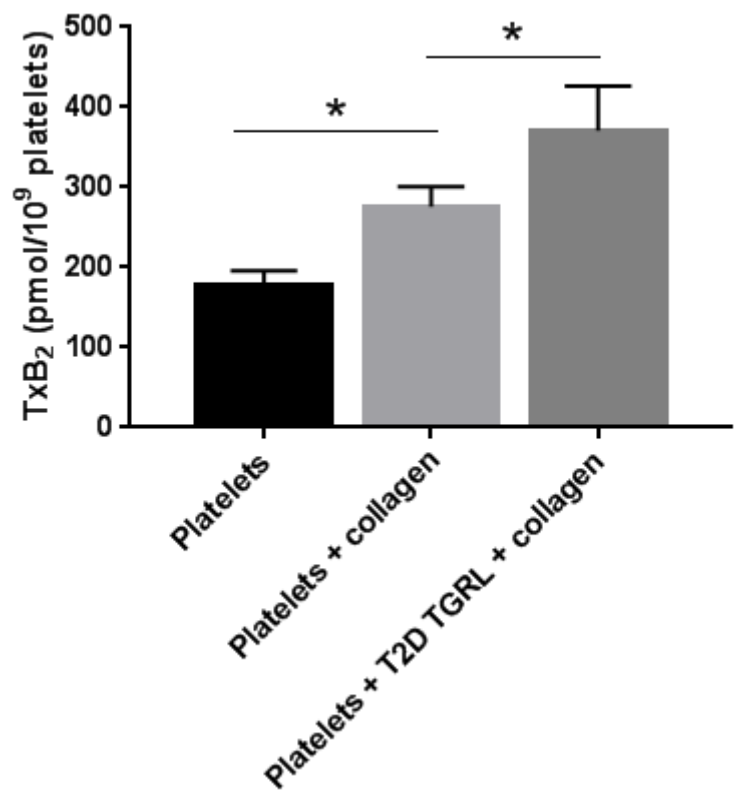
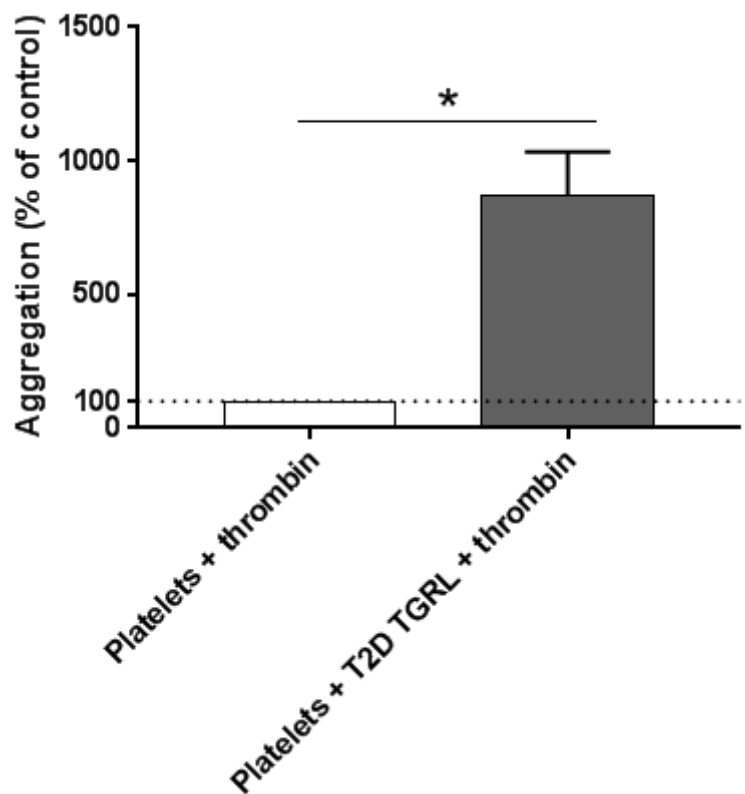
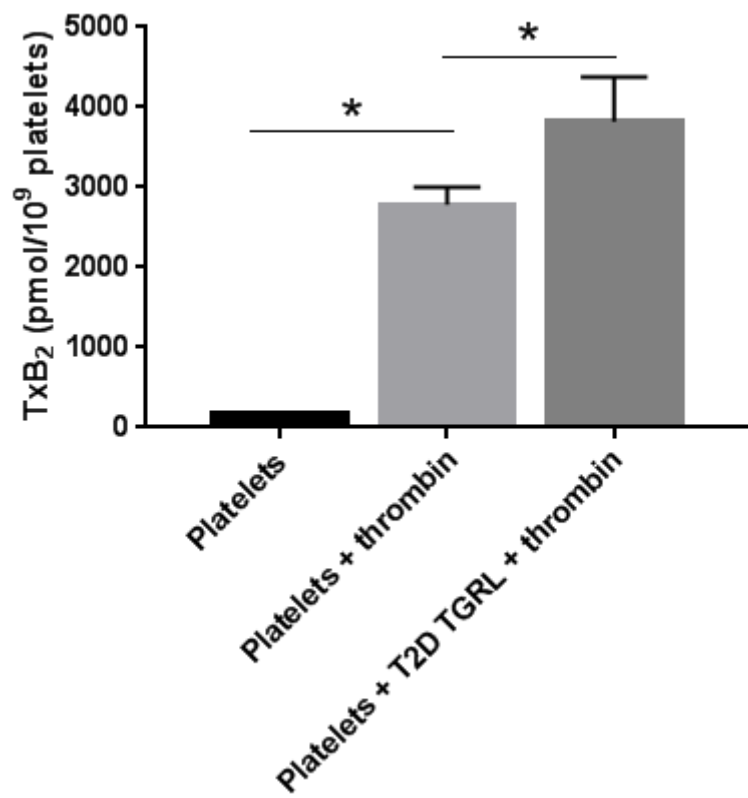


Figure 2A.



552 **Figure 2B.**



553

Figure 2C.

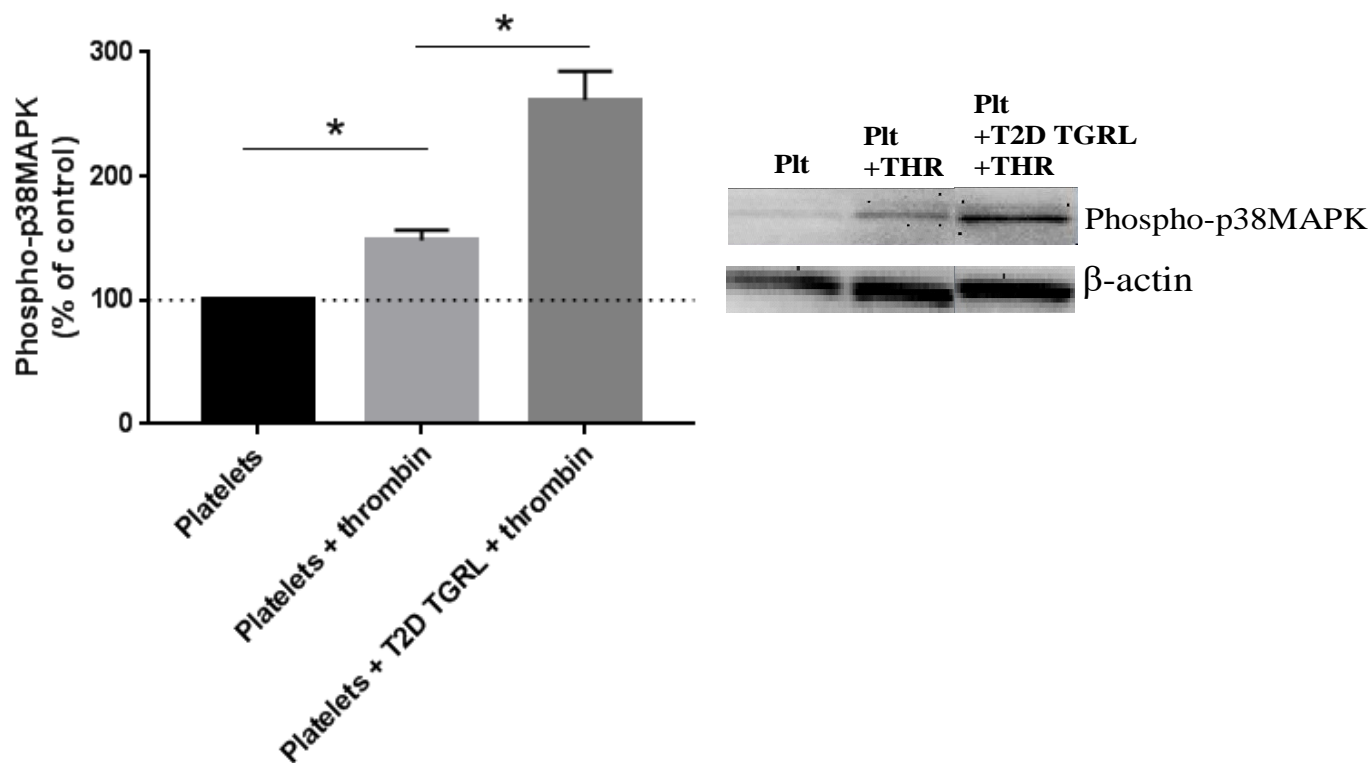


Figure 2D.

