In vivo platelet activation and aspirin responsiveness in type 1 diabetes mellitus

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Brief title: Platelet activation, aspirin and type 1 diabetes

Word count: 2,000

References: 25

Abstract word count: 187 words

Number of figures: 4

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Abstract

Platelet activation is persistently enhanced and its inhibition by low-dose aspirin is impaired in type 2 diabetes mellitus (T2DM). We investigated in vivo thromboxane (TX) and prostacyclin (PGI₂) biosynthesis and their determinants, as well as aspirin responsiveness in young adult subjects with type 1 diabetes mellitus (T1DM) without overt cardiovascular disease and stable glycemic control. The biosynthesis of TXA₂ was persistently increased in T1DM versus matched healthy subjects, with female showing higher urinary TX metabolite (TXM) excretion than male T1DM subjects. Microalbuminuria and urinary 8-iso-PGF₂α, an index of in vivo oxidative stress, independently predicted TXM excretion in T1DM. No homeostatic increase in PGI₂ biosynthesis was detected. Platelet COX-1 suppression by low-dose aspirin and the kinetics of its recovery following drug withdrawal were similar in patients and controls, and were unaffected by glucose variability.

We conclude T1DM patients with stable glycemic control, display enhanced platelet activation correlating with female gender, microvascular and oxidative damages. Moreover, aspirin responsiveness is unimpaired in T1DM, suggesting that the metabolic disturbance per se is unrelated to altered pharmacodynamics. The efficacy and safety of low-dose aspirin in T1DM warrants further clinical investigation.

Key words: type 1 diabetes, thromboxane, aspirin, platelet activation, prostacyclin, isoprostane.
Introduction

Type 1 diabetes mellitus (T1DM) is associated with an increased risk of early micro- and macro-vascular complications which shorten life expectancy (1-5). Although the increased cardiovascular risk is common to T1DM and type 2 diabetes (T2DM), the pathophysiology underlying early atherothrombosis in T1DM is less understood as compared to T2DM (1; 4). Platelet activation is known to contribute to the development and progression of atherothrombosis (6). Experimental animal models suggest platelet hyperactivity in T1DM (7; 8), but studies of platelet function in T1DM patients appear inconsistent (summarized in the online Table 1).

Aspirin is effective in atherothrombosis treatment and prevention (1; 5). However, the duration of the antiplatelet effect of low-dose aspirin may be reduced in T2DM patients, and a twice-daily dosing improves inhibition of T2DM platelets versus the standard once-daily regimen (9; 10). Whether this applies to T1DM remains unexplored.

The aims of our study were to investigate in vivo thromboxane (TX) and prostacyclin (PGI₂) biosynthesis and their determinants, as well as aspirin responsiveness in young adult T1DM patients without overt cardiovascular disease and stable glycemic control.

Research Design and Methods

Design of the studies

We performed a cross-sectional study of platelet and endothelial activation, as well as a short-term aspirin intervention study to assess drug responsiveness.

The cross-sectional study included 51 T1DM patients (online Table 2) diagnosed according to the American Diabetes Association (2). Exclusion criteria were: poorly controlled hypertension or hypercholesterolemia, cigarette smoking, pregnancy, obesity (body mass index >30kg/m²), aspirin intolerance, recent (<6 months) major bleeding, bleeding disorders, platelets <150,000/µL, use of antiplatelet, anticoagulant, nonsteroidal antiinflammatory and/or oral hypoglycemic drugs.
The intra-subject coefficient of variation (CV) of each measured biomarker was assessed in 10 patients by repeating blood and urine samplings 3 times over 10 days.

The intervention study included 31 T1DM and 10 matched healthy subjects (online Table 2) who were given enteric-coated aspirin 100 mg (Cardioaspirin®, Bayer, Italy) once daily at 8 pm for 21 days. In the evening of the last day on treatment, they underwent blood and urine sampling and then a witnessed aspirin intake. A continuous glucose monitor (CGM) (CGMS® System Gold TM, Medtronic MiniMed, Northridge, CA) was inserted subcutaneously in the abdomen for measuring interstitial glucose every 5-min over 24 hours. Blood and urine samples were collected 12, 24, 48, 72 hours, and 7 days thereafter. The study was approved by the Ethics Committee. Subjects signed an informed consent.

**Analytical measurements**

Routine haematology, routine chemistry, immature platelets, high-sensitivity C-reactive protein (CRP), soluble receptor of the advanced glycation end products (sRAGE), interleukin (IL)-15 and IL-6 levels were measured with commercial kits (online material).

The rate of in vivo TXA₂ biosynthesis was assessed by the urinary excretion of its major enzymatic metabolite, 11-dehydro-TXB₂ (TXM), as previously described (9; 11). In vivo oxidant stress was assessed by the urinary excretion of the F₂-isoprostane, 8-iso-PGF₂α (12) as previously described (9); the major urinary prostacyclin metabolite (PGIM), 2,3-dinor-6-keto-PGF₁α was measured as previously described (13) (online material). The degree of platelet COX-1 inhibition by aspirin and the time-course of post-aspirin recovery were assessed by serum TXB₂ a validated index of the maximal biosynthetic capacity of platelet COX-1, as previously described (14). Ex vivo platelet function in whole blood was assessed by the VerifyNow Aspirin System (Accumetrics, San Diego, CA).
**Statistical analysis**

Considering serum TXB$_2$ levels previously measured in healthy subjects at 12 and 24 hours post-aspirin (15), 30 T1DM patients and 10 matched healthy subjects would allow detecting a difference of 1.4 ng/ml between the groups 24 hours post-dosing (two-sided $\alpha=0.05$, power=0.90). Based on our previous measurements of TXM excretion in healthy subjects (15), 50 T1DM patients and 10 healthy subjects would allow detecting an absolute difference $\geq$100 pg/mg creatinine, equivalent to 25% higher values in diabetics (two-sided $\alpha=0.05$, power=0.90). We also compared urinary TXM, 8-iso-PGF$_{2\alpha}$ and PGIM excretion of T1DM subjects with previously published, matched healthy subjects (online Table 3).

Data are presented as mean ± standard deviation (SD) or median and interquartile range [IQR] based on distribution; differences were evaluated with parametric (analysis of variance) or non-parametric (Mann-Whitney) tests, and correlations estimated with Spearman rank test. The kinetics of serum TXB$_2$ and TXM recovery post-aspirin were fitted using GraFit (3.0, Erithacus Software Ltd., Horley, UK), where medians or means were plotted against time. Different equations were evaluated by F-test. Analyses were performed with Stata (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX) and SigmaPlot (version 3.1; Systat Software, San Jose, CA). A $p<0.05$ was considered statistically significant.

**Results**

*Platelet activation and its determinants in T1DM subjects*

The baseline clinical characteristics, biochemical and hematological variables and their correlations are detailed in online Tables 2 and 4.

The urinary excretion rates of TXM and 8-iso-PGF$_{2\alpha}$, *in vivo* indexes of platelet activation and oxidative stress, respectively, were quite stable over time (online Fig. 1 and 2; TXM CV: 17±8%; 8-iso-PGF$_{2\alpha}$ CV: 29±15%). T1DM subjects had significantly higher TXM excretion and inter-individual variability as compared to the pooled group of 63 (including 53 previously
published) subjects (Fig. 1A), and to the 10 healthy subjects recruited for this study (p<0.01). Urinary TXM excretion in T1DM was significantly higher in female versus male patients (Fig. 1B) and correlated inversely with body weight (rho=-0.32, p=0.030), and directly with microalbuminuria (Fig. 1C) and urinary 8-iso-PGF$_{2\alpha}$ (Fig. 1D). A multivariate analysis including urinary 8-iso-PGF$_{2\alpha}$, microalbuminuria and gender as independent variables, showed that TXM could be predicted by 8-iso-PGF$_{2\alpha}$ (p<0.001), microalbuminuria (p<0.001), and female gender (p=0.06) (adjusted $R^2=0.66$ for the entire model). Urinary 8-iso-PGF$_{2\alpha}$ excretion of T1DM subjects was significantly higher than the pooled group of healthy subjects (Fig. 2A), without gender-related differences. Urinary PGIM excretion, an index of endothelial thromboresistance, was comparable in T1DM and healthy subjects (Fig. 2B), without gender-related differences.

The intra-subject CV of serum TXB$_2$ upon repeated measurements was 16±6% (online Fig. 3); its median values were similar in T1DM and healthy subjects (online Table 2, p=0.26). Platelet count and volume, ex vivo platelet function, hs-CRP, IL-6, IL-15 and sRAGE were comparable in T1DM and healthy subjects, while immature platelets were significantly lower in T1DM (online Table 2). However, none of these biomarkers correlated with urinary prostanoid metabolites or serum TXB$_2$ (data not shown). Other correlations are shown in the online Table 5.

**Platelet COX-1 inhibition and recovery following aspirin**

Thirty-one T1DM and 10 healthy subjects (see online Table 2) were given aspirin 100 mg once daily for 21 days. Compliance was assessed by pill count and by comparing serum TXB$_2$ immediately before and 24 hours after the witnessed intake (p=0.7 for paired comparisons, and online material). Platelet COX-1 activity, as reflected by serum TXB$_2$, was suppressed by 99.2 [98-99.6]% and 99.3 [98-99.5]%, in healthy and T1DM subjects, respectively, at 12 hours, and by 98.6[98.3-99.3]% and 98.4[98-99.2]% at 24 hours after witnessed aspirin (Fig. 3). In both T1DM and healthy subjects, <1% of the pre-aspirin level (serum TXB$_2$ 1.5 and 1.1 ng/ml, respectively) was recovered between 12 and 24 hours after the witnessed intake, consistent with permanent
suppression of platelet COX-1 throughout the dosing interval. The recovery kinetics of serum TXB₂ up to 7 days following aspirin withdrawal showed a similar exponential pattern in T1DM and healthy subjects (Fig. 4A). *Ex vivo* platelet function, as assessed by the VerifyNow assay, showed similar inhibition and recovery in T1DM and healthy subjects (online Fig. 4).

Urinary TXM excretion was also reduced by aspirin in both groups. Consistent with an increased baseline rate, urinary TXM excretion remained significantly higher in T1DM as compared to healthy subjects following aspirin withdrawal (online Fig. 5). In healthy subjects, urinary TXM largely (72±27%) recovered by 72 hours, and the recovery was described by a first-order equation (Fig. 4B). In T1DM, TXM recovery at 72 hours averaged 47±21% of pre-aspirin values (p=0.012 vs. healthy subjects), but the kinetic parameters of the recovery best fitting were similar to controls (Fig. 4B, F-test, p=0.7).

Urinary 8-iso-PGF₂α and PGIM excretion rates were not affected by aspirin intake to any statistically significant extent (online Fig. 6). Moreover, CGM-derived mean glucose values and their SD, an index of short-term glucose variability (16), were not associated with the degree of TXA₂ inhibition by aspirin or with its recovery following drug withdrawal (online Table 6).

**Discussion**

We showed that well-controlled, adult T1DM subjects have persistently enhanced *in vivo* platelet activation, as reflected by TXA₂ biosynthesis, and oxidative stress, as reflected by F₂-isoprostane biosynthesis, despite relatively young age and absence of symptomatic cardiovascular disease. Our data substantially extend and clarify previous contradictory findings on platelet function in T1DM (online Table 1). In our T1DM cohort, with stable glycemic control, the rate of *in vivo* TXA₂ biosynthesis appeared largely driven by endothelial dysfunction (17) and oxidative stress. Moreover, TXA₂ biosynthesis was significantly higher in female than male patients. Higher ischemic heart disease has been reported in young T1DM women versus T1DM men or healthy
women (3). Differences in platelet activation might contribute, at least in part, to gender-related differences in atherothrombotic risk of T1DM.

At variance with platelet TXA₂, the in vivo biosynthesis of PGI₂ was comparable in T1DM and healthy subjects. Endothelial PGI₂ physiologically inhibits platelet activation, thus, unchanged PGI₂ biosynthesis in the face of persistently enhanced TXA₂-dependent platelet activation in T1DM might be interpreted as a failure of the endothelial response to platelet activation. Indeed, an interplay between enhanced in vivo TXA₂ biosynthesis from activated platelets and a parallel increase in vascular PGI₂ has been reported in patients and mice with severe atherosclerosis (18; 19). Moreover, COX-2 inhibition doubles the risk of myocardial infarction (20), consistent with COX-2-dependent PGI₂ biosynthesis acting as an important mechanism of endothelial thromboresistance. Hypoestrogenism has been described in young T1DM women (21). PGI₂ biosynthesis is modulated by estrogens via COX-2 (22) and, in animal models, estrogen-dependent atheroprotection largely relies on COX-2-derived PGI₂ (23).

Despite persistent platelet activation in T1DM, aspirin responsiveness appeared substantially unchanged during the 24-hour dosing interval. Moreover, platelet COX-1 recovery up to 7 days post-aspirin, an index of platelet turnover-dependent renewal of the drug target (24), was comparable in T1DM and healthy subjects. Consistently, we found no evidence of accelerated platelet turnover in T1DM as shown by a number of reticulated platelets which were even slightly decreased in patients (online Table 2). These findings demonstrate, for the first time, that T1DM does not share the abnormal aspirin pharmacodynamics, previously described in T2DM (9-10). Neither glycemic control nor its 24-hour variability influenced aspirin responsiveness of T1DM, providing indirect evidence that the impaired platelet COX-1 inhibition reported in T2DM is not a consequence of hyperglycemia.

Recent studies describing excess cardiovascular morbidity and mortality in T1DM (3; 25) emphasize the importance of early preventive strategies. In principle, the benefit/risk profile of low-dose aspirin could be more favorable in T1DM than T2DM due to unimpaired antiplatelet
pharmacodynamics, lower bleeding liability because of younger age, and under-representation of other bleeding risk factors.

Study limitations include: i) lack of clinical endpoints due to the small sample size and mechanistic nature of the investigation. However, our results provide important information and rationale for a trial of antiplatelet prophylaxis in T1DM; ii) highly selected nature of our T1DM population, whereby inclusion and exclusion criteria excluded potential confounders affecting platelet activation while maximizing patients’ safety. However, the study allowed characterizing the major determinants of platelet activation and safely exploring low-dose aspirin pharmacodynamics in T1DM. Having excluded obese subjects, these results cannot be extrapolated to obese T1DM patients; finally, iii) we did not explore other platelet activatory signaling pathways in addition to TXA$_2$ and their pathophysiologic importance in T1DM cannot be excluded.

In conclusion, asymptomatic, young T1DM subjects show persistently enhanced TXA$_2$-dependent platelet activation and oxidant stress in vivo uncoupled to a homeostatic increase in vascular PGI$_2$ biosynthesis. Persistent platelet activation in T1DM patients with stable glycemic control, is possibly related to female gender, microvascular and oxidative damages. Differently from T2DM, aspirin responsiveness is unimpaired in T1DM, suggesting that the metabolic disturbance per se is not responsible for altered pharmacodynamics. The efficacy and safety of low-dose aspirin in T1DM warrants further investigation.

**Author Contribution:** F.Z.: acquisition, statistical analysis, interpretation of data, paper drafting; A.R.: data acquisition, analysis and interpretation of data, paper drafting; G.P.: acquisition and analysis of data; F.C.: analysis, interpretation of data, paper drafting; L.T.: data acquisition and analysis; F.P.: data acquisition and analysis; V.C.: data acquisition and analysis; A.C.: data acquisition and analysis; A.H.: analysis and interpretation of data; I.S.: sample analysis; P.R.: data acquisition; E.T.: interpretation of data, paper drafting; B.R.: design, statistical analysis,
interpretation of data, paper drafting; D.P.: data acquisition and interpretation; C.P.: conception, design, analysis, interpretation of data, paper drafting.

**Acknowledgements.** The Authors thank Prof. Raimondo De Cristofaro for experimental data fitting. The enthusiastic and generous participation of all T1DM and healthy subjects is gratefully acknowledged.

Manuscript Guarantors: Carlo Patrono, Dario Pitocco, Bianca Rocca

**Financial supports:** The study was supported by grants from: the Innovative Medicines Initiative Joint Undertaking under grant agreement n°115006, the SUMMIT Consortium, to C.P.; Catholic University, Linea D3.2 2013-70201169 to B.R.; and Italian Ministry for University and Research ‘Fondo per il Sostegno dei Giovani’ 2012 to A.R.

**Disclosures:** C.P. has received an institutional grant from Bayer AG for investigator-initiated research and is an unpaid member of the Scientific Advisory Board of the Aspirin Foundation.
References


consistently by platelet function assays: implications for aspirin "resistance". J Am Coll Cardiol 53:667-677, 2009
Figure legends

Figure 1. Urinary TXM excretion and its determinants in T1DM. A and B: Box-whisker plots representing median, IQR, minimum and maximum values of urinary TXM excretion rates in 51 T1DM and 63 healthy subjects (A); and a comparison of urinary TXM excretion in 32 male and 19 female T1DM subjects (B). C and D: The plots represent individual microalbuminuria (n=46) (C) and 8-iso-PGF$_{2\alpha}$ measurements (n=51) (D) and the corresponding urinary TXM excretion rates in T1DM patients. Rho and p values are indicated in each plot.

Figure 2. Urinary 8-iso-PGF$_{2\alpha}$ and PGIM excretion in T1DM and healthy subjects. A: Box-whisker plots representing median, IQR, minimum and maximum values of urinary 8-iso-PGF$_{2\alpha}$ excretion rates in 51 T1DM and 57 healthy subjects. B) Box-whisker plots representing median, IQR, minimum and maximum values of urinary PGIM excretion rates in 46 T1DM and 31 healthy subjects.

Figure 3. Effects of low-dose aspirin on platelet thromboxane production in T1DM and healthy subjects. Mean and SD of serum TXB$_2$ measured at baseline (before aspirin), and 12 and 24 hours after the last witnessed aspirin intake in 31 T1DM (A) and 10 healthy (B) subjects. *p<0.001 versus baseline values; #p<0.05 versus 12-hour values. The insert in each panel magnifies the serum TXB$_2$ values at 12 and 24 hrs after dosing.

Figure 4. Kinetics of serum TXB$_2$ and TXM recovery following aspirin withdrawal in T1DM and healthy subjects. A) Mean and SD of serum TXB$_2$ values measured at 12, 24, 48, 72 hours and 7 days after the last witnessed aspirin intake in 31 T1DM and 10 healthy subjects. Data were fitted according to a previously described equation (15). *p<0.05 vs. healthy subjects. B) Mean and
SD of urinary TXM excretion, expressed as % of baseline, measured at 12, 24, 48, 72 hours and 7 days after the last witnessed aspirin intake in 31 T1DM and 10 healthy subjects. Data were fitted according to a previously described equation (15). *p<0.05 vs. healthy subjects.
Figure 2

A.
 Urinary 8iso-PGF$_{2\alpha}$ pg/mg creatinine

- Healthy subjects
- T1DM subjects

P < 0.001

B.
 Urinary PGIM pg/mg creatinine

- Healthy subjects
- T1DM subjects

P = 0.57
Figure 3

A

T1DM subjects

Serum TXB$_2$ ng/ml

baseline 12 hours 24 hours

Post-Aspirin

B

Healthy subjects

Serum TXB$_2$ ng/ml

baseline 12 hours 24 hours

Post-Aspirin
A

Serum TXB$_2$ ng/ml

Healthy subjects

T1DM subjects

Time following aspirin withdrawal

B

Urinary TXM % of baseline

Healthy subjects

T1DM subjects

Time following aspirin withdrawal

Figure 4
ONLINE SUPPLEMENTARY MATERIAL

In vivo platelet activation and aspirin responsiveness in type 1 diabetes mellitus

Materials for analytical measurements

Immature platelets were counted by the Sysmex® XE-500 Instrument (Sysmex Corporation, Kobe, Japan); high-sensitivity C-reactive protein (CRP) was measured by an ELISA Kit (Cycllex Co.Ltd, Nagano, Japan), sRAGE by Quantikine® ELISA human RAGE kit (R&D System, Minneapolis, MN), interleukin (IL)-15 by Quantikine® ELISA kit (R&D Systems) and IL-6 by an EIA kit-human (Cayman Europe, Tallinn, Estonia). All measurements were made according to the manufacturer’s instructions. For serum thromboxane (TX) B2 measurements, briefly, 2 ml of venous blood were collected into a glass tube without anticoagulant, immediately clotted for 1 hour at 37° C, centrifuged at 1,200 g for 10 min and the supernatant serum was stored at -20°C until assayed. Serum TXB2 was measured using an enzyme immunoassay (EIA), as previously described (1; 2). Urinary 11-dehydro-TXB2 (TXM) and 8-iso-PGF2α were extracted by a chromatographic method adapted to 1 ml of urine, and measured by EIA assays (3; 4). Urinary 2,3-dinor-6-keto-PGF1α (PGIM) was measured by as previously described by liquid chromatography-tandem mass spectrometry (5). All urinary metabolite measurements were expressed as pg/mg of urinary creatinine, assessed by a colorimetric kit (Creatinine Colorimetric Detection Kit; Enzo Life Sciences, Farmingdale, NY).

Check of compliance

Compliance was assessed by pill count at the last visit, before witnessed aspirin administration, as well as by comparing serum TXB2 levels in each patients measured at two time points: immediately before the in-hospital witnessed administration, i.e. at 8 pm of the 20th day
on treatment and 24 hours after the witnessed administration. The serum TXB₂ levels measured before the witnessed administration would likely reflect compliance of the patient at home during the previous 3-4 days. This method not only reflects compliance, but might also unmask recent intake of NSAIDs. If the value of serum TXB₂ at 8pm of day 20 differed by >20% from the corresponding value at 8pm on day 21 (post-witnessed) the patient was further investigated and questioned about compliance, time of aspirin intake at home and possible NSAID use. The cutoff of 20% took into account the variability of the serum TXB₂ analytical assay, i.e. ~15%. None of our T1DM or healthy subjects had differences between the two time points > 20%. In particular, we measured the following values of serum TXB₂ in the T1DM cohort: 3.4 [2.3-6.3] ng/ml on day 20th and 3.5 [2.1-5.5] ng/ml on day 21st (p=0.7 for paired comparisons).

**Online References**

Online Figure legends

**Online Figure 1. Consistency of thromboxane biosynthesis over time in T1DM.** The figure shows individual urinary TXM excretion values measured in 9 T1DM subjects who were sampled 3 times over 7 to 10 days (visits 1 to 3). Horizontal bars represent the mean value for each visit. Intra-patient values were not significantly different across different visits.

**Online Figure 2. Consistency of F2-isoprostane biosynthesis over time in T1DM.** The figure shows individual urinary 8-iso-PGF$_2\alpha$ excretion values measured in 10 T1DM subjects who were sampled 3 times over 7 to 10 days (visits 1 to 3). Horizontal bars represent the mean value for each visit. Intra-patient values were not significantly different across different visits.

**Online Figure 3. Consistency of platelet thromboxane production over time in T1DM.** The figure shows individual serum TXB$_2$ values measured in 9 T1DM subjects who were sampled 3 times over 7 to 10 days (visits 1 to 3). Horizontal bars represent the mean value for each visit. Intra-patient values were not significantly different across different visits.

**Online Figure 4. Ex vivo platelet function as assessed by the VerifyNow Aspirin assay at baseline and post-aspirin in T1DM and healthy subjects.** The figure depicts mean and SD values of Aspirin Reaction Units (ARUs) measured in T1DM (n=6) and healthy subjects (n=10) at baseline (pre-aspirin) and between 12 hours and 7 days after the last witnessed aspirin intake. The dotted line represents the upper threshold of ‘aspirin responsiveness’ (550 ARU). *p<0.05 versus healthy subjects.
Online Figure 5. Kinetics of the recovery of thromboxane biosynthesis following aspirin withdrawal in T1DM and healthy subjects. The figure depicts mean (±SD) values of urinary TXM excretion measured in 31 T1DM and 10 healthy subjects, between 12 hours and 7 days after the last witnessed aspirin intake. *p<0.05 versus the corresponding value of healthy subjects.

Online Figure 6. Unchanged F₂-isoprostone and PGIM biosynthesis in T1DM subjects following aspirin administration. A) Box-whisker plots represent medians and IQR of urinary 8-iso-PGF₂α excretion values measured in 31 T1DM subjects at baseline (pre-aspirin) and between 12 hrs and 7 days after the last witnessed aspirin intake. Values were not-significantly different among the different time-points. B) The box-whisker plots represent medians and IQR of urinary PGIM excretion rates in T1DM subjects (n=31) before starting a three-week course of 100 mg daily aspirin and 24 hrs after the last witnessed intake. Pre- and post-aspirin values were not significantly different.
Online Table 1. Summary of published studies exploring platelet function or platelet-related indexes in T1DM

<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Population</th>
<th>Methodology</th>
<th>Results</th>
</tr>
</thead>
</table>
| **Alessandrini P et al**  
*(N Engl J Med 319:208-212, 1988)* | 28 T1DM with and without retinopathy, 20 controls | Urinary excretion of 2,3-dinor-TXB₂ and 2,3-dinor-6-keto-prostaglandin F₁α, platelet granule constituents, the aggregation response to ADP or arachidonic acid, and levels of serum TXB₂ | 2,3-dinor-TXB₂ and 2,3-dinor-6-keto-prostaglandin F₁α did not differ between diabetics with or without retinopathy and controls. Platelet granule constituents, the aggregation response to ADP or arachidonic acid and serum TXB₂ failed to discriminate between the groups |
| **Hu H et al**  
*(Thromb Res 106:91-95, 2002)* | Platelets isolated from 10 patients and 10 controls | Platelets pre-incubated with insulin, C-peptide or both and then ADP-stimulated. Platelet-fibrinogen binding and P-selectin with FACS analysis | Insulin pre-incubation did not influence in vitro platelet reactivity |
| **Davi’ G et al**  
*(Circulation 107:3199-3203, 2003)* | Pediatric patients: 23 new-onset T1DM, 23 T1DM > 1 yr, 23 controls | Urinary 8-iso-PGF₂α, TXM, plasma IL-6, TNF-α, serum CRP. | Urinary 8-iso-PGF₂α, TXM, IL-6, CRP and TNF-α higher in patients vs. controls. Newly-diagnosed patients had higher prostanoid excretion than patients with established disease. Disease duration and IL-6 predicted 8-iso-PGF₂α |
| **Yngen M et al**  
*(Diabetologia 47:537-540, 2004)* | T1DM adult patients without (n=19) and with (n=20) microangiopathy; 27 controls | Thrombin-induced platelet P-selectin; soluble CD40 ligand and P-selectin, CRP, soluble E-selectin. | All parameters increased only in patients with microangiopathy. No differences between controls and patients without microangiopathy. |
| **Harding SA et al**  
*(Atherosclerosis 176:321-325, 2004)* | 22 patients with uncomplicated T1DM and 22 controls | CRP, soluble CD40 ligand, platelet CD40 expression, platelet-monocyte aggregates | All parameters higher in patients than controls. CRP and platelet-monocyte aggregates correlated with glycemia |
| **Tarnow I et al**  
*(Platelets 20:513-519, 2009)* | Adult T1DM patients: 35 with nephropathy, 51 without nephropathy, 30 controls | Platelet membrane P-selectin, GPIIb/IIIa, platelet monocyte aggregates, platelet neutrophils aggregates after ADP- or TRAP-induced aggregation (flow cytometry analysis) | P-selectin higher in response to ADP in nephropatic vs. non-nephropatic patients or healthy controls. No differences for GPIIb/IIIa expression and no differences upon TRAP stimulation |
| **Vignini A et al**  
(Diabetes Metab Res Rev 27:277-285, 2011) | 30 newly-diagnosed pediatric T1DM patients and 20 controls | Platelet membrane fluidity, NO and peroxynitrite production, Na⁺/K⁺ ATP-ase activity | Increased rigidity of inner platelet membrane; NO and Na⁺/K⁺ ATP-ase activity reduced; peroxynitrite increased. |
| **Schlingemann RO et al**  
(Diabetes Care 36:1629-1634, 2013) | 64 T1DM patients, 21 controls | Plasma beta-thromboglobulin, PF-4, VEGF | No differences in beta-thromboglobulin in patients and controls |

**Abbreviations:** CRP: C-reactive protein; sRAGE: soluble receptor of advanced glycation end-products; IL: interleukin; TNF: tumor necrosis factor; 8-iso-PGF₂α: 8-iso-prostaglandin F₂α; TXM: urinary thromboxane metabolite; GPIIb/IIIa: glycoprotein IIb/IIIa; ADP: adenosine diphosphate; TRAP: thrombin receptor activating peptide; FACS: fluorescence activated cell sorting; VEGF: vascular endothelial growth factor; PF-4: platelet factor-4; TX: thromboxane.
Online Table 2. Baseline characteristics of T1DM and healthy subjects enrolled in the cross-sectional and intervention studies.

<table>
<thead>
<tr>
<th>Variable</th>
<th>All T1DM subjects (n=51)</th>
<th>T1DM subjects in the aspirin study (n=31)</th>
<th>Healthy subjects (n=10)</th>
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<td>Fasting plasma glucose - mmol/L</td>
<td>7.8±4.2</td>
<td>7.4±4.1</td>
<td>5.2 ± 1</td>
</tr>
<tr>
<td>Glycated hemoglobin (HbA1c)-%</td>
<td>7.3±1 (56±3)</td>
<td>7.3 ± 1 (56±3)</td>
<td>5.4 ± 0.3 (36±1)</td>
</tr>
<tr>
<td>Diabetes duration - years</td>
<td>14 [8-23]</td>
<td>14 [8-24]</td>
<td>NA</td>
</tr>
<tr>
<td>Retinopathy- no. (%)</td>
<td>1 (2%)</td>
<td>1 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>Total cholesterol - mmol/L</td>
<td>4.8 ± 1</td>
<td>4.8 ± 0.9</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>LDL cholesterol - mmol/L</td>
<td>2.7 ± 0.8</td>
<td>2.8 ± 0.8</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>HDL cholesterol - mmol/L</td>
<td>1.6 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>Triglycerides - mmol/L</td>
<td>0.7 [0.6-1]</td>
<td>0.7 [0.6-1]</td>
<td>0.6 [0.6-1]</td>
</tr>
<tr>
<td>eGFR - mL/min</td>
<td>121 ± 28</td>
<td>116 ± 27</td>
<td>112 ± 24</td>
</tr>
<tr>
<td>Microalbuminuria - mg/L</td>
<td>7 [3-12]</td>
<td>7 [4-12]</td>
<td>NA</td>
</tr>
<tr>
<td>Mean platelet volume - fl</td>
<td>10.8 [10.3-11.5]</td>
<td>10.8 [10.3-11.5]</td>
<td>10.9 [10.3-11]</td>
</tr>
<tr>
<td>Reticulated platelets - %</td>
<td>2.9 [1.9-3.8]</td>
<td>3.0 [2.0-3.9]</td>
<td>3.9 [3.5-5.3]</td>
</tr>
<tr>
<td>Reticulated platelets - 10^9/µL</td>
<td>6.6 [4.6-8.8]*</td>
<td>6.7 [4.8-8.8]</td>
<td>10.7 [8.8-14.7]</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>0.8 [0.4-1.5]</td>
<td>0.7 [0.4-1.4]</td>
<td>0.6 [0.2-1]</td>
</tr>
<tr>
<td>sRAGE (pg/mL)</td>
<td>1,339±378</td>
<td>1,307±353</td>
<td>1,410±504</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>2.2 [2-3.6]</td>
<td>1.3 [0.8-2.8]</td>
<td>1.4 [0.8-2.8]</td>
</tr>
<tr>
<td>IL-15 (pg/mL)</td>
<td>1.9 [1.4-3.1]</td>
<td>1.9 [1.2-3.0]</td>
<td>NA</td>
</tr>
<tr>
<td>Serum TXB2 (ng/ml)</td>
<td>257 [210-306]</td>
<td>256 [209-305]</td>
<td>301 [241-425]</td>
</tr>
<tr>
<td>Verify-Now (ARU)</td>
<td>NA</td>
<td>642±41#</td>
<td>659±5</td>
</tr>
</tbody>
</table>
Values are mean ± SD or median and IQR. None of the differences is statistically significant unless indicated.

**Abbreviations:** AST: Aspartate Transaminase; ALT: Alanine Transaminase; ARU: Aspirin Reactive Units; BMI: Body Mass Index; CRP: C-reactive protein; eGFR: estimated glomerular filtration rate according to the Cockcroft-Gault formula; HDL: High-Density Lipoprotein; IL: interleukin; LDL: Low-Density Lipoprotein; NA: not applicable/available; sRAGE: soluble receptor of advanced glycation end-products.

*p<0.05 versus healthy controls;  + p=0.05 versus healthy controls; #n=6
## Online Table 3. Urinary prostanoid excretion rates in healthy subjects from published (TXM and 8-iso-PGF$_{2\alpha}$) or unpublished (PGIM) studies and enrolled in the present study.

<table>
<thead>
<tr>
<th></th>
<th>Previously published, matched healthy subjects (n=53; 16F, 37M)#</th>
<th>Unpublished matched healthy subjects (n=21; 8F, 13M)§</th>
<th>Current controls (n=10; 3F, 7M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, yr</strong></td>
<td>37±11</td>
<td>37±6</td>
<td>33±7</td>
</tr>
<tr>
<td><strong>Urinary TXM</strong></td>
<td>513±433</td>
<td>NA</td>
<td>584±153</td>
</tr>
<tr>
<td>(pg/mg creatinine)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Urinary 8-iso-</strong></td>
<td>417±274 (n=47; 10F, 37M)</td>
<td>NA</td>
<td>519±200</td>
</tr>
<tr>
<td><strong>PGF$_{2\alpha}$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pg/mg creatinine)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Urinary PGIM</strong></td>
<td>NA</td>
<td>342±351</td>
<td>259±138</td>
</tr>
<tr>
<td>(pg/mg creatinine)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD. The differences among different groups of healthy controls for the same parameter are all non-significant. NA: not available.

**Abbreviations:** TXM: thromboxane metabolite; 8-iso-PGF$_{2\alpha}$: 8-iso-prostaglandin F$_{2\alpha}$; PGIM: prostacyclin metabolite.


§individual data from: Cavalca V and Tremoli E, unpublished data.
Online Table 4. Univariate correlations between anthropometric, clinical and routine biochemical indexes in 51 T1DM subjects

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>Age</th>
<th>SBP</th>
<th>DBP</th>
<th>Diabetes Duration</th>
<th>eGFR</th>
<th>Total Cholesterol</th>
<th>LDL Cholesterol</th>
<th>FPG</th>
<th>HbA1c</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP</td>
<td>0.43</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBP</td>
<td>0.29</td>
<td>0.63</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes Duration</td>
<td>0.49</td>
<td>0.04</td>
<td>0.03</td>
<td></td>
<td>&lt;0.001</td>
<td>0.779</td>
<td>0.840</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR</td>
<td>-0.35</td>
<td>0.12</td>
<td>0.31</td>
<td>-0.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.022</td>
<td>0.446</td>
<td>0.039</td>
<td>0.029</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>0.52</td>
<td>0.10</td>
<td>0.07</td>
<td>0.26</td>
<td>-0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>0.485</td>
<td>0.650</td>
<td>0.081</td>
<td>0.661</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL Cholesterol</td>
<td>0.51</td>
<td>0.16</td>
<td>0.23</td>
<td>0.34</td>
<td>0.03</td>
<td>0.87</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>0.295</td>
<td>0.115</td>
<td>0.025</td>
<td>0.829</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPG</td>
<td>0.09</td>
<td>0.01</td>
<td>-0.07</td>
<td>0.10</td>
<td>0.04</td>
<td>0.01</td>
<td>-0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.541</td>
<td>0.952</td>
<td>0.665</td>
<td>0.516</td>
<td>0.789</td>
<td>0.964</td>
<td>0.811</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.33</td>
<td>-0.01</td>
<td>-0.07</td>
<td>0.35</td>
<td>-0.16</td>
<td>0.13</td>
<td>0.16</td>
<td>0.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.016</td>
<td>0.937</td>
<td>0.633</td>
<td>0.018</td>
<td>0.298</td>
<td>0.385</td>
<td>0.278</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.22</td>
<td>0.33</td>
<td>0.34</td>
<td>0.03</td>
<td>0.54</td>
<td>0.33</td>
<td>0.32</td>
<td>0.01</td>
<td>-0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.119</td>
<td>0.018</td>
<td>0.015</td>
<td>0.831</td>
<td>&lt;0.001</td>
<td>0.019</td>
<td>0.032</td>
<td>0.949</td>
<td>0.239</td>
<td></td>
</tr>
<tr>
<td>WHR</td>
<td>0.27</td>
<td>0.35</td>
<td>0.49</td>
<td>-0.05</td>
<td>0.23</td>
<td>0.09</td>
<td>0.18</td>
<td>-0.24</td>
<td>-0.25</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.056</td>
<td>0.011</td>
<td>&lt;0.001</td>
<td>0.717</td>
<td>0.149</td>
<td>0.513</td>
<td>0.234</td>
<td>0.124</td>
<td>0.081</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Data are reported as Spearman’s coefficients and p values.

**Abbreviations:** SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; LDL: low density lipoprotein; FPG: fasting plasma glucose; HbA1c: hemoglobin A1c; BMI: body mass index; WHR: waist-hip ratio
Online Table 5. Univariate correlations between inflammatory biomarkers, sRAGE and anthropometric, clinical and routine biochemical indexes in 51 T1DM subjects

<table>
<thead>
<tr>
<th></th>
<th>sRAGE</th>
<th>hs-CRP</th>
<th>IL-15</th>
<th>IL-6</th>
<th>Age</th>
<th>Diabetes duration</th>
<th>BMI</th>
<th>Waist circumference</th>
<th>Platelet count</th>
<th>Neutrophil count</th>
<th>IPF</th>
<th>HbA1c</th>
<th>FPG</th>
<th>Total cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>sRAGE</td>
<td>-</td>
<td>-0.22</td>
<td>0.03</td>
<td>-0.09</td>
<td>0.01</td>
<td>-0.09</td>
<td>-0.03</td>
<td>0.00</td>
<td>-0.14</td>
<td>0.02</td>
<td>-0.12</td>
<td>-0.04</td>
<td>-0.12</td>
<td>0.22</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>-0.22</td>
<td>-</td>
<td>0.09</td>
<td>0.22</td>
<td>0.10</td>
<td>0.27</td>
<td>0.16</td>
<td>0.33*</td>
<td>0.13</td>
<td>0.23</td>
<td>-0.21</td>
<td>-0.19</td>
<td>-0.02</td>
<td>0.13</td>
</tr>
<tr>
<td>IL-15</td>
<td>0.03</td>
<td>0.03</td>
<td>-</td>
<td>0.35*</td>
<td>0.01</td>
<td>-0.12</td>
<td>0.36*</td>
<td>0.32</td>
<td>0.21</td>
<td>0.08</td>
<td>-0.02</td>
<td>0.02</td>
<td>0.04</td>
<td>0.20</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.09</td>
<td>-0.09</td>
<td>-0.09</td>
<td>-</td>
<td>-0.09</td>
<td>0.27</td>
<td>0.30</td>
<td>0.34*</td>
<td>0.12</td>
<td>0.32</td>
<td>-0.22</td>
<td>0.12</td>
<td>0.25</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Abbreviations:** sRAGE: soluble receptor of advanced glycation end-products; hs-CRP: high-sensitivity C-reactive protein; IL: interleukin; BMI: body mass index; IPF: immature platelet fraction (%); HbA1c: hemoglobin A1c; FPG: fasting plasma glucose. * P<0.05
Online Table 6. Association between CGM-derived glucose variables and TXA2-related indexes following the last aspirin intake

<table>
<thead>
<tr>
<th>CGM-Variable</th>
<th>Urinary TXM</th>
<th>Serum TXB₂</th>
<th>Urinary 8-iso-PGF₂α</th>
<th>Δ24h</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12h 24h 48h 72h 168h</td>
<td>12h 24h 48h 72h 168h</td>
<td>12h 24h 48h 72h 168h</td>
<td>TXM</td>
<td>TXB₂</td>
</tr>
<tr>
<td>24-hour Mean Glucose</td>
<td>0.149 0.016 0.100 0.004 0.005</td>
<td>0.003 0.010 0.035 0.059 0.031</td>
<td>0.084 0.154 0.468 0.238 0.235</td>
<td>0.023</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>0.52 0.94 0.71 0.99 0.98</td>
<td>0.99 0.96 0.87 0.78 0.88</td>
<td>0.72 0.46 0.08 0.30 0.31</td>
<td>0.92</td>
<td>0.90</td>
</tr>
<tr>
<td>24-hour SD Glucose</td>
<td>0.009 0.033 0.074 0.227 0.160</td>
<td>0.150 0.009 0.010 0.097 0.074</td>
<td>0.151 0.122 0.561 0.175 0.009</td>
<td>0.135</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>0.97 0.87 0.79 0.32 0.49</td>
<td>0.48 0.97 0.96 0.64 0.73</td>
<td>0.51 0.56 0.03 0.45 0.97</td>
<td>0.56</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Data are reported as Spearman’s coefficients and p values (bold). Δ24h: difference between 24 hour and 12 hour values.

**Abbreviations:** CGM: continuous glucose monitoring; TXM: thromboxane metabolite; TX: thromboxane; 8-iso-PGF₂α: 8-iso-prostaglandinF₂α.
Online Figure 1
Online Figure 2

Urinary 8-iso-PFG$_{2\alpha}$ pg/mg creatinine

visit 1 visit 2 visit 3
Online Figure 3
Online Figure 4

ARU

T1DM subjects
healthy subjects

Time following aspirin withdrawal

pre-aspirin

hours

*
Online Figure 5

Witnessed aspirin intake

Time following aspirin withdrawal

Urinary TXM pg/mg creatinine

Healthy subjects

T1DM

* *

Witnessed aspirin intake

Online Figure 5
Urinary 8-iso-PGF$_{2\alpha}$ pg/mg creatinine

Time following aspirin withdrawal

Pre-aspirin 12 24 48 72 168 hours

Urinary PGIM pg/mg creatinine

Pre-aspirin 24 hours post-aspirin