

Reduced platelet glycoprotein Iba shedding accelerates thrombopoiesis and COX-1 recovery: implications for aspirin dosing regimen

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Title: Reduced platelet glycoprotein Iba shedding accelerates thrombopoiesis and COX-1 recovery: implications for aspirin dosing regimen

Short Title: Platelet lifespan imbalance and aspirin response

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Abstract

Cardiovascular prevention with low-dose aspirin can be less effective in patients with a faster recovery of platelet cyclooxygenase (COX)-1 activity during the 24 hours dosing interval. We previously showed that incomplete suppression of TXA₂ over 24 hours can be rescued by a twice daily aspirin regimen.

Here we show that reduced platelet glycoprotein (GP)Ib α shedding characterizes patients with accelerated COX-1 recovery and may contribute to higher thrombopoietin production and higher rates of newly-formed platelets, escaping aspirin inhibition over 24 hours.

Two-hundred aspirin-treated patients with high cardiovascular risk (100 with type 2 diabetes mellitus) were stratified according to the kinetics of platelet COX-1 activity recovery during the 10-24h dosing interval. Whole proteome analysis showed that platelets from patients with accelerated COX-1 recovery were enriched in proteins involved in cell survival, inhibition of apoptosis and cellular protrusion formation. In agreement, we documented increased plasma thrombopoietin, megakaryocyte maturation and proplatelet formation, and conversely increased platelet galactose and reduced caspase 3, phosphatidylserine exposure and ADAM17 activation, translating into diminished GPIb α cleavage and glycojalicin release. Treatment of HepG2 cells with recombinant glycojalicin led to a dose-dependent reduction in liver thrombopoietin mRNA, suggesting that reduced GPIb α ectodomain shedding may unleash thrombopoiesis. A cluster of clinical markers, including younger age, non-alcoholic fatty liver disease, visceral obesity and higher thrombopoietin/glycojalicin ratio, predicted with significant accuracy the likelihood of faster COX-1 recovery and suboptimal aspirin response. Circulating thrombopoietin/glycojalicin ratio, reflecting a dysregulation of platelet lifespan and production, may provide a simple tool to identify patients amenable to more frequent aspirin daily dosing.

INTRODUCTION

Platelets play key roles in atherothrombosis, by acting as inflammatory mediators, and participating in the processes of forming and extending atherosclerotic plaques, and in thrombus growth and vascular occlusion.¹ Thromboxane (TX)-dependent platelet activation, as reflected by 11-dehydro-TXB₂ urinary excretion, has been associated with several conditions, including cardio-cerebrovascular diseases and cardiovascular risk factors, such as diabetes mellitus.^{2,3}

The antiplatelet and cardioprotective properties of low-dose (81 to 100 mg daily) aspirin rely on its capacity to irreversibly inactivate cyclooxygenase (COX)-1 in the anucleate platelets and TXA₂ biosynthesis over 24 hours.³ Over the last decades, the concept of suboptimal response to aspirin, firstly inadequately referred to as aspirin resistance, has affirmed, based on the evidence of lower-than-expected inhibition of platelet function assays, which however poorly reflect the mechanism of action of aspirin, and display scarce reproducibility over repeated measurements and poor correlation with COX-1 inhibition, as reflected by serum TXB₂ (sTXB₂).⁴⁻⁶

The variable turnover rate of aspirin target, platelet COX-1, is the most convincing determinant of the interindividual variability in aspirin response. Under several pathologic conditions, such as essential thrombocythemia, polycythemia vera, on-pump coronary artery bypass surgery, as well as in patients at high cardiovascular risk, with or without T2DM,⁷⁻¹⁰ a proportion of platelets with uninhibited COX-1 may determine faster recovery of COX-1 activity and TXA₂ generation and limit the extent and duration of aspirin effect during the 12-24 hour dosing interval^{7,8} leading to substantial platelet activation and recovery of platelet function. Because full suppression of TXA₂-dependent platelet function requires >97% inhibition of COX-1 activity,⁶ even a modest recovery of this activity can sustain a substantial platelet activation responsible for the excess cardiovascular (CV) events in aspirin-treated subjects.

Shorter duration of TXA₂ suppression during the 24h dosing interval can be rescued by a twice daily low-dose aspirin regimen^{7,9,10}, leading to persistently inhibited sTXB₂ over 24

hours. An accelerated platelet turnover has been historically advocated as the underlying mechanism, although no direct evidence has been provided to substantiate enhanced megakaryopoiesis and accelerated destruction in subjects with shorter duration of aspirin effect. Patients' classification based on the clinical setting, including diabetes or acute coronary syndrome, failed to accurately identify patients escaping low-dose aspirin inhibition and for whom different strategies or aspirin dosing regimens may be required. Understanding the mechanisms driving faster COX-1 recovery in the usual dosing interval may help identifying novel biomarkers of suboptimal drug response.

Thrombopoietin (TPO) is the primary regulator of platelet production from megakaryocytes (Mks)¹¹ and is produced by the liver under a number of stimuli including inflammation.¹¹ Conversely, platelet lifespan, glycan degradation and apoptosis mediate platelet clearance.¹¹ Senescent platelets are cleared upon exposure of galactose to hepatic Ashwell-Morrell receptors (AMR), in turn stimulating TPO production.^{11,13} Glycoprotein Ia (GPIb α) shedding by metalloproteinases such as a disintegrin and metalloprotease (ADAM) 17 is another mechanism of platelet clearance,¹² and plasma glycaliccin (GC), an extra cellular domain of GPIb α , released during platelet clearance, is an index of destruction and platelet turnover.¹⁴ Platelet extracellular GPIb α domain is required for platelet-mediated TPO generation, as underscored in GPIb α ^{-/-} mice and patients with Bernard-Soulier syndrome.¹⁵

By employing an integrated approach including biochemistry, proteomics, flow-cytometry, cell biology, and a mechanism-based endpoint to monitor aspirin pharmacodynamics, we analyzed the platelet proteome, platelet turnover, as reflected by TPO and GC circulating levels, galactose exposure and GPIb α ectodomain shedding, Mk maturation and proplatelet formation in patients at high CV risk with or without type 2 diabetes mellitus (T2DM) stratified according to the kinetics of COX-1 recovery. We show that reduced platelet GPIb α shedding characterizes patients with accelerated COX-1 recovery and may contribute to higher TPO production and higher rates of newly-formed platelets,

escaping aspirin inhibition over 24 hours. The TPO/GC ratio, a relatively simple, mechanism-based biochemical tool, may identify with significant diagnostic accuracy aspirin-poor responders due to accelerated renewal of the drug target.

METHODS

Study design and participants

One-hundred T2DM patients diagnosed according to the European Society of Cardiology (ESC) guidelines,¹⁶ with or without prior vascular disease, on aspirin 100 mg (enteric coated, Cardioaspirin, the antiplatelet dosage and formulation employed in Italy) once daily for at least 1 year and 100 patients without T2DM with comparable characteristics (Online Supplementary Table S1, study participants) were enrolled at the Diabetes and CV Prevention Clinics, Chieti SS. Annunziata Hospital, Italy. Five healthy subjects were enrolled as control. All patients underwent liver ultrasound (see Online Supplementary Methods).

The study was preceded by a 7-day run-in during which patients were instructed to take aspirin at 8 pm.

Blood was collected before witnessed aspirin intake (8 am, day 1), and repeated at 10 and 24 hours after the last intake, to assess the kinetics of COX-1 activity recovery, as reflected by the slope of sTXB₂ levels throughout the 24 hours dosing interval between two witnessed aspirin administrations (Figure 1A). sTXB₂ is indeed a marker of COX-1 activity *ex vivo*.¹⁷ We previously reported that the absolute increase in sTXB₂ levels between 12 and 24 hours after dosing predicts ~90% slope variability, as assessed by measuring sTXB₂ every 3 hours in the 12- to 24 hours dosing interval.¹⁰

Based on sTXB₂ slope (sTXB₂ T24 – sTXB₂ T10)/14, we stratified patients in tertiles (n~33 each) and carried out a cross-sectional comparison of platelet-related study variables between first (good aspirin responders) and third tertile (poor responders) within patients with and without T2DM.

Platelet isolation

Platelet rich plasma (PRP) was separated (100 xg, 15 min) from ACD-A anticoagulated blood, mixed with prostaglandin E₁ (PGE₁, 4uM) and EDTA (10mM), and filtered on Pall Purecell PL (Pall Medical, New York, USA) to remove leukocyte contaminant. Filtered PRP was analyzed with flow cytometer to exclude red blood cells (CD235⁺) contamination and platelet activation (CD62P⁺). Platelets were lysed with DIGE buffer for Western Blot (WB) and proteomics analysis.

Statistics

We estimated that at least 30 patients would be required in each group to detect a mean difference in any of the investigated parameter ≥ 1 standard deviation between the first and the third sTXB₂ slope tertiles with alpha=0.01 and power=90%.

All the statistical analyses have been carried out separately in individuals with and without T2DM.

Univariable comparisons between groups were performed by χ^2 tests or Mann-Whitney U or Spearman rank correlation test.

Multivariable logistic regression models were constructed to identify factors associated with the likelihood of being in the third vs. first tertile for sTXB₂ recovery slope. A parsimonious backward-stepwise elimination of variables with p<0.20 was deemed as appropriate in our setting. ROC curves were constructed for the predicted probabilities derived from the logistic regression models.

The data analysis was generated using SAS software.

Study approval. The protocol was approved by the institutional Ethics Committee (Prot 1129/2015, GR-2011-02350450). Participants provided written informed consent, and were identified by number, not by name.

See Online Supplementary Methods (pages 2-8) for more details on study participants and extended description of experimental procedures.

RESULTS

Clinical characteristics

Clinical characteristics of patients are in Online Supplementary Table S1 and detailed in Online Supplementary Results. Patients' characteristics according to sTXB₂ recovery slope tertiles are reported in Table 1 and detailed in Online Supplementary Table S2 and Results.

A fraction of patients shows faster recovery of COX-1 activity over the 10–24 hours aspirin dosing interval, higher COX-1 mRNA expression and thromboxane-dependent platelet activation

In the first tertile of recovery slope, sTXB₂ was steadily suppressed over the 10–24 hours dosing interval (Figure 1, B and C), as observed in healthy subjects,⁶ while patients in the third tertile (slope \geq 0.17 ngmL⁻¹h⁻¹ and slope \geq 0.18 ngmL⁻¹h⁻¹ in patients with and without diabetes, respectively) showed a significantly faster recovery of COX-1 activity, as reflected by the sTXB₂ increase between 10 and 24 h post-aspirin (Figure 1, B and C).

This indicates that at least a fraction of aspirin-treated patients at risk for CV events has an accelerated recovery of the drug target, and that the “poor aspirin responder” phenotype is not specific for diabetes. In addition, the TXB₂ recovery slope was not related to duration of aspirin treatment (Online Supplementary Table S2).

Platelet COX-1 mRNA was significantly higher in third vs. first tertile (p=0.014, Figure 1D), indicating a faster renewal of the drug target in the patients with suboptimal response to aspirin. To assess whether an accelerated recovery of sTXB₂ *ex vivo* translated into enhanced *in vivo* TX-dependent platelet activation, we measured urinary 11-dehydro-TXB₂ levels, that were higher in patients of third vs. first tertile in the whole group (p=0.022, Figure 1E) and in T2DM (p=0.049, data not shown).

Platelet proteome indicates increased cell survival and inhibition of apoptosis in patients with faster COX-1 activity recovery

We characterized the functional proteomic profile of platelets from third vs. first tertile

within patients with or without T2DM, using pooled samples as described in method section. The quantified proteins are reported in Online Supplementary Table S4 and S5. In order to ensure the quality of our proteomics dataset we compared it to the reference platelet proteomic repository, obtaining more than 95% of correlation (data not shown). Volcano Plots show proteins differentially identified between first vs. third in patients without and with T2DM (Online Supplementary Figure S3, S3A and S3B).

The protein ratio was used for “Core Analysis” through the Ingenuity Pathway Analysis (IPA software). We found a significant increase in the pathways “cell survival” (z-score=1.54 for noT2DM, z-score=2.01 for T2DM) “formation of cellular protrusions” (z-score=2.43 for noT2DM, z-score=1.92 for T2DM), and “production of reactive oxygen species” (z-score=2.06 for noT2DM, z-score=2.37 for T2DM), and a simultaneous inhibition of “apoptosis” (z-score=-0.58 for noT2DM, z-score=-1.86 for T2DM) in both clinical conditions in platelets from third tertile subjects in comparison to those from first tertile (Figure 2, A and B). We validated the proteomic data by WB analysis and confirmed a significant up-regulation of the endoplasmic reticulum stress marker 78 kDa glucose-regulated protein (GRP78, $p=0.042$; Figure 2C) and COX-1 ($p=0.051$; Figure 2D). Patients of third tertile showed consistently higher levels of GRP78 ($p=0.042$, $p=0.017$; Figure 2C) and COX-1 ($p=0.051$, $p=0.004$; Figure 2D) compared to first tertile patients and healthy subjects. To further explore a potential hypothesis about the formation of protrusions in platelets, we used Molecule Activity Predictor (MAP) function of IPA by selecting “formation of proplatelets” as downstream of interest. Our proteomic dataset was able to simulate directional consequences on this function by inferring its activation in platelets from third tertile subjects in both clinical conditions analyzed as shown by subnetworks in Figure 2E, with a significant up-regulation of Rab27B, a protein actively involved in platelet biogenesis and proplatelet formation,¹⁸ in platelets of third vs. first tertile ($p=0.038$; Figure 2F) and vs. healthy subjects ($p<0.001$; Figure 2F).

Enhanced Mk maturation and proplatelet formation in patients with faster COX-1 activity recovery

Based on the proteomic findings, we investigated whether the different recovery of COX-1 activity reflected differences in megakaryopoiesis and proplatelet production by differentiating Mks *in vitro* from the haematopoietic progenitors derived from 18 peripheral blood samples of patients with and without T2DM belonging to first and third sTXB₂ tertile and from 4 healthy subjects (Figure 3, A and B). Patients' groups analyzed were comparable for clinical characteristics (Online Supplementary Table S6).

Mks differentiated from the third tertile patients appeared more mature with a significantly higher staining of CD41 and CD42b (GPIb α) Mk¹⁹ compared to the first tertile and healthy subjects (Figure 3, C and D), while the percentages of CD41+ and GPIb α + Mks on the total cultured cells were comparable (Figure 3, C and D). Mks from the third tertile patients extended more proplatelets compared to the first tertile or healthy subjects (Figure 3E). These data demonstrated that terminal Mk maturation and proplatelet formation were increased in patients presenting a faster recovery of platelet COX-1 activity.

Faster COX-1 activity recovery is associated with increased platelet production and decreased destruction

We next evaluated the balance between platelet production and destruction by measuring TPO, GC and GC index (GCI), an index normalizing GC for platelet count [$GC \text{ concentration } (\mu\text{g/mL}) \times 250 \times 10^9 \text{ platelet/L} / \text{individual platelet count } \times 10^9 / \text{L}$], 24 hours after witnessed aspirin intake. Consistent with the proteomic findings, plasma TPO levels were higher ($p=0.004$, Figure 4A and $p=0.023$, Online Supplementary Figure 4SA), while plasma GC ($p<0.001$ both; Figure 4B and Online Supplementary Figure 4SB) and GCI ($p<0.001$ both; Figure 4C and Online Supplementary Figure 4SC) were lower in patients of third *vs.* first tertile, in the whole group and in subjects with T2DM, with an inverse correlation between TPO, GC ($\rho=-0.216$, $p=0.013$, data not shown) and GCI (whole group: $\rho=-0.245$,

p=0.006; T2DM: rho=-0.358, p=0.004; Figure 4D and Online Supplementary Figure 4SD). Similarly, among patients without T2DM, GC (p<0.001; Online Supplementary Figure 4SB) and GCI (p<0.001; Online Supplementary Figure 4SC) were significantly reduced in the third vs. first sTXB₂ tertile, with a nonsignificant trend for increased TPO (Online Supplementary Figure 4SA). Levels of TPO were lower (p=0.001, p<0.001, p=0.009; Figure 4A) and levels of GC were higher (p<0.001, p<0.001, p=0.005; Figure 4 B and Online Supplementary Figure 4SB) in healthy subjects vs. third tertile, in the whole group and in subjects without and with T2DM.

Consistently, platelet count was higher in the third vs. first tertile in all patients (p=0.024, Figure 4E), and in T2DM patients (p=0.047, Online Supplementary Figure 4SE). In the whole group, the TXB₂ recovery slope correlated directly with TPO (rho=0.252, p=0.003), and inversely with GC (rho=-0.432, p<0.001) and GCI (rho=-0.495, p<0.001) (data not shown).

Together, these results indicated that patients in the third TXB₂ slope tertile presented increased platelet production and reduced destruction.

Lower levels of circulating GC depend on reduced activation of ADAM17 and GPIIb/IIIa ectodomain shedding in patients with faster COX-1 activity recovery

To determine the mechanisms underlying lower GC in the third sTXB₂ slope tertile patients, we measured GPIIb/IIIa N-terminal fragment and ADAM17 expression in platelets.²⁰

In all patients, we observed enhanced levels of GPIIb/IIIa (p=0.016 and p<0.001; Figure 5, A) and lower levels of ADAM17²¹ (p=0.015 and p=ns, Figure 5, B) in third vs. first tertile and vs. healthy subjects, respectively, which may explain the lower levels of circulating GC in the third slope tertile (Figure 4B and Online Supplementary Figure 4SB).

To further understand the mechanisms underlying reduced GPIIb/IIIa shedding, we analyzed the percentage of annexin V⁺ platelets exposing phosphatidylserine (PS), since PS exposure is a signal for clearance of apoptotic platelets²² and is required for ADAM17 activation²⁰. The percentage of annexin V⁺ platelets was significantly lower in the third vs. first

tertile in all patients ($p=0.007$, Figure 5C) and in patients with T2DM ($p<0.001$, data not shown) with a nonsignificant trend for patients without T2DM (data not shown).

In healthy volunteers, aspirin administration was associated with PS exposure increase ($p=0.002$, Figure 5D), and activation of ADAM17²³ after 10 and 24 hours (Figure 5E). Thus, reduced PS exposure and ADAM17 activation, translating into diminished GPIIb α cleavage and GC release, characterizes patients with accelerated recovery of platelet COX-1, and may be related to lower aspirin effect.

Soluble GC inhibits TPO expression

We next hypothesized a role for the GPIIb α ectodomain in the regulation of TPO expression, since its uncleaved form on the surface of platelets is a trigger of TPO synthesis in hepatocytes.¹⁵ We asked whether the fragment of GC *per se* was able to modulate TPO expression.

Treatment of HepG2 cells with an increasing concentration of human recombinant GC was associated, after 1 hour of incubation, with a significant dose-dependent reduction in TPO mRNA (Figure 5F). Thus, lower GC levels, as observed in the third TXB₂ slope tertile, may explain the higher liver expression and circulating levels of TPO.

Higher platelet galactose exposure in patients with faster COX-1 activity recovery

Since TPO production is regulated by the binding of galactose exposed on aged platelets to the AMR in hepatocytes, we analyzed galactose-recognizing lectins, RCA-I and ECL, to assess platelet expression of the terminal β 4-N-acetyllactosamine (LacNAc).²⁴ Platelets of the third tertile were characterized by higher ECL ($p=0.034$, $p=0.064$) and RCA-I levels ($p=0.002$, $p=0.004$) *vs.* first tertile and healthy subjects (Figure 6, A and B), indicating an increase of platelet galactose exposure in aspirin poor responders.

More pronounced platelet galactose exposure in the third *vs.* first tertile was accompanied by higher expression of neuraminidase (Neu)1 ($p=0.061$, Figure 6C), the sialidase that removes

sialic acid from GPIIb/IIIa.²⁵ These data suggested that platelets from patients with accelerated COX-1 recovery are characterized by a higher degree of terminal galactose.

Determinants of the accelerated recovery of COX-1 and predictive value of the TPO/GC ratio

Finally, we carried out a multivariable analysis to identify clinical and biochemical determinants of accelerated recovery of COX-1.

In T2DM patients, starting from a panel of potential determinants including age, gender, BMI=body mass index (BMI), HbA1c, Nonalcoholic Fatty Liver Disease (NAFLD), established atherosclerotic cardiovascular disease (ASCVD), glomerular filtration rate, platelet count, mean platelet volume (MPV), PDW and tertiles of TPO/GC ratio, stepwise multivariable logistic regression analysis identified younger age, presence of NAFLD, higher platelet count and higher TPO/GC ratio as independent predictors of the likelihood of being in the third sTXB₂ slope tertile (Figure 7A).

In patients without T2DM, starting from a panel of potential determinants including age, gender, BMI, WHR, platelet count, MPV, PDW, hemoglobin, statin treatment, and tertiles of TPO/GC ratio, stepwise multivariable logistic regression analysis identified higher WHR, lower MPV and higher TPO/GC ratio as independent predictors of the likelihood of being in the third sTXB₂ slope tertile (Figure 7A).

Analysis of the ROC curves revealed an outstanding diagnostic accuracy ($AUC \geq 0.88$) for the two models in the prediction of the third tertile status vs. first tertile (Figure 7B).

To translate into a clinically useful tool our mechanistic findings of increased platelet production and reduced clearance characterizing accelerated COX-1 recovery, we challenged the predictive value of TPO/GC ratio. Multivariable logistic regression analysis revealed that among subjects with T2DM, those in the third tertile of the TPO/GC ratio (threshold=138 pg/ug) were 33 times more likely (Figure 7A) to be in the third tertile for sTXB₂ slope, while those with TPO/GC between 60 and 137 were 11 times more likely (Figure 7A) to be in the third tertile. The addition of tertiles of TPO/GC ratio to the model including only clinical or

hemocromocytometric variables yielded a significant increase in AUC (from 0.754 to 0.883; P for difference 0.015).

Among patients without T2DM, those with TPO/GC above 147 were 10 times more likely (Figure 7A) to be in the third tertile, while those with TPO/GC between 76 and 146 were 9 times more likely (Figure 7A) to be in the third tertile.

Thus, a clinical and biochemical signature may unravel patients with shorter duration of sTXA₂ inhibition for whom more frequent dosing regimens may prevent the steep recovery of platelet COX-1 activity.

See Online Supplementary Results (pages 9-10) for more details on clinical characteristics and study findings.

DISCUSSION

In this work we demonstrate that the shorter duration of TXB₂ suppression by aspirin over the 10 to 24-hour dosing interval, in a fraction of high-risk patients on chronic aspirin treatment, may be explained by 1. accelerated recovery of COX-1 through increased functionally active COX-1 and COX-1 mRNA, higher TX-dependent platelet activation, enhanced TPO production, megakaryocyte maturation and proplatelet formation, leading to increased platelet number; 2. reduced platelet PS exposure, GPIIb/IIIa ectodomain shedding and higher galactose exposure, fostering thrombopoiesis through liver TPO synthesis; 3. a clinical and molecular signature, including younger age, NAFLD, visceral obesity and high TPO/GCI, identifying with high accuracy aspirin-poor responders.

Over the last decades, the concept of suboptimal response to aspirin has affirmed, but the prevalence of this phenomenon is unclear, due to the heterogeneity of methods used to quantitate the antiplatelet effect of aspirin in these studies, which poorly reflect the biochemical pathway affected by aspirin, i.e. platelet COX-1 activity⁶ and variably reflect the aspirin-sensitive TX-dependent component of platelet aggregation.¹ Even when using sTXB₂, a mechanism-based endpoint with the highest specificity and sensitivity to monitor aspirin pharmacodynamics, we and others have previously characterized an interindividual variability in platelet COX-1 recovery during the 12–24 h dosing interval in patients at high CV risk, or undergoing coronary artery by-pass surgery or with essential thrombocythemia.^{7,9,10} This phenomenon was reverted by shortening the dosing interval, suggesting an accelerated COX-1 renewal within the dosing interval. While previous evidence was largely indirect and based on increased MPV or higher levels of reticulated platelets,^{10,26} here we demonstrated increased circulating TPO, enhanced *in vitro* Mk maturation and proplatelet formation in patients with accelerated kinetics of platelet COX-1. Our data substantiate the hypothesis that, during the 24h dosing interval, newly generated platelets entering the circulation, after aspirin effect waning, bear COX-1 unacetylated and synthesize new TXA₂.

Accelerated MK maturation and proplatelet formation may be driven, at least in part, by higher TPO circulating concentrations. We hypothesize that the higher TPO *in vivo* biases the commitment of haematopoietic progenitors toward the Mk lineages that we observe *in vitro* cultures.²⁷

We next sought to analyze circulating TPO and GC, markers of platelet production and destruction, respectively. We unraveled that reduced platelet destruction, as reflected by lower circulating GC, lower levels of caspase 3²⁸ (Online Supplementary Figure S5) and platelet PS exposure, identify platelets from patients with shorter durability of aspirin response. Indeed, proteomics profiling indicated activation of cell survival and inhibition of apoptosis pathways in third sTXB₂ tertile patients, which may characterize younger platelets.²⁹

The observation that platelets from patients with accelerated recovery of COX-1 are less prone to apoptosis was corroborated by lower caspase 3, lower annexin V staining, reduced ADAM17 activation, increased expression of uncleaved GPIb α , mirroring reduced destruction³⁰⁻³³ leading to lower circulating GC and GCI and higher platelet count. Even if PS alone is regarded as a common marker for both procoagulant or apoptotic platelets, our results on caspase 3, along with proteomics analysis, corroborate our conclusion that third slope tertile patients have less apoptotic platelets.³⁴ Of interest, PS exposure is required for ADAM17-mediated cleavage of GPIb α ²⁰ whose constitutive proteolysis is considered as a signature event of platelet aging. Indeed, treatment with artificial agents mimicking platelet aging induces GC release^{30,31} and accelerated removal of transfused platelets occurs following GPIb α proteolysis from stored platelets.^{32,33} Inhibition of GPIb α shedding by kinase inhibitors³³ or antibodies³² can mitigate platelet clearance and prolong the lifespan of transfused platelets in mice.

Our present results do not allow to make final conclusions regarding platelet lifespan. Although circulating GC has been consistently regarded as an index of platelet destruction, and several lines of evidence converge to support inhibited apoptosis, no direct demonstration of reduced platelet clearance in the third COX-1 recovery tertile has been provided in our cohort. The

reduced percentage of PS-exposing platelets and reduced apoptosis may alternatively be regarded as a feature of young, newly released platelets, or may be the result of earlier platelet clearance over the 24-hour time interval. However, MPV was not higher in third tertile of either group, despite the higher prevalence of larger, newly formed platelets, and lower, rather than higher, MPV was a significant predictor of belonging to the upper sTXB₂ slope tertile among non-diabetic patients, raising the hypothesis of longer platelet lifespan in aspirin poor responders, with coexistence of larger and smaller size platelets.

Whether increased platelet survival/reduced apoptosis is a feature of the “poor-responder” platelets or a consequence of poor aspirin response, is not unraveled. Aspirin induces platelet apoptosis³⁵ and shedding of GPIb α and GPV through activation of ADAM17.²³ Consistently, in a small number of our healthy volunteers, aspirin treatment was associated with enhanced annexin V⁺ platelets and increased expression of active ADAM17. *Vice versa*, patients with accelerated COX-1 recovery displayed lower annexin V+ platelets and lower platelets ADAM17 expression, concomitant with higher expression of platelet GPIb α N-terminal domain and lower GC and GCI, *vs.* normal COX-1 recovery patients, suggesting lack of apoptosis induction by aspirin.

In order to establish a link between the extent of COX-1 acetylation or inhibition and GPIb α clustering, on the one hand, and PS exposure, on the other hand, we measured complexes of the adapter protein 14-3-3 ξ with GPIb α and COX-1 in platelets from first and third tertile patients. It was previously shown that arachidonic acid (AA) accumulation due to COX-1 inactivation in cold-stored platelets induce 14-3-3 ζ -GPIb α association, 14-3-3 ζ release from phospho-Bad, Bad activation, PS exposure, and apoptosis.³⁶ GPIb α clustering is also linked to galactose exposure.³⁷ In our setting, 14-3-3 ξ :GPIb α complexes were significantly less in third tertile patients, while 14-3-3 ξ :COX-1 complexes were significantly higher (Online Supplementary Figure S5). Therefore, it is possible that in first tertile patients, treatment with aspirin, which leads to arachidonic acid accumulation due to inhibition of its

biochemical utilization by platelet COX-1, results in displacement of 14-3-3 ξ from the proapoptotic protein Bad in favor of GPIb α and subsequent activation of platelet death. In contrast, in third tertile patients, which have a faster recovery of COX-1 activity, conversion of AA into TXA₂ may determine a lower degree of interaction of 14-3-3 ξ with GPIb α and a reduced activation of apoptosis. In keeping with this, we found that apoptosis was inactivated in platelets from third tertile patients.

Increased GPIb α and reduced GC characterize and predict poor aspirin response and may play a role in promoting platelet activation and escape from aspirin. While it is assumed that ADAM17 restrains continuous GPIb α -mediated platelet activation,³⁸ the phenotype observed in third tertile patients may indicate hyperreactive platelets since GPIb α clustering triggers TXA₂.³⁹ Along this line, aspirin poor responders with high GPIb α have higher platelet COX-1 and persistent TXA₂ biosynthesis.

The unexpected inverse relationship between platelet destruction and production, as reflected by GC or GCI and TPO, respectively, prompted us to hypothesize that defective GPIb α ectodomain shedding may contribute to sustain enhanced thrombopoiesis in patients with shorter duration of COX-1 inhibition. Indeed, the extracellular domain of GPIb α *per se*, independently of platelet clearance, is required for liver TPO production.¹² Thus, we challenged the effect of a commercially available recombinant human soluble GC expressed in murine myeloma cells on liver cells *in vitro*, showing a dose-dependent inhibition in TPO mRNA expression. Together our findings suggest that GPIb α ectodomain shedding by ADAM17 leads to soluble GC binding to hepatocytes, thus reducing liver TPO release. Conversely, low levels of GC shedding in subjects with accelerated COX-1 recovery, may unleash TPO mRNA transcription. We recognize that sugar additions on rGC synthesized in murine myelomas may differ from GC found in circulating human GC, including non-human N-glycolylneuraminic acids, which could affect the binding and recognition of rGC by hepatocytes. Further studies, which will require a careful glycoproteomics approach, are

necessary to understand the role of the protein backbone and sugar additions in the binding of GC to hepatocytes. However, glycoproteomics of rGC and human GC is out of scope of this report.

Platelet galactose exposure also triggers thrombopoiesis through the interaction with AMR.⁹ In our study, patients with shorter duration of aspirin response showed increased lectin binding and Neu-1 expression, suggesting a possible further mechanism activating TPO production in these subjects. Thus, both platelet galactose exposure and GPIb α expression may contribute, with a feed-forward mechanism, to accelerated thrombopoiesis escaping aspirin inhibition at the usual dosing interval. More terminal galactose moieties would be expected to lead to increased platelet clearance and decreased circulating platelet count. Other evidence shows that platelets isolated from myeloproliferative (MPNs), often associated with change in circulating platelet count, have a significant increase in terminal galactose expression that correlated with the high allele burden regardless of the underlying identified mutation. Megakaryocytes derived in vitro from these patients showed an increased expression of the B4GALT1 gene encoding β -1,4-galactosyltransferase 1 (β 4GalT1) and terminal galactose expression relative to healthy controls. Altered expression of B4GALT1 in mutant megakaryocytes can lead to the production of platelets with aberrant galactosylation, which in turn promote hepatic TPO synthesis regardless of platelet mass.²⁷ These data suggest a more complex role for B4GALT1 dependent galactose decorations to balance platelet clearance and production. A pathologic increase in galactose could result in both increased platelet production and platelet clearance to perpetuate TPO production.

Finally, we identified a cluster of clinical and biochemical markers predicting the likelihood of suboptimal aspirin response, with particular reference to the TPO/GC ratio, mirroring our mechanistic findings.

This may help identifying those patients for whom a more frequent aspirin dosing regimen (*bis in die*) may be required. The twice daily regimen has already been suggested for the

management of myeloproliferative neoplasms⁴⁰ and a phase 2 trial is ongoing to assess the safety of this approach in this setting. Moreover, the ongoing ANDAMAN trial is testing the efficacy and safety of aspirin twice a day in patients with acute coronary syndrome and diabetes, obesity, or aspirin failure (<https://clinicaltrials.gov/ct2/show/NCT02520921>). However, no clinical setting has been shown to accurately identify those with faster recovery of COX-1 activity. Obesity is known to impair aspirin responsiveness by affecting systemic drug availability, ie absorption and biotransformation, leading to reduced, albeit steady, 24h inhibition of COX-1–dependent TX production⁴. Here we show an additional role of obesity in shortening the effect of aspirin to less than 24 hours. Indeed, in our study, visceral obesity and NAFLD, in patients without and with T2DM, respectively, were independent clinical predictors of shorter duration of aspirin effect, suggesting a role for insulin resistance as the pathophysiological hallmark of both conditions. Indeed, plasma TPO was directly related to waist circumference, hs-CRP, insulinemia, and HOMA-IR.

Diabetes *per se* is also a recognized setting of platelet hyperreactivity⁴¹, persistent TX-dependent platelet activation^{42,43} and enhanced platelet turnover, with suboptimal platelet responsiveness.⁴⁴ Hyperglycemia is a trigger of IL-6 mediated liver TPO production.⁴⁵ Not surprisingly, the predictive power of the TPO/GC ratio is substantially higher in patients with T2DM *vs.* nondiabetic subjects, regardless of underlying CV risk: indeed, patients in the upper tertile for the TPO/GC ratio, have a 33-fold higher risk to be poor aspirin responders, *vs.* 11-fold higher risk in subjects without T2DM.

However, the diagnostic accuracy of single clinical features, such as obesity, or diabetes, in discriminating subjects with faster COX-1 recovery is poor and does not allow a personalized, disease-based approach. On the other hand, assessment of aspirin response in the individual patient based on the kinetics of COX-1 recovery is complex and requires repeated measurements. At variance, the TPO/GC ratio identified here is calculated with one blood sampling and provides alone good diagnostic accuracy in detecting subjects with faster

COX-1 recovery and for whom the efficacy and safety of more frequent antiplatelet dosing regimens should be tested.

Limitations of the study include its observational nature, and lack of reticulated platelet data, although previously shown by our group and others and overcome by a direct evaluation of Mk maturation and proplatelet formation. Information about COX-1 acetylation or salicylate measurement is lacking. In addition, we performed lectin blots using galactose binding lectins, showing all proteins with terminal galactose, instead of flow cytometry, that would have revealed most of surface exposed terminal galactose moieties. It is noteworthy that most intracellular proteins are not glycosylated, exception being the Golgi apparatus and O-GlcNAcylated proteins, which seem to be relatively low expressed in platelets (not shown) and platelets contain only few Golgi-like granule.⁴⁶ Hence, we speculate that most proteins with exposed galactose would reside on the platelet surface. The healthy subject group is very small, although results pre vs. post aspirin administration are quite evident. Strengths are accurate clinical and biochemical characterization and CV risk stratification; ascertainment of compliance to low-dose aspirin; accurate timing of blood sampling; use of a mechanism-based biochemical endpoint to monitor aspirin pharmacodynamics and renewal of the drug target; a combined approach including biochemistry, proteomics, flow-cytometry, cell biology.

Conclusions

In conclusion, an imbalance between platelet production and clearance, with accelerated megakaryopoiesis/platelet production and reduced clearance/prolonged survival, characterizes patients with poor aspirin response, as reflected by the accelerated recovery of platelet COX-1 activity, with or without diabetes (Figure 7C). This imbalance translated into increased platelet count (especially in patients with T2DM) and enhanced TX-dependent platelet activation. Integration of clinical data with TPO to GC ratio may provide a relatively simple tool to identify patients amenable to more frequent aspirin daily dosing, and should be tested in larger, independent cohorts.

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Table 1: Characteristics of patients with and without type 2 diabetes in relation to tertiles of sTXB₂ recovery slope

Variable	T2DM				noT2DM			
	1 st tertile	2 nd tertile	3 rd tertile	P value	1 st tertile	2 nd tertile	3 rd tertile	P value
Age – years	69.0 (66.0-74.5)	69.0 (63.7-73.5)	67.0 (61.0-69.5)	0.047	69.0 (62.5-76.5)	67.0 (61.8-71.0)	69.0 (65.5-73.0)	0.468
Male gender, n. (%)	20 (60.6)	22 (64.7)	22 (66.7)	0.872	17 (51.5)	19 (55.9)	21 (63.6)	0.602
Smoke, n. (%)	4 (12.1)	5 (14.7)	4 (12.1)	0.936	4 (12.1)	2 (5.9)	4 (12.1)	0.616
Weight – Kg	80.0 (70.5-90.0)	80.0 (71.3-95.5)	85.0 (73.3-92.5)	0.505	80.0 (70.5-90.0)	80.0 (71.3-95.5)	85.0 (73.3-92.5)	0.505
BMI - Kg/m ²	29.3 (25.5-31.5)	29.5 (25.7-33.7)	31.0 (28.1-34.7)	0.148	26.3 (25.4-30.0)	29.4 (24.4-32.5)	28.0 (25.9-32.7)	0.248
WC – cm	100.0 (100.0-110.0)	100.0 (90.0-110.0)	110.0 (100.0-110.0)	0.120	100.0 (90.0-110.0)	100.0 (90.0-110.0)	110.0 (100.0-120.0)	0.026
WHR	1.0 (0.9-1.0)	1.0 (0.9-1.0)	1.0 (0.9-1.0)	0.747	0.9 (0.9-1.0)	1.0 (0.9-1.0)	1.0 (0.9-1.0)	0.003
Obesity, n. (%)	13 (39.4)	15 (44.1)	21 (63.6)	0.112	10 (30.3)	16 (47.1)	14 (42.4)	0.354
SAP – mmHg	145.0 (134.0-150.0)	146.0 (134.0-150.0)	148.0 (134.0-160.0)	0.675	140.0 (128.0-151.5)	138.5 (125.0-152.5)	142.0 (130.0-159.0)	0.413
DAP – mmHg	75.0 (70.0-80.0)	74.0 (69.0-80.0)	78.0 (70.0-83.0)	0.593	74.0 (67.0-80.0)	78.5 (70.0-81.5)	80.0 (70.5-86.0)	0.341
Hypertension, n. (%)	29 (87.9)	30 (88.2)	31 (93.9)	0.653	24 (72.7)	28 (82.4)	28 (84.8)	0.429
Dyslipidemia, n. (%)	17 (51.5)	22 (64.7)	21 (63.6)	0.476	24 (72.7)	13 (38.2)	17 (51.5)	0.017
Diabetes duration – years	4.0 (2.0-13.0)	5.0 (3.0-10.0)	6.5 (1.0-11.0)	0.983	-	-	-	-
NAFLD, n. (%)	19 (70.4)	30 (90.9)	27 (87.1)	0.040	21 (63.6)	13 (38.2)	18 (54.5)	0.322
HbA1c - mmol/mol	50.8 (42.1-56.3)	50.8 (45.4-56.3)	53.0 (45.4-61.7)	0.312	38.8 (34.4-41.0)	38.8 (34.4-42.1)	38.8 (36.6-41.0)	0.831
- %	6.8 (6.0-7.3)	6.8 (6.3-7.3)	7.0 (6.3-7.8)		5.7 (5.3-5.9)	5.7 (5.3-6.0)	5.7 (5.5-5.9)	
Total cholesterol – mmol/L	4.5 (3.7-5.0)	4.2 (3.7-4.7)	4.6 (4.1-5.3)	0.223	4.9 (4.1-5.4)	5.3 (4.5-5.6)	4.4 (3.9-5.3)	0.130
HDL cholesterol – mmol/L	1.2 (1.0-1.5)	1.3 (1.0-1.4)	1.3 (1.1-1.5)	0.562	1.4 (1.1-1.5)	1.4 (1.2-1.6)	1.2 (1.1-1.4)	0.088
Triglycerides – mmol/L	1.4 (1.0-2.0)	1.4 (1.0-1.8)	1.5 (1.0-2.1)	0.914	1.2 (1.0-1.7)	1.2 (0.9- 1.6)	1.3 (1.0-1.9)	0.494
AST - U/L	21.0 (19.0-33.0)	24.5 (20.0-28.2)	26.0 (20.0-32.5)	0.513	25.0 (22.0-30.0)	22.5 (21.0-26.3)	24.0 (19.5-29.5)	0.593
ALT - U/L	28.0 (24.0-41.0)	31.0 (24.0-39.2)	34.0 (27.0-43.0)	0.270	31.0 (25.0-36.0)	25.5 (22.0-31.0)	30.0 (25.5-36.0)	0.005
Total Bilirubin – umol/L	10.0 (7.0-14.0)	10.0 (9.0-14.0)	12.0 (9.0-1.0)	0.257	12.0 (10.0-15.0)	14.0 (0.6-15.0)	12.0 (10.0-17.0)	0.957
hs-PCR – nmol/L	19.0 (9.5-38.1)	28.6 (9.5-38.1)	19.0 (9.5-57.1)	0.592	19.0 (9.5-28.6)	19.0 (9.5-38.1)	28.6 (9.5-47.6)	0.546
Platelet count – x10 ⁹ /L	222.0 (179.0-241.0)	221.0 (193.0-251.0)	234.0 (206.0-284.0)	0.080	214.5 (178.2-240.5)	243.0 (193.8-288.8)	223.0 (198.5-278.8)	0.196
Mean Platelet Volume – fL	11.3 (10.6-11.9)	11.2 (10.5-11.7)	11.3 (10.7-11.8)	0.673	11.4 (11.1-12.0)	11.1 (10.2-11.6)	11.0 (10.2-11.5)	0.029
FPG – mmol/L	7.0 (5.7-7.6)	6.6 (5.7-7.2)	6.7 (5.6-7.8)	0.469	5.3 (4.7-5.5)	5.2 (4.8-5.5)	5.3 (4.9-5.7)	0.533
Serum Creatinine – umol/L	79.2 (61.6-88.0)	70.4 (61.6-79.2)	70.4 (61.6-79.2)	0.689	70.4 (61.6-79.2)	70.4 (61.6-88.0)	70.4 (70.4-96.8)	0.396
eGFR – ml/min	85.0 (67.4-97.0)	90.1 (75.5-95.2)	91.0 (86.5-109.6)	0.034	88.6 (65.3-106.0)	88.3 (75.8-96.6)	82.0 (70.7-93.5)	0.570

Reference range of “normal values”: AST, 5-34 U/L; ALT, 0-55 U/L; hs-C-reactive protein, 0-47.62 nmol/L; Platelet count, 150-450 x10⁹/L; Total cholesterol, 0.0-5.17 mmol/L; HDL cholesterol, 1.03-1.55 mmol/L; Triglycerides, 0-1.69 mmol/L; Fasting plasma glucose, 4.61-6.11 mmol/L; Serum Creatinine, 50.2-97.7 umol/L; Fasting plasma glucose, 3.9-5.5 mmol/L; eGFR, >60 mL/min.

Abbreviations: BMI= body mass index, WHR= Waist to Hip ratio, SAP= systolic arterial pressure, DAP= diastolic arterial pressure, NAFLD= Nonalcoholic Fatty Liver Disease, HbA1c= glycated hemoglobin, HDL= high-density lipoproteins, AST= Aspartate Aminotransferase, ALT= Alanine amino Transferase, hs-PCR= hs-C-reactive protein, FPG= fasting plasma glucose, eGFR= estimated glomerular filtration rate. Data are median (25th – 75th percentile).

†Determined by Kruskal-Wallis or χ^2 test, as appropriate.

Figure 1. Subjects with faster kinetics of recovery of serum thromboxane B₂ (sTXB₂) during the 24-hour aspirin dosing interval, display increased platelet COX-1 expression and urinary 11-dehydro-TXB₂ excretion as compared to those with normal sTXB₂ recovery.

Study design (A). Linear fitting of sTXB₂ measured 10 and 24h post-aspirin intake in patients without (B, n=100), and with T2DM (C, n=100) stratified in tertiles according to sTXB₂ (*ex vivo* index of COX-1-dependent TXA₂ production) slope (n=33/tertile) Patients in the third sTXB₂ slope tertile display significantly faster recovery of sTXB₂ vs. first tertile (p<0.001), during 24 hours between two witnessed aspirin administrations. sTXB₂ values are as median and interquartile range. Comparison of subjects in the third vs. first sTXB₂ slope tertile for platelet transcript levels of COX-1 mRNA (D, n= 20 vs. n=24) and urinary 11-dehydro-TXB₂ (E, n=66 vs. n=66). Significance was calculated by Mann-Whitney *U* test.

Figure 2. Proteomic Analysis shows activation of pathways “formation of cellular protrusions”, “formation of proplatelets”, “cell survival”, “production of reactive oxygen species”, and inhibition of “apoptosis” in platelets from patients with faster COX-1 recovery.

Proteomic Analysis using Ingenuity Pathway Analysis (IPA) revealed activation of “formation of cellular protrusions”, “formation of proplatelets”, “cell survival”, “production of reactive oxygen species”, and inhibition of “apoptosis” pathways in platelets of third vs. first sTXB₂ tertile in patients without (A and E), and with T2DM (B and E). Further details are reported in the Online Figure Supplementary S1, S2 and S3. Validation of proteomic data by western blot, assessing 78 kDa glucose-regulated protein (GRP78) (C, n=4/tertile), COX-1 (D, n=4/tertile) and Rab27B (F, n=4/tertile) in patients from third tertile vs. first tertile and healthy subjects (HS, n=4), using β-Actin as loading control. Significance was calculated by Student’s t-test.

Figure 3. Enhanced megakaryocytes (Mks) maturation and proplatelet formation (PPF) in patients with faster COX-1 recovery

Representative immunofluorescence of Mks (A) and PPF (B). The proportion of mature Mks was measured by flow cytometry, as the percentage and mean fluorescence intensity (MFI) of CD41-positive cells (healthy subjects n=5; first tertile: all n=8, T2DM n=4, noT2DM n=4; third tertile: all n=10, T2DM n=4, noT2DM n=6) (C-D); and as the percentage and MFI of GPIb α (CD42b)-positive cells (healthy subjects n=5; first tertile: all n=8, T2DM n=4, noT2DM n=4; third tertile: all n=11, T2DM n=5, noT2DM n=6) (C-D). PPF was quantified as the proportion of Mks displaying at least one proplatelet with respect to the total number of adhered Mks (healthy subjects n=5; first tertile: all n=7, T2DM n=4, noT2DM n=3; third tertile: all n=8, T2DM n=4, noT2DM n=4) (E).

Figure 4. Higher circulating levels of thrombopoietin (TPO) and platelet count and lower glyocalicin (GC) and glyocalicin index (GCI) at 24h after witnessed aspirin intake in patients with faster COX-1 recovery.

Comparison of TPO (A), GC (B) and GCI (C) between first vs. third sTXB₂ slope tertile in all patients (n=132). Comparison of TPO (A) and GC (B) between healthy subjects (HS, n=5) vs. first and vs. third sTXB₂ slope tertile in all patients (n=132). Significance was calculated by Mann-Whitney *U* test. Correlation between GCI and TPO in all investigated patients (D). Spearman correlation coefficient and p-value are reported. Platelet count in first vs. third tertile (C) in all patients (n=132). Significance was calculated by Mann-Whitney *U* test.

Figure 5. Lower GC circulating levels in platelets from patients with faster COX-1 recovery depend on higher GPIb α expression, lower phosphatidylserine expression and lower ADAM17 activation, and enhance TPO mRNA transcription in liver cells.

GPIb α protein levels in platelets of healthy subjects (HS, n=4) vs. first (n=4) vs. third (n=4) tertile in all patients (A). ADAM17 levels in platelets of healthy subjects (HS, n=3) vs. first

(n=4) vs. third sTXB₂ slope tertile (n=4) in all patients (B). PS-positive platelets (%CD41a+/AnV+) in the first (n=34) vs. third tertile (n=20) in all patients (C). PS-positive platelets (%CD41a+/AnV+) (D) and active-ADAM17 cleaved form (E) in 4 healthy subjects treated with low-dose aspirin, at 10h and 24h post-aspirin. Treatment of HepG2 cells (n=4) with an increasing concentrations of human recombinant GC (rGC, 0.5, 1 and 2 µg/ml) is associated, after 1 hour of incubation, with a significant dose-dependent reduction in TPO mRNA (F). Significance was calculated by Mann-Whitney *U* test or by Student's t-test.

Figure 6. Higher platelet desialylation rate in patients with faster COX-1 recovery.

Expression levels of galactose-recognizing lectins, *Erythrina cristagalli* agglutinin (ECL, A), and *Ricinus communis* agglutinin I (RCA-I, B), in platelets of healthy subjects (HS, n=4) vs. first (n=4) vs. third (n=4) tertile in all patients. Expression levels of the sialidase Neu1 in the same subset (C). β-Actin was used as loading control. Significance was calculated by Student's t-test.

Figure 7. Multivariable logistic regression analyses, receiver operating characteristic (ROC) curve for the prediction of poor aspirin response and proposed model depicting the mechanisms involving platelet lifespan that may limit the extent and duration of aspirin effect over 24 hours.

Determinants of the accelerated recovery of COX-1 activity in patients with (A) and without T2DM (B) assessed by multivariable logistic regression analysis. Receiver operating characteristic (ROC) curve for the prediction of poor aspirin response (C and D). ROC and the relative area under the curve (AUC) showing the ability of the model in discriminating between the third vs. first sTXB₂ slope tertile. Among patients with T2DM, the combination of younger age (SD=6.38 years), presence of NAFLD, higher platelet count (SD=55.66 µL) and higher TPO/GC ratio (1st tertile: <60; 2nd tertile: from 60 to 138; 3rd tertile >138) yielded

an AUC value of 0.883 (95%CI: 0.799 to 0.966) in distinguishing patients in third sTXB₂ slope tertile from first tertile patients (C). In comparison with a model including only clinical/hemocromocytometric variables (age, NAFLD and platelet count) the addition of TPO/GC ratio yielded a significant increase in AUC (from 0.754 to 0.883; P for difference 0.015). Among patients without T2DM, higher WHR (SD=0.066), lower mean platelet volume

(SD=0.93 μL) and higher TPO/GC ratio (1st tertile: <76; 2nd tertile: from 76 to 147; 3rd tertile >147) yielded an AUC value of 0.880 (95%CI: 0.794 to 0.966) in distinguishing patients in third sTXB₂ slope tertile from first tertile patients (D). Aspirin-treated patients were stratified according to the kinetics of COX-1 recovery over the 10-24h dosing interval. In poor aspirin responders we showed: i. increased plasma thrombopoietin, megakaryocyte maturation and proplatelet formation reflecting enhanced platelet production; ii: increased platelet desialylation, lower phosphatidylserine exposure, lower platelet sheddase ADAM17 and plasma glyocalicin and increased glycoprotein (GP)Ibα expression, altogether reflecting defective platelet GPIbα shedding; iii: a proteomic signature characterized by activation of cell survival and inhibition of apoptosis. Younger age, non-alcoholic fatty liver disease and visceral obesity, higher platelet count together with higher thrombopoietin-to-glyocalicin ratio, predict suboptimal aspirin response. PLT, platelets; PPF, proplatelet formation; GC, glyocalicin; MK, megakaryocyte. sTXB₂, serum thromboxane B₂ (E).

Fig.1

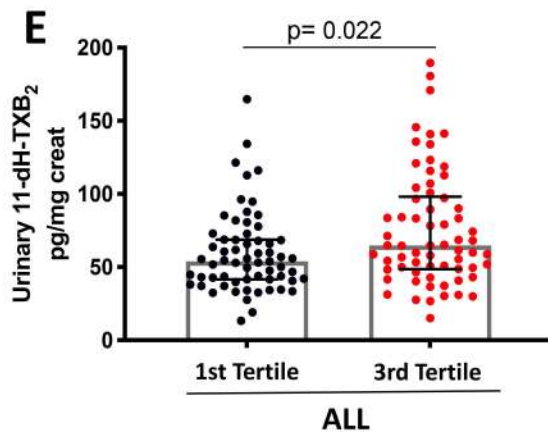
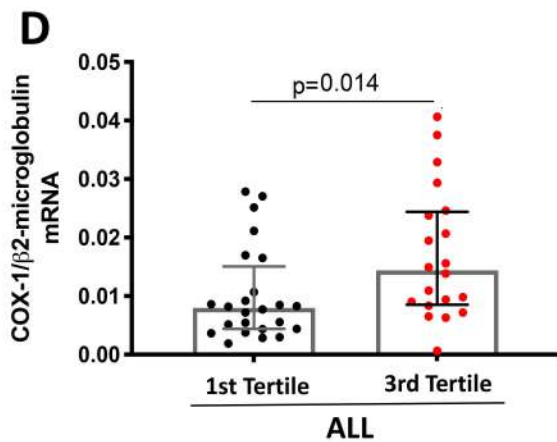
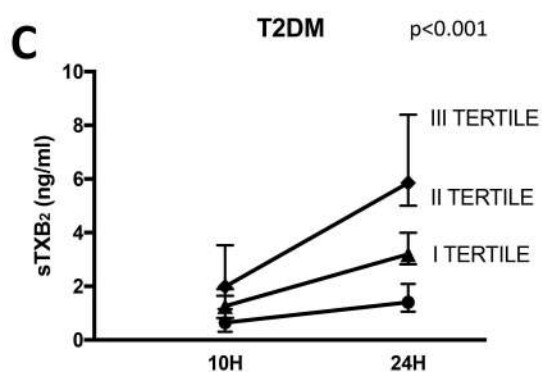
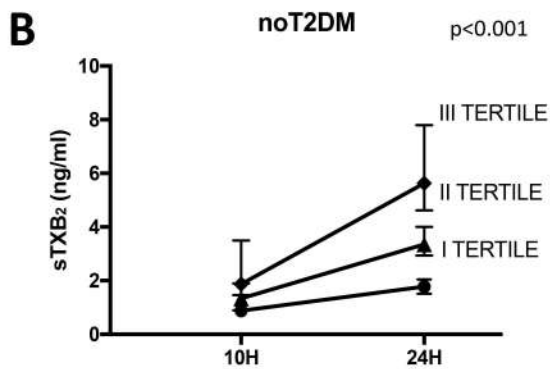
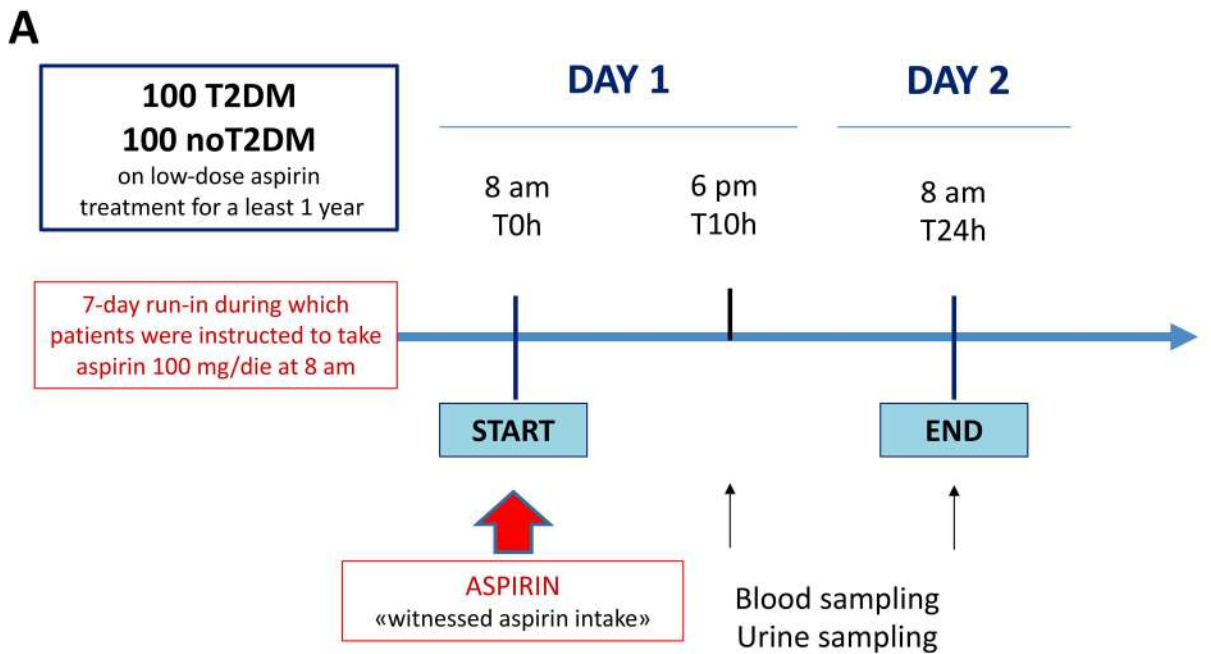


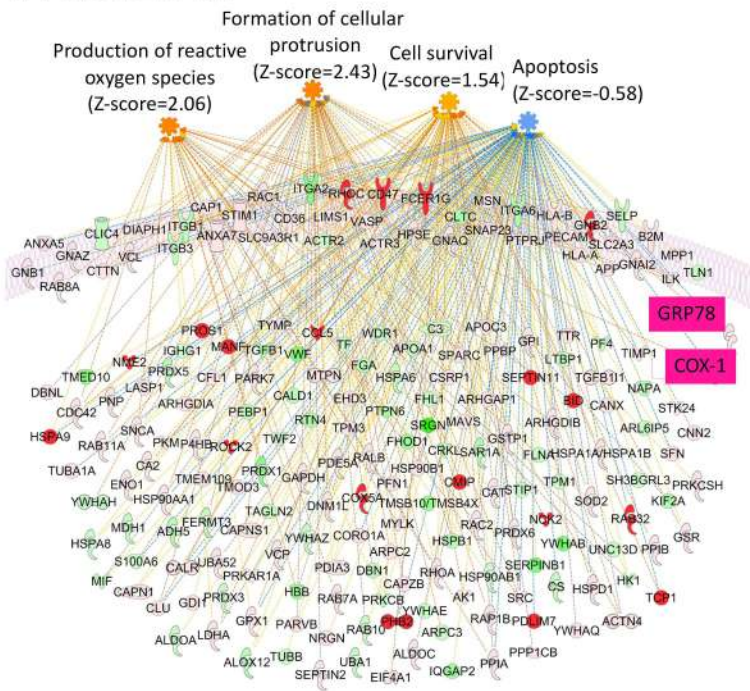
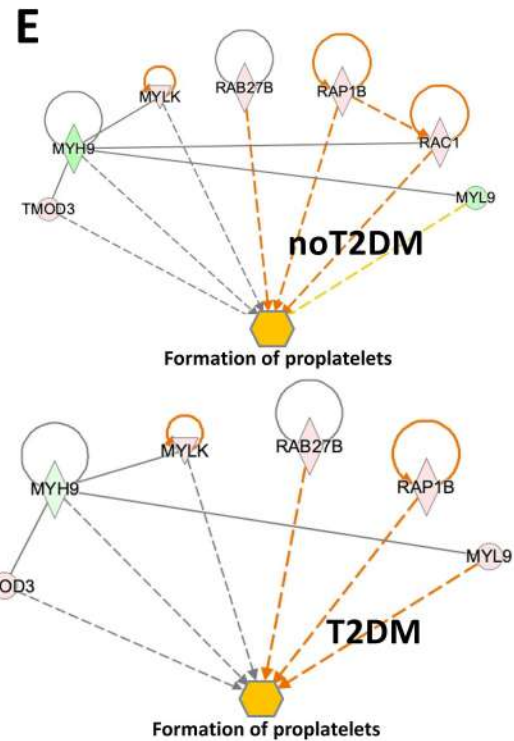
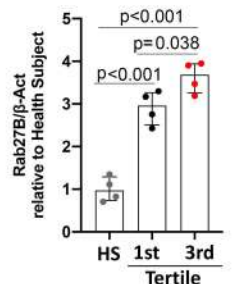
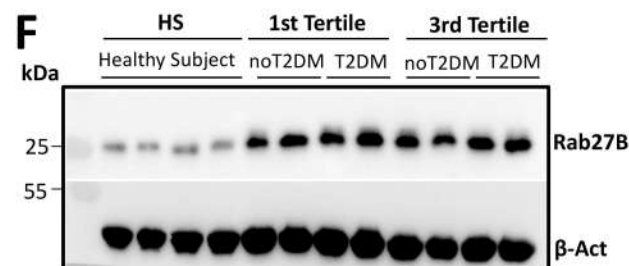
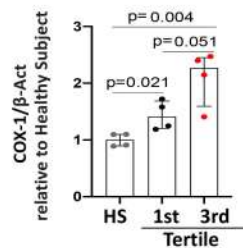
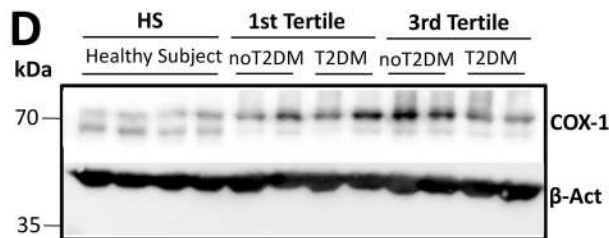
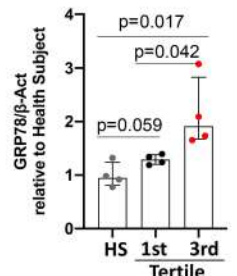
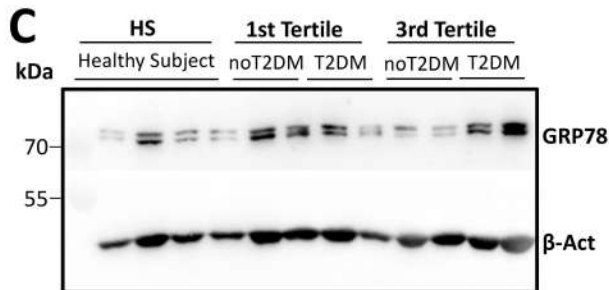
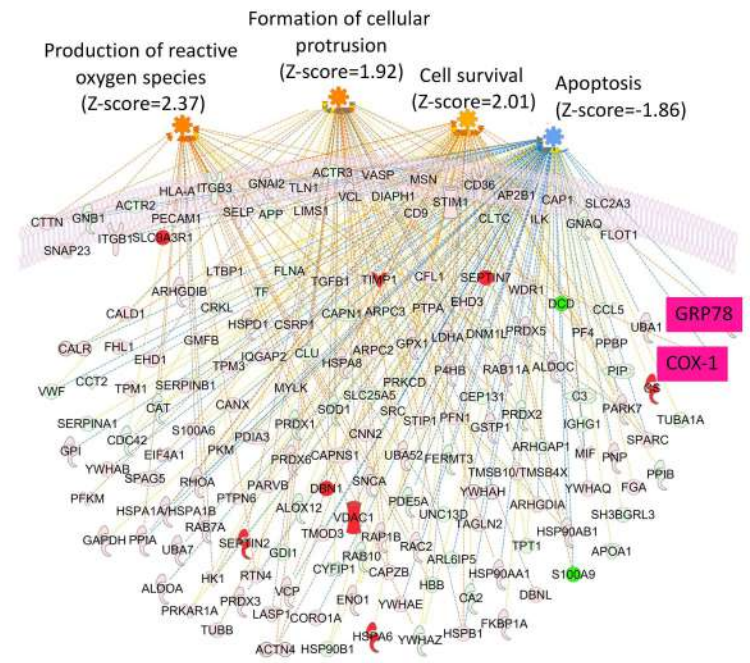
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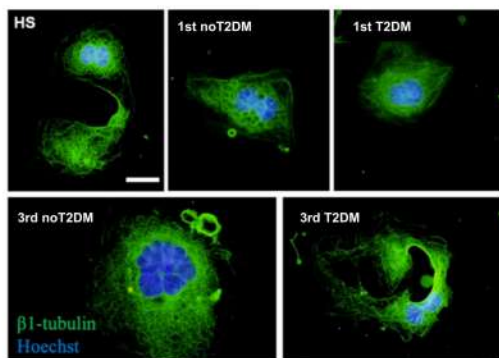
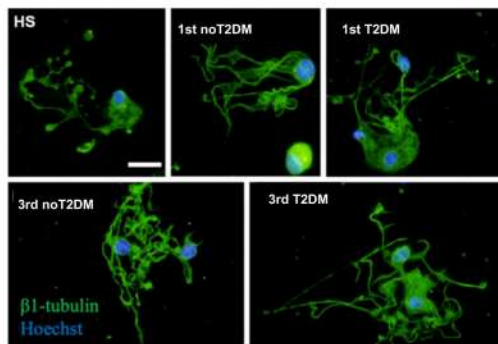
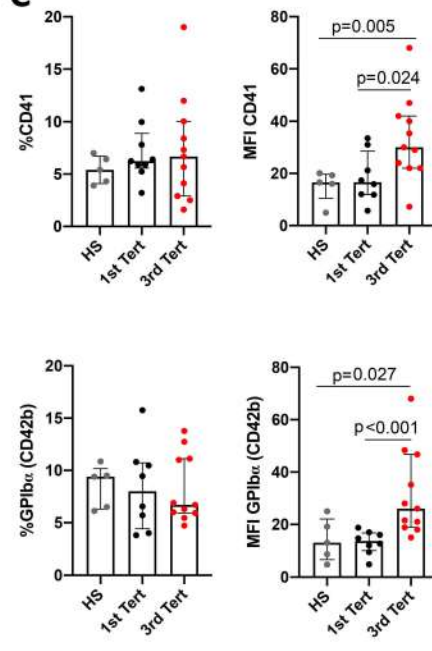
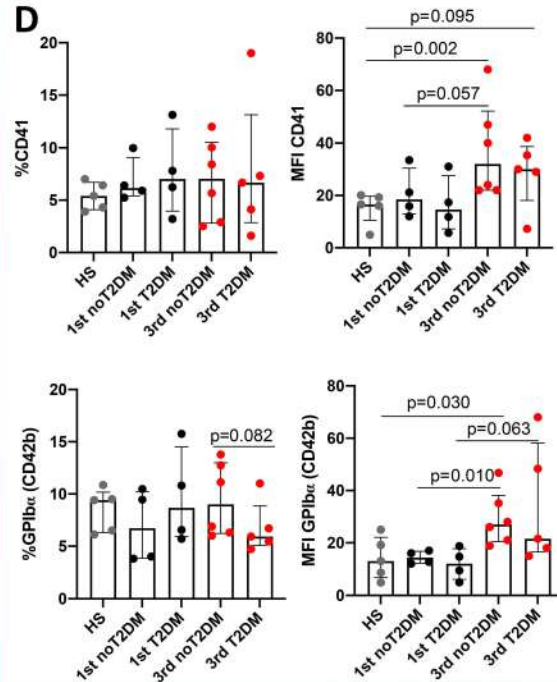
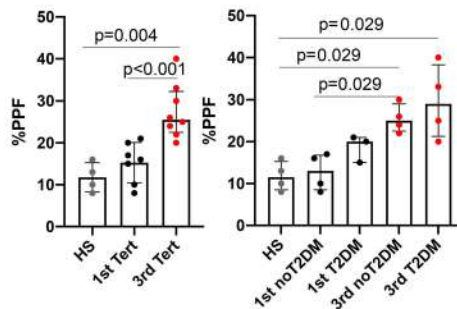
Fig.3**A****B****C****D****E**

Fig.4

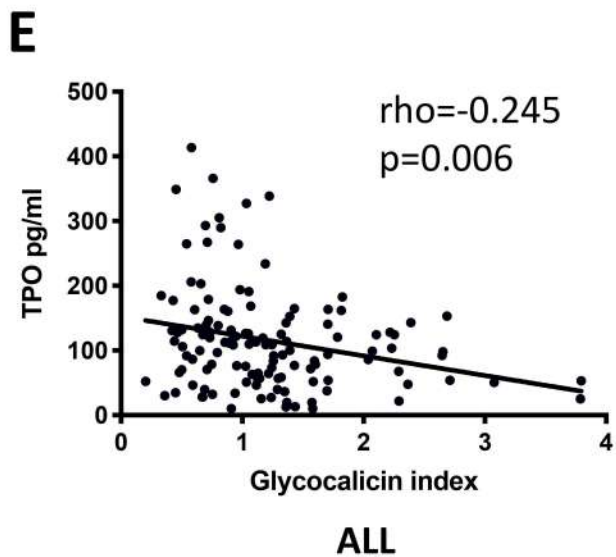
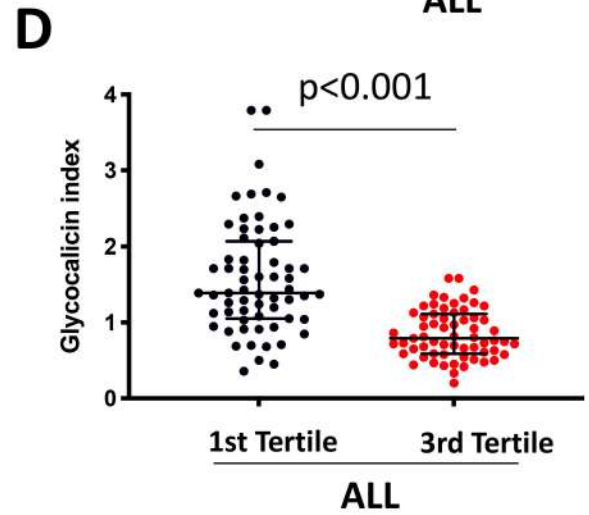
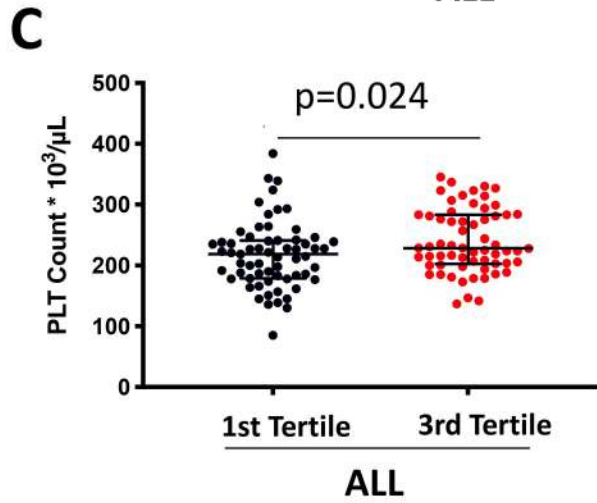
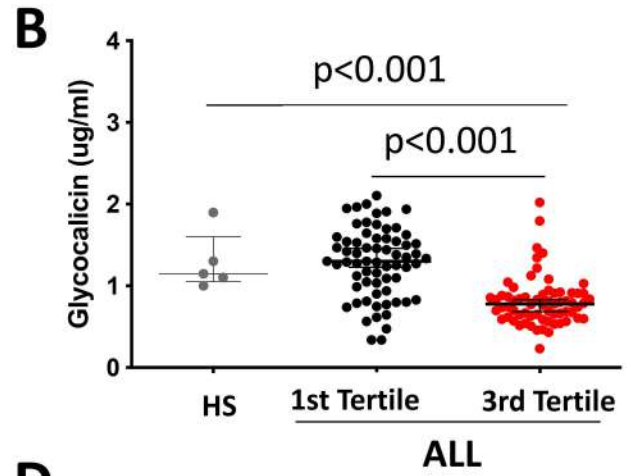
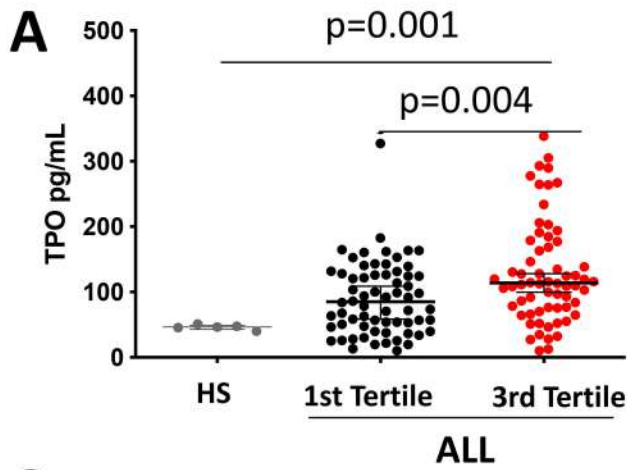


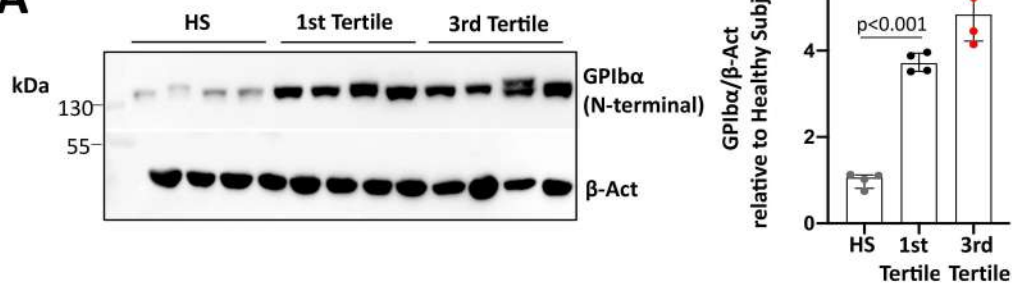
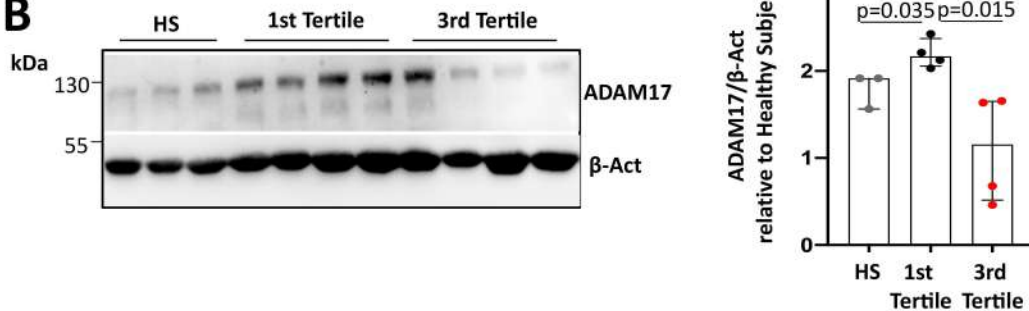
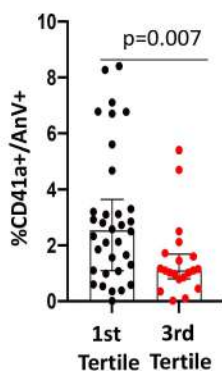
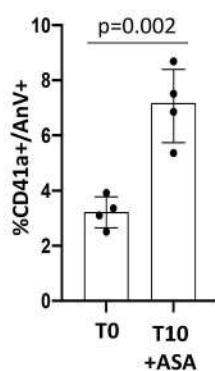
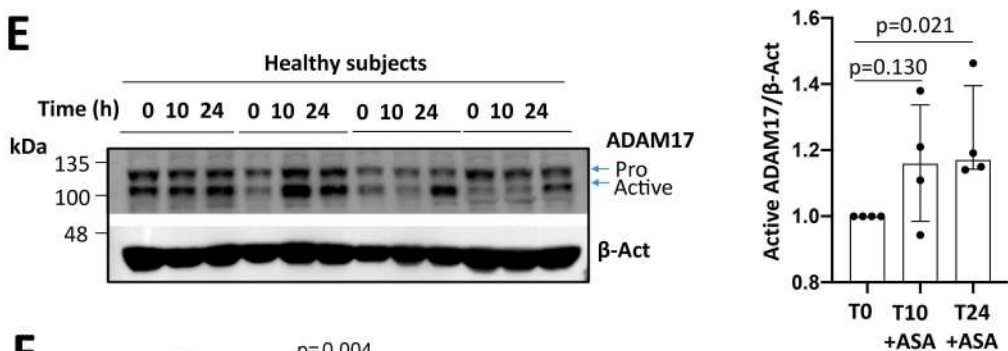
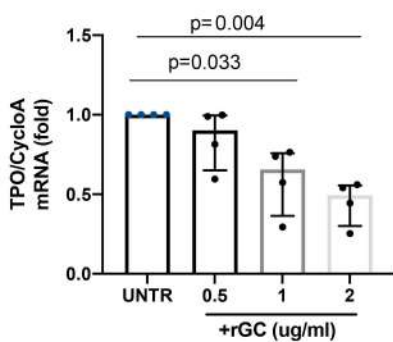
Fig.5**A****B****C****D****E****F**

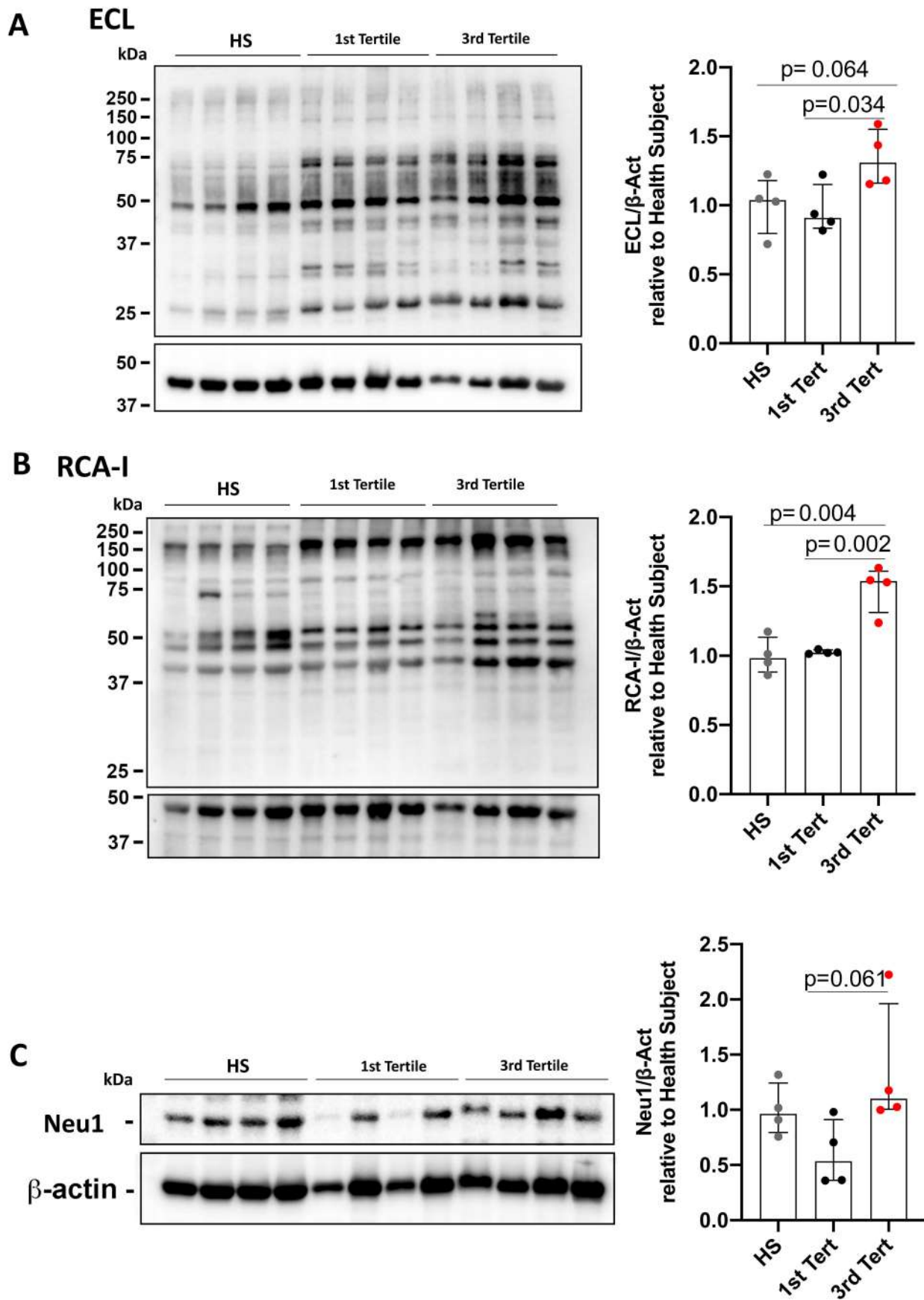
Fig.6

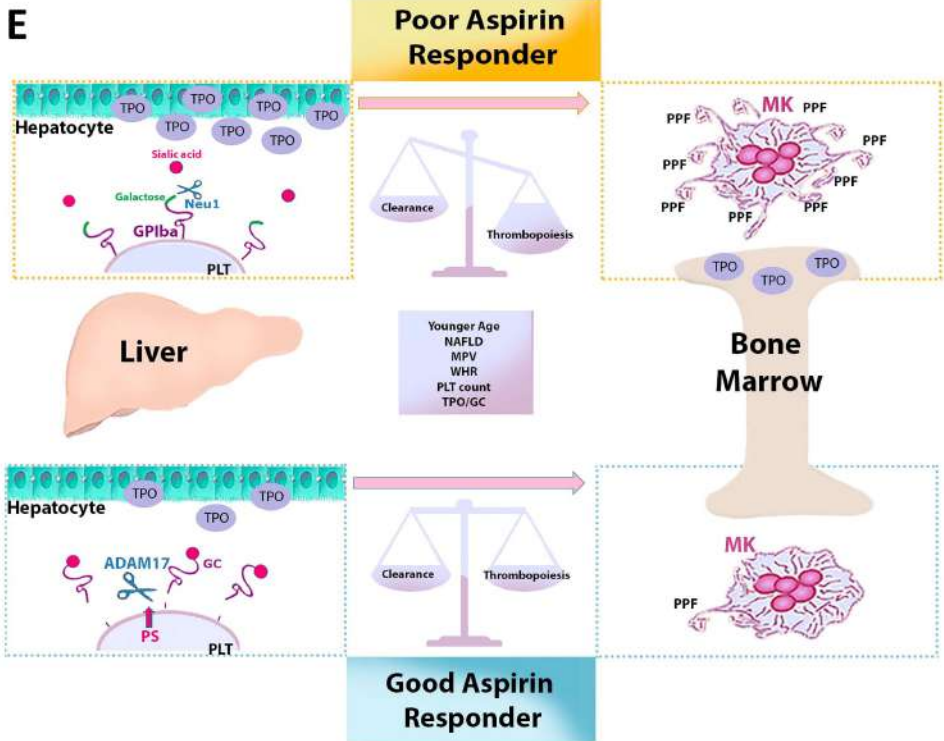
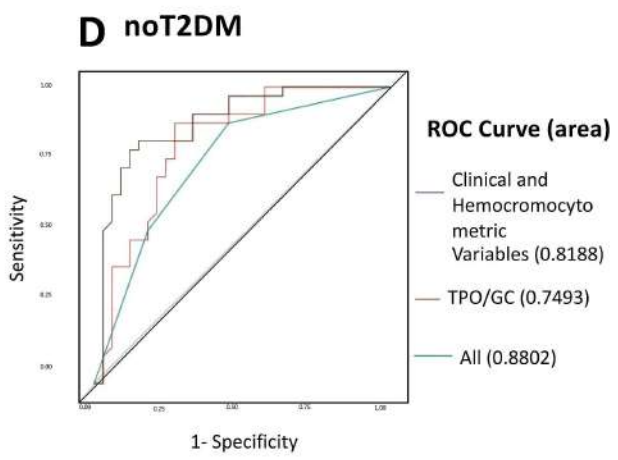
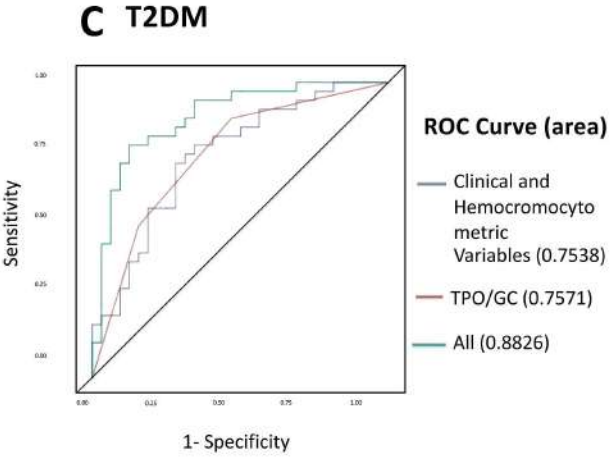
Fig.7

A

T2DM			
Variable	OR	95% CI	P-value
Age (1 SD increment)	0.44	0.21-0.93	0.031
NAFLD (ys vs no)	3.74	0.65-21.5	0.14
Platelet Count (1 SD increment)	1.79	0.83-3.88	0.14
TPO/GC II tertile vs. I tertile	12.30	2.14-70.8	0.0049
TPO/GC III tertile vs. I tertile	34.05	4.94-234.6	0.0003

B

noT2DM			
Variable	OR	95%CI	P-value
WHR (1 SD increment)	3.43	1.49-7.88	0.0037
MPV (1 SD increment)	0.50	0.25-0.99	0.049
TPO/GC II tertile vs. I tertile	10.56	1.82-61.38	0.0086
TPO/GC III tertile vs. I tertile	11.06	2.20-55.72	0.0036



Supplementary extended study participants, methods and results Appendix

Title: Reduced platelet glycoprotein Iba shedding accelerates thrombopoiesis and COX-1 recovery: implications for aspirin dosing regimen

Short Title: Platelet lifespan imbalance and aspirin response

Online Supplementary Methods: pages 2-8

Online Supplementary Results: page 9-10

Online Supplementary References: page 11

Online Supplementary Figure S1: page 12

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Online Supplementary Methods

Study participants

For all patients, inclusion criteria included: age between 45 and 80 years, body mass index (BMI) between 20 and 45 kg/m². A diagnosis of overt type 2 diabetes mellitus (T2DM), according with the ADA criteria¹, was also required for the T2DM group.

Exclusion criteria were poorly controlled hypertension or hypercholesterolemia, smoking, pregnancy or lactation, impaired liver or renal function, history of malignant neoplasms (diagnosed and treated within the last 5 years), history of malabsorption, regular (daily) consumption of alcohol, or treatment with non-steroidal anti-inflammatory drugs (NSAIDs), anticoagulants or other antiplatelet drugs. Type 1 diabetes was suspected and excluded with islet autoantibodies evaluation (anti-glutamic acid decarboxylase, islet cell cytoplasmic, and IA-2 antibodies), when one of the following applied: family history of type 1 diabetes, age lower than 40, lean phenotype, precocious requirement for insulin therapy. No patient was diagnosed as having MODY (Maturity Onset Diabetes of the Young).

Liver Ultrasound

The grading of liver steatosis has been obtained using a high-resolution ultrasound System (GE Healthcare), equipped with a 3.5-MHz convex- array probe. Subjects were examined in the supine position. Ultrasound features included liver brightness, contrast between the liver and the kidney, US appearance of the intrahepatic vessels, liver parenchyma and diaphragm. Steatosis was graded as follows: absent (score 0) when the echotexture of the liver is normal; mild (score 1), when there is a slight and diffuse increase of liver echogenicity with normal visualization of the diaphragm and of the portal vein wall; moderate (score 2), in case of a moderate increase of liver echogenicity with slightly impaired appearance of the portal vein wall and the diaphragm; severe (score 3), in case of marked increase of liver echogenicity with poor or no visualization of portal vein wall, diaphragm, and posterior part of the right liver lobe². The examinations were assessed by the same operator, to reduce the variability.

Biochemical measurements and glycolalicin index (GCI)

Serum thromboxane B₂ (sTXB₂) produced during whole blood clotting *ex vivo*³ was measured with

an enzyme-immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI).⁴ Urinary 11-dehydro-TXB₂, a major TXA₂ metabolite reflecting TXA₂ biosynthesis rate, was measured with validated liquid chromatography–tandem mass spectrometry (LC-MS-MS).⁴

Thrombopoietin (TPO) (R&D Systems, Minneapolis, MN) and glycoalbumin (GA) (Cusabio Technology, Houston, TX), ELISA kits were used on ethylenediamine tetraacetic acid (EDTA) plasma.

The glycoalbumin index (GI), a parameter of platelet destruction,^{5,6} was calculated as: $[GA \text{ concentration } (\mu\text{g/mL}) \times 250 \times 10^9 \text{ platelet/L} / \text{individual platelet count} \times 10^9/\text{L}]$.^{7,5} Normal GI is 0.7 (0.6–0.9).

Proteomics data processing and bioinformatics parameters

Proteomics analysis was performed on pooled platelet samples by selecting 10 patients (with or without T2DM) per group according to their homogeneous clinical and demographical parameters. Samples were analyzed in triplicate (third vs. first tertile) by LC-MS/MS and processed using MaxQuant 1.6.6.0 as previously reported.^{8,9}

For protein identification and quantification carbamidomethylation of cysteines I was defined as fixed modification, while oxidation of methionines (M), deamidation of asparagines (N) and glutamines (Q) were set as variable modifications. Mass tolerances were set by default to 0.07 Da in the first search and 0.006 Da in the main search, while TOF MS/MS match tolerance was set to 0.05 Da. A retention time tolerance of 0.7 min was used to align any time shift in acquisition between samples. Match-between-runs (MBR) algorithm was used to transfer the peptide identifications from one LC-MS/MS run to all others using its default settings (match window of 0.7 min and alignment time of 20 min). False discovery rate (FDR) at the protein level was set at 2%, on the contrary at peptide level was set at 1%. Intensity-based absolute quantification (iBAQ) was used to quantify protein abundance in each sample in the bioinformatics analysis performed with Perseus,¹⁰ version 1.6.10.50. (Max-Planck Institute for Biochemistry, Martinsried, Germany) uploading the protein groups generated by MaxQuant. Data were log₂ transformed in order to facilitate the calculation of the protein expression. Site only, reverse and contaminant peptides were removed from the dataset. Then, the missing and invalid values were removed.

The minimum number of valid values accepted was set at 2 in at least one treatment condition. In this way we have evaluated not only the different protein expression, but also the presence and absence of proteins between the different clinical conditions. The expression of common proteins between two clinical groups (third vs. first tertile) in both patients without T2DM (Online Supplementary Figure S1A) and with T2DM (Online Supplementary Figure S1B) was evaluated comparing the iBAQ (\log_2 transformed) of the proteins in the density plot with Pearson correlation (R^2). A Volcano plot function was used to identify the differentially regulated proteins by performing a T-test with a false discovery rate (FDR) of 0.22 and a S_0 of 0.05 (Online Supplementary Figure S2).

The quality of our proteomic data is evaluated by using Platelet W-b - Systems Biology Workbench. Gene Ontology and Comparison Analysis were performed using Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) by loading the protein ratio for each comparison (third vs. first tertile) both for patients with T2DM and without T2DM. We considered molecules and/or relationships in all species and a confidence setting as “high predicted” or “experimental observed” (excluding medium predicted). The predicted activation or inhibition of each transcriptional regulator or downstream was inferred by the IPA-generated z-scores¹¹ (Online Supplementary Figure S2).

Immunoprecipitation

For immunoprecipitation, platelet lysates (200 μ g of total protein) were precleared by incubation with protein A-Sepharose (Sigma Aldrich). Precleared lysates were incubated with 2 μ g of anti-14-3-3 ξ antibody (Santa Cruz Biotechnology) for 2 h at 4°C on a rotatory shaker, followed by adding 100 μ L of 50 mg/ml protein A-Sepharose and incubation for 1 hour at 4°C on a rotatory shaker. Beads were washed three times in lysis buffer and samples were eluted with Laemmli buffer at 95°C for 5 minutes. Protein lysates were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane.

Megakaryocyte maturation

CD45⁺ cells were separated from peripheral blood samples obtained from healthy subjects and patients by immunomagnetic bead selection (Miltenyi Biotech, Bologna, Italy) and cultured in Stem Span

medium (Stem Cell Technologies, Canada) supplemented with 1% L-glutamine, 1% penicillin-streptomycin, 10 ng/mL recombinant human thrombopoietin and 10 ng/mL recombinant human interleukin-11 (PeproTech, London, UK) for 14 days as previously described.¹² To evaluate Mk maturation, at the end of the culture, aliquots of 50×10^3 cells were collected, washed in PBS, and double-stained with the FITC-conjugated monoclonal antibody HIP8 against CD41 (eBioscience, ThermoFisher Scientific) and the PE-conjugated monoclonal antibody HIP1 against glycoprotein (GP)Ib α (CD42b, Abcam, Cambridge, UK). Cells were analyzed using a Navios flow cytometer (Beckman Coulter). A minimum of 10,000 events was acquired. Off-line data analysis was performed using the Beckman Coulter Navios software package. The maturation of Mks was measured as the percentage and mean fluorescence intensity of CD41 and GPIb α positive cells.¹³

Proplatelet formation (PPF) assay

PPF was investigated in adhesion to fibrinogen according to a previously described protocol¹². At the end of the culture, large Mks were separated by a bovine serum albumin gradient (3-4%). 1×10^5 Mks were allowed to adhere at 37°C and 5% CO₂ for 16 hours onto glass coverslips coated with 100 μ g/mL fibrinogen (Merck-Millipore, Milan, Italy). Samples were then fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 5% BSA and stained with the anti- β 1-tubulin antibody (Abcam). An Alexa Fluor 488-conjugated goat anti-rabbit antibody (Life Technologies, Monza, Italy) was used as secondary antibody. Hoechst was used for counterstaining nuclei. Proplatelet forming Mk were identified by fluorescence microscopy as the β 1-tubulin positive cells displaying at least one proplatelet with respect to the total number of β 1-tubulin positive cells. At least 10 fields per sample were analyzed using an Olympus IX53 microscope (Olympus Deutschland GmbH, Hamburg, Germany).

Western Blot Antibodies

The used primary antibodies were: anti-78 kDa glucose-regulated protein (GRP78, 1:1000; clone 76-E6, sc-13539, Santa Cruz Biotechnology), anti- β -actin (1:5000; Sigma Aldrich), anti-cyclooxygenase (COX)-1 (1:1000; clone CX111, Cayman Chemical), anti-Rab27B (1:1000; 44813 Cell Signaling), anti-GPIb α (1:10000; EPR6995, ab27901, Abcam), anti- α disintegrin and metalloprotease 17 (ADAM17,

1:1000; Ab2051, Abcam), and anti-neuraminidase (Neu)1 (1:1000; Invitrogen), anti-caspase 3 (Caspase 3, 1:1000; sc-7272, Santa Cruz). The used horseradish peroxidase-conjugated secondary antibodies were: goat anti-mouse (1:3000, Calbiochem) or goat anti-rabbit (1:5000, Calbiochem).

The used lectins were: biotinylated *Erythrina cristagalli* (ECL, 1:3000), and biotinylated *Ricinus communis agglutinin I* (RCA-I, 1:3000) as previously described.¹⁴ Immunoreactive bands were detected by horseradish peroxidase-labeled streptavidin (1:3000).

Finally, the blots were developed using Western Lightning Chemiluminescence Reagent (PerkinElmer) and immunoblot images were acquired with Alliance 4.7 (UVITEC, Cambridge, UK). The intensity of the relevant bands was evaluated using the ImageJ NIH Image Analysis Program.

Quantitative Real-time PCR (qRT-PCR)

Platelet RNA was extracted by using Total RNA Purification Kit (Norgen), and the retrotranscription of 500 ng was performed by Omniscript RT Kit (Qiagen). qRT-PCRs were performed using miSCRIPT Syber Green PCR Kit (Qiagen). Primers (5' to 3') used were:

-COX-1 forward TCATCAGGGAGTCTCGGGAG, reverse ATTCCTCCAACCTCTGCTGCC;

-β2-microglobulin forward GCTCGCGCTACTCTCTCTTT, reverse
TGTCGGATGGATGAAACCCA;

-thrombopoietin (TPO) forward, ACTGCTTCGTGACTCCCATG, reverse
AGGAGGGATGAGAGGCAAGT;

-cyclophilin (cyclo) A forward, AGTCCATCTATGGGGAGAAATTTG reverse
GCCTCCACAATATTCATGCCTTC.

Platelet Annexin V

Platelet staining for flow cytometry analyses were carried out by adding 5 μL of peripheral blood samples to the reagent mix detailed in Supplementary Table S3. 1×10^6 events/sample were acquired by flow cytometry (FACSVerse, BD Biosciences), and data were analyzed using FACSuite v 1.0.6.5230 and FlowJo X v 10.0.7 software (BD Biosciences). Platelets were identified on an FSC-H/SSC-H dot plot and then they were analyzed for their positivity to CD41a. CD41a+ platelets were gated and analyzed for their

Annexin V positivity¹⁵.

Total and platelet derived Annexin V Extracellular vesicles

Extracellular-vesicles, total and platelet derived, for flow cytometry analyses were carried out by adding 5 µL of peripheral blood samples to the reagent mix¹⁵ prepared by adding to 195 µL of binding buffer 1X (BD Biosciences) the dyes and antibodies (1 µL Lyophilic Cationic Dye (LCD) APC 626267 BD Biosciences; 5 µL CD41a BV510 563250 BD Biosciences; 1 µL AnnexinV V450 560506 BD Biosciences) and incubated for 45 minutes (RT, in the dark). Finally, samples were adequately diluted with binding buffer 1X (1:143), and no swarm effects occurred¹⁵ when they were acquired (1 x 10⁶ events/samples) by flow cytometry (FACSVerse, BD Biosciences). All requirements imposed for polychromatic flow cytometry EV analysis were considered¹⁶. The trigger threshold was placed on the channel in which the dye used to stain EVs emits (lipophilic cationic dye, allophycocyanin—APC—channel, threshold value = 200/262.144). For all used parameters the height (H) signals and bi-exponential or logarithmic modes were selected. Instrument performances, data reproducibility, and fluorescence calibrations were sustained by the Cytometer Setup & Tracking Module (BD Biosciences). The evaluation of non-specific fluorescence was obtained by acquiring fluorescence minus one control combined with the respective isotype control. Compensation was automatically calculated. Data were analyzed using FACSuite v 1.0.6.5230 (BD Biosciences) and Flow Jo X v 10.0.7 (BD Biosciences) software. EV concentrations were obtained by the volumetric count function¹⁷. EVs were identified and subtyped as already reported.¹⁵ Briefly, within the gate of intact EVs (LCD+/Phalloidin-), platelets derived EVs were identified as CD41a⁺ events. Each of the above-mentioned EV subsets were also analyzed for the surface expression of phosphatidylserine (AnV⁺).

In vitro HepG2 cells treatment

Human HepG2 hepatocarcinoma cells were grown as previously reported¹⁸ in DMEM high glucose, pyruvate (GIBCO, Thermo Fisher Scientific), 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Thermo Fisher Scientific), 10,000 U ml⁻¹ penicillin G and 10 mg ml⁻¹ streptomycin sulphate. For *in vitro* assay, the HepG2 cells were transferred to 12-well plates (10⁶ per well), allowed to adhere for 24 hours,

and treated with a commercially available recombinant human GPIIb/IIIa peptide (rGC, 4067-GP R&D Systems) at the indicated concentration for 1 hour and for 24 hours at 37°C. DMSO was used as control. After the incubation period, the HepG2 cells were lysed in order to harvest the mRNA (after 1 hours) and culture supernatants (after 24 hours) were collected for TPO assay as described above. The rGC protein (His 17-Leu 505) was expressed in mouse myeloma cells (90-120 kDa apparent molecular weight under SDS-PAGE).

Online Supplementary Results

Clinical characteristics

All subjects with T2DM were at high (n=64) or very high (n=36) CV risk, according to the 2019 ESC guidelines.¹⁹ Among those without T2DM, 53 patients had very-high CV risk, 28 high risk, 18 moderate and 1 low risk. Patients without T2DM in primary prevention (n=78) had an average 10-year risk of fatal CVD of 12.3% according to European SCORE.²⁰

Demographic, anthropometric, and clinical parameters of two groups of patients with vs. without diabetes were fairly balanced, except for higher BMI (p=0.026), HOMA-IR (p=0.038), higher prevalence of non-alcoholic-fatty-liver disease (NAFLD) (p<0.001) and retinopathy (p=0.007), lower total cholesterol (p=0.003) levels (with higher prevalence of statin treatment, p=0.015), lower total bilirubin (p=0.006), red blood cell count (p<0.001), hemoglobin (p<0.001), and hematocrit (p<0.001) in T2DM patients (Online Supplementary Table S1).

As expected, patients were significantly different for diabetes-specific treatment such as metformin (p<0.001), glinides (p=0.001), PPAR- γ agonists (p<0.001), insulin (p=0.029), for glycated hemoglobin % (p<0.001), and fasting plasma glucose (p<0.001). Notably, median HbA1c in T2DM patients was 51.0 mmol/mol (6.8%), reflecting good glycemic control in T2DM patients in the group of patients with diabetes (Online Supplementary Table S1).

T2DM patients of the third tertile had higher platelet counts (p=0.080 across the three groups, p=0.047 third vs. first tertile), a higher prevalence of NAFLD (p=0.04) and a nonsignificant trend for higher HOMA-IR (p=0.071) third vs. first tertile (Table 1).

Among patients without T2DM, while platelet count (p=ns) did not change across sTXB₂ recovery slope tertiles, MPV (p=0.029) was lower in patients of third vs. first tertile. Moreover, patients in the upper tertile had a lower prevalence of dyslipidemia (p=0.017) and lower rate of statin treatment (p=0.014) vs. first tertile (Table 1 and Online Supplementary Table S2).

sTXB₂ recovery slope was inversely related to age and directly related to BMI, waist circumference, estimated glomerular filtration rate (eGFR) in T2DM patients (Online Supplementary

TableS7). Among patients without T2DM, the sTXB₂ recovery slope was directly related to weight, waist circumference, WHR and diastolic arterial pressure (Online Supplementary TableS7).

Inhibited apoptosis in patients of the third tertile

We found no differences in annexin-V-positive, both total and platelet-derived, extracellular vesicles, in patients of first (n=35) vs. third (n=21) tertile (Online Supplementary Figure S5A), to corroborate that the faster recovery of COX-1 and differential PS exposure is not linked to different pro-coagulant properties of third tertile platelets.

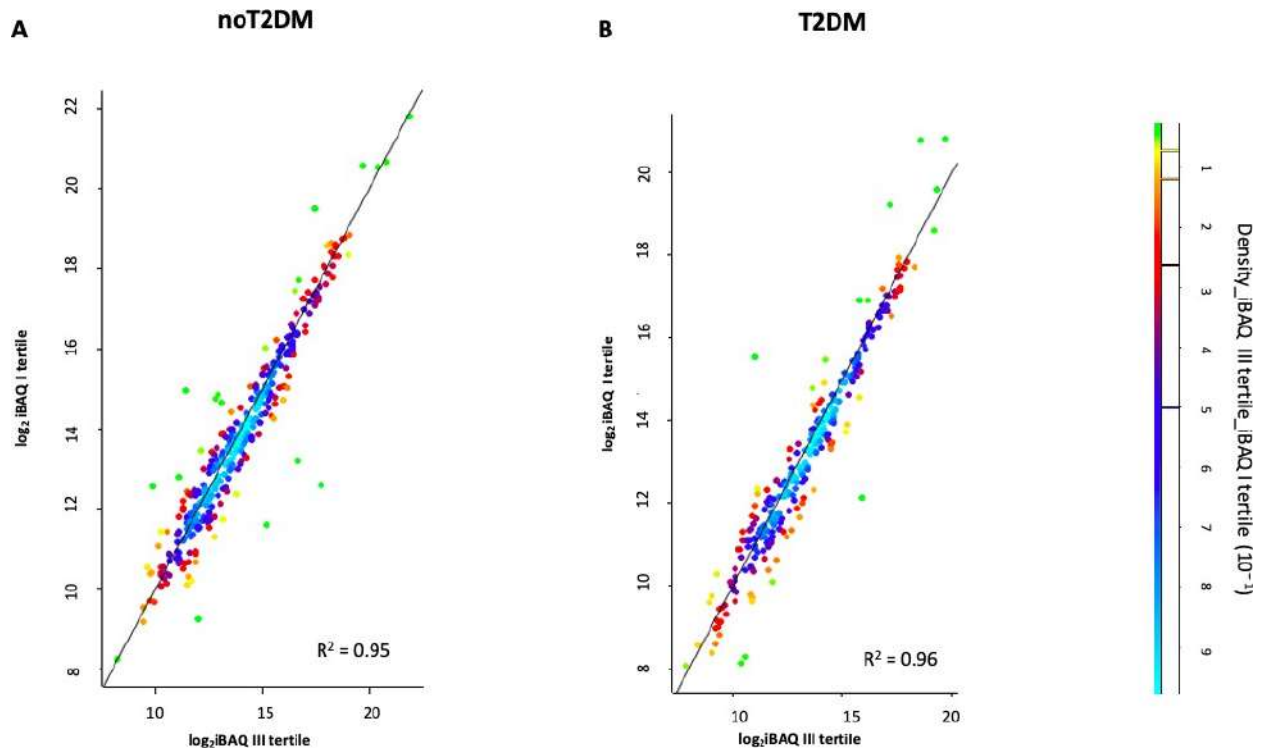
In the intrinsic apoptotic pathway of PLTs, the activation of the effector caspases (Caspase-3/7) determines the subsequent exposure of PS on the membrane.²¹⁻²³ We observed lower levels of caspase 3 in patients of third vs. first tertile (p=0.042, Online Supplementary Figure S5B) and vs. healthy subjects (p=0.015, Online Supplementary Figure S5B), supporting inhibition of apoptosis in our patients with accelerated platelet COX-1 recovery.

Based on data showing “inhibition of apoptosis” and enhanced GPIIb/IIIa receptor levels in poor aspirin responder, we investigated whether the different recovery of COX-1 activity reflected differences in 14-3-3 ζ platelet immunoprecipitates of complexes 14-3-3 ζ -GPIIb/IIIa and 14-3-3 ζ -COX-1 in patients of first (n=3) vs. third tertile (n=3) (Online Supplementary Figure S5C). We observed significantly lower levels of 14-3-3 ζ :GPIIb/IIIa complexes (p=0.016, Online Supplementary Figure S5C) and higher levels of 14-3-3 ζ :COX-1 complexes (p=0.019, Online Supplementary Figure S5C) in patients of third vs. first tertile, thus confirming inactivated apoptosis in patients of third tertile and providing a mechanistic link between COX-1 acetylation or inhibition and GPIIb/IIIa clustering.

Online Supplementary References

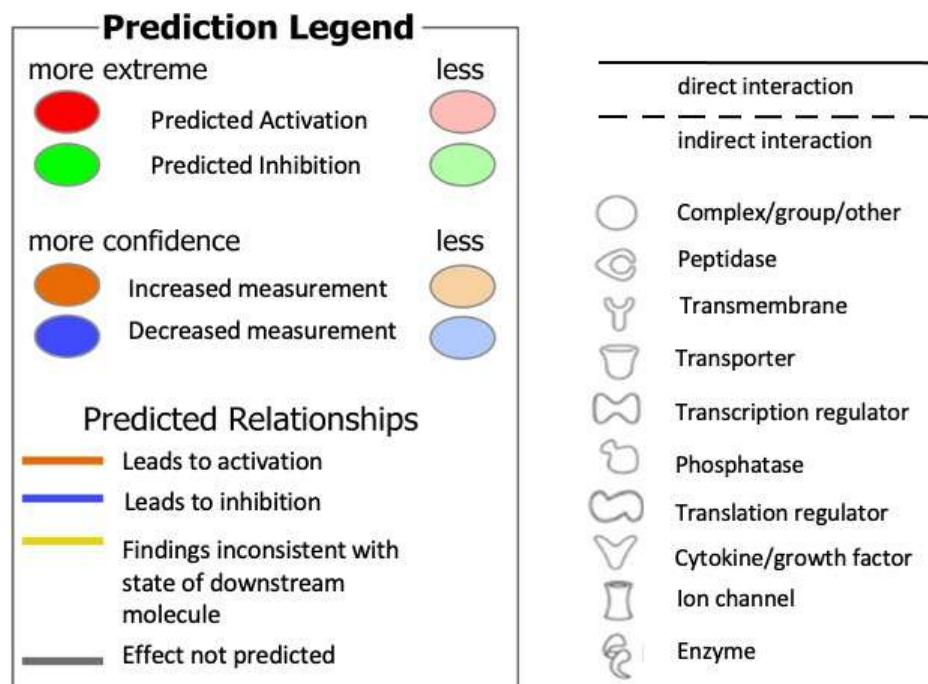
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Online Supplementary Figure S1



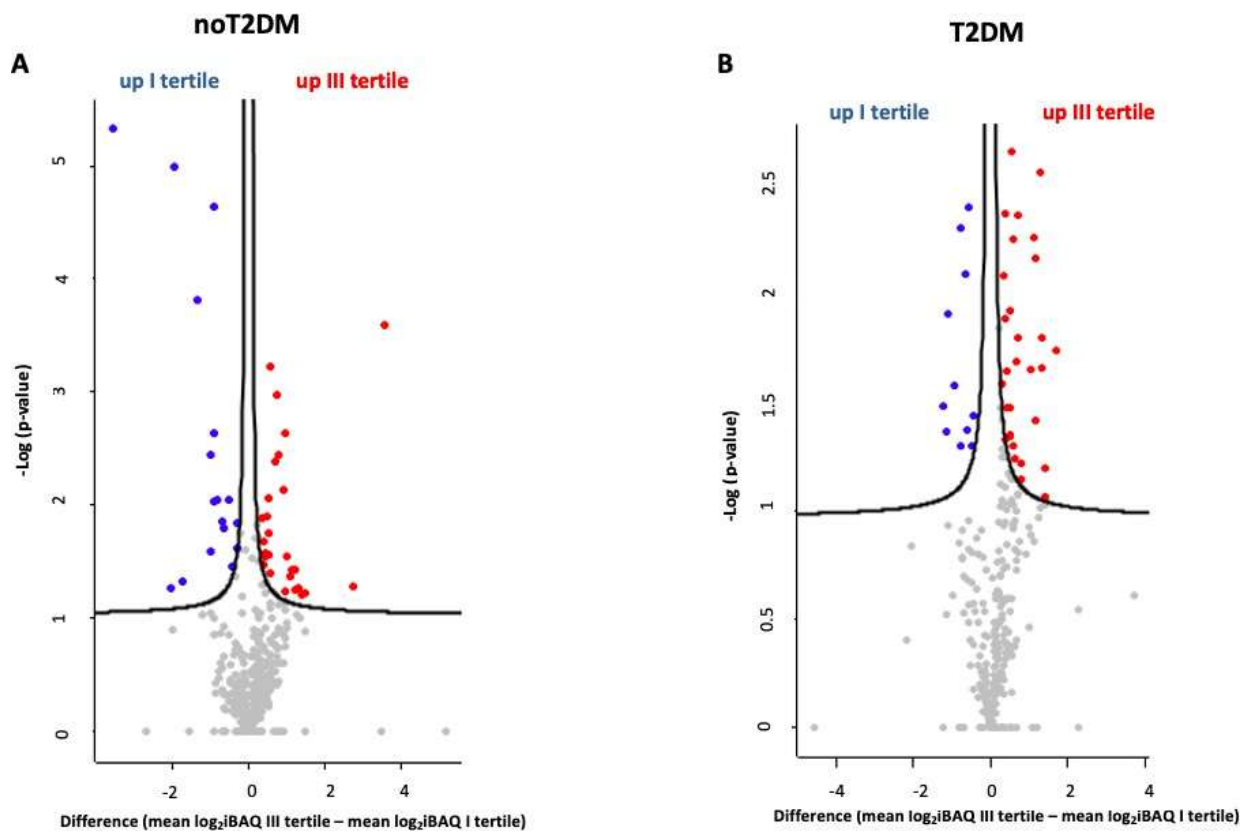
Online Supplementary Figure S1: Density Plot of protein expression in patients without T2DM (A) and with T2DM (B). Protein expression is reported as density value of iBAQ: data points with the highest density are light blue, on the contrary data points with lowest density are bright green. The color gradient is report in the legend made on comparison between third vs. first tertile both in patients without and with T2DM.

Online Supplementary Figure S2



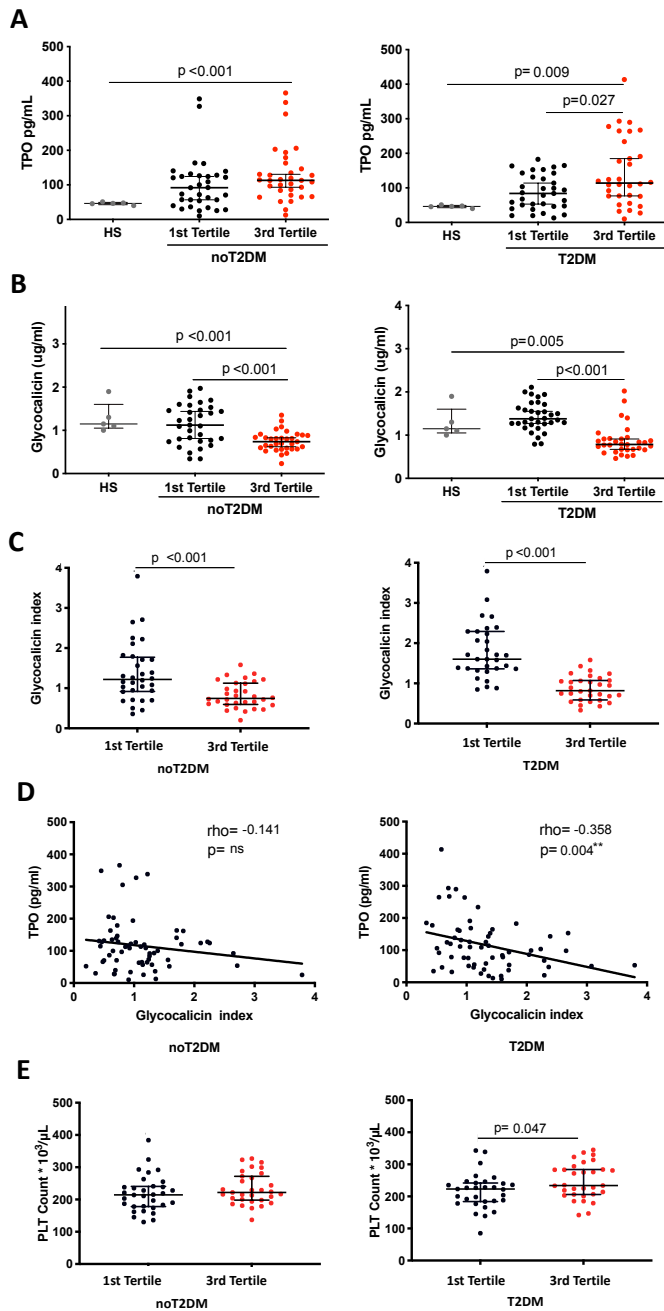
Online Supplementary Figure S2: IPA networks legend. The figure shows the color and shape key to read the IPA networks reported in the work. Red and green color: increased or decreased measurements of quantified proteins. Blue and orange shapes: predicted inhibition (z-scores ≤ -2.0) and activation (z-scores ≥ 2.0). Direct activation: orange solid lines; direct inhibition: blue solid lines. Indirect relationships: dotted lines. Yellow and grey lines: “inconsistent” or “not predicted” relationship effects.

Online Supplementary Figure S3



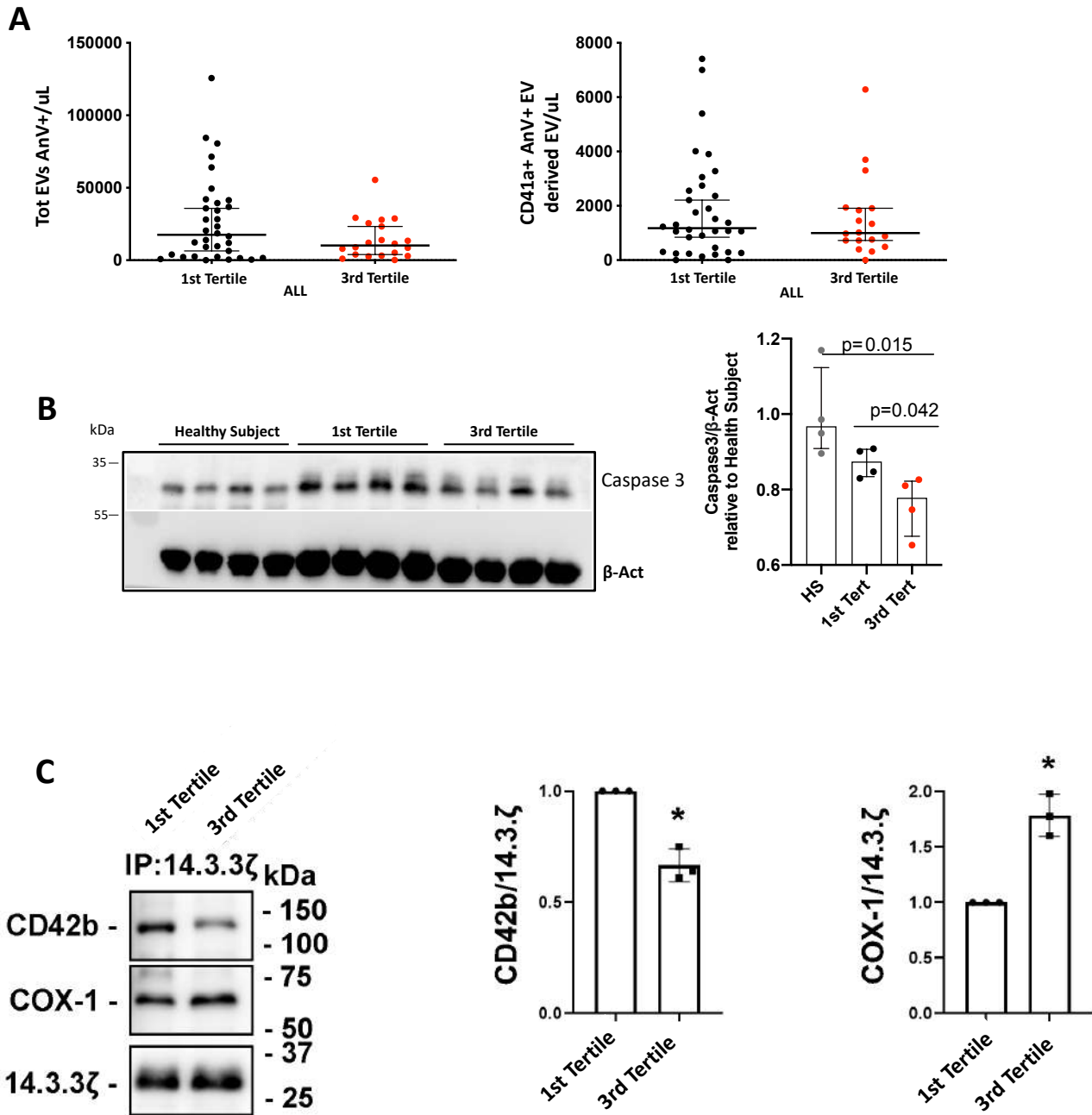
Online Supplementary Figure S3: Volcano Plots of differentially expressed proteins in the different clinical groups, in patients without and with T2DM (A and B, respectively). Proteins were graphed by fold change (Difference) and -Log (p value) using a false discovery rate (FDR) of 0.22 and an S_0 of 0.05 for both graphs. Grey dots represent proteins that are not differentially expressed; while red and blue dots represent proteins that are significantly up-regulated in the pooled platelets of third tertile and up-regulated in the pooled platelets of first tertile, respectively, in both the clinical conditions.

Online Supplementary Figure S4



Online Supplementary Figure S4: Higher circulating levels of thrombopoietin (TPO) and platelet (PLT) count and lower glycoalcin (GC) and glycoalcin index (GCI) at 24h after witnessed aspirin intake in patients with faster COX-1 recovery. Comparison of TPO (A), GC (B), GCI (C) and PLT count (E) between first and third sTXB₂ slope tertile in patients without (n=66) and with T2DM (n=66). Comparison of TPO (A) and GC (B) in third vs. first sTXB₂ slope tertile and vs. healthy subjects (HS, n=5) in patients without (n=66) and with T2DM (n=66). Significance was calculated by Mann-Whitney *U* test. Correlation between GCI and TPO in patients without and with T2DM (D). Spearman correlation coefficient and p-value are reported.

Online Supplementary Figure S5



Online Supplementary Figure S5: Inhibited apoptosis in patients of the third tertile. The number of AnnexinV-positive extracellular vesicles (Total EVs AnV+ and, CD41a+ PLT AnV+ derived) was measured in patients of first (n=35) and third tertile (n=21) (A); Expression levels of caspase 3 in healthy subjects (n=4), patients of first (n=4) and third tertile (n=4). β -Actin was used as loading control (B); Measurement in 14-3-3 ζ platelet immunoprecipitates of complexes 14-3-3 ζ -COX-1 and 14-3-3 ζ -GPIIb/IIIa in patients of first (n=3) and third tertile (n=3) (C). Significance was calculated by Mann-Whitney *U* test or by Student's *t*-test.

Online Supplementary Table S1. Characteristics of patients with and without type 2 diabetes mellitus (T2DM)

Variable	noT2DM	T2DM	P value*
N.	100	100	1.000
Age – years	69.0 (63.0-74.0)	68.0 (64.0-72.8)	0.989
Male gender, n.	57	64	0.386
Smoke, n.	10	13	0.658
Weight – Kg	77.0 (67.0-85.8)	80.0 (72.4-91.8)	0.084
BMI - Kg/m ²	27.9 (25.2-31.1)	29.9 (25.8-33.0)	0.026
Waist circumference – cm	100.0 (92.3-110.0)	104.0 (96.0- 111.0)	0.069
WHR	1.0 (0.9-1.0)	1.0 (0.9-1.0)	0.290
Obesity, n.	40	49	0.255
Systolic arterial pressure – mmHg	140.0 (128.3-153.8)	146.0 (134.3-152.0)	0.120
Diastolic arterial pressure – mmHg	77.0 (70.0-83.0)	75.0 (70.0-80.0)	0.626
Hypertension, n.	80	90	0.073
Glycated hemoglobin - mmol/mol	38.8 (35.5-42.1)	50.8 (45.4-58.5)	0.000
- %	5.7 (5.4-6.0)	6.8 (6.3-7.5)	
Diabetes duration – years	-	5.0 (2.0-10.0)	-
Total cholesterol – mmol/L	4.9 (4.1-5.5)	4.4 (3.8-5.0)	0.003
HDL cholesterol – mmol/L	1.3 (1.1-1.5)	1.3 (1.0-1.5)	0.160
Triglycerides – mmol/L	1.3 (0.9-1.7)	1.4 (1.0-1.9)	0.198
Dyslipidemia, n	54	60	0.475
AST - U/L	24.0 (21.0-28.0)	24.0 (20.0-31.0)	0.851
ALT - U/L	28.5 (24.0-33.0)	31.0 (25.0-40.8)	0.076
Total Bilirubin – umol/L	12 (10-15)	10 (9-14)	0.006
NAFLD, n.	52	76	0.000
hs-C-reactive protein – nmol/L	19.0 (9.5-38.1)	19.0 (9.5-47.6)	0.619
Red Blood Cell count, x10 ¹² /L	4.9 (4.5-5.1)	4.6 (4.4- 4.9)	0.000
Hemoglobin – g/dL	14.3 (13.5-15.3)	13.7 (12.8-14.4)	0.000
Hematocrit – %	42.6 (40.0-45.1)	38.3 (40.1-42.4)	0.000
Red cell Distribution Width –	13.2 (12.8-13.8)	12.8 (13.3- 13.9)	0.491

%			
Platelet count – '10 ⁹ /L	223.5 (189.3-266.0)	227.0 (191.0-264.3)	0.963
Platelet Distribution Width – fL	13.7 (12.3-15.0)	13.8 (12.4-15.4)	0.538
Mean Platelet Volume – fL	11.2 (10.5-11.7)	11.3 (10.6-11.9)	0.500
White Blood Cell count – '10 ⁹ /L	6.6 (5.6-7.8)	7.0 (5.8-8.3)	0.130
Fasting plasma glucose – mmol/L	5.29 (4.89-5.67)	6.75 (5.79-7.61)	0.000
Fasting plasma Insulin – pmol/L	67.4 (54.2-95.8)	72.9 (51.4-102.7)	0.784
HOMA-IR	2.4 (1.7-3.2)	3.2 (1.9-5.0)	0.038
Serum Creatinine – umol/L	70.4 (61.6-88.0)	70.4 (61.6-79.2)	0.221
eGFR – ml/min	88.0 (72.5-99.6)	88.8 (78.0-98.6)	0.222
Uric Acid – umol/L	327.1 (267.7-410.4)	333.1 (273.6-386.6)	0.990
Aspirin treatment duration - years	4.0 (2.0-8.0)	5.0 (2.0-10.0)	0.190
Cardiovascular disease, n.			
Stable CAD	6	11	0.311
Carotid stenosis (>50%), n.	30	42	0.105
MI or revascularization	11	16	0.308
Stroke, TIA or revascularization	11	9	0.814
Peripheral artery disease	1	6	0.118
Diabetic microvascular disease, n.			
Retinopathy	0	8	0.007
Nephropathy	0	2	0.497
Neuropathy	0	1	1.000
Therapy, n.			
Metformin	1	63	0.000
Glinide	0	11	0.001
PPAR- γ agonists	0	12	0.000
Sulfonylurea	0	4	0.121
Insulin	0	6	0.029

sGLT2 inhibitors	0	3	0.246
GLP-1 RA	0	2	0.497
DPP-IV	0	5	0.059
Acarbose	0	2	0.497
ACE-I	27	31	0.640
ARBs	34	41	0.381
Diuretics	28	31	0.757
β -blockers	34	32	0.881
CCA	22	26	0.602
Other antihypertensives	3	9	0.134
Statins	36	54	0.015
Fibrates	1	2	1.000
Ezetimibe	8	8	1.000
Omega 3	4	3	1.000
Proton Pump Inhibitors	45	39	0.474
ASA	100	100	1.000

Abbreviations: BMI= body mass index, WHR= Waist to Hip ratio, HDL= high-density lipoproteins, AST= Aspartate Aminotransferase, ALT= Alanine amino Transferase, NAFLD= non alcoholic fatty liver disease, HOMA-IR= Homeostatic Model Assessment of Insulin Resistance, eGFR= estimated glomerular filtration rate, CAD= coronary artery disease, CCA= Common carotid artery, MI= myocardial infarction, TIA= transient ischemic attack, PPAR-Y= peroxisome proliferator-activated receptor gamma, SGLT2= Sodium-glucose co-transporter-2, GLP1 RA= glucagon-like peptide 1 receptor agonist, DPP-IV= dipeptidyl peptidase IV, ACE-I= angiotensin-converting-enzyme -inhibitors, ARBs= angiotensin receptor blockers, CCA= calcium channel antagonists, ASA= acetylsalicylic acid.

Data are median (25th – 75th percentile). †Determined by Kruskal-Wallis or Mann-Whitney or Chi Square test, as appropriate.

Online Supplementary Table S2. Characteristics of patients with and without type 2 diabetes in relation to tertiles of sTXB₂ recovery slope

Variable	T2DM				noT2DM			
	1 st tertile	2 nd tertile	3 rd tertile	P value	1 st tertile	2 nd tertile	3 rd tertile	P value
Cardiovascular disease, n. (%)								
Carotid stenosis (>50%)	12 (36.4)	15 (44.1)	15 (45.5)	0.721	10 (30.3)	0 (0.0)	11 (33.3)	0.828
MI or revascularization	6 (18.2)	3 (8.8)	7 (21.2)	0.329	8 (24.2)	9 (26.5)	2 (6.1)	0.011
Stroke, TIA or revascularization	4 (12.1)	1 (2.9)	4 (12.1)	0.315	1 (3.0)	3 (8.8)	7 (21.2)	0.054
Peripheral artery disease	3 (9.1)	2 (5.9)	1 (3.0)	0.584	0 (0.0)	0 (0.0)	1 (3.0)	0.359
Diabetic microvascular disease, n. (%)								
Retinopathy	3 (9.1)	1 (2.9)	4 (12.1)	0.368	-	-	-	-
Nephropathy	0 (0.0)	0 (0.0)	2 (6.1)	0.126	-	-	-	-
Neuropathy	0 (0.0)	0 (0.0)	1 (3.0)	0.359	-	-	-	-
Therapy, n. (%)								
Metformin	21 (63.6)	21 (61.8)	21 (63.6)	0.983	-	-	-	-
Glinide	4 (12.1)	3 (8.8)	4 (12.1)	0.883	-	-	-	-
PPAR- γ agonists	4 (12.1)	4 (11.8)	4 (12.1)	0.999	-	-	-	-
Sulfonylurea	2 (6.1)	0 (0.0)	2 (6.1)	0.342	-	-	-	-
Insulin	1 (3.0)	2 (5.9)	3 (9.1)	0.584	-	-	-	-
sGLT2 inhibitors	0 (0.0)	1 (2.9)	2 (6.1)	0.353	-	-	-	-
GLP-1 RA	0 (0.0)	1 (2.9)	1 (3.0)	0.605	-	-	-	-
DPP-IV	2 (6.1)	1 (2.9)	2 (6.1)	0.795	-	-	-	-
Acarbose	0 (0.0)	2 (5.9)	0 (0.0)	0.138	-	-	-	-
ACE-I	8 (24.2)	9 (26.5)	14 (42.4)	0.218	6 (18.2)	12 (35.3)	9 (27.3)	0.288
ARBs	12 (36.4)	16 (47.1)	13 (39.4)	0.656	11 (33.3)	9 (26.5)	14 (42.4)	0.385
Diuretics	8 (24.2)	11 (32.4)	12 (36.4)	0.555	10 (30.3)	12 (35.3)	6 (18.2)	0.278
β -blockers	12 (36.4)	11 (32.4)	9 (27.3)	0.730	12 (36.4)	12 (35.3)	10 (30.3)	0.857
CCA	9 (27.3)	8 (23.5)	9 (27.3)	0.922	7 (21.2)	8 (23.5)	7 (21.2)	0.965
Other antihypertensives	2 (6.1)	4 (11.8)	3 (9.1)	0.717	0 (0.0)	2 (5.9)	1 (3.0)	0.369
Statins	16 (48.5)	20 (58.8)	18 (54.4)	0.695	17 (51.5)	6 (17.6)	13 (39.4)	0.014
Fibrates	0 (0.0)	2 (5.9)	0 (0.0)	0.138	0 (0.0)	0 (0.0)	1 (3.0)	0.359
Ezetimibe	4 (12.1)	2 (5.9)	2 (6.1)	0.566	6 (18.2)	1 (2.9)	1 (3.0)	0.031
Omega 3	1 (3.0)	2 (5.9)	0 (0.0)	0.373	3 (9.1)	1 (2.9)	0 (0.0)	0.157
Proton Pump Inhibitors	12 (36.4)	16 (47.1)	11 (33.3)	0.480	16 (48.5)	15 (44.1)	14 (42.4)	0.878
ASA	33 (100.0)	34 (100.0)	33 (100.0)	-	33 (100.0)	34 (100.0)	33 (100.0)	-
Aspirin treatment duration – year	5.0 (2.0-10.0)	4.0 (2.0-10.0)	5.0 (2.0-9.5)	0.579	4.0 (1.5-10.0)	3.0 (1.0-7.3)	5.0 (2.0-9.0)	0.464

Abbreviations: CAD= coronary artery disease, CCA= Common carotid artery, MI= myocardial infarction, TIA= transient ischemic attack, PPAR- γ = peroxisome proliferator-activated receptor gamma, SGLT2= Sodium-glucose co-transporter-2, GLP1 RA= glucagon-like peptide 1 receptor agonist, DPP-IV= dipeptidyl peptidase IV, ACE-I= angiotensin-converting-enzyme - inhibitors, ARBs= angiotensin receptor blockers, CCA= calcium channel antagonists, ASA= acetylsalicylic acid. Data are median (25th – 75th percentile).

†Determined by Kruskal-Wallis or χ^2 test, as appropriate.

Online Supplementary Table S3. List of flow cytometry specificities and reagents.

Detection	Fluorochrome	Vendor	Ab Clone	Catalog	Amount per Test
Lipophilic Cationic Dye (LCD)	-	BD Biosciences	-	626267 custom	0.5
Phalloidin-FITC	FITC	BD Biosciences	-	626267 custom	0.5
CD41a	PE	BD Biosciences	HIP8	555467	2,5 µl
AnnexV	PerCP-Cy5.5	BD Biosciences		561431	0.25 µl

Keys: Peridinin-Chlorophyll-protein-Cyanine 5.5 (PerCP-Cy5.5). Becton Dickison (BD) Bioscience (San Jose, CA, USA)

Online Supplementary Table S4. Significantly differential proteins at univariate statistical analysis (Volcano Plot) in pooled patients without diabetes (noT2DM). A positive value of “Difference” indicates an up-regulation in third tertile respect to first tertile, while a negative value indicates a down-regulation in the same comparison.

Protein IDs	Protein names	Gene names	-LOG(P-value)	Difference	Peptides	Score	log2 iBAQ	log2 iBAQ	log2 iBAQ	log2 iBAQ	log2 iBAQ	log2 iBAQ
							1 st tertile noT2DMC	1 st tertile noT2DMC	1 st tertile noT2DMC	3 rd tertile noT2DMC	3 rd tertile noT2DMC	3 rd tertile noT2DMC
							02	03	04	02	03	04
Myosin light chain 1/3, skeletal												
P05976	muscle isoform	MYL1	5.332177	-3.54049	1	2.6151	14.95606	15.01581	14.9036	0	11.4417	11.3943
P69905	Hemoglobin subunit alpha	HBA1	1.258716	-2.03383	10	192.99	19.16416	19.6849	19.60449	16.31771	17.19017	18.84417
P10124	Serglycin	SRGN	5.001477	-1.95195	2	24.377	14.80972	14.86767	14.88216	12.88108	0	12.92139
Q9Y6C2	EMILIN-1	EMILIN1	1.325754	-1.71139	13	25.248	12.65988	13.05371	12.66831	11.8011	9.91247	11.53416
P04275	von Willebrand factor	VWF	3.813111	-1.31896	43	89.557	13.47193	13.36782	13.50643	11.96391	12.24487	12.18053
P62328	Thymosin beta-4	TMSB4X	2.448961	-0.98798	5	21.013	17.6404	17.49748	17.97146	16.63254	16.87183	16.64104
Glycogen phosphorylase, brain												
P11216	form	PYGB	1.596722	-0.9733	10	17.113	12.34452	12.59038	12.37278	11.74239	10.9257	11.71969
Tyrosine-protein phosphatase												
P29350	non-receptor type 6	PTPN6	2.641331	-0.90101	3	25.222	11.43327	11.48553	11.40243	10.69427	10.28575	10.63816
P02042	Hemoglobin subunit delta	HBD	4.641929	-0.8987	12	31.915	15.97123	16.09809	16.00904	15.14534	15.13495	15.10198
Ras GTPase-activating-like												
Q13576	protein IQGAP2	IQGAP2	2.029018	-0.89803	4	8.8637	10.49725	10.50661	10.62388	9.438646	9.477212	10.01778
P68871	Hemoglobin subunit beta	HBB	2.040805	-0.8288	14	323.31	20.89386	20.37571	20.37103	19.69129	19.696	19.76691
P12259	Coagulation factor V	F5	1.848569	-0.67294	17	39.206	12.35113	12.71031	12.17068	11.67424	11.75951	11.77956

Sarcoplasmic/endoplasmic												
Q93084	reticulum calcium ATPase 3	ATP2A3	1.796691	-0.62606	21	145.32	14.84711	15.00325	15.33494	14.31465	14.49648	14.49598
P35579	Myosin-9	MYH9	2.044834	-0.52842	130	323.31	18.54762	18.53727	18.62724	18.15837	17.82706	18.14146
P02679	Fibrinogen gamma chain	FGG	1.457894	-0.42144	20	323.31	18.73218	18.62607	18.52187	18.0146	18.17524	18.42595
P05106	Integrin beta-3	ITGB3	1.846059	-0.30173	29	323.31	17.77462	17.72521	17.6447	17.37653	17.32869	17.53412
Q7Z406	Myosin-14	MYH14	1.613068	-0.29379	11	3.7569	12.92928	0	12.99435	12.76754	12.63411	12.60242
P02656	Apolipoprotein C-III	APOC3	1.882155	0.352379	1	14.545	12.80306	12.70016	12.64908	12.95456	13.06056	13.19431
Q71U36	Tubulin alpha-1A chain	TUBA1A	1.674371	0.378992	17	3.33	12.98865	12.87233	12.77748	13.37041	13.30834	13.09668
P06753	Tropomyosin alpha-3 chain	TPM3	1.475808	0.384619	12	59.094	17.01944	17.19806	17.42431	17.65536	17.58461	17.5557
P06733	Alpha-enolase	ENO1	1.57891	0.430053	16	228.3	16.76811	16.55817	16.38956	17.0268	16.88691	17.09228
F-actin-capping protein subunit												
P52907	alpha-1	CAPZA1	1.546087	0.458621	6	33.976	14.11772	14.45725	14.1055	14.54352	14.78948	14.72334
Glyceraldehyde-3-phosphate												
P04406	dehydrogenase	GAPDH	1.894752	0.490143	13	317.94	17.77514	17.63616	17.91992	18.33704	18.10835	18.35625
P61225	Ras-related protein Rap-2b	RAP2B	1.556436	0.523111	2	3.8337	0	11.65374	11.37488	12.03738	12.143	11.93188
P01876	Ig alpha-1 chain C region	IGHA1	1.751109	0.525279	4	12.817	13.47814	13.50569	13.88408	14.21371	14.12444	14.10558
P00491	Purine nucleoside phosphorylase	PNP	2.055927	0.539813	6	28.786	13.73904	13.78187	13.80413	14.50265	14.3246	14.11724
Actin-related protein 2/3 complex												
O15511	subunit 5	ARPC5	1.394136	0.578421	4	14.082	14.54702	15.14669	14.88741	15.53992	15.5073	15.26916
Chloride intracellular channel												
O00299	protein 1	CLIC1	3.224727	0.58839	10	79.555	16.41005	16.51297	16.32745	16.95651	17.04655	17.01258
P14625	Endoplasmic	HSP90B1	2.384741	0.719527	22	120.9	13.91083	14.27801	14.2746	14.8825	14.872	14.86752
Delta-aminolevulinic acid												
P13716	dehydratase	ALAD	2.98044	0.75905	2	6.0967	0	11.66787	11.67225	0	12.45358	12.40464

P60174	Triosephosphate isomerase	TPI1	2.451393	0.806739	11	37.299	14.78872	14.59881	15.00233	15.71893	15.57344	15.5177
	Peptidyl-prolyl cis-trans											
P23284	isomerase B	PIIB	2.130531	0.895937	8	21.934	15.38903	14.9917	15.55408	16.12155	16.16938	16.33169
Q99832	T-complex protein 1 subunit eta	CCT7	1.242115	0.948009	5	7.8265	10.90516	0	10.95768	0	11.64372	12.11514
Q15084	Protein disulfide-isomerase A6	PDIA6	2.629273	0.951978	7	41.456	14.77427	14.43169	14.6775	15.39238	15.66828	15.67874
P11021	78 kDa glucose-regulated protein	HSPA5	1.553379	0.99763	22	126.49	14.71505	15.56066	14.91961	16.35741	15.86375	15.96705
P61158	Actin-related protein 3	ACTR3	1.363059	1.08742	11	32.722	14.38283	13.67937	14.89174	15.64931	15.24481	15.32207
Q9H0U4	Ras-related protein Rab-1B	RAB1B	1.426842	1.140889	7	36.248	15.7072	14.72051	14.6625	16.46415	15.94622	16.10251
P13489	Ribonuclease inhibitor	RNH1	1.253269	1.229427	4	11.132	10.11048	9.931639	10.93921	11.48472	0	11.62835
P40227	T-complex protein 1 subunit zeta	CCT6A	1.421799	1.232392	5	8.5281	10.25136	11.46817	10.27053	11.88023	11.95143	11.85557
	Nucleosome assembly protein 1-											
P55209	like 1	NAP1L1	1.260657	1.288467	2	13.287	13.74599	13.83368	15.22596	15.58942	15.53898	15.54264
P11234	Ras-related protein Ral-B	RALB	1.206523	1.410002	4	6.1192	12.44964	12.97061	11.71643	0	13.62605	13.95174
P29401	Transketolase	TKT	1.216089	1.498017	3	4.0548	0	10.21359	10.18339	12.08348	0	11.30953
O60610	Protein diaphanous homolog 1	DIAPH1	1.277175	2.734471	13	22.023	0	8.606442	9.923476	10.96441	12.90834	12.12554
	Superoxide dismutase [Mn],											
P04179	mitochondrial	SOD2	3.592551	3.566264	4	13.7	11.7945	11.41891	0	15.04286	15.32024	15.15581

Online Supplementary Table S5. Significantly differential proteins at univariate statistical analysis (Volcano Plot) in pooled patients with diabetes (T2DM). A positive value of “Difference” indicates an up-regulation in third tertile respect to first tertile, while a negative value indicates a down-regulation in the same comparison.

Protein IDs	Protein names	Gene names	-LOG(P-value)	Difference	Peptides	Score	log2 iBAQ	log2 iBAQ	log2 iBAQ	log2 iBAQ	log2 iBAQ	log2 iBAQ
							3 rd tertile	3 rd tertile	3 rd tertile	1 st tertile	1 st tertile	1 st tertile
							T2DM 01	T2DM 02	T2DM 03	T2DM 01	T2DM 02	T2DM 03
P02042	Hemoglobin subunit delta	HBD	1.485667	-1.23763	12	56.441	14.08697	13.70271	14.92105	15.6418	15.58552	15.19629
O75915	PRA1 family protein 3	ARL6IP5	1.362992	-1.13315	2	19.28	14.20533	13.84353	12.92151	14.88832	14.65654	14.82496
P47755	F-actin-capping protein subunit alpha-2	CAPZA2	1.907236	-1.123	2	6.7226	11.48165	11.20316	10.67384	12.28326	12.042	12.40237
P01009	Alpha-1-antitrypsin	SERPINA1	1.574752	-0.94506	2	31.074	11.17636	11.09764	10.7151	11.54868	12.36506	11.91053
P40197	Platelet glycoprotein V	GP5	1.30206	-0.77677	6	35.965	12.77569	12.31967	12.54134	13.801	13.18587	12.98014
P32119	Peroxiredoxin-2	PRDX2	2.307579	-0.76107	5	31.728	13.78259	13.64959	13.40248	14.33427	14.52099	14.26261
P01877	Ig alpha-2 chain C region	IGHA2	2.090789	-0.65005	1	6.7504	10.4292	10.28019	10.62571	11.19722	10.92072	11.16729
Q71U36	Tubulin alpha-1A chain	TUBA1A	1.374359	-0.60008	16	9.8418	11.23829	10.81306	10.70977	11.7608	11.45389	11.34668
Q00325	Phosphate carrier protein, mitochondrial	SLC25A3	2.397486	-0.56923	1	7.3322	10.81137	10.69357	10.94017	11.31882	11.32193	11.51205
Q93084	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	ATP2A3	1.297035	-0.46689	14	143.33	14.22362	14.07079	13.82665	14.72542	14.49611	14.30021
P61026	Ras-related protein Rab-10	RAB10	1.439938	-0.44629	4	19.822	12.94171	12.84223	13.16101	13.50581	13.56546	13.21256
A0A087WV48	Peptidyl-prolyl cis-trans isomerase FKBP1A	FKBP1A	1.582264	0.314351	1	7.7167	13.10617	13.36318	13.38073	12.96842	12.93257	13.00605
P21926	CD9 antigen	CD9	2.086679	0.347439	2	11.463	14.79563	14.76523	14.94105	14.43111	14.57831	14.45018

P11021	78 kDa glucose-regulated protein	HSPA5	2.371004	0.385367	14	95.684	14.86104	14.86868	14.79609	14.41283	14.57837	14.3785
P11142	Heat shock cognate 71 kDa protein	HSPA8	1.890217	0.39384	17	128.76	15.40171	15.65749	15.52616	15.02484	15.19568	15.18332
P09486	SPARC	SPARC	1.325489	0.39593	4	30.946	15.64329	15.3549	15.20201	15.09959	14.99643	14.91639
P06703	Protein S100-A6	S100A6	1.478966	0.402231	1	6.0352	13.07494	0	13.29519	12.68753	12.788	12.87298
P14770	Platelet glycoprotein IX	GP9	1.647101	0.413659	4	29.441	15.80735	15.66069	15.77316	15.17094	15.53162	15.29767
P19105	Myosin regulatory light chain 12A	MYL12A	1.475887	0.425207	8	84.722	17.65425	17.72507	17.58152	17.34906	16.97455	17.36162
P61224	Ras-related protein Rap-1b	RAP1B	1.345179	0.486839	10	88.933	17.68414	17.37101	17.82637	17.34577	17.03596	17.03928
P30101	Protein disulfide-isomerase A3	PDIA3	1.477568	0.506003	6	40.336	13.37545	13.65508	13.15477	13.00692	12.78195	12.87842
P06753	Tropomyosin alpha-3 chain	TPM3	1.351964	0.510431	11	63.08	15.52506	15.46531	15.33696	15.08986	15.10906	14.59712
Q14766	Latent-transforming growth factor beta-binding protein 1	LTBP1	1.924697	0.51086	12	96.839	12.99327	12.7426	12.85722	12.48902	12.39218	12.17932
P01834	Ig kappa chain C region	IGKC	2.657308	0.559691	1	7.796	13.25441	13.36932	13.51015	12.75776	12.84015	12.85689
Q9UBW5	Bridging integrator 2	BIN2	1.300584	0.563637	8	51.553	13.58848	14.01498	13.77982	13.44061	13.33818	12.91358
P10124	Serglycin	SRGN	2.25759	0.569873	2	20.092	15.6434	15.67777	15.55597	14.98686	14.93111	15.24956
P62491	Ras-related protein Rab-11A	RAB11A	1.239257	0.610461	4	29.32	14.61896	14.54243	14.53491	14.35218	13.95647	13.55627
Q9NZN3	EH domain-containing protein 3	EHD3	1.691269	0.683522	10	73.056	14.34935	13.89945	13.85565	13.52906	13.21193	13.31288
P10809	60 kDa heat shock protein, mitochondrial	HSPD1	1.801825	0.692291	4	27.759	12.35508	11.79576	12.12069	11.51062	11.36008	11.32396
P09493	Tropomyosin alpha-1 chain	TPM1	2.366772	0.721876	7	6.4116	11.78324	11.6475	11.86213	11.14275	11.15526	10.82925
P06744	Glucose-6-phosphate isomerase	GPI	1.218757	0.781909	2	12.827	10.86751	11.75121	11.7309	10.8157	10.6359	10.55229
P78417	Glutathione S-transferase omega-	GSTO1	1.145498	0.792027	5	30.831	14.34748	14.42947	14.20358	13.89851	13.80564	12.9003

P09211	Glutathione S-transferase P	GSTP1	1.654674	1.026776	3	21.064	14.82063	14.62445	14.0612	13.72973	13.53782	13.1584
P10720	Platelet factor 4 variant	PF4V1	2.258485	1.125004	3	38.881	14.25274	14.58959	14.52326	13.02649	13.64611	13.31798
P0DP25			1.418752	1.156385	3	18.644	15.72355	15.71473	15.74405	15.32952	14.21021	14.17344
P04632	Calpain small subunit 1	CAPNS1	2.16573	1.166851	3	21.31	13.11399	12.97302	13.41389	11.9115	12.35824	11.7306
O15144	Actin-related protein 2/3 complex subunit 2	ARPC2	2.560653	1.274261	6	35.028	12.30318	12.80613	12.29809	11.07808	11.38306	11.12348
P07237	Protein disulfide-isomerase	P4HB	1.661223	1.309026	5	31.242	12.37648	12.74201	12.8865	11.75104	11.61305	10.71382
Q13561	Dynactin subunit 2	DCTN2	1.801636	1.312723	2	12.557	12.29769	11.72626	11.85981	10.84101	10.45605	0
P09972	Fructose-bisphosphate aldolase C	ALDOC	1.194812	1.396106	3	8.3192	13.33762	0	12.77604	11.36271	12.33829	11.28118
P30740	Leukocyte elastase inhibitor	SERPINB1	1.061225	1.397216	7	41.743	13.28944	13.97127	13.84931	12.00748	11.48109	13.4298
Q01813	ATP-dependent 6- phosphofructokinase, platelet type	PFKP	1.737466	1.69225	3	17.686	11.95815	11.64273	0	10.596	10.03974	9.688828

Online Supplementary Table S6. Characteristics of patients with and without type 2 diabetes analyzed for MK and proplatelet studies in relation to tertiles of sTXB₂ recovery slope.

	noT2DM 1 st tertile	noT2DM 3 rd tertile	P value	T2DM 1 st tertile	T2DM 3 rd tertile	P value
N.	4	6		4	5	
Age – years	58.0 (44.3-73.3)	66.5 (53.8-68.0)	0.521	67.0 (60.8-73.3)	68.0 (60.5-70.0)	0.902
Male gender, n. (%)	3 (75)	4 (66)	1.000	2 (50)	4 (20)	0.524
Weight – Kg	72.0 (51.0-98.3)	79.5 (68.8-107.0)	0.522	82.5 (74.3-93.8)	100.0 (84.5-101.5)	0.110
BMI - Kg/m ²	25.4 (20.3-33.4)	27.0 (25.6-33.9)	0.522	30.2 (26.8-37.0)	32.2 (30.9-34.8)	0.462
Waist circumference – cm	96.5 (79.3-116.8)	103.5 (98.0-117.3)	0.240	103.5 (90.8-121.5)	107.0 (106.5-116.0)	0.621
WHR	0.97 (0.86-1.07)	0.99 (0.96-1.01)	0.667	0.92 (0.88-0.98)	0.97 (0.95-1.02)	0.110
Obesity, n. (%)	1 (25)	2 (33)	1.000	2 (50)	5 (100)	0.167
Systolic arterial pressure – mmHg	128.5 (113.8-168.0)	155.0 (140.5-167.3)	0.201	143.0 (135.5-152.8)	148.0 (137.0-170.0)	0.461
Diastolic arterial pressure – mmHg	78.0 (63.0-90.0)	88.5 (84.3-94.0)	0.281	75.5 (70.8-79.5)	79.0 (74.5-83.5)	0.327
Hypertension, n. (%)	2 (50)	6 (100)	.133	3 (75)	4 (20)	1.000
Glycated hemoglobin - mmol/mol	39 (36-44)	39 (33-45)	0.747	53 (41-72)	56 (50-64)	0.461
Glycated hemoglobin - %	5.7 (5.4-6.2)	5.7 (5.2-6.3)	0.747	7.0 (5.9-8.7)	7.3 (6.7-8.0)	0.461
Dyslipidemia, n. (%)	2 (50)	3 (50)	1.000	4 (100)	4 (20)	1.000
AST - U/L	30.0 (21.0-42.8)	27.0 (21.3-31.8)	0.669	20.5 (16.8-25.8)	25.0 (20.0-26.0)	0.537
ALT - U/L	43.0 (30.5-54.0)	31.0 (27.0-38.3)	0.240	28.0 (19.8-37.8)	32.0 (26.5-41.5)	0.268
Total Bilirubin – umol/L	0.65 (0.50-0.88)	0.95 (0.55-1.20)	0.333	0.50 (0.43-0.88)	0.60 (0.45-0.60)	0.702
NAFLD, n. (%)	4 (100)	3 (50)	.444	3 (75)	5 (100)	.126
hs-C-reactive protein – nmol/L	0.27 (0.07-0.60)	0.23 (0.06-0.40)	0.670	0.08 (0.05-0.30)	0.23 (0.09-0.29)	0.389
Red Blood Cell count, x10 ¹² /L	4.8 (4.5-5.0)	5.4 (4.7-5.5)	0.219	4.6 (4.5-4.7)	4.7 (4.3-4.9)	0.624
Hemoglobin – g/dL	14 (14-15)	15 (14-16)	0.806	13 (13-14)	14 (13-15)	0.142
Hematocrit – %	43 (42-45)	45 (42-48)	0.221	39 (38-41)	41 (40-42)	0.142
Red cell Distribution Width – %	13.7 (12.5-14.3)	14.3 (12.9-14.8)	0.387	14.1 (13.7-14.4)	13.2 (13.2-13.6)	0.026
Platelet count – 10 ⁹ /L	201 (138-348)	224 (213-229)	1.000	230 (169-241)	228 (194-259)	1.000
Platelet Distribution Width – fL	13.6 (10.8-16.8)	14.7 (13.5-15.0)	0.461	12.5 (11.6-15.2)	15.3 (12.9-16.1)	0.221
Mean Platelet Volume – fL	11.6 (9.7-12.7)	11.7 (11.1-11.8)	0.806	10.7 (10.1-11.9)	11.7 (10.9-16.6)	0.176
White Blood Cell count – 10 ⁹ /L	6.44 (5.08-8.81)	7.02 (5.27-9.58)	0.624	6.36 (4.68-9.26)	6.22 (5.52-6.76)	0.806
Fasting plasma glucose – mmol/L	86.5 (77.8-100.5)	90.5 (86.8-104.3)	0.454	130.0 (96.0-150.5)	130.0 (110.5-147.5)	0.806
Fasting plasma Insulin – pmol/L	10.2 (7.27-10.37)	10.2 (7.67-20.57)	0.67	6.4 (4.5-12.57)	14.2 (11.8-23.85)	0.086
HOMA-IR	2.21 (1.4-3.33)	2.45 (1.69-4.97)	0.67	2.32 (1.43-2.99)	4.8 (3.81-7.1)	0.014
Serum Creatinine – umol/L	61.6 (48.4-88.0)	74.8 (66.0-96.8)	0.190	83.6 (52.8-83.6)	79.2 (55.4-83.6)	0.537
eGFR – ml/min	109.5 (73.5-126.1)	90.8 (74.4-102.0)	0.394	91.0 (79.8-97.8)	87.0 (82.4-104.0)	0.806

Uric Acid – umol/L	285.5 (279.6-297.4)	333.1 (237.9-535.3)	0.453	321.1 (243.9-362.8)	327.1 (303.3-440.1)	0.624
Cardiovascular disease, n. (%)	2 (50)	2 (33)	1.000	2 (50)	4 (20)	0.524
Stable CAD	0 (0)	0 (0)		1 (25)	1 (20)	1.000
Carotid stenosis (>50%)	0 (0)	2 (33)	0.467	0 (0)	2 (40)	0.444
MI or revascularization	1 (25)	0 (0)	0.400	1 (25)	1 (20)	1.000
Stroke, TIA or revascularization	1 (25)	1 (16)	1.000	1 (25)	1 (20)	1.000
Peripheral artery disease	0 (0)	0 (0)		1 (25)	0 (0)	0.444
Therapy, n. (%)						
Metformin	0 (0)	0 (0)	-	4 (100)	3 (60)	0.444
Glinide	0 (0)	0 (0)	-	1 (25)	1 (20)	1.000
PPAR- γ agonists	0 (0)	0 (0)	-	1 (25)	1 (20)	1.000
Sulfonylurea	0 (0)	0 (0)	-	0 (0)	0 (0)	-
Insulin	0 (0)	0 (0)	-	0 (0)	0 (0)	-
sGLT2 inhibitors	0 (0)	0 (0)	-	0 (0)	1 (20)	1.000
GLP-1 RA	0 (0)	0 (0)	-	0 (0)	0 (0)	-
DPP-IV	0 (0)	0 (0)	-	1 (25)	0 (0)	0.444
Acarbose	0 (0)	0 (0)	-	0 (0)	0 (0)	-
ACE-I	1 (25)	1 (16)	1.000	0 (0)	1 (20)	-
ARBs	1 (25)	2 (33)	1.000	1 (25)	2 (40)	1.000
Diuretics	0 (0)	1 (16)	1.000	0 (0)	2 (40)	0.444
β -blockers	1 (25)	5	0.190	2 (50)	2 (40)	1.000
CCA	1 (25)	1 (16)	1.000	2 (50)	1 (20)	0.524
Other antihypertensives						-
Statins	2 (50)	3 (50)	1.000	3 (75)	1 (20)	0.206
Fibrates	0 (0)	0 (0)	-	0 (0)	0 (0)	-
Ezetimibe	0 (0)	0 (0)	-	0 (0)	0 (0)	-
Omega 3	0 (0)	0 (0)	-	1 (25)	0 (0)	0.444
Proton Pump Inhibitors	3 (75)	3 (50)	0.571	1 (25)	1 (20)	1.000
ASA	4 (100)	6 (100)	1.000	4 (100)	5 (100)	-
sTXB ₂ T0	3.20 (1.17-5.56)	12.74 (8.31-18.06)	0.055	1.42 (1.05-2.50)	4.88 (3.76-12.77)	0.014
sTXB ₂ T10	1.57 (0.35-2.47)	3.53 (1.56-5.16)	0.088	0.88 (0.47-1.52)	1.99 (1.93-5.48)	0.014
sTXB ₂ T24	2.71 (0.95-3.49)	7.72 (5.37-11.46)	0.011	1.51 (1.26-2.58)	5.83 (5.12-13.91)	0.014
Slope_sTXB ₂ ngmL ⁻¹ h ⁻¹	0.07 (0.03-0.09)	0.270.25-0.51)	0.011	0.05 (0.04-0.08)	0.28 (0.22-0.60)	0.014

Reference range of “normal values”: AST, 5-34 U/L; ALT, 0-55 U/L; hs-C-reactive protein, 0-47.62 nmol/L; Platelet count, 150-450 x10⁹/L; Total cholesterol, 0.0-5.17 mmol/L; HDL cholesterol, 1.03-1.55 mmol/L; Triglycerides, 0-1.69 mmol/L; Fasting plasma glucose, 4.61-6.11 mmol/L; Serum Creatinine, 50.2-97.7 umol/L; Fasting plasma glucose, 3.9-5.5 mmol/L; eGFR, >60 mL/min.

Abbreviations: BMI= body mass index, WHR= Waist to Hip ratio, HDL= high-density lipoproteins, AST= Aspartate Aminotransferase, ALT= Alanine amino Transferase, NAFLD= non alcoholic fatty liver disease, HOMA-IR= Homeostatic Model Assessment of Insulin Resistance, eGFR= estimated glomerular filtration rate, CAD= coronary artery disease, CCA= Common carotid artery, MI= myocardial infarction, TIA= transient ischemic attack, PPAR- γ = peroxisome proliferator-activated receptor gamma, SGLT2= Sodium-glucose co-transporter-2, GLP1 RA= glucagon-like peptide 1 receptor agonist, DPP-IV= dipeptidyl peptidase IV, ACE-I= angiotensin-converting-enzyme -inhibitors, ARBs= angiotensin receptor blockers, CCA= calcium channel antagonists, ASA= acetylsalicylic acid.

Data are median (25th – 75th percentile). †Determined by Kruskal-Wallis or Mann-Whitney or Chi Square test, as appropriate.

Online Supplementary Table S7. Correlations between sTXB₂ recovery slope and clinical variables in patients with and without type 2 diabetes mellitus.

T2DM				
	Age	BMI	WC	eGFR
sTXB₂ slope	rho=-0.215 p=0.030	rho=0.217 p=0.027	rho=0.229 p=0.020	rho=0.256 p=0.009
noT2DM				
	Weight	WHR	WC	DAP
sTXB₂ slope	rho=0.217 p=0.029	rho=0.267 p=0.007	rho=0.211 p=0.034	rho=0.227 p=0.022

Abbreviations: BMI=body mass index; WC=waist circumference; eGFR=estimated glomerular filtration rate; WHR=waist-to-hip ratio; DAP=diastolic arterial pressure.

Spearman correlation coefficient and p-value are reported.