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

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Abstract

Cardiovascular (CV) disease prevention with low-dose aspirin can be less effective in patients with a faster recovery of platelet (PLT) cyclooxygenase (COX)-1 activity during the 24-hour dosing interval. We previously showed that incomplete suppression of TXA_2 over 24 hours can be rescued by a twice daily aspirin regimen. Here we show that reduced PLT glycoprotein (GP)Ibα shedding characterizes patients with accelerated COX-1 recovery and may contribute to higher thrombopoietin (TPO) production and higher rates of newly formed PLT, escaping aspirin inhibition over 24 hours. Two hundred aspirin-treated patients with high CV risk (100 with type 2 diabetes mellitus) were stratified according to the kinetics of PLT COX-1 activity recovery during the 10- to 24-hour dosing interval. Whole proteome analysis showed that PLT 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 TPO, megakaryocyte maturation and proplatelet formation, and conversely increased PLT galactose and reduced caspase 3, phosphatidylserine exposure and ADAM17 activation, translating into diminished GPIbα cleavage and glycocalicin (GC) release. Treatment of HepG2 cells with recombinant GC led to a dose-dependent reduction of *TPO* mRNA in the liver, suggesting that reduced GPIbα ectodomain shedding may unleash thrombopoiesis. A cluster of clinical markers, including younger age, non-alcoholic fatty liver disease, visceral obesity and higher TPO/GC ratio, predicted with significant accuracy the likelihood of faster COX-1 recovery and suboptimal aspirin response. Circulating TPO/GC ratio, reflecting a dysregulation of PLT 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 (PLT) activation, as reflected by 11-dehydro-TXB_2 urinary excretion, has been associated with several conditions, including cardio-cerebrovascular diseases and cardiovascular (CV) risk factors,

such as diabetes mellitus.[2,3]

The anti-PLT and cardioprotective properties of low-dose (81-100 mg daily) aspirin rely on its capacity to irreversibly inactivate cyclooxygenase (COX)-1 in anucleate PLT and TXA_2 biosynthesis over 24 hours.[3] Over the last decades, the concept of suboptimal response to aspirin, firstly inadequately referred to as aspirin resistance, has been affirmed, based on the evidence of lower-than-expected inhibition of PLT 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 TXB_2 ($sTXB_2$).[4–6]

The variable turnover rate of the aspirin target, PLT COX-1, is the most convincing determinant of the interindividual variability in the aspirin response. Under several pathological conditions, such as essential thrombocythemia, polycythemia vera, on-pump coronary artery by-pass surgery, as well as in patients at high CV risk, with or without type 2 diabetes mellitus (T2DM),[7–10] a proportion of PLT with uninhibited COX-1 may determine faster recovery of COX-1 activity and TXA_2 generation and limit the extent and duration of aspirin effect during the 12- to 24-hour dosing interval[7,8] leading to substantial PLT activation and recovery of PLT function. As full suppression of TXA_2-dependent PLT function requires >97% inhibition of COX-1 activity,[6] even a modest recovery of this activity can sustain a substantial PLT activation responsible for the excess CV events in aspirin-treated subjects.

Shorter duration of TXA_2 suppression during the 24-hour dosing interval can be rescued by a twice daily low-dose aspirin regimen,[7,9,10] leading to persistently inhibited $sTXB_2$ over 24 hours. An accelerated PLT 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 the effect of aspirin. Patients' classification based on clinical setting, including diabetes or acute coronary syndrome, failed to accurately identify patients escaping low-dose aspirin inhibition 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 to identify novel biomarkers of suboptimal drug response.

Thrombopoietin (TPO) is the primary regulator of PLT production from megakaryocytes (Mk)[11] and is produced by the liver due to a number of stimuli including inflammation.[11] Conversely, PLT lifespan, glycan degradation and apoptosis mediate PLT clearance.[11] Senescent PLT are cleared upon exposure of galactose to hepatic Ashwell-Morrell receptors (AMR), in turn stimulating TPO production.[11,13] Glycoprotein Iα (GPIbα) shedding by metalloproteinases such as a disintegrin and metalloprotease (ADAM) 17 is another mechanism of PLT clearance,[12] and plasma glycocalicin (GC), an extra cellular domain of GPIbα, released during PLT clearance, is an index of destruction and PLT turnover.[14] PLT extracellular GPIbα domain is required for PLT-mediated TPO generation, as underscored in GPIb$\alpha^{-/-}$ 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 PLT proteome, platelet turnover, as reflected by TPO and GC circulating levels, galactose exposure and GPIbα ectodomain shedding, Mk maturation and proplatelet formation (PPF) in patients at high CV risk with or without T2DM stratified according to the kinetics of COX-1 recovery. We show that reduced platelet GPIbα shedding characterizes patients with accelerated COX-1 recovery and may contribute to higher TPO production and higher rates of newly formed PLT, 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 100 mg aspirin (enteric-coated, Cardioaspirin, the anti-PLT 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 in Italy. Five healthy subjects were enrolled as control. All patients underwent a liver ultrasound (see the *Online Supplementary Appendix*). The study was preceded by a 7-day run-in during which patients were instructed to take aspirin at 8 am. 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 $sTXB_2$ levels throughout the 24-hour dosing interval between two witnessed aspirin administrations (Figure 1A). $sTXB_2$ is indeed a marker of COX-1 activity *ex vivo*.[17] We previously reported that the absolute increase in $sTXB_2$ levels between 12 and 24 hours after dosing predicts ~90% slope variability, as assessed by measuring $sTXB_2$ every 3 hours in the 12- to 24-hour dosing interval.[10]

Based on the $sTXB_2$ slope ($sTXB_2$ T24 – $sTXB_2$ T10)/14, we

stratified patients in tertiles (n=~33 each) and carried out a cross-sectional comparison of PLT-related study variables between first (good aspirin responders) and third tertile (poor responders) within patients with and without T2DM.

Platelet isolation

PLT rich plasma (PRP) was separated (centrifugation 100xg, 15 minutes) from ACD-A anticoagulated blood, mixed with prostaglandin E_1 (PGE_1, 4 uM) and EDTA (10 mM), and filtered on Pall Purecell PL (Pall Medical, New York, USA) to remove leukocyte contaminants. Filtered PRP was analyzed by flow cytometery to exclude red blood cell ($CD235^+$) contamination and platelet activation ($CD62P^+$). PLT 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 parameters with ≥1 standard deviation between the first and the third $sTXB_2$ slope tertiles with α=0.01 and power=90%. All the statistical analyses were 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 *versus* the first tertile for the $sTXB_2$ recovery slope. A parsimonious backward-stepwise elimination of variables with P<0.20 was deemed 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 the *Online Supplementary Appendix* (pages 2-8) for more details on study participants and an extended description of experimental procedures.

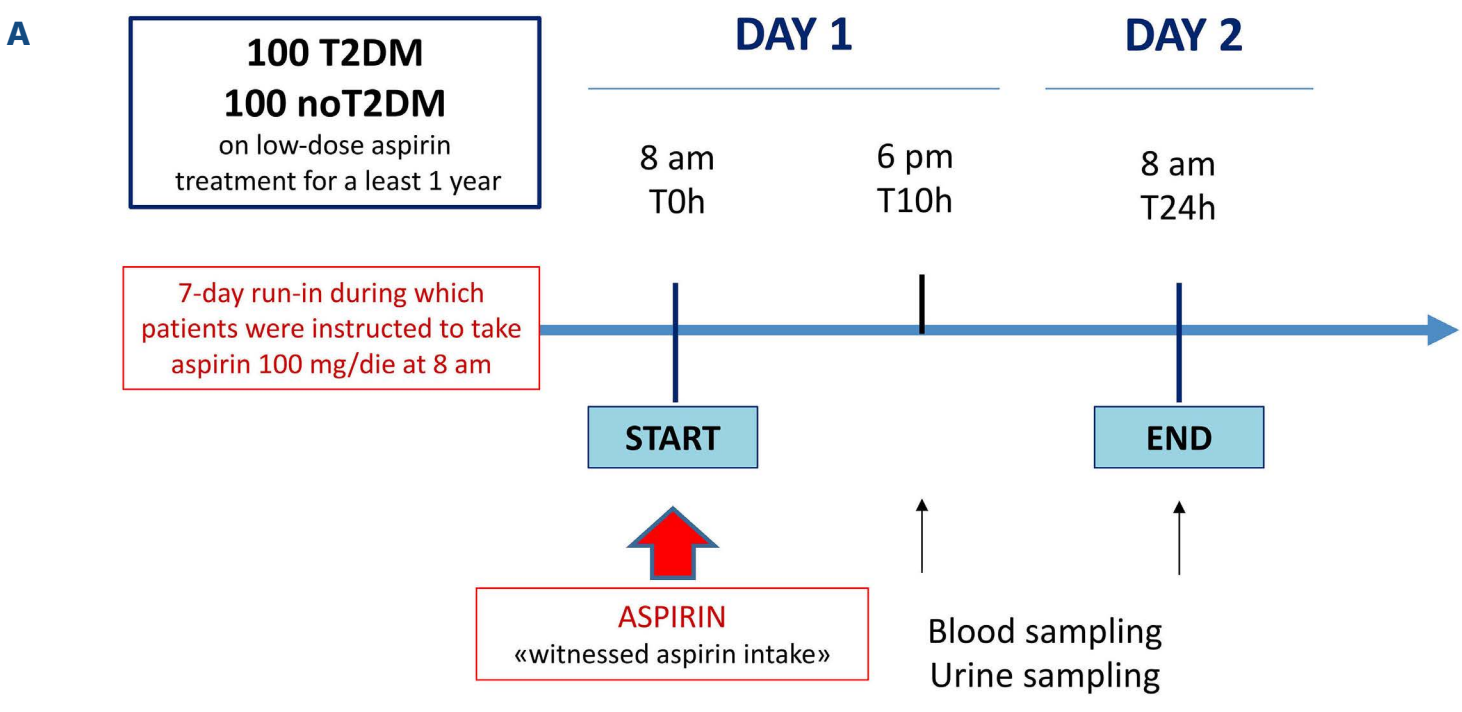
Results

Clinical characteristics

Clinical characteristics of patients are listed in the *Online Supplementary Table S1* and detailed in the *Online Supplementary Appendix*. Patients' characteristics according to $sTXB_2$ recovery slope tertiles are reported in Table 1 and detailed in the *Online Supplementary Table S2* and the *Online Supplementary Appendix*.

COX-1 activity recovery

A fraction of patients showed faster recovery of COX-1 activity over the 10– to 24-hour aspirin dosing interval, higher *COX-1* mRNA expression and TX-dependent platelet activation. In the first tertile of the recovery slope, $sTXB_2$ was steadily suppressed over the 10– to 24-hour dosing interval (Figure 1B, C), as observed in healthy subjects,[6] while patients in the third tertile (slope >=0.17 $ngmL^{-1}h^{-1}$ and slope >=0.18 $ngmL^{-1}h^{-1}$ in patients with and without diabetes, respectively) showed a significantly faster recovery of COX-1 activity, as reflected by the $sTXB_2$ increase between 10 and 24 hours post-aspirin (Figure 1B, 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"



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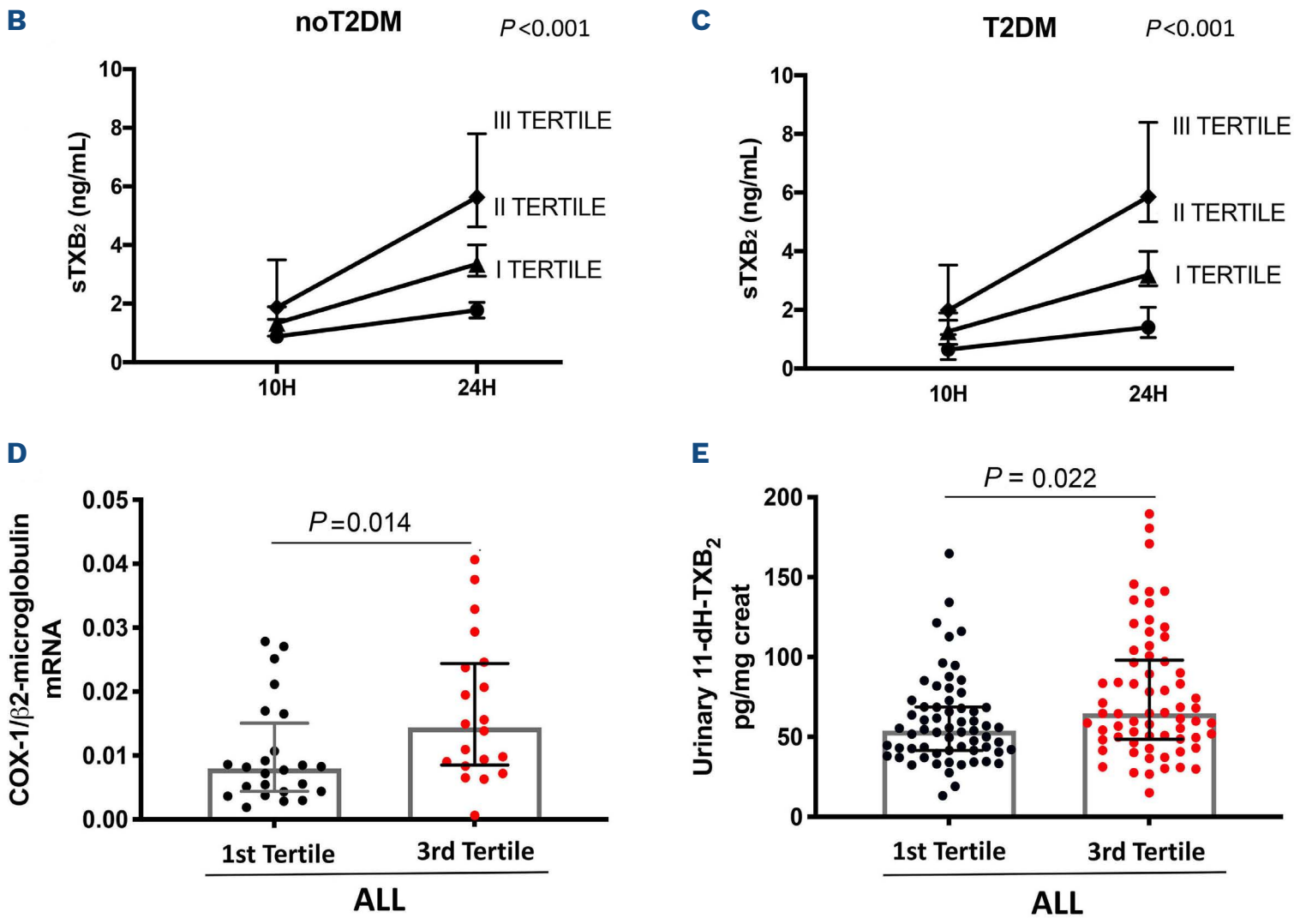


Figure 1. Subjects with faster kinetics of recovery of serum thromboxane B_2 during the 24-hour aspirin dosing interval, display increased platelet COX-1 expression and urinary 11-dehydro-thromboxane B_2 excretion as compared to those with normal serum thromboxane B_2 recovery. (A) Study design. Linear fitting of serum thromboxane B_2 ($sTXB_2$) measured 10 and 24 hours post-aspirin intake in patients without (B) (n=100), and with type 2 diabetes mellitus (T2DM) (C) (n=100) stratified in tertiles according to $sTXB_2$ (*ex vivo* index of COX-1-dependent TXA_2 production) slope (n=33/tertile). Patients in the third $sTXB_2$ slope tertile display significantly faster recovery of $sTXB_2$ *vs.* first tertile (P<0.001), during the 24 hours between 2 witnessed aspirin administrations. $sTXB_2$ values are as median and interquartile range. Comparison of subjects in the third *vs.* first $sTXB_2$ slope tertile for platelet transcript levels of *COX*-1 mRNA (D) (n=20 *vs.* n=24) and urinary 11-dehydro-TXB_2 (E) (n=66 *vs.* n=66). Significance was calculated by Mann-Whitney U test.

phenotype is not specific for diabetes. In addition, the TXB_2 recovery slope was not related to the duration of the aspirin treatment (*Online Supplementary Table S2*).

PLT *COX-1* mRNA was significantly higher in the third *versus* the first tertile (P=0.014; Figure 1D), indicating a faster renewal of the drug target in the patients with suboptimal response to aspirin. In order to assess whether an accelerated recovery of $sTXB_2$ *ex vivo* translated into enhanced *in vivo* TX-dependent PLT activation, we measured urinary 11-dehydro-TXB_2 levels, that were higher in patients of the third *versus* the first tertile in the whole group (P=0.022; Figure 1E) and in T2DM (P=0.049; *data not shown*).

Characterization of the proteomic platelet profile

PLT proteome indicated increased cell survival and inhibition of apoptosis in patients with faster COX-1 activity recovery. We characterized the functional proteomic profile of PLT from the third *versus* the first tertile within patients with or without T2DM, using pooled samples as described in the method section. The quantified proteins identified are reported in the *Online Supplementary Tables S4, S5*. In order to ensure the quality of our proteomics dataset we compared it to the reference PLT proteomic repository, obtaining more than 95% correlation (*data not shown*). Volcano plots show proteins differentially identified between the first *versus* the third tertile patients without and with T2DM (*Online Supplementary Figure S3A, B*).

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 the first tertile (Figure 2A, B). We validated the proteomic data by WB analysis and

Table 1. Characteristics of patients with and without type 2 diabetes mellitus in relation to tertiles of serum thromboxane B_2 recovery slope.

Variable	T2DM				noT2DM			
	1st tertile	2nd tertile	3rd tertile	*P* value	1st tertile	2nd tertile	3rd tertile	*P* value
Age in 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 sex, N (%)	20 (60.6)	22 (64.7)	22 (66.7)	0.872	17 (51.5)	19 (55.9)	21 (63.6)	0.602
Smokers, 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 in 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
HbA1c, %	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 x10^9/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. BMI: body mass index; WC: waist circumference; WHR: waist-to-hip ratio; SAP: systolic arterial pressure; DAP: diastolic arterial pressure; NAFLD: non-alcoholic 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 x^2 test, as appropriate.

confirmed a significant upregulation 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 the 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. In order to further explore the potential hypothesis of the formation of protrusions in PLT, we used the 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 PLT from third tertile subjects in both clinical conditions analyzed as shown by subnetworks in Figure 2E, with a significant upregulation of Rab27B, a protein actively involved in platelet biogenesis and proplatelet formation,[18] in PLT of the third *versus* the first tertile (*P*=0.038; Figure 2F) and *versus* healthy subjects (*P*<0.001; Figure 2F).

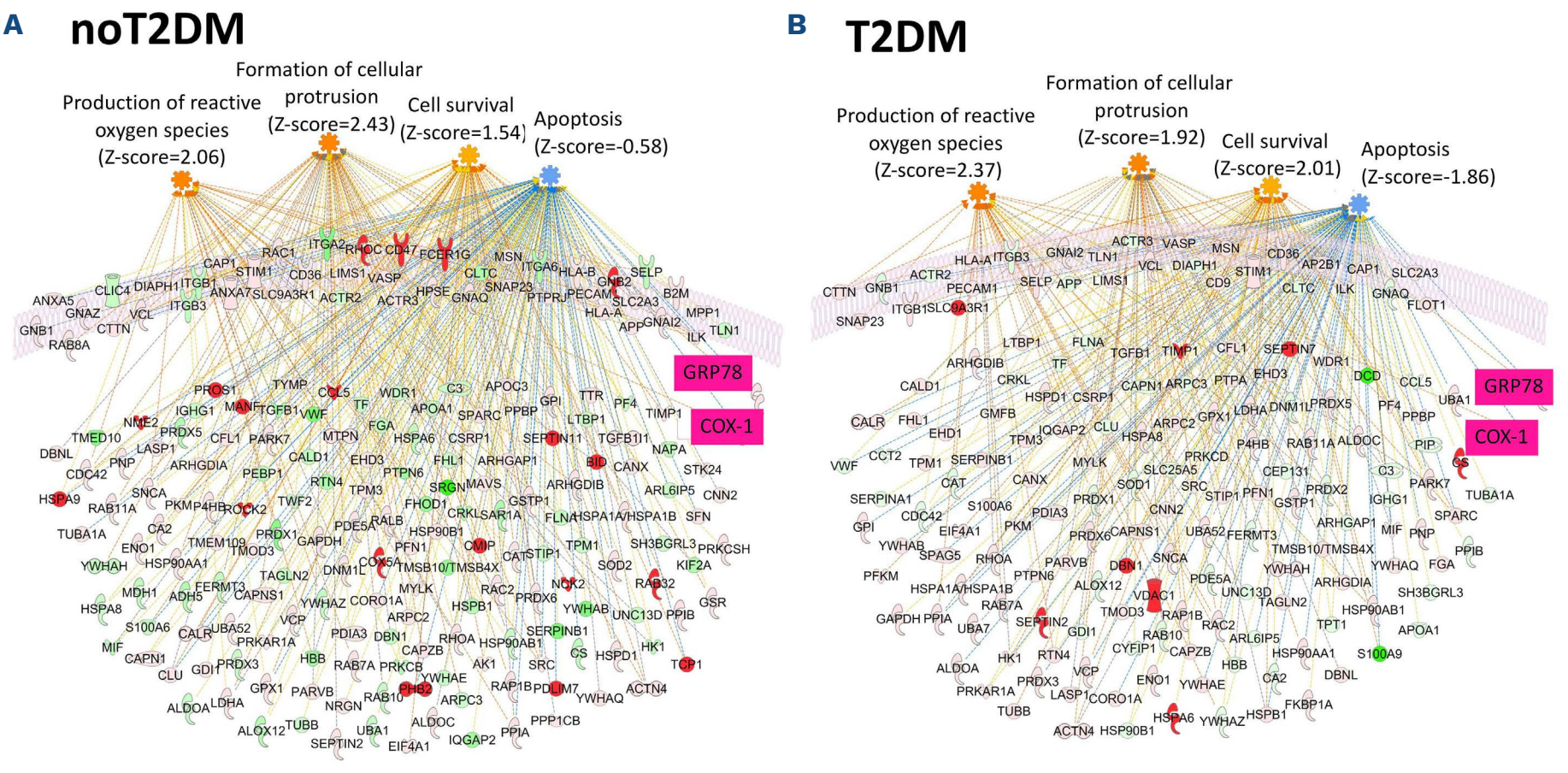
Megakaryocyte maturation and proplatelet formation in patients with faster COX-1 activity recovery

Based on the proteomic findings, we investigated whether the different recovery time of COX-1 activity reflects differences in megakaryopoiesis and proplatelet production by differentiating Mk *in vitro* from the hematopoietic progenitors derived from 18 peripheral blood samples of patients with and without T2DM belonging to the first and third $sTXB_2$ tertile and from four healthy subjects (Figure 3A, B). The clinical characteristics of the patients' groups analyzed were comparable (*Online Supplementary Table S6*).

Mk differentiated from third tertile patients appeared more mature with a significantly higher staining of CD41 and CD42b (GPIbα) Mk[19] compared to first tertile and healthy subjects (Figure 3C, D), while the percentages of CD41+ and GPIbα+ Mk on the total cultured cells were comparable (Figure 3C, D). Mk from third tertile patients extended more proplatelets compared to the first tertile or healthy subjects (Figure 3E). These data demonstrate that terminal Mk maturation and proplatelet formation were increased in patients presenting a faster recovery of platelet COX-1 activity.

Association between COX-1 activity recovery and platelet production and destruction

We next evaluated the balance between PLT production and destruction by measuring TPO, GC and GC index (GCI), an index normalizing GC for PLT count (GC concentration µg/mLx250x10^9 PLT/L/individual platelet count x10^9/L), 24 hours after witnessed aspirin intake. Consistent with the proteomic findings, plasma TPO levels were higher (*P*=0.004, *P*=0.023; Figure 4A; *Online Supplementary Figure 4SA*), while plasma GC (*P*<0.001 both; Figure 4B; *Online Supplementary Figure 4SB*) and GCI (*P*<0.001 both; Figure 4D; *Online Supplementary Figure 4SC*) were lower in patients of the third *versus* the 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:



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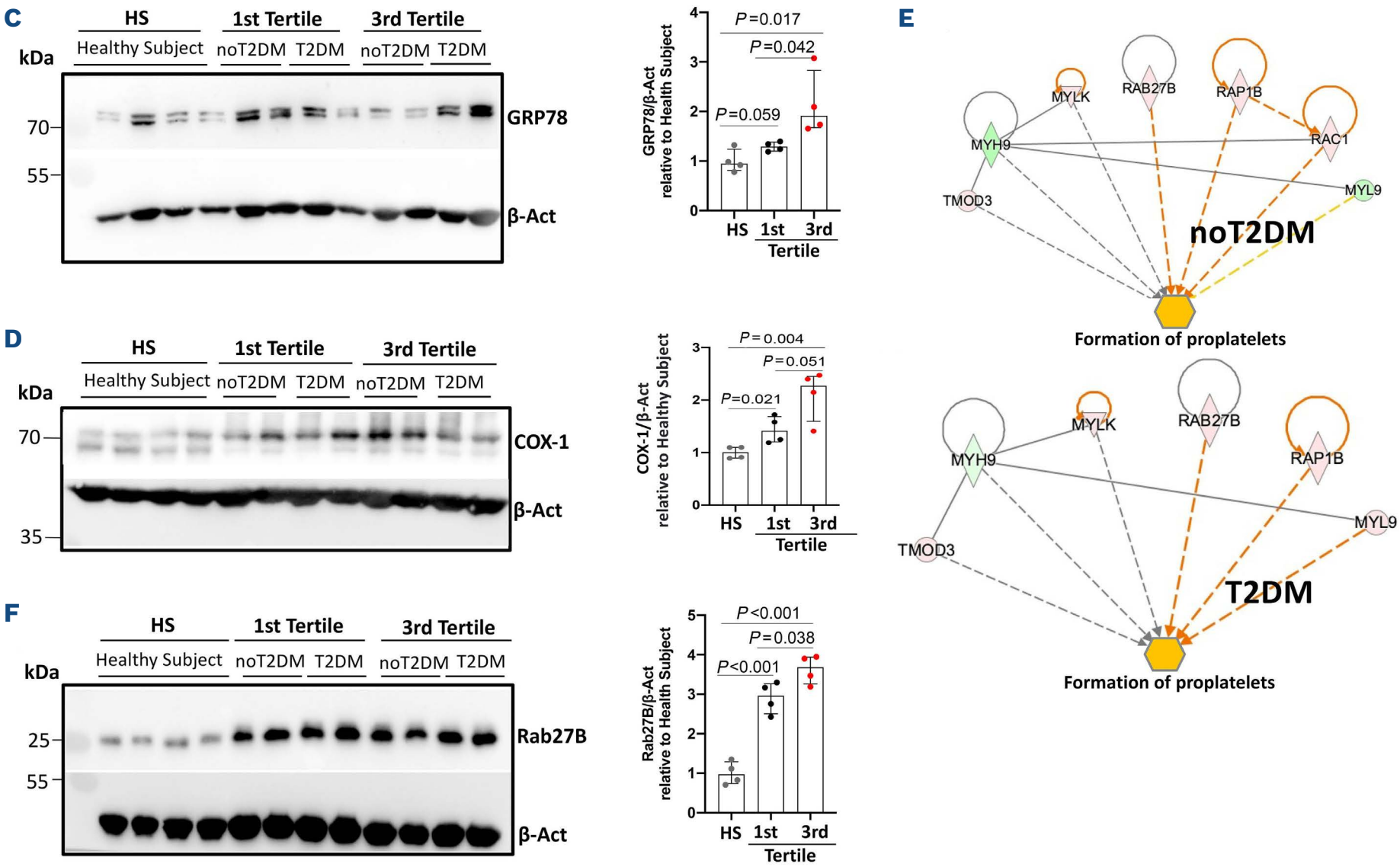


Figure 2. Proteomic analysis shows activation of pathways "formation of cellular protrusions", "formation of proplatelets", "cell survival", "production of reactive oxygen species", and inhibition of "apoptosis" in platelets from patients with faster COX-1 recovery. Proteomic analysis using ingenuity 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 serum thromboxane B_2 ($sTXB_2$) tertile in patients without (A, E), and with type 2 diabetes mellitus (T2DM) (B, E). Further details are reported in the *Online Supplementary Figures 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.

rho=-0.358, *P*=0.004; Figure 4E; *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 *versus* the first $sTXB_2$ tertile, with a non-significant trend for increased TPO (*Online Supplementary Figure 4SA*). Levels of TPO were lower (*P*=0.001, *P*<0.001, *P*=0.009; Figure 4A; *Online Supplementary Figure 4SA*) and levels of GC were higher (*P*<0.001, *P*<0.001, *P*=0.005; Figure 4B; *Online Supplementary Figure 4SB)* in healthy subjects *versus* third tertile patients, in the whole group and in subjects without and with T2DM.

Consistently, the platelet count was higher in the third *versus* first tertile in all patients (*P*=0.024; Figure 4C), and in T2DM patients (*P*=0.047; *Online Supplementary Figure 4SE*). In the whole group, the TXB_2 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 indicate that patients in the third TXB_2 slope tertile presented increased platelet production and reduced destruction.

Mechanisms underlying low circulating glycocalicin levels

In order to determine the mechanisms underlying lower GC in the third $sTXB_2$ slope tertile patients, we measured GPIbα N-terminal fragment and ADAM17 expression in platelets.[20] In all patients, we observed enhanced levels of GPIbα (*P*=0.016, *P*<0.001; Figure 5A) and lower levels of ADAM17[21] (*P*=0.015, *P*=ns; Figure 5B) in the third *versus* first tertile and *versus* healthy subjects, respectively, which may explain the lower levels of circulating GC in the third slope tertile (Figure 4B; *Online Supplementary Figure 4SB*). In order to further understand the mechanisms underlying reduced GPIbα shedding, we analyzed the percentage of Annexin V^+ platelets exposing phosphatidylserine (PS), since PS exposure is a signal for clearance of apoptotic platelets[22] and is required for ADAM17 activation.[20] The

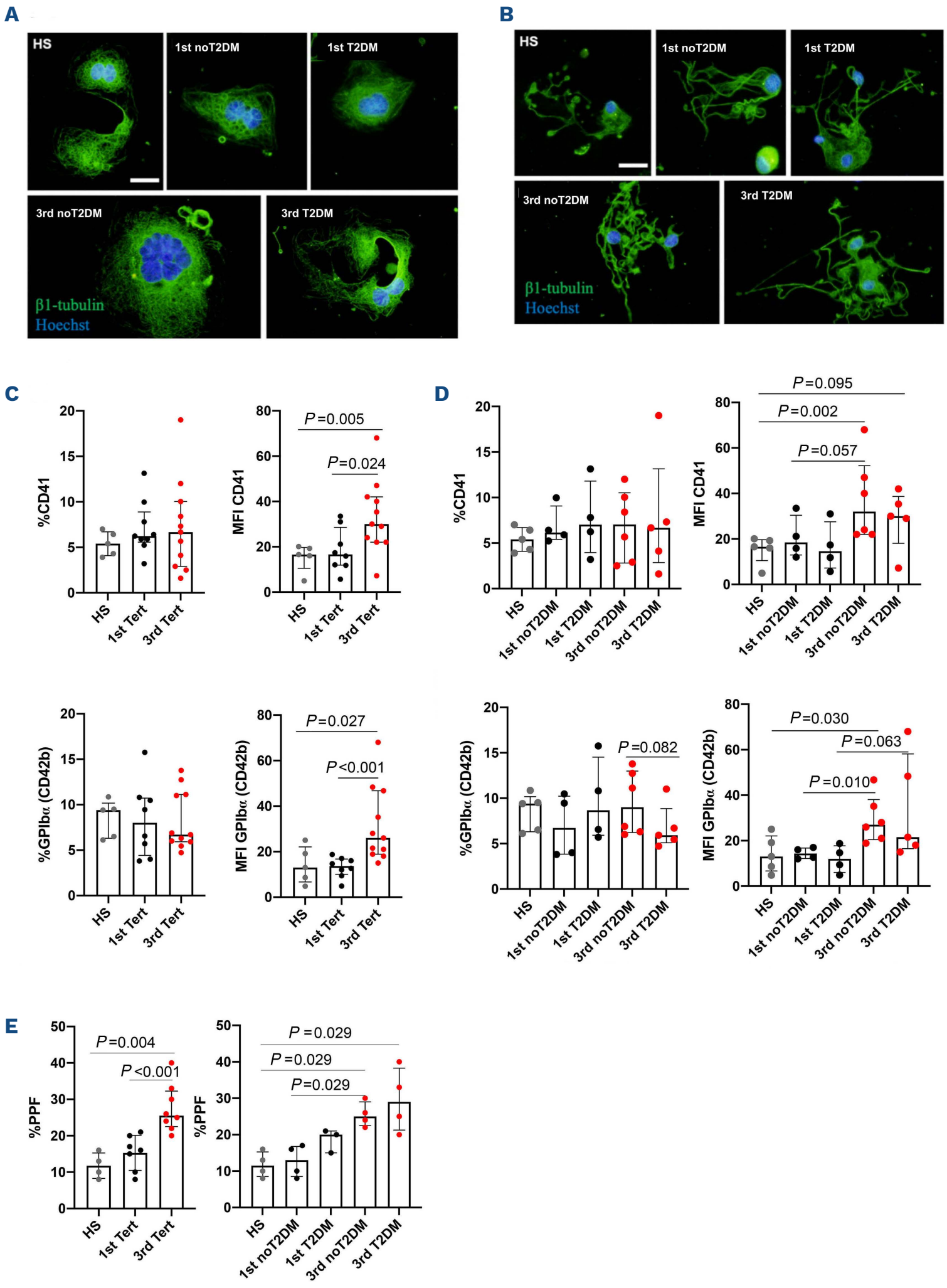


Figure 3. Enhanced megakaryocyte maturation and proplatelet formation in patients with faster COX-1 recovery. Representative immunofluorescence of megakaryocytes (Mk) (A) and proplatelet formation (PPF) (B). The proportion of mature Mk was measured by flow cytometry, as the percentage and mean fluorescence intensity (MFI) of CD41-positive cells (healthy subjects n=5; first tertile: all n=8, type 2 diabetes mellitus (T2DM) n=4, no T2DM n=4; third tertile: all n=10, T2DM n=4, no T2DM n=6) (C, D); and as the percentage and MFI of GPIbα (CD42b)-positive cells (healthy subjects n=5; first tertile: all n=8, T2DM n=4, no T2DM n=4; third tertile: all n=11, T2DM n=5, no T2DM n=6) (C, D). PPF was quantified as the proportion of Mk displaying at least one proplatelet with respect to the total number of adhered Mk (healthy subjects n=5; first tertile: all n=7, T2DM n=4, no T2DM n=3; third tertile: all n=8, T2DM n=4, no T2DM n=4) (E).

percentage of Annexin V$^+$ platelets was significantly lower in the third *versus* first tertile in all patients (*P*=0.007; Figure 5C) and in patients with T2DM (*P*<0.001; *data not shown*) with a non-significant trend for patients without T2DM (*data not shown*).

In healthy volunteers, aspirin administration was associated with an increase of PS exposure (*P*=0.002; Figure 5D), and activation of ADAM17[23] after 10 and 24 hours (Figure 5E). Thus, reduced PS exposure and ADAM17 activation, translating into diminished GPIbα cleavage and GC release, characterizes patients with accelerated recovery of platelet COX-1, and may be related to a lower aspirin effect.

Regulation of thrombopoietin expression

We next hypothesized a role for the GPIbα 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 TXB_2 slope tertile, may explain the higher liver expression and circulating levels of TPO.

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 β4-N-acetyllactosamine (LacNAc).[24] PLT 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) *versus* first tertile and healthy subjects (Figure 6A, B), indicating an increase of PLT galactose exposure in aspirin poor responders. More pronounced PLT galactose exposure in the third *versus* first tertile was accompanied by higher expression of neuraminidase (Neu)1 (*P*=0.061; Figure 6C), the sialidase that removes sialic acid from GPIbα.[25] These data suggest that PLT 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 thrombopoietin/ glycocalicin 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, sex, body mass index (BMI), HbA1c, non-alcoholic fatty liver disease

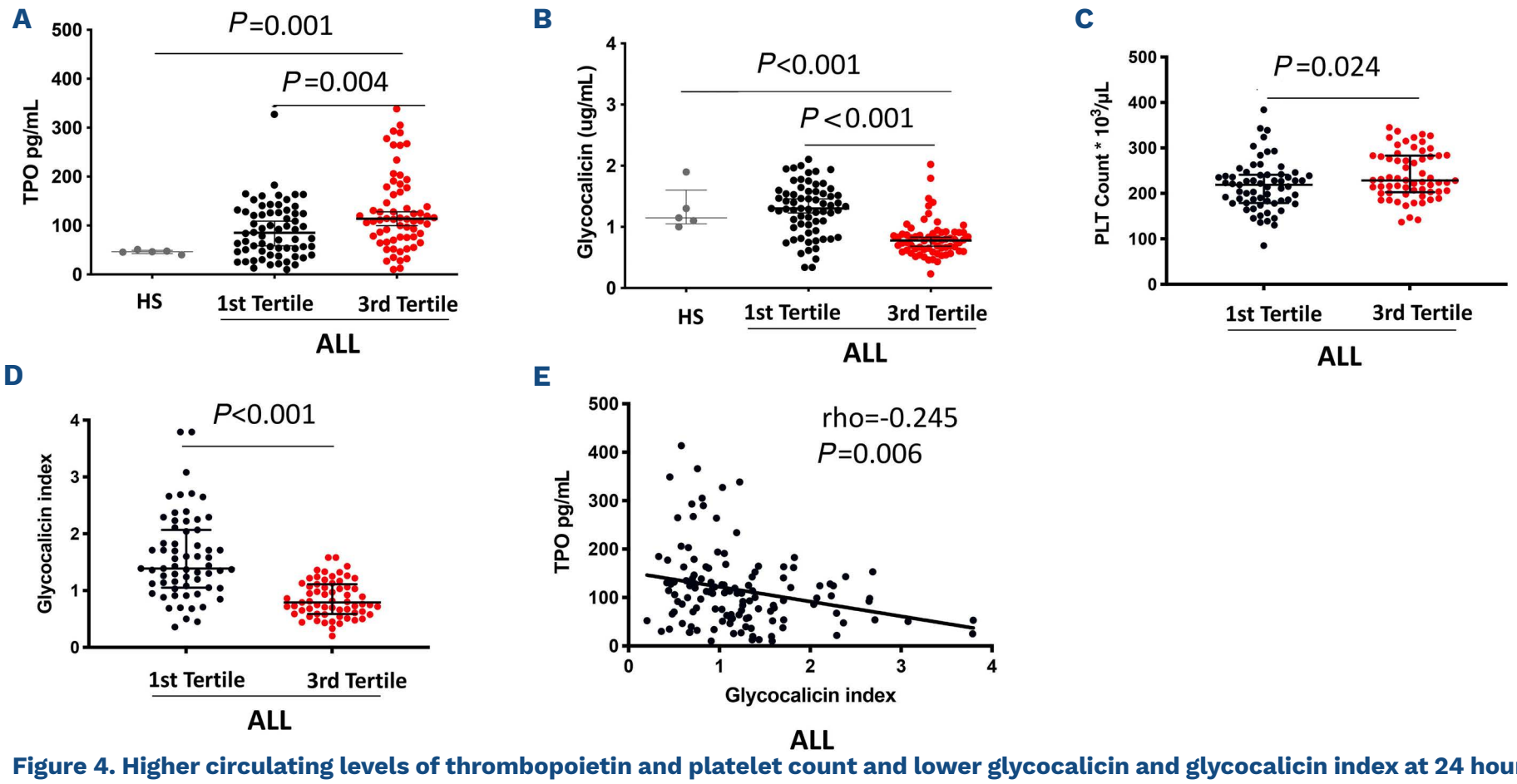


Figure 4. Higher circulating levels of thrombopoietin and platelet count and lower glycocalicin and glycocalicin index at 24 hours after witnessed aspirin intake in patients with faster COX-1 recovery. Comparison of thrombopoietin (TPO) (A), glycocalicin (GC) (B), platelet (PLT) count (C) and glycocalicin index (GCI) (D) between first *vs.* third serum thromboxane B_2 ($sTXB_2$) 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 $sTXB_2$ slope tertile in all patients (n=132). Significance was calculated by Mann-Whitney U test. Correlation between GCI and TPO in all investigated patients (E). Spearman correlation coefficient and *P* value are reported. Significance was calculated by Mann-Whitney U test.

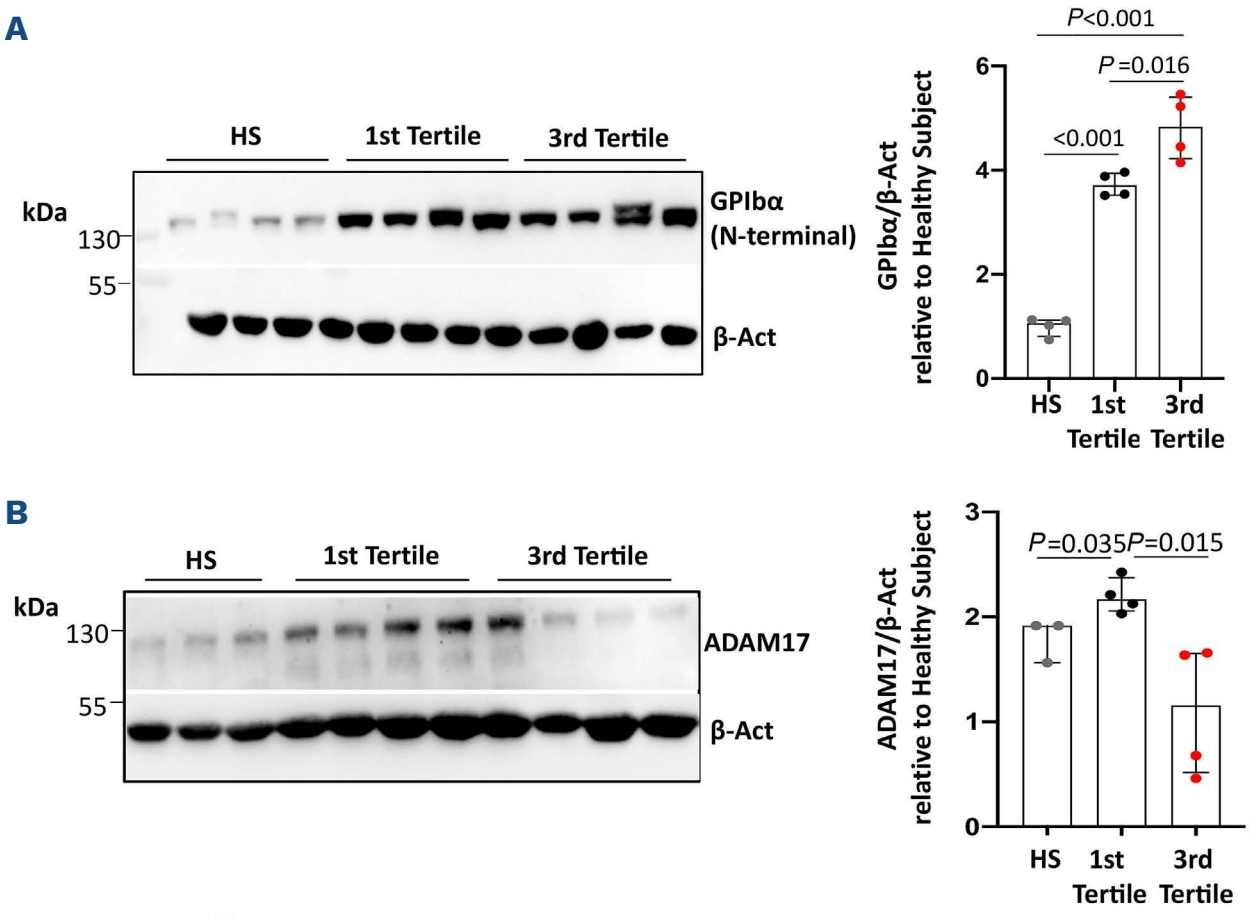


Figure 5. Lower glycocalicin circulating levels in platelets from patients with faster COX-1 recovery depend on higher GPIbα expression, lower phosphatidylserine expression and lower ADAM17 activation, and enhance thrombopoietin mRNA transcription in liver cells. GPIbα protein levels in platelets of healthy subjects (HS) (n=4) *vs.* first (n=4) *vs.* third (n=4) tertile in all patients (A). ADAM17 levels in platelets of HS (n=3) *vs.* first (n=4) *vs.* third serum thromboxane B_2 ($sTXB_2$) slope tertile (n=4) in all patients (B). Phosphatidylserine (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 10 and 24 hours post aspirin. Treatment of HepG2 cells (n=4) with an increasing concentrations of human recombinant glycocalicin (rGC, 0.5, 1 and 2 µg/mL) is associated, after 1 hour of incubation, with a significant dose-dependent reduction in thrombopoietin (*TPO*) mRNA (F). Significance was calculated by Mann-Whitney U test or by Student's *t* test.

(NAFLD), established atherosclerotic CV disease (ASCVD), glomerular filtration rate, PLT 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 $sTXB_2$ slope tertile (Figure 7A). In patients without T2DM, starting from a panel of potential determinants including age, sex, BMI, waist-to-hip ratio (WHR), PLT 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 $sTXB_2$ slope tertile (Figure 7B). Analysis of the ROC curves revealed an outstanding diagnostic accuracy (AUC ≥0.88) for the two models in the prediction of the third tertile status *versus* first tertile (Figure 7 C, D).

In order to translate our mechanistic findings of increased PLT production and reduced clearance characterizing accelerated COX-1 recovery into a clinically useful tool, we challenged the predictive value of TPO/GC ratio. Multivari-

able 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 $sTXB_2$ slope tertile, 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 area under the curve (AUC) (from 0.754 to 0.883; *P* for difference 0.015). Among patients without T2DM, those with TPO/GC above 147 were ten times more likely (Figure 7B) to be in the third tertile, while those with TPO/GC between 76 and 146 were nine times more likely (Figure 7B) to be in the third tertile. Thus, a clinical and biochemical signature may unravel patients with shorter duration of $sTXA_2$ inhibition for whom more frequent dosing regimens may prevent the steep recovery of platelet COX-1 activity. See the *Online Supplementary Appendix* (pages 9-10) for more details on clinical characteristics and study findings.

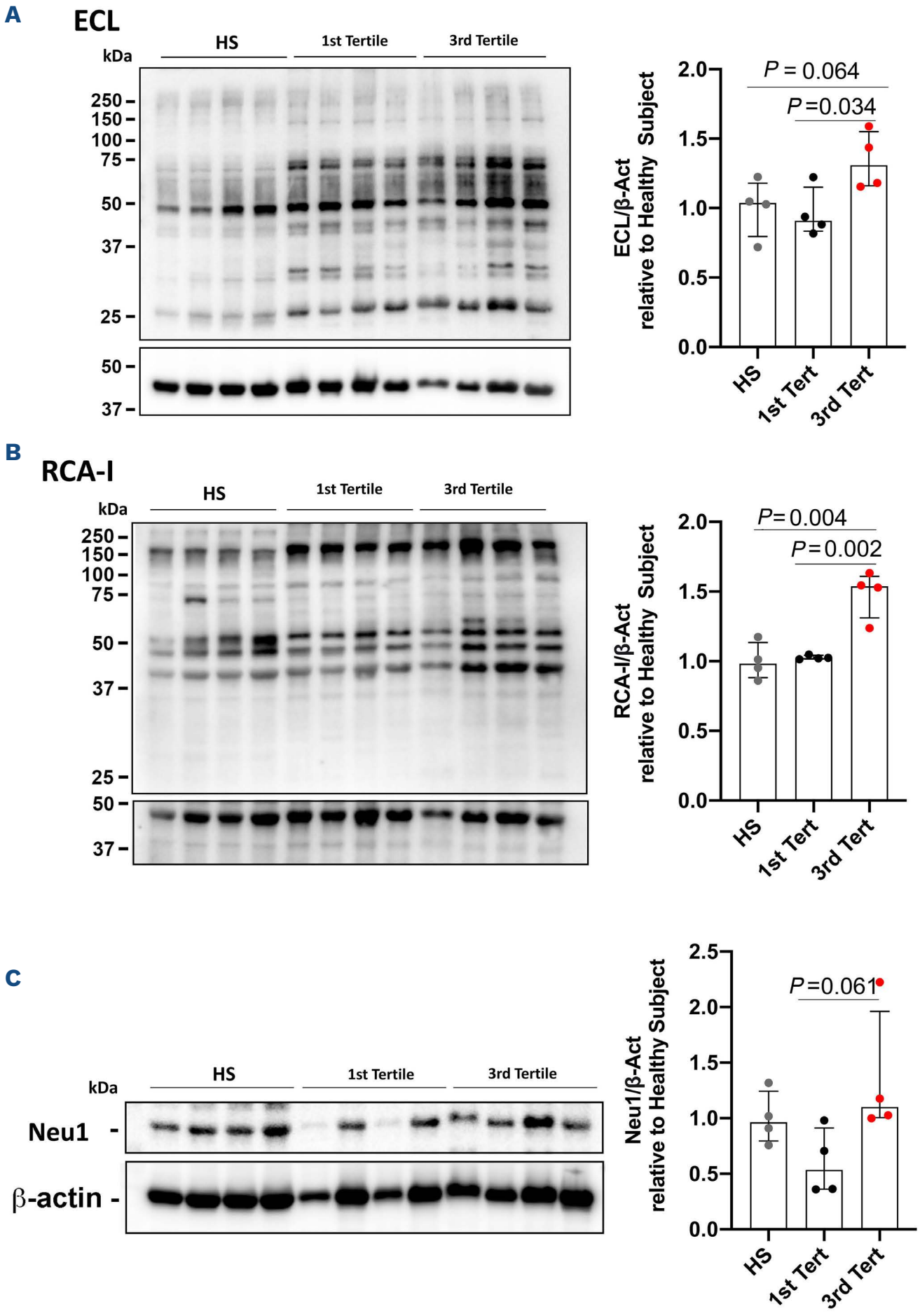


Figure 6. Higher platelet desialylation rate in patients with faster COX-1 recovery. Expression levels of galactose-recognizing lectins, *Erythrina cristagalli* agglutinin (ECL) (A), and *Ricinus communis* agglutinin I (RCA-I) (B), in platelets of healthy subjects (HS) (n=4) *vs.* first (n=4) *vs.* third (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.

Discussion

In this work we demonstrate that the shorter duration of TXB_2 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 i) accelerated recovery of COX-1 through increased functionally active COX-1 and COX-1 mRNA, higher TX-dependent PLT activation, enhanced TPO production, Mk maturation and proplatelet formation, leading to increased PLT number; ii) reduced PLT PS exposure, GPIbα ectodomain shedding and higher galactose exposure, fostering thrombopoiesis through liver TPO synthesis; iii) a clinical and molecular signature, including younger age, NAFLD, visceral obesity and high TPO/GCI, identifying with high accuracy aspirin-poor responders.

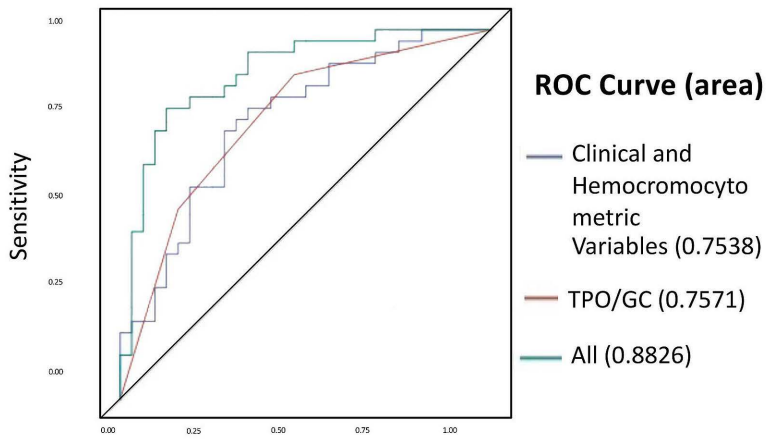
A

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

C



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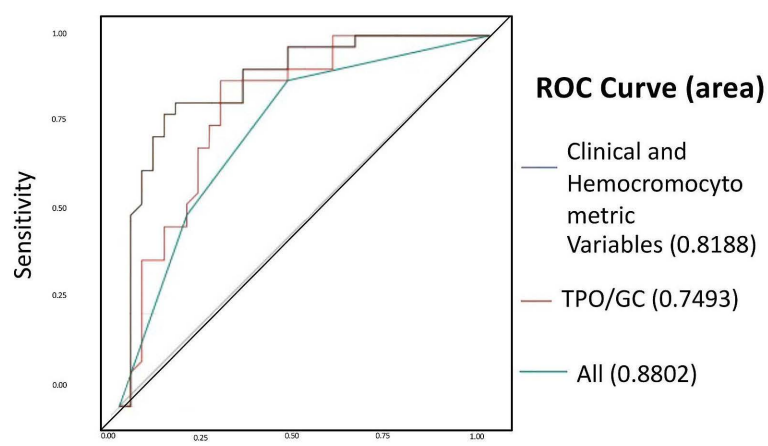

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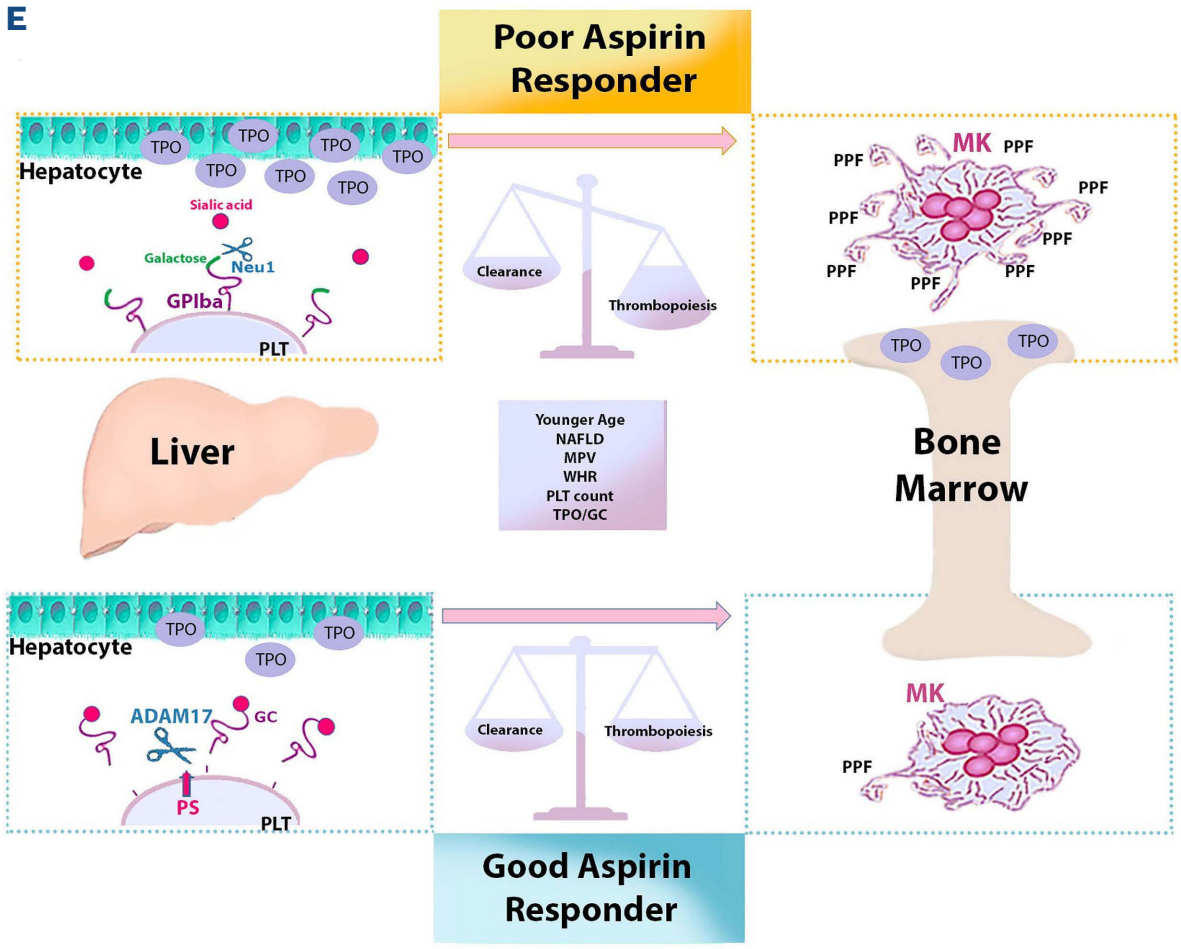

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Figure 7. Multivariable logistic regression analyses, receiver operating characteristic curve for the prediction of poor aspirin response and proposed model depicting the mechanisms involving platelet lifespan that may limit the extent and duration of aspirin effect over 24 hours. Determinants of the accelerated recovery of COX-1 activity in patients with (A) and without type 2 diabetes mellitus (T2DM) (B) assessed by multivariable logistic regression analysis. Receiver operating characteristic (ROC) curve for the prediction of poor aspirin response (C, D). ROC and the relative area under the curve (AUC) showing the ability of the model in discriminating between the third *vs.* first serum thromboxane B_2 ($sTXB_2$) slope tertile. Among patients with T2DM, the combination of younger age (standard deviation [SD]=6.38 years), presence of non-alcoholic fatty liver disease (NAFLD), higher platelet (PLT) count (SD=55.66 μL) and higher thrombopoietin/glycocalicin (TPO/GC) ratio (1st tertile: <60; 2nd tertile: from 60 to 138; third tertile >138) yielded an AUC value of 0.883 (95% confidence interval [CI]: 0.799-0.966) in distinguishing patients in third $sTXB_2$ slope tertile from first tertile patients (C). In comparison with a model including only clinical/hemocromocytometric variables (age, NAFLD and PLT 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 waist-to-hip ratio (WHR) (SD=0.066), lower mean platelet volume (SD=0.93 μL) and higher TPO/GC ratio (first tertile: <76; second tertile: from 76 to 147; third tertile >147) yielded an AUC value of 0.880 (95% CI: 0.794-0.966) in distinguishing patients in third $sTXB_2$ slope tertile from first tertile patients (D). Aspirin-treated patients were stratified according to the kinetics of COX-1 recovery over the 10- to 24-hour dosing interval. In poor aspirin responders we showed: i) increased plasma thrombopoietin, megakaryocyte (Mk) maturation and proplatelet formation (PPF) reflecting enhanced PLT production; ii) increased PLT desialylation, lower phosphatidylserine exposure, lower PLT sheddase ADAM17 and plasma glycocalicin and increased glycoprotein (GP)Ibα expression, altogether reflecting defective PLT GPIbα shedding; iii) a proteomic signature characterized by activation of cell survival and inhibition of apoptosis. Younger age, NAFLD and visceral obesity, higher PLT count together with higher thrombopoietin-to-glycocalicin ratio, predict suboptimal aspirin response (E).

Over the last decades, the concept of suboptimal response to aspirin has been affirmed, but the prevalence of this phenomenon is unclear. This is due to the heterogeneity of methods used to quantity the anti-PLT effect of aspirin in these studies, which poorly reflect the biochemical pathway affected by aspirin, i.e., PLT COX-1 activity[6] and variably reflect the aspirin-sensitive TX-dependent component of PLT aggregation.[1] Even when using $sTXB_2$, a mechanism-based endpoint with the highest specificity and sensitivity to monitor aspirin pharmacodynamics, we and others have previously characterized an interindividual variability in PLT COX-1 recovery during the 12- to 24-hour 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 PLT,[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 24-hour dosing interval, newly generated PLT entering circulation, after aspirin effect waning, contain unacetylated COX-1 and synthesize new TXA_2.

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

We next sought to analyze circulating TPO and GC, markers of PLT production and destruction, respectively. We unraveled that reduced PLT destruction, as reflected by lower circulating GC, lower levels of caspase 3[28] (*Online Supplementary Figure S5*) and platelet PS exposure, identify platelets from patients with a shorter response durability to aspirin. Indeed, proteomics profiling indicated activation of cell survival and inhibition of apoptosis pathways in third $sTXB_2$ tertile patients, which may characterize younger platelets.[29]

The observation that PLT 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 GPIbα, mirroring reduced destruction[30–33] leading to lower circulating GC and GCI and higher PLT 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 GPIbα[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 GPIbα proteolysis from stored platelets.[32,33] Inhibition of GPIbα shedding by kinase inhibitors[33] or antibodies[32] can mitigate PLT clearance and prolong the lifespan of transfused PLT in mice.

Our present results do not allow to draw final conclusions regarding the lifespan of PLT. Although circulating GC has been consistently regarded as an index of PLT destruction, and several lines of evidence converge to support inhibited apoptosis, no direct demonstration of reduced PLT clearance in the third COX-1 recovery tertile has been obtained in our cohort. The reduced percentage of PS-exposing platelets and reduced apoptosis may alternatively be regarded as a feature of young, newly released PLT, or may be the result of earlier PLT 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 PLT,

and lower, rather than higher, MPV was a significant predictor of belonging to the upper $sTXB_2$ slope tertile among non-diabetic patients, raising the hypothesis of a longer PLT lifespan in poor aspirin responders, with coexistence of larger and smaller size PLT.

Whether increased PLT survival/reduced apoptosis is a feature of the "poor-responder" PLT or a consequence of poor aspirin response, is not unraveled. Aspirin induces PLT apoptosis[35] and shedding of GPIbα and GPV through activation of ADAM17.[23] Consistently, in a small number of our healthy volunteers, aspirin treatment was associated with enhanced Annexin V^+ PLT and increased expression of active ADAM17. *Vice versa*, patients with accelerated COX-1 recovery displayed lower Annexin V+ platelets and lower PLT ADAM17 expression, concomitant with higher expression of platelet GPIbα N-terminal domain and lower GC and GCI, *versus* 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 GPIbα clustering, on the one hand, and PS exposure, on the other hand, we measured complexes of the adapter protein 14-3-3ξ with GPIbα 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 PLT induce 14-3-3ζ-GPIbα association, 14-3-3ζ release from phospho-Bad, Bad activation, PS exposure, and apoptosis.[36] GPIbα clustering is also linked to galactose exposure.[37] In our setting, 14-3-3ξ:GPIbα complexes were significantly less in third tertile patients, while 14-3-3ξ: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 14-3-3ξ from the proapoptotic protein Bad in favor of GPIbα 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 TXA_2 may determine a lower degree of interaction of 14-3-3ξ with GPIbα and a reduced activation of apoptosis. In keeping with this, we found that apoptosis was inactivated in PLT from third tertile patients.

Increased GPIbα and reduced GC characterize and predict poor aspirin response and may play a role in promoting PLT activation and escape from aspirin. While it is assumed that ADAM17 restrains continuous GPIbα-mediated PLT activation,[38] the phenotype observed in third tertile patients may indicate hyperreactive PLT since GPIbα clustering triggers TXA_2.[39] Along this line, poor aspirin responders with high GPIbα have higher platelet COX-1 and persistent TXA_2 biosynthesis.

The unexpected inverse relationship between PLT destruction and production, as reflected by GC or GCI and TPO, respectively, prompted us to hypothesize that defective GPIbα ectodomain shedding may contribute to sustain enhanced thrombopoiesis in patients with shorter duration of COX-1 inhibition. Indeed, the extracellular domain of GPIbα *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 GP1bα 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.

PLT galactose exposure also triggers thrombopoiesis through the interaction with AMR.[9] In our study, patients with a shorter duration of the aspirin response showed increased lectin binding and Neu-1 expression, suggesting a possible further mechanism activating TPO production in these subjects. Thus, both PLT galactose exposure and GPIbα 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 PLT clearance and decreased circulating platelet count. Other evidence shows that PLT isolated from myeloproliferative diseases, 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. Mk derived *in vitro* from these patients showed an increased expression of the B4GALT1 gene encoding β-1,4-galactosyltransferase 1 (β4GalT1) and terminal galactose expression relative to healthy controls. Altered expression of B4GALT1 in mutant Mk 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 PLT production and PLT 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 neoplasms[40] and a phase II 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 (*clinicaltrials gov. Identifier: 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, i.e., absorption and biotransformation, leading to reduced, albeit steady, 24-hour inhibition of COX-1–dependent TX production.[4] 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 hyperreactivity,[41] persistent TX-dependent platelet activation[42,43] and enhanced PLT turnover, with suboptimal PLT 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 *versus* non-diabetic 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, *versus* 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 anti-PLT dosing regimens should be tested.

Limitations of the study include its observational nature and the lack of reticulated PLT data, although previously shown by our group and others and overcome by a direct evaluation of Mk maturation and proplatelet formation. Also information about COX-1 acetylation or salicylate measurement is lacking. However, in addition, we performed lectin blots using galactose binding lectins, showing all proteins with terminal galactose, instead of flow cytometry, which 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 PLT (*data not shown*) and PLT contain only few Golgi-like granules.[46] Hence, we speculate that most proteins with exposed galactose would reside on the platelet surface. The healthy subject group is very small, although differences in results pre *versus* 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.

In conclusion, an imbalance between platelet production and clearance, with accelerated megakaryopoiesis/ PLT 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 7E). This imbalance translated into increased PLT count (especially in patients with T2DM) and enhanced TX-dependent PLT 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.

Disclosures

FS has received research grant support and consulting fees from Bayer, all unrelated to this manuscript. The remaining authors have no conflicts of interest to disclose.

Contributions

FS conceived and designed the research studies and obtained funds for the project; PS enrolled patients and acquired data; RL, RT, SC, AR, VA, BP, MLS, ML, RA, GB, PDB, DP, and MCC conducted experiments; AdC, RL, RT, SC and PS analyzed data; FS, PS, RL, AR and AB wrote the manuscript with input from the other authors; FS, AB, PL, MC, DP, ET, KMH, PDB and FC revised and provided critical interpretation for important intellectual content. All authors approved the final version.

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Data-sharing statement

For original data, please contact the corresponding author.

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