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The Recovery of Platelet Cyclooxygenase Activity Explains Interindividual Variability in Responsiveness to Low-Dose Aspirin in Patients With and Without Diabetes

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Running title: Diabetes and variability in aspirin response

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Summary

Background. Interindividual variability in response to aspirin has been popularized as "resistance". We hypothesized that faster recovery of platelet cyclooxygenase-1 activity may explain incomplete thromboxane (TX) inhibition during the 24-hour dosing interval.

Objective. Characterize the kinetics and determinants of platelet cyclooxygenase-1 recovery in aspirin-treated diabetic and non-diabetic patients.

Patients/Methods. One-hundred type 2 diabetic and 73 non-diabetic patients on chronic aspirin 100 mg daily were studied. Serum TXB_2 was measured every 3 hours, between 12 and 24 hours after a witnessed aspirin intake, to characterize the kinetics of platelet cyclooxygenase-1 recovery. Patients with the fastest TXB_2 recovery were randomized to aspirin 100 mg once daily, 200 mg once daily or 100 mg twice daily, for 28 days and TXB_2 recovery was reassessed.

Results and Conclusions. Platelet TXB_2 production was profoundly suppressed at 12 hours in both groups. Serum TXB_2 recovered linearly, with a large interindividual variability in slope. Diabetic patients in the third tertile of recovery slopes ($\geq$0.10 ng/ml hr^{-1}) showed significantly higher mean platelet volume, body mass index, and younger age. Higher body weight was the only independent predictor of a faster recovery in non-diabetics. Aspirin 100 mg twice daily completely reversed the abnormal TXB_2 recovery in both groups. Interindividual variability in the recovery platelet cyclooxygenase activity during the dosing interval may limit the duration of the antiplatelet effect of low-dose aspirin in patients with and without diabetes. Inadequate thromboxane inhibition can be easily diagnosed and corrected by a twice daily regimen.

Keywords: aspirin, diabetes, thromboxane, platelets.

Introduction

Interindividual variability in the response to antiplatelet drugs has been popularized under the term “resistance” [1]. While pharmacokinetic and pharmacogenetic mechanisms can explain the large variability in clopidogrel response [2], no such mechanisms have been characterized to explain the variability in response to aspirin [3]. In particular, a diminished responsiveness to the antiplatelet effects of aspirin has been reported in patients with type 2 diabetes mellitus (T2DM) [4-6], with the suggestion that this might explain the apparent failure of the drug in reducing the risk of atherothrombotic events in individual trials [7,8] and meta-analyses of aspirin in diabetes [9,10].

Based on our previous studies of the antiplatelet effects of low-dose aspirin in healthy subjects [11-13] and patients with myeloproliferative neoplasms and high platelet turnover [14,15], we developed a novel investigative approach to describe the kinetics of the recovery of platelet cyclooxygenase (COX) activity – the primary drug target [16] – during the 12 to 24 hour dosing interval after a witnessed administration of aspirin 100 mg. With this approach we tested the hypothesis that an accelerated renewal of the drug target might explain response variability in patients with versus those without T2DM. Moreover, we explored the effects of increasing the aspirin dose and shortening its dosing interval in an attempt to reduce response variability in this setting.

Methods

Study participants

One hundred T2DM patients (66 males, median age 64.6 years) diagnosed according to the American Diabetes Association [17], with or without prior vascular disease, on aspirin 100 mg once daily (od) for ≥1 month, were recruited at two Institutions. Exclusion criteria were: poorly-controlled hypertension or hypercholesterolemia, smoking, pregnancy, impaired liver or renal function, treatment with non-steroidal anti-inflammatory drugs (NSAIDs), anticoagulants or other antiplatelet drugs.

Moreover, we studied 73 patients (42 males, median age 68.5 years) without T2DM, comparable for demographic, anthropometric and clinical characteristics, with particular reference to cardiovascular risk factors and concurrent treatments, on aspirin 100 mg od since ≥1 month for primary or secondary cardiovascular prevention. Inclusion criteria included: age between 45 and 80 years, body mass index (BMI) between 20 and 45 Kg/m^2, absence of T2DM [17], besides the other criteria used for diabetics.

The protocols were approved by the institutional Ethics Committees (Approvals: n. 1589/08 Center of Excellence on Aging, n. A/287/CE/2008 and P/244/CE2010 Catholic University). Participants provided written informed consent.

Study design

The study consisted of 2 phases, preceded by a 7-day run-in during which patients were instructed to take aspirin (enteric-coated 100 mg, Cardioaspirin®, Bayer, Italy) at 8 pm.

Phase 1 was designed to assess the kinetics of recovery of platelet COX-1 activity during the dosing interval. Phase 2 was a randomized, open-label intervention study that tested different dosing schedules in one third of patients selected based on the results of phase 1.

Phase 1

At 8 pm on the last day of the run-in, eligible patients underwent blood and urine sampling, and a witnessed aspirin intake. The following day, blood was sampled every 3 hours and urine collected every 4 hours, between 8 am and 8 pm. Before phase 2, two patients with T2DM dropped out because of clopidogrel requirement, and clinically relevant bleeding, respectively.

Phase 2

Patients with diabetes

Based on the slope of recovery of platelet COX-1 activity during the 12 to 24 hour dosing interval measured in phase 1, 33 patients with slopes in the upper tertile entered phase 2. Eligibility was re-assessed and patients randomized to one of the following aspirin regimens: 1) 100 mg od (8pm, n=11); 2) 100 mg twice daily (bid) (8am-8pm, n=11); 3) 200 mg od (8 pm, n=11). Blood and urine sampling and witnessed aspirin administration were performed at 8 pm of day 28, and every 3 (blood) or 4 (urine) hours between 8 am and 8 pm on day 29.

Patients without diabetes

Based on the same criteria, 18 out of 24 patients with recovery slopes in the upper tertile entered phase 2. Six patients were ineligible at re-assessment due to anticoagulant or NSAID requirement, or refusal to continue. Patients were randomized to one of the following aspirin regimens: 1) 100 mg od (8 pm, n=6); 2) 100 mg bid (8am-8pm, n=12). Blood and urine sampling and witnessed aspirin administration were performed as in diabetic patients. One patient assigned to the 100 mg bid regimen could not complete the study at day 28 because of intercurrent illness.

Analytical measurements

Serum TXB_2 [18,19], urinary 11-dehydro- TXB_2 [20], plasma IL-6 and CRP, glycocalycin index [21] and platelet function measurements are detailed in Supporting information.

Statistical analyses

We hypothesized that a shorter interval of aspirin administration would restore a 24-hour profile of TXB_2 suppression, comparable to healthy volunteers in whom we detected a consistent and profound (>99%) suppression of platelet TXB_2 at 24 hours after aspirin intake [13]. We estimated that 8 T2DM patients would be required in each arm of phase 2 to detect a mean difference in serum TXB_2 of 3 ng/mL between aspirin 100 mg od (assuming a mean level of serum TXB_2 of 5±2 ng/mL) and 100 mg bid, 24 hours after dosing, with a 2-tailed alpha of 0.05 and 95% power. Considering a 20% drop-out, we calculated the need of 10 patients for the 100 mg once daily group (standard regimen) and 10 patients for the 100 mg twice-daily group (experimental regimen). In addition to this main comparison, we also planned a third group of diabetics randomized to 200 mg once daily as a control for the doubling of the dose. Therefore, 100 patients were recruited to provide at least 30 patients (upper tertile of TXB_2 recovery slope) for phase 2. With 73 patients without T2DM recruited, the study had 99% power to detect a mean difference in serum TXB_2 recovery slope between the two patient groups of 0.06 ng/ml hr^{-1}, with a 2-tailed alpha of 0.05. With 12 and 6 patients without T2DM randomized to 100 mg bid and 100 mg od, respectively, the study had at least 80% power to detect a 50% difference in TXB_2 recovery slope between the two aspirin regimens, with a 2-tailed alpha of 0.05.

All variables were tested for normal distribution, and non-normally distributed variables were log- or square-root-transformed. The time course of each variable was analyzed by ANOVA with

repeated measurements. Serum TXB_2 values were fitted with a simple linear model ($y=a+bx$) (Grafit Software, Erithacus Software, Staines, UK), calculating the slope of the fitting. Additional analyses are detailed in Supporting Information.

Results

Baseline characteristics of patients with and without T2DM are detailed in Table 1. The two groups of patients were fairly balanced for demographic, anthropometric and clinical parameters, except for a slightly older age, higher total, LDL and HDL cholesterol in the non diabetics.

Patients with diabetes

Phase 1

Recovery of serum TXB_2 during the 12 to 24 hour dosing interval displayed linear kinetics (F-testing of linear versus different grade fittings: $P<0.001$). Due to the skewed distribution of serum TXB_2 recovery slopes (Fig 1A), data were analyzed by tertiles. In the first tertile of recovery slopes, serum TXB_2 was steadily suppressed over the 12 to 24 hour dosing interval (Fig 2A), as observed in healthy subjects [11,13], while diabetic patients in the third tertile (slope ≥ 0.10 ng/ml hr^{-1}) showed a significantly faster recovery of platelet COX-1 activity ($P<0.0001$, Fig 2C). Patients' characteristics according to TXB_2 recovery slope tertiles are reported in Table 1 of Supporting Information.

The TXB_2 recovery slope was inversely related to age (rho=-0.21, P=0.037) and directly to BMI (rho=0.288, P=0.004), waist circumference (rho=0.277, P=0.005), cholesterol (rho=0.26, P=0.010), aspartate transaminase (rho=0.295, P=0.009), and mean platelet volume (MPV) (rho=0.44, $P<0.0001$; Figure 3A). On multiple linear regression analysis, MPV (Beta Coefficient=0.42, SEM=0.005, $P<0.0001$), higher BMI quartiles (Beta Coefficient=0.26, SEM=0.022, P=0.007), and age (Beta Coefficient=-0.18, SEM=0.002, P=0.049) independently predicted the serum TXB_2 recovery slope ($R^2=0.28$ for the entire model, and Fig 1A of Supporting Information). Interestingly, MPV was

significantly correlated with serum TXB_2 levels 24 hours, but not 12 hours post-aspirin administration.

Urinary 11-dehydro-TXB_2 did not change over the 24-hour dosing interval, or across serum TXB_2 recovery slope tertiles (data not shown).

Platelet COX-1 and COX-2 expression levels were comparable at 12 and 18 hours post-aspirin administration (data not shown), and were not correlated with MPV or serum TXB_2 recovery slopes (Table 2 of Supporting Information). The percentage of thiazole orange-positive platelets, which represent the newly-released, mRNA-positive platelets, was significantly higher in patients with the fastest recovery slopes at both 12 and 18 hours after aspirin dosing (Figure 3B). The percentage of thiazole orange (TO)-positive platelets was also positively correlated with platelet CD36 levels (Figure 3C), MPV (Figure 3 D) and TXB_2 recovery slopes, and inversely correlated with surface P-selectin (Supporting Information, Table 2). Platelet COX-2 was directly correlated with surface P-selectin only (Supporting Information, Table 2). Thus in our diabetic population TO positivity was associated with higher platelet volume, expression of CD36 and COX-1 enzymatic activity.

CRP, IL-6 and the glycocalicin index did not correlate with serum TXB_2 recovery slopes (Supporting Information) or urinary 11-dehydro-TXB_2.

Phase 2

The baseline clinical characteristics were comparable across the randomized treatments (Supporting Information, Table 3). The TXB_2 recovery slope over the 12 to 24 hour interval post-aspirin was approximately 90% lower in patients randomized to 100 mg bid as compared to 100 mg od ($P<0.0001$) (Fig 4). The 200 mg od regimen displayed an intermediate pattern of recovery. The median (IQR) changes versus phase 1 slopes were 33% (0-50), 55% (40-61) and 88% (69-100) decrease in patients treated with 100 mg od, 200 mg od and 100 mg bid, respectively.

The excretion rate of urinary 11-dehydro-TXB_2 (Figure 5 A) and the platelet function measured

by the Verify-Now Aspirin (Figure 5 B) were not significantly affected by the randomized treatment.

Patients without diabetes

Phase 1

Recovery of serum TXB_2 during the 12 to 24 hour dosing interval was also linear and the median recovery slope was similar to T2DM patients [0.075 (0.046-0.12) vs. 0.070 (0.03-0.11) ng/ml hr^{-1}, P=0.11]. Serum TXB_2 recovery slopes were also analyzed by tertiles (Fig 1B). In the first tertile, serum TXB_2 was steadily suppressed over the 12 to 24 hour dosing interval (Fig 2D), while patients in the third tertile (slope ≥0.093 ng/ml hr^{-1}) showed a significantly faster recovery of platelet COX-1 activity (Fig 2F). Patients' characteristics in relation to TXB_2 recovery slope tertiles are reported in Table 4 of Supporting Information. Interindividual variability in the recovery slopes appeared to be somewhat greater in the non diabetic as compared to the diabetic patients.

The TXB_2 recovery slope was inversely related to age (rho=-0.256, P=0.029) and hemoglobin A1c (rho=-0.28, P=0.017), and directly to body weight (rho=0.276, P=0.018) and height (rho=0.258, P=0.027). On multiple linear regression analysis, higher body weight (Beta Coefficient=0.325, SEM=0.001, P=0.006) was the only independent predictor of the serum TXB_2 recovery slope (R^2=0.10 for the entire model).

Urinary 11-dehydro-TXB_2 was stable over the 24-hour dosing interval, and comparable across serum TXB_2 recovery slope tertiles (data not shown).

Phase 2

The baseline characteristics of patients randomized to different aspirin regimens are detailed in Supporting Information (Table 5).

The TXB_2 recovery slope over the 12 to 24 hour interval post-aspirin was approximately 75% lower in patients randomized to 100 mg bid as compared to 100 mg od (P=0.02) (Fig 6). The median (IQR) changes versus phase 1 slopes were 35% (7-101) increase and 74% (51-106) decrease in patients

treated with 100 mg od and 100 mg bid, respectively.

Discussion

An extensive literature has described lower-than-expected inhibition of platelet function (often referred to as "resistance") in a variable proportion of aspirin-treated patients [1]. However, neither the mechanisms of response variability nor its reversibility have been established. Major limitations of previous studies are related to: i) inadequate ascertainment of compliance; ii) largely undefined time interval between aspirin dosing and measurements of platelet inhibition; iii) dichotomous definition of responder vs non-responder status based on a single determination of platelet function using arbitrary thresholds of response; and iv) lack of intervention studies to clarify the underlying mechanisms of variability in aspirin responsiveness.

To overcome these limitations, we have developed an investigative approach based on the following innovative features: i) a witnessed drug administration; ii) accurate timing of blood sampling at 12, 15, 18, 21 and 24 hours after dosing in order to assess the maximal level of platelet inhibition and the kinetics of its reversal; iii) use of a mechanism-based biochemical end-point, ie serum TXB_2, with the highest specificity and sensitivity to monitor aspirin pharmacodynamics; and iv) randomized intervention studies to test the reproducibility of the abnormal biochemical phenotype, and its potential correction by increasing the aspirin dose or shortening its dosing interval.

We have characterized substantial interindividual variability in the recovery rate of platelet COX-1 activity during the 12 to 24 hours dosing interval of aspirin administration in well controlled T2DM patients and in patients without diabetes with an indication for antiplatelet therapy for primary or secondary prevention. While very profound suppression of platelet thromboxane production was measurable in the vast majority of patients at 12 hours after a witnessed administration of aspirin, a variable linear increase in serum TXB_2 production between 12 and 24 hours was unraveled by repeated determinations. In 2006, Perneby et al [22] had reported that once daily dosing was associated with

considerable recovery of arachidonate-induced platelet aggregation in whole blood after 24 hours, even after 960 mg aspirin, in a group of 15 healthy male volunteers. More recently Henry [23] et al and Lordkipanidze et al [24] studied the recovery of platelet activity as a function of time within the 24 hr dosing interval in coronary artery disease (CAD) patients. Both studies reported reported a time-dependent increase in the percentage of aspirin poor responders between 2 and 24 hours after low-dose aspirin in stable CAD patients studied with different methods.

A serum TXB_2 recovery slope of 0.15 ng/ml hr^{-1}, as calculated in the upper tertile of patients with and without diabetes, would generate higher TXB_2 levels than the reported threshold (2.2 ng/ml) for loss of functional inhibition [25] as well as the cut-off (3.1 ng/ml) associated with increased risk of vascular events [26]. Whereas virtually complete suppression of platelet COX-1 activity is ensured by presystemic acetylation of the enzyme [27], the long-lasting duration of this effect is determined by systemic acetylation of the enzyme in bone marrow megakaryocytes and by the rate of platelet turnover [11,12,28]. Our finding that the median serum TXB_2 concentration measured at 12 hours after aspirin dosing in 100 patients with and 73 without T2DM was comparable to the median value that we reported in 48 healthy subjects treated with the same dose and formulation of aspirin [13] strongly argues for unimpaired presystemic acetylation of platelet COX-1, ie, no “resistance” of the drug target. In contrast, the shorter-than-expected duration of COX-1 suppression in about one third of the studied population is consistent with the hypothesis of accelerated renewal of the drug target during the dosing interval.

Our finding that the glycocalicin index, a validated measure of platelet destruction [21], did not correlate with the TXB_2 recovery slope of diabetic patients suggests that peripheral platelet destruction does not affect aspirin pharmacodynamics. Instead, the newly-released mRNA-positive platelet fraction [29] and the MPV both positively correlated with the rate of recovery of platelet cyclooxygenase activity (Supporting Information and Fig 3). Notably, both biomarkers were associated with high platelet reactivity in aspirin-treated diabetic patients in the recent study by Spectre et al [30]. The latter study

also showed that a twice-daily aspirin regimen was more effective in suppressing platelet function than the standard once daily regimen.

The contribution of abnormal megakaryopoiesis to accelerated renewal of the drug target is exemplified by patients with essential thrombocythemia, most of whom displayed levels of serum TXB_2 $\geq$ 4.0 ng/ml 24 hours after aspirin dosing [14], an abnormal biochemical phenotype that could be normalized by a bid regimen of aspirin but not by doubling the dose given od [15]. In the present study, MPV was the strongest predictor of the TXB_2 recovery slope in patients with diabetes, suggesting that the diabetic milieu may account for faster *de novo* synthesis of COX-1 in the bone marrow progenitors or their accelerated turn-over [31-33], as seen in essential thrombocythemia [14,15].

Body weight appeared the major determinant of the rate recovery of platelet COX-1 activity during 12 to 24 hour dosing interval in patients without diabetes providing a mechanistic explanation for the previously reported association between obesity and diminished responsiveness to low-dose aspirin [25]. Smith et al. have recently characterized the contribution of a high BMI to poor aspirin response in CAD patients with the metabolic symdrome [34]. Among both diabetics and non-diabetics, obese patients had a significantly higher level of serum TXB_2 at 12 hours after dosing as compared to non-obese patients (data not shown). This finding would strongly suggest that obesity might affect aspirin pharmacokinetics, possibly reducing its bioavailability, as is the case for several lipophilic drugs administered to obese subjects [35]. A lower aspirin bioavailability would be expected to limit the maximal acetylation of megakaryocyte COX-1, a time-dependent cumulative process determining the rate of recovery of platelet TXB_2 production upon aspirin withdrawal [13], and the two-day lag before a detectable linear recovery in platelet COX-1 activity previously characterized in healthy subjects [11,28].

One major limitation of the present study is represented by the lack of reticulated platelet data in the group of non-diabetic subjects. Thus, we cannot exclude changes in platelet turnover in this setting.

To clarify the potential mechanisms of the accelerated recovery of platelet COX-1 activity and to assess its reversibility, we performed two randomized studies in the 33 diabetic and 18 non-diabetic patients with the steepest recovery slopes, and tested the effects of bid (both groups) or double-dose (only in diabetics) regimens versus the standard od regimen. The consistency of the kinetics of recovery of serum TXB_2 in those patients who continued their standard aspirin regimen (Figs 4A and B and 6A and B) demonstrates the stability of the abnormal biochemical phenotype in patients with and without diabetes. The finding that 200 mg od halved the recovery slope in patients with diabetes is consistent with the hypothesis that pharmacokinetic abnormalities associated with diabetes only partially explain faster recovery of platelet COX-1 during the dosing interval. The results depicted in Figs 3 and 4 clearly demonstrate complete reversal of the accelerated recovery pattern with a bid aspirin administration, consistently with faster renewal of platelet COX-1 representing the main mechanism underlying this phenomenon in both patients with and without diabetes. Three different studies have recently reported a greater inhibition of platelet function and/or platelet TXB_2 production by bid versus single dosing in T2DM [30,36,37]. However, a control group was not included in these studies and the underlying mechanism was not investigated.

The failure to detect further improvement in the inhibition of arachidonate-induced platelet aggregation, as monitored by the VerifyNow Aspirin or 11-dehydro-TXB_2, may reflect the non-linearity of this functional response and its larger intraindividual variability as compared to serum TXB_2 [13, 15]. Similar findings (i.e. the absence of a linear relationship between urinary 11-dehydro-TXB_2 and serum TXB_2) have been reported in a recent study on CAD patients with the metabolic syndrome [34]. Thus this urinary index, which reflects heterogeneous biosynthetic compartments (kidney, leukocytes, platelets), should not be used to measure the adequacy of platelet COX-1 inactivation by aspirin [34].

Our findings may explain, independently of non-compliance, a variable dilution of the maximal theoretical effect of aspirin as an antithrombotic agent, depending on the clinical setting and prevalence

of the factors influencing aspirin pharmacokinetics (eg, obesity) or pharmacodynamics (eg, T2DM).

While increasing the dose of aspirin may, at least in part, correct pharmacokinetic abnormalities, this is not going to affect pharmacodynamic abnormalities related to faster renewal of the drug target. In contrast, decreasing the dosing interval (eg, bid regimen) appears to correct the abnormal biochemical phenotype described in the present study. This may have important clinical implications for the management of the individual patient (ie, personalized dosing regimen), as well as for the four ongoing trials of aspirin in primary prevention [38-41], all of which employ the same enteric-coated 100 mg od regimen and are powered to detect a 15 to 25% relative risk reduction.

We conclude that in aspirin-treated patients: i) the variable rate of turn-over of the drug target represents the main mechanism contributing to the interindividual variability in drug response; ii) the rate of recovery of platelet COX-1 during the 24-hour dosing interval is likely to be influenced by different determinants in patients with T2DM versus those without diabetes; iii) inadequate thromboxane inhibition by low-dose aspirin can be easily diagnosed and corrected by a twice-daily regimen.

Addendum for Author's contribution.

Drs. Patrono and Davì had full access to all the data in this study and take responsibility for the integrity of the data and the accuracy of the data analysis. Drs. Davì, Ghirlanda and Pitocco take responsibilities for the integrity of the clinical data. The funding sources had no role in the design of the study, analysis of the results and drafting of the manuscript.

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Figure Legends

Figure 1. Individual recovery slopes of serum TXB_2 over the 12 to 24 hour interval of low-dose aspirin administration in patients with and without type 2 diabetes.

Panel A: Individual values of serum TXB_2 recovery slopes measured during phase 1 of the study following a witnessed intake of aspirin 100 mg are depicted for 100 patients with T2DM. *Panel B*: Individual values of serum TXB_2 recovery slopes measured during phase 1 of the study are similarly represented for 73 patients without T2DM.

Figure 2. Linear recovery of serum TXB_2 between 12 and 24 hours after aspirin dosing, according to recovery slope tertiles.

Panel A: linear fitting of serum TXB_2 values measured every 3 hours between the 12 to 24 hour interval post-aspirin intake, in diabetic patients (n=34) in the lower tertile of serum TXB_2 recovery slopes. The red diamond represents the median (interquartile range) serum TXB_2 value previously measured in aspirin-treated healthy subjects (HS) [13]. *Panel B*: linear fitting of serum TXB_2 values measured every 3 hours between the 12 to 24 hour interval post-aspirin intake, in diabetic patients

(n=33) in the mid tertile of serum TXB_2 recovery slopes. *Panel C*: linear fitting of serum TXB_2 values measured every 3 hours between 12 and 24 hour post-aspirin intake, in diabetic patients (n=33) in the upper tertile of serum TXB_2 recovery slopes. *Panel D*: linear fitting of serum TXB_2 values measured every 3 hours between the 12 to 24 hour interval post-aspirin intake, in non-diabetic patients (n=24) in the lower tertile of serum TXB_2 recovery slopes. The red diamond represents the median (interquartile range) serum TXB_2 previously measured in aspirin-treated healthy subjects (HS) [13]. *Panel E*: linear fitting of serum TXB_2 values measured every 3 hours between the 12 to 24 hour interval post-aspirin intake, in non-diabetic patients (n=25) in the mid tertile of serum TXB_2 recovery slopes. *Panel F*: linear fitting of serum TXB_2 values measured every 3 hours between 12 and 24 hour post-aspirin intake, in non-diabetic patients (n=24) in the upper tertile of serum TXB_2 recovery slopes.
Serum TXB_2 values are represented as median and interquartile range with the best linear fitting of the five values for each tertile. The median and interquartile range of recovery slopes for each tertile is indicated in the panel.

Figure 3. Correlations between platelet size, serum TXB_2 slopes, percentage of thiazole-orange (TO)-positive platelets and CD36 expression in diabetic patients.

Panel A: Mean Platelet Volume (MPV) values according to distribution of tertiles of serum TXB_2 slopes. *Panel B*: platelets from 41 diabetic patients were studied for TO expression by flow cytometry at 12 and 18 hours after aspirin intake. The figure represents the box-whisker plots of platelet thiazole orange positive fraction at 8 am and 2 pm of day 1 of the study. $^{*}p<0.05$ vs lower tertile. *Panels C and D* depict the correlations between the fraction of TO-positive platelets (presumably, young platelets) and membrane expression of CD36 or MPV, respectively. MFI: mean fluorescence intensity.

Figure 4. Serum TXB_2 recovery slopes in diabetic patients in the upper tertile, before and after the randomized phase of the study.

Thirty-three patients in the upper tertile of serum TXB_2 recovery slopes were randomized to receive aspirin 100 mg once daily, 200 mg once daily or 100 mg twice daily for 28 days. *Panels A and B* depict the linear fittings of the median serum TXB_2 values measured every 3 hours between 12 and 24 hours post-aspirin intake, in phase 1 (A) and phase 2 (B) in the eleven patients randomized to 100 mg once daily. *Panels C and D* depict similar measurements performed in the eleven patients randomized to 200 mg once daily. *Panels E and F* depict similar measurements performed in the eleven patients randomized to 100 mg twice daily. The red diamond represents the median (interquartile range) serum TXB_2 value previously measured in aspirin-treated healthy subjects (HS) [13].

Figure 5. Urinary 11-dehydro TXB_2 excretion and functional platelet response to arachidonate between 12 and 24 hours after aspirin dosing, in diabetic patients according to the randomized aspirin treatments of phase 2.

Thirty-three patients in the upper tertile of serum TXB_2 recovery slopes were randomized to receive aspirin 100 mg once daily, 200 mg once daily or 100 mg twice daily for 28 days. *Panels A and B* depict the median urinary 11-dehydro TXB_2 excretion rates and Verify-Now aspirin response units (ARU) measured between 12 and 24 hours post-aspirin intake.

Figure 6. Serum TXB_2 recovery slopes in non-diabetic patients in the upper tertile, before and after the randomized phase of the study.

Eighteen patients in the upper tertile of serum TXB_2 recovery slopes were randomized to receive aspirin 100 mg once daily or 100 mg twice daily for 28 days. *Panels A and B* depict the linear fittings of the median serum TXB_2 values measured every 3 hours between 12 and 24 hours post-aspirin intake, in phase 1 (A) and phase 2 (B) in the six patients randomized to 100 mg once daily. *Panels C and D* depict similar measurements performed in the eleven patients randomized to 100 mg twice daily. The red diamond represents the median (interquartile range) serum TXB_2 value previously measured in aspirin-treated healthy subjects (HS) [13]

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Table 1. Baseline characteristics of patients with type 2 diabetes (n=100) and non-diabetic (n=73) patients

Variable	Type 2 diabetes	No Diabetes	P value*
Age - years	64.6 (60.7 – 69.0)	68.5 (62.0 – 75.2)	0.014
Male gender – no. (%)	66 (66)	42 (57.5)	0.258
Weight - Kg	74 (68-87)	76 (67-81)	0.515
BMI - Kg/m^2	28.0 (24.2 – 31.0)	27.5 (25.3 - 29)	0.640
Waist circumference - m	0.995 (0.93 – 1.1)	1.02 (0.94 – 1.06)	0.984
WHR	0.95 (0.90 - 1.01)	0.96 (0.88 - 0.99)	0.353
Systolic arterial pressure - mmHg	130 (129 – 140)	135 (122.5 – 145)	0.239
Diastolic arterial pressure - mmHg	80.0 (70 - 80)	80 (70- 87.5)	0.239
Fasting plasma glucose - mmol/L	7.29 (5.89 – 8.53)	4.99 (4.34-5.5)	0.000
Glycated hemoglobin – %	6.6 (6.2-7.1)	5.9 (5.6-6.2)	<0.0001
Diabetes duration – years	9.5 (4- 20)	NA	
Total cholesterol - mmol/L	4.18 (3.66–4.97)	4.94 (4.43–5.64)	<0.0001
LDL cholesterol - mmol/L	2.25 (1.84–2.96)	2.92 (2.38-3.63)	<0.0001
HDL cholesterol - mmol/L	1.16 (0.98–1.45)	1.32 (1.12–1.55)	0.005
Triglycerides - mmol/L	1.41 (0.99–1.9)	1.25 (0.94–1.82)	0.682
AST - U/L	23 (19 – 31)	23 (20-27.5)	0.755
ALT - U/L	24 (18 – 41.5)	27 (22 – 34)	0.473
Platelet count - $10^3/\mu L$	225 (198 - 282)	218 (171-269.8)	0.041
Mean platelet volume - fL	10.6 (9.1-11.5)	10.8 (10.2-11.4)	0.302
Hypertension - no. (%)	74 (74)	57 (78.1)	0.537
Dyslipidemia - no. (%)	43 (43)	40 (54.8)	0.125
Aspirin treatment duration - years	2 (0.8-4)	4 (1-10)	0.004
Cardiovascular disease – n. (%)	46 (46)	35 (47.9)	0.858
Stable CAD	6 (6)	3 (4.1)	0.581
Carotid stenosis (>50%)	14 (14)	7 (9.6)	0.382
MI or revascularization	18 (18)	14 (19.2)	0.844
Stroke, TIA or revascularization	8 (8)	15 (20.5)	0.017

Peripheral artery disease	8 (8)	8 (11.0)	0.508
Diabetic microvascular disease-n. (%)	34 (34)	NA	
Retinopathy	14 (14)	NA	
Nephropathy	21 (21)	NA	
Neuropathy	13 (13)	NA	
Therapy - n. (%)			
Metformin	67 (67)	1 (1.4)	<0.0001
PPAR-γ agonist	10 (10)	0	
Sulfonylurea	14 (14)	0	
Insulin	11 (11)	0	
Meglitinide	6 (6)	0	
Incretins	3 (3)	0	
ACE-I	37 (37)	20 (27.4)	0.186
ARBs	32 (32)	17 (23.3)	0.210
Diuretics	28 (28)	22 (30.1)	0.760
β-blockers	30 (30)	24 (32.9)	0.688
CCB	25 (25)	19 (26.0)	0.879
Other antihypertensive	10 (10)	7 (9.6)	0.929
Statins	55 (55)	33 (48.1)	0.181
Fibrates	5 (5)	1 (1.4)	0.195
PUFA	10 (10)	3 (4.1)	0.143
Proton pump inhibitors	17 (17)	29 (39.7)	0.001

*By Mann-Whitney or Chi-square, as appropriate. Data are expressed as medians and IQR unless otherwise indicated. **Abbreviations**: ACE-I: Angiotensin Converting Enzyme-I; ALT: Alanine Transaminase; ARBs: Angiotensin Receptors Blockers; AST: Aspartate Transaminase; CAD: Coronary Artery Disease; CCB: Calcium channel blockers; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; MI: Myocardial Infarction; PPAR-γ: Peroxisome Proliferator-Activated Receptor-γ; PUFA: Polyunsaturated Fatty Acid; TIA: Transient Ischemic Attack. WHR: Waist-Hip Ratio

Table 2. Correlations between platelet antigens, serum TXB_2 recovery slope and MPV in patients with diabetes.

	COX-1	COX-2	P-selectin	CD36	MPV	TO	Recovery slope
COX-1	--	-0.048 (0.78)	-0.104 (0.62)	-0.223 (0.37)	-0,178 (0.34)	0.024 (0.88)	-0.016 (0.92)
COX-2	-0.048 (0.78)	---	0.671 *(0.001)*	-0.54 *(0.04)*	-0.039 (0.82)	-0.311 (0.06)	-0.074 (0.70)
P-selectin	-0.104 (0.62)	0.671 *(0.001)*	---	-0.55 *(0.018)*	-0.18 (0.39)	-0.53 *(0.008)*	-0.284 (0.17)
CD36	-0.22 (0.37)	-0.54 *(0.04)*	-0.55 *(0.018)*	--	-0.07 (0.71)	0.71 *(0.001)*	0.21 (0.41)
MPV	-0,178 (0.34)	-0.039 (0.82)	-0.18 (0.39)	-0.07 (0.71)	---	0.53 *(0.002)*	0.4 *(0.04)*
TO	0.024 (0.88)	-0.311 (0.06)	-0.53 *(0.008)*	0.71 *(0.001)*	0.53 *(0.002)*	---	0.41 *(0.015)*
Recovery slope	-0.016 (0.92)	-0.074 (0.70)	-0.284 (0.17)	0.21 (0.41)	0.4 *(0.04)*	0.41 *(0.015)*	---

The correlation coefficient (according to Spearman correlation) value is shown and the correspondent P value is given in brackets. For antigens and TO fraction that were measured at 8 am and 2 pm, the values used for assessing correlations are the mean of two determinations. Abbreviations: COX: cyclooxygenase; MPV: mean platelet volume; TO: thiazole orange; TX: thromboxane

The correlation coefficient (according to Spearman correlation) value is shown and the correspondent P value is given in brackets. For antigens and TO fraction that were measured at 8 am and 2 pm, the values used for assessing correlations are the mean of two determinations. Abbreviations: COX: cyclooxygenase; MPV: mean platelet volume; TO: thiazole orange; TX: thromboxane.

Table 3. Baseline characteristics of patients with diabetes according to the randomized aspirin regimen

Variable	**100 mg od**	**200 mg od**	**100 mg bid**	**P value***
No.	11	11	11	
Age - years	60 (53-70)	61 (58-66)	64 (61-68)	0.425
Male gender – no. (%)	8 (72.7)	9 (81.8)	9 (81.8)	0.834
Weight – Kg	81 (66-87)	84 (74-96)	87 (74-93)	0.486
Body-mass index - Kg/m^2	28.1 (23.1-31)	29.7 (28.1-31.4)	31 (25.6-33.8)	0.446
Waist circumference - m	0.99 (0.92-1.07)	1.04 (0.97-1.14)	1.10 (0.97-1.14)	0.165
WHR	0.92 (0.9-1.04)	0.96 (0.94-1.02)	0.99 (0.94-1)	0.383
Systolic arterial pressure - mmHg	130 (120-130)	135 (120-140)	140 (125-140)	0.173
Diastolic arterial pressure - mmHg	80 (70-80)	80 (70-90)	73 (70-80)	0.375
Fasting plasma glucose – mmol/L	6.93 (6.49-8.03)	7.64 (6.71-8.41)	8.47 (7.31-9.46)	0.274
Glycated hemoglobin – %	6.6 (5.8-8.1)	6.7 (6.4-7.3)	6.5 (6.3-7.1)	0.967
Diabetes duration – years	9 (7-15)	5 (2-8)	15 (6-28)	0.088
Total cholesterol - mmol/L	4.37 (3.35-4.70)	4.10 (3.61-6.08)	4.52 (3.43-4.91)	0.963
LDL cholesterol - mmol/L	2.28 (1.87-2.91)	2.18 (1.66-3.56)	2.39 (1.53-3.07)	0.923
HDL cholesterol - mmol/L	1.30 (1.01-1.46)	1.14 (0.96-1.38)	1.06 (0.99-1.61)	0.927
Triglycerides - mmol/L	0.98 (0.76-1.67)	1.69 (1.14-2.05)	1.21 (0.78-1.75)	0.240
AST - U/L	24 (20-31)	31 (19-40)	24 (23-29)	0.868
ALT - U/L	44 (23-45)	32 (24-42)	42 (22-44)	0.860
Platelet count - $10^3/\mu L$	233 (223-296)	217 (187-294)	191 (186-206)	0.151
Mean platelet volume - fL	10.3 (9-10.7)	11.1 (10.6-12.2)	11.9 (10.8-13.5)	0.075
Hypertension - no. (%)	5 (45.5)	9 (81.8)	8 (72.7)	0.170
Dyslipidemia - no. (%)	5 (45.5)	5 (45.5)	5 (45.5)	1.00
Aspirin duration - years	0.8 (0.8-1-5)	1.6 (0.6-3.9)	4 (2.3-4)	0.386
Cardiovascular disease - no. (%)	3 (27.3)	3 (27.3)	4 (36.4)	0.866

Stable CAD	1 (9.1)	1 (9,1)	0	0.587
Carotid stenosis (>50%)				0.137
MI or revascularization	2 (18.2)	1 (9.1)	1 (9.1)	0.752
Stroke, TIA or revascularization	0	0	2 (18.2)	0.119
Peripheral artery disease	0	0	1 (9.1)	0.357
Diabetic microvascular disease – no. (%)	3 (27.3)	6 (54.5)	6 (54.5)	0.333
Retinopathy	2 (18.2)	1 (9.1)	3 (27.3)	0.543
Nephropathy	1 (9.1)	5 (45.5)	5 (45.5)	0.113
Neuropathy	0	1 (9.1)	4 (36.4)	0.047
Therapy - no. (%)				
Metformin	8 (72.7)	9 (81.8)	8 (72.7)	0.848
PPAR-γ agonist	1 (9.1)	2 (18.2)	2 (18.2)	0.790
Sulfonylurea	1 (9.1)	2 (18.2)	5 (45.5)	0.117
Insulin	5 (45.5)	1 (9.1)	5 (45.5)	0.113
Meglitinide	1 (9.1)	2 (18.2)	1 (9.1)	0.752
Incretins	0	1 (9.1)	0	0.357
ACE-I	3 (27.3)	5 (45.5)	5 (45.5)	0.602
ARBs	2 (18.2)	4 (36.4)	3 (27.3)	0.632
Diuretics	3 (27.3)	6 (54.5)	2 (18.2)	0.170
β-blockers	3 (27.3)	4 (36.4)	2 (18.2)	0.632
CCB	0	2 (18.2)	5 (45.5)	0.032
Other antihypertensive	0	3 (27.3)	1 (9.1)	0.137
Statins	5 (45.5)	5 (45.5)	5 (45.5)	1.00
Fibrates	1 (9.1)	0	0	0.357
PUFA	1 (9.1)	1 (9.1)	0	0.587
Proton pump inhibitors	3 (27.3)	2 (18.2)	3 (27.3)	0.848

*By Kruskal-Wallis or Chi-square, as appropriate. Data are expressed as medians and IQR unless otherwise indicated. **Abbreviations**: ACE-I: Angiotensin Converting Enzyme-I; ALT: Alanine Transaminase; ARBs: Angiotensin Receptors Blockers; AST: Aspartate Transaminase; CAD: Coronary

Artery Disease; CCB: Calcium channel blockers; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; MI: Myocardial Infarction; PPAR-γ: Peroxisome Proliferator-Activated Receptor-γ; PUFA: Polyunsaturated Fatty Acid; TIA: Transient Ischemic Attack. WHR: Waist-Hip Ratio.

Table 4. Baseline characteristics of the patients without diabetes in relation to tertiles of serum TXB_2 recovery slope

Variable	1st tertile	2nd tertile	3th tertile	P value*
No.	24	25	24	
Age - years	71 (67-76)	68 (62-76)	63 (58-72)	0.05
Male gender – no. (%)	13 (54.2)	12 (48.0)	17 (70.8)	0.25
Weight - Kg	71 (64-78)	77 (68-80)	79 (69-87)	0.10
Body-mass index - Kg/m^2	27.2 (23.9-28.7)	26.4 (25.3-30.6)	28.3 (25.5-28.9)	0.43
Waist circumference - m	99 (91-104)	103 (96-107)	103 (98-107)	**0.39**
WHR	0.96 (0.88-0.98)	0.94 (0.88-1.00)	0.95 (0.88-0.99)	0.96
Systolic arterial pressure - mmHg	130 (120-143)	135 (128-150)	135 (121-148)	0.62
Diastolic arterial pressure - mmHg	78 (70-87)	82 (78-86)	81 (70-89.5)	0.46
Fasting plasma glucose - mmol/L	5.39 (4.79-5.78)	4.84 (4.24-5.40)	4.90 (4.30-5.34)	0.11
Glycated hemoglobin – %	6.0 (5.8-6.5)	5.8 (5.5-6.2)	5.7 (5.6-5.9)	0.02
Total cholesterol - mmol/L	5.04 (4.52-6.24)	4.94 (4.50-5.62)	4.86 (3.93-5.64)	0.31
LDL cholesterol - mmol/L	3.22 (2.47-3.85)	2.86 (2.50-3.33)	2.78 (1.85-3.61)	0.27
HDL cholesterol - mmol/L	1.38 (1.19-1.56)	1.27 (1.14-1.61)	1.33 (1.09-1.56)	0.79
Triglycerides - mmol/L	1.20 (0.90-2.05)	1.29 (0.88-1.79)	1.21 (1.04-1.47)	0.98
AST - U/L	24 (21-31)	24 (20-25)	22 (18-25)	0.24
ALT - U/L	28 (22-34)	27 (24-31)	27 (21-39)	0.97
Platelet count - $10^3/\mu L$	181 (126-259)	209 (166-275)	228 (186-273)	0.39
Mean platelet volume - fL	10.8 (10.3-11.3)	10.7 (10.3-11.5)	10.7 (8.8-11.5)	0.82
Hypertension - no. (%)	18 (75.0)	21 (84.0)	18 (75.0)	0.67
Dyslipidemia - no. (%)	15 (65.2)	13 (52.0)	12 (50.0)	0.52
Aspirin treatment duration –	3.0 (1.0-10.0)	4.0 (2.0-9.2)	4.0 (0.8-10.0)	0.60

years

Cardiovascular disease - no (%)	9 (37.5)	7 (28)	5 (20.8)	0.44
Stable CAD	3 (12.5)	0	0	0.041
Carotid stenosis (>50%)	3 (12.5)	2 (8.0)	2 (8.3)	0.83
MI or revascularization	4 (16.7)	5 (20.0)	5 (20.8)	0.93
Stroke, TIA or revascularization	6 (25.0)	5 (20.0)	4 (16.7)	0.77
Peripheral artery disease	1 (4.2)	4 (16.0)	3 (12.5)	0.39
Therapy - no. (%)				
ACE-I	6 (25.0)	7 (28)	7 (29.2)	0.95
ARBs	5 (20.8)	8 (32.0)	4 (16.7)	0.42
Diuretics	8 (33.3)	9 (36.0)	5 (20.8)	0.47
β-blockers	7 (29.2)	9 (36.0)	8 (33.3)	0.87
CCB	5 (20.8)	7 (28.0)	7 (29.2)	0.77
Other antihypertensive	0	2 (8.0)	5 (20.8)	0.047
Statins	10 (41.7)	12 (48.0)	11 (45.8)	0.90
Fibrates	0	0	1 (4.2)	0.35
PUFA	1 (4.2)	0	2 (8.3)	0.34
Proton pump inhibitors	11 (45.8)	11 (44.0)	7 (29.2)	0.43

*By Kruskal-Wallis or Chi-square, as appropriate.

Abbreviations: ACE-I: Angiotensin Converting Enzyme-I; ALT: Alanine Transaminase; ARBs: Angiotensin Receptors Blockers; AST: Aspartate Transaminase; CAD: Coronary Artery Disease; CCB: Calcium channel blockers; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; MI: Myocardial Infarction; PUFA: Polyunsaturated Fatty Acid; TIA: Transient Ischemic Attack. WHR: Waist-Hip Ratio.

Table 5. Baseline characteristics of patients without diabetes according to the randomized aspirin regimen

Variable	100 mg od	100 mg bid	P value*
No.	6	11	
Age - years	62 (58-74)	63 (53-67)	0.687
Male gender – no. (%)	4 (66.7)	8 (72.7)	0.799
Weight - Kg	81 (77-103)	79 (66-85)	0.350
Body-mass index - Kg/m^2	28.8 (25.7-40.2)	28.3 (24.2-29.0)	0.482
Waist circumference - m	1.06 (1.03-1.09)	1.01 (0.82-1.04)	0.144
WHR	0.97 (0.86-1.03)	0.98 (0.78-0.99)	0.763
Systolic arterial pressure - mmHg	145 (135-156)	125 (120-132)	0.049
Diastolic arterial pressure - mmHg	81 (68-90)	82 (70-85)	0.685
Fasting plasma glucose - mmol/L	5.00 (3.86-5.61)	4.96 (4.18-5.17)	0.840
Glycated hemoglobin – %	5.6 (5.5-5.8)	5.7 (5.6-6.0)	0.168
Total cholesterol - mmol/L	4.18 (3.69-4.73)	5.09 (4.55-5.67)	0.088
LDL cholesterol - mmol/L	1.98 (1.90-2.86)	3.40 (2.70-4.00)	0.027
HDL cholesterol - mmol/L	1.30 (1.22-1.56)	1.22 (1.12-1.51)	0.545
Triglycerides - mmol/L	1.13 (1.12-1.84)	1.16 (0.94-1.41)	0.801
AST - U/L	23 (21-25)	22 (19-24)	0.880
ALT - U/L	28 (19-39)	30 (21-39)	0.880
Platelet count - $10^3/\mu L$	199 (161-235)	227 (212-271)	0.366
Mean platelet volume - fL	10.4 (8.1-11.5)	11.3 (10.7-11.4)	0.594
Hypertension - no. (%)	4 (66.7)	6 (54.6)	0.638
Dyslipidemia - no. (%)	2 (33.3)	3 (27.3)	0.799
Aspirin treatment duration – years	10 (7-14)	4 (1-10)	0.213
Cardiovascular disease - no. (%)	3 (50)	4 (36.4)	0.596
Stable CAD	0	0	
Carotid stenosis (>50%)	0	1 (9.0)	0.460
MI or revascularization	1 (16.7)	1 (9.0)	0.653
Stroke, TIA or revascularization	2 (33.3)	2 (18.2)	0.495
Peripheral artery disease	1 (16.7)	0	0.176

Therapy - no. (%)			
ACE-I	1 (16.7)	2 (18.2)	0.939
ARBs	1 (16.7)	1 (9.0)	0.653
Diuretics	2 (33.3)	3 (27.3)	0.799
β-blockers	0	4 (36.4)	0.101
CCB	2 (33.3)	2 (18.2)	0.495
Other antihypertensive	2 (33.3)	0	0.048
Statins	3 (50)	4 (36.4)	0.596
Fibrates	0	1 (9.0)	0.460
PUFA	0	0	
Proton pump inhibitors	4 (66.7)	3 (27.3)	0.126

*By Kruskal-Wallis or Chi-square, as appropriate.

Abbreviations: ACE-I: Angiotensin Converting Enzyme-I; ALT: Alanine Transaminase; ARBs: Angiotensin Receptors Blockers; AST: Aspartate Transaminase; CAD: Coronary Artery Disease; CCB: Calcium channel blockers; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; MI: Myocardial Infarction; PUFA: Polyunsaturated Fatty Acid; TIA: Transient Ischemic Attack; WHR: Waist-Hip Ratio.

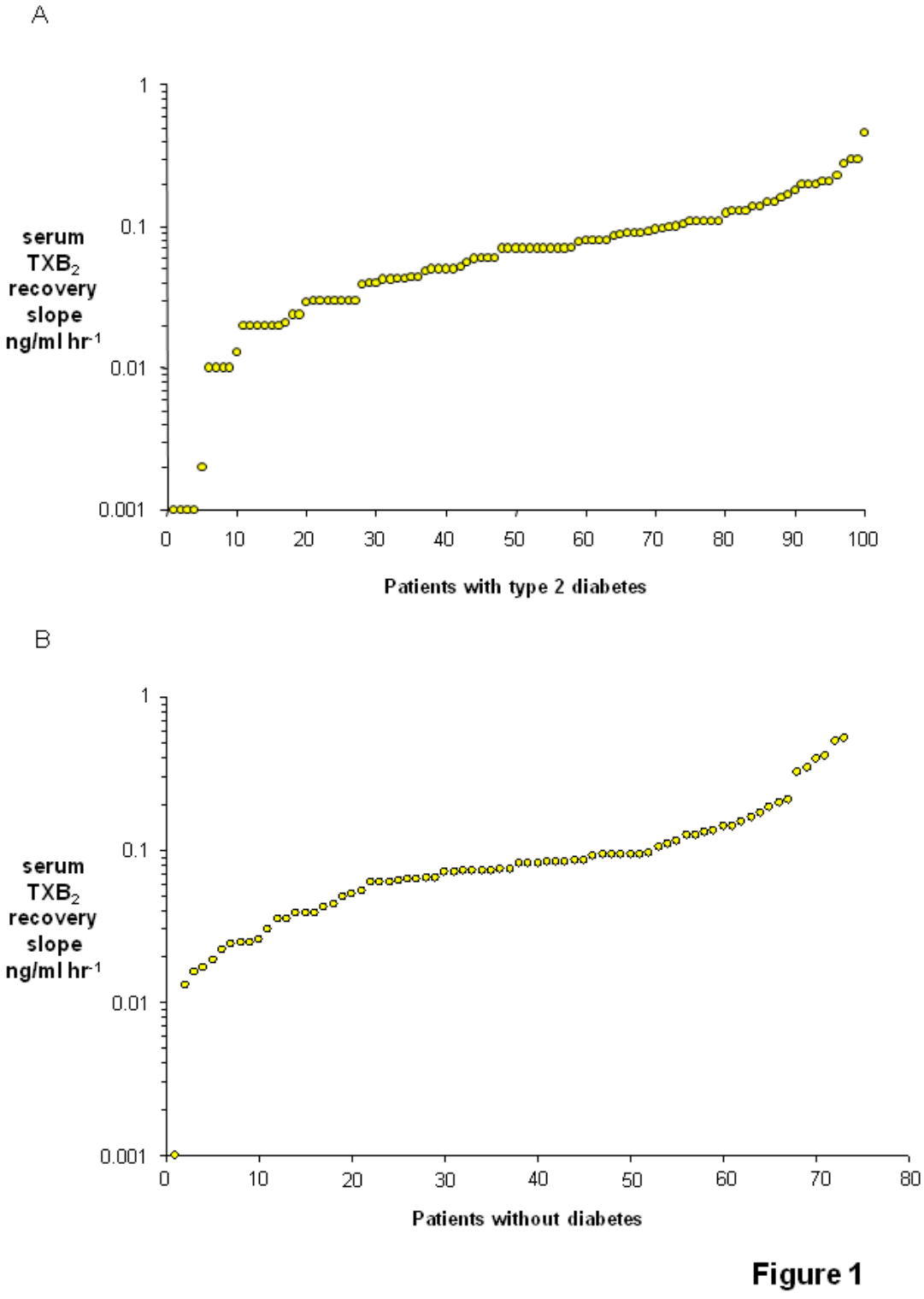


Figure 1

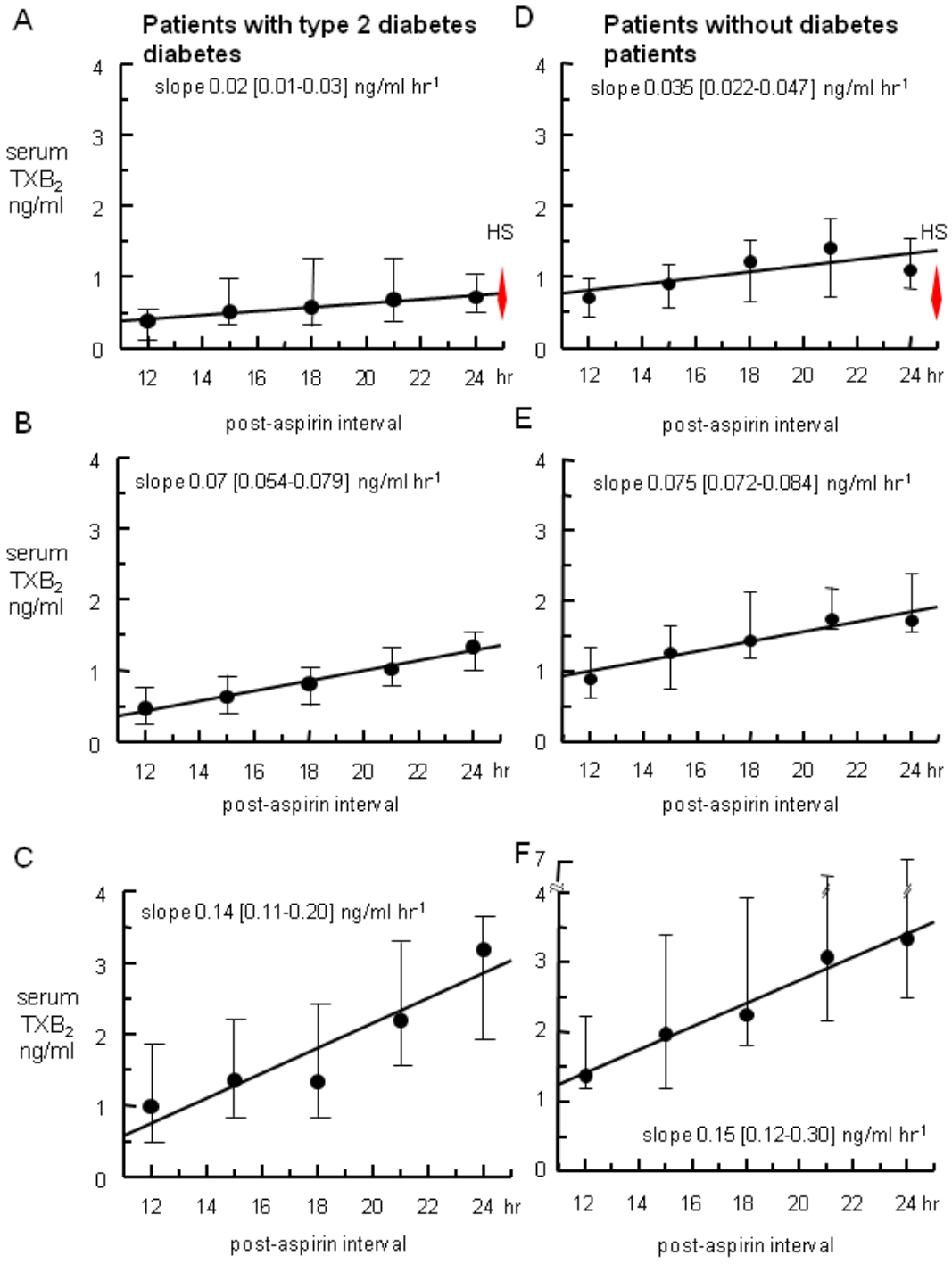


Figure 2

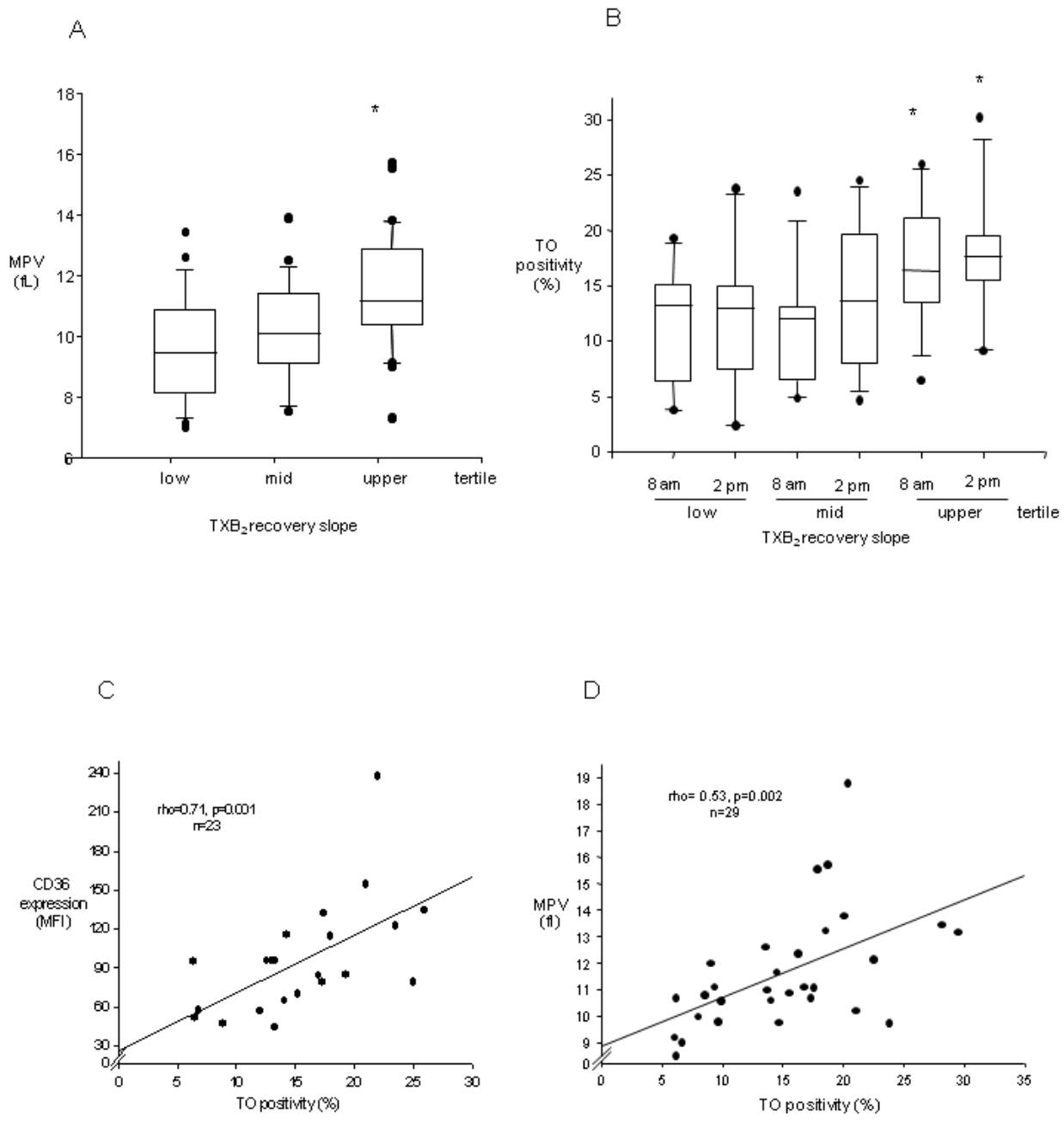


Figure 3

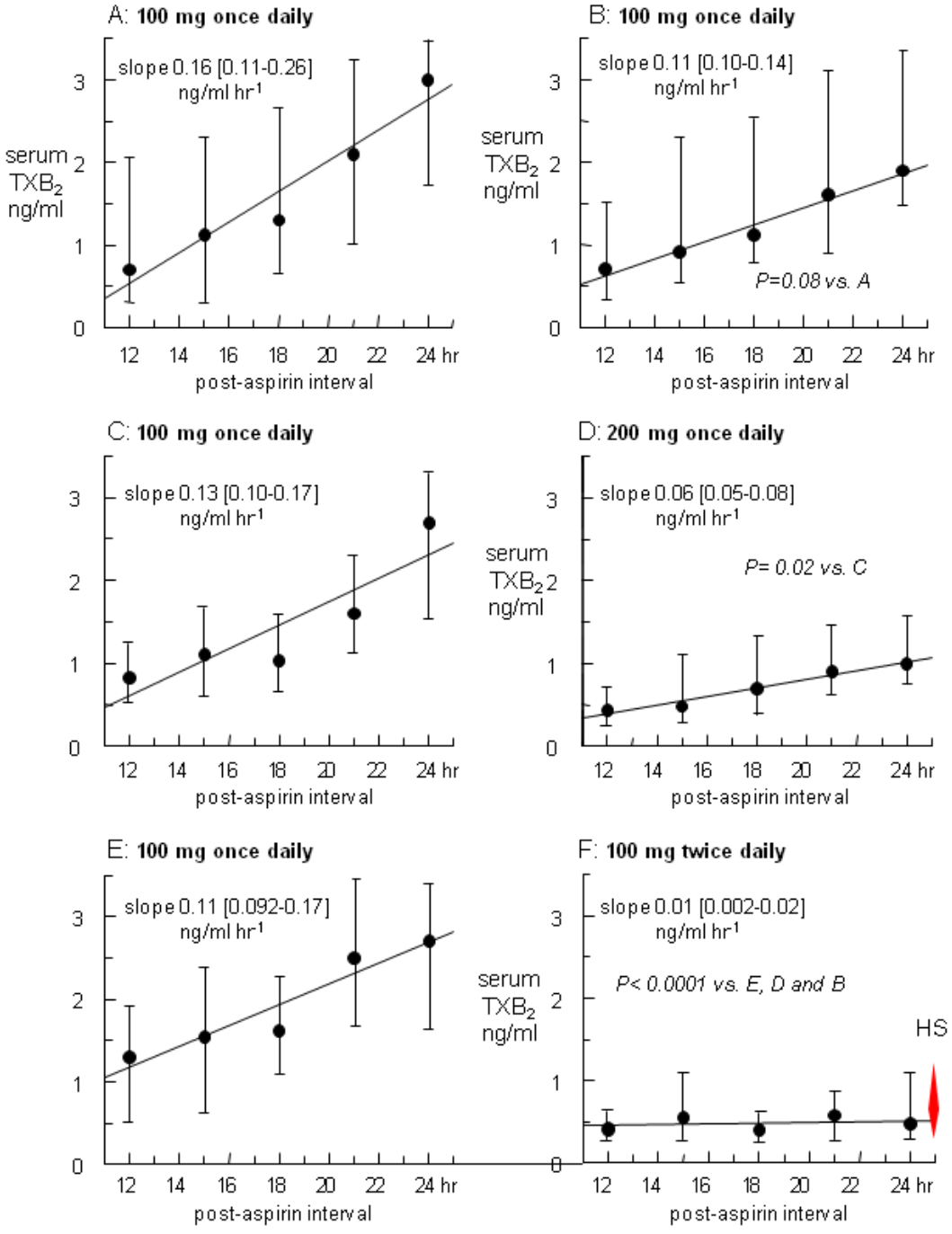
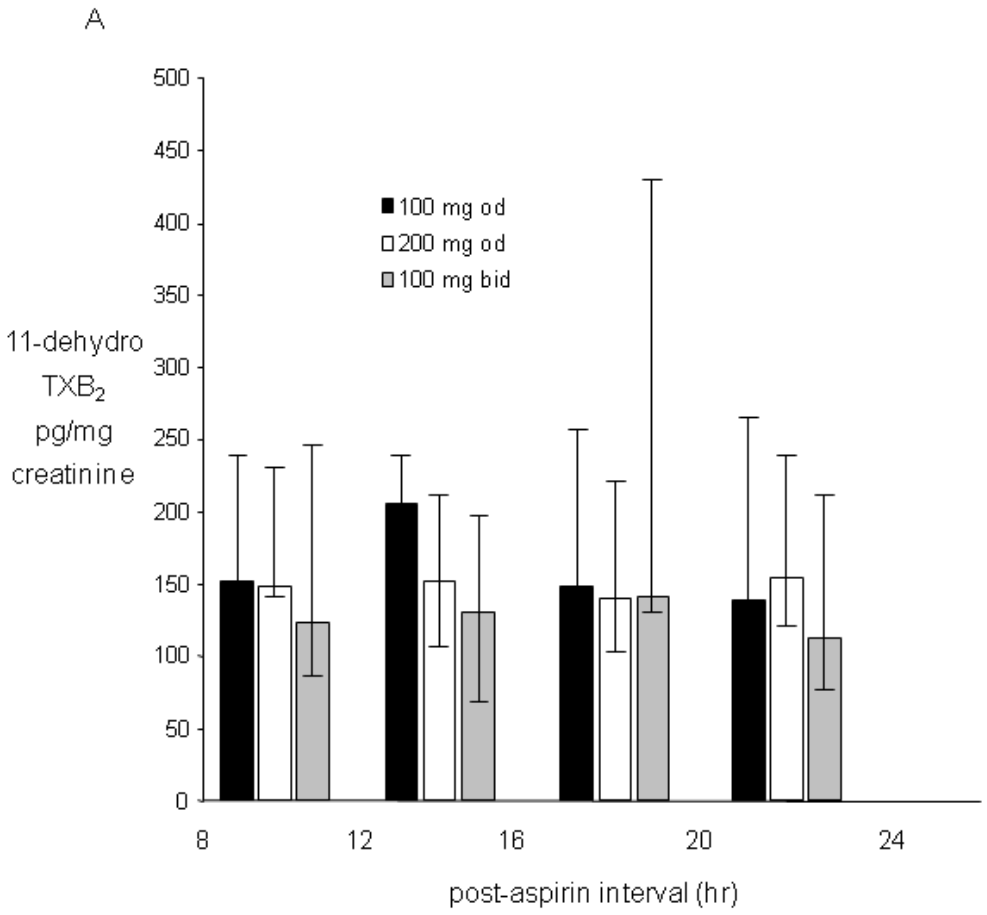


Figure 4



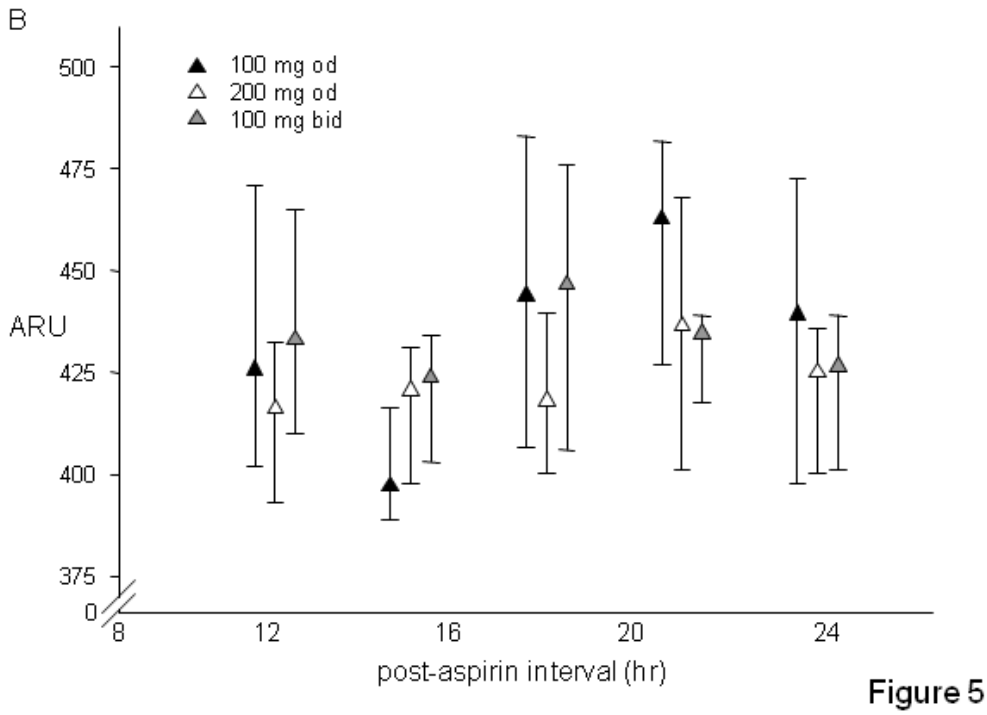


Figure 5

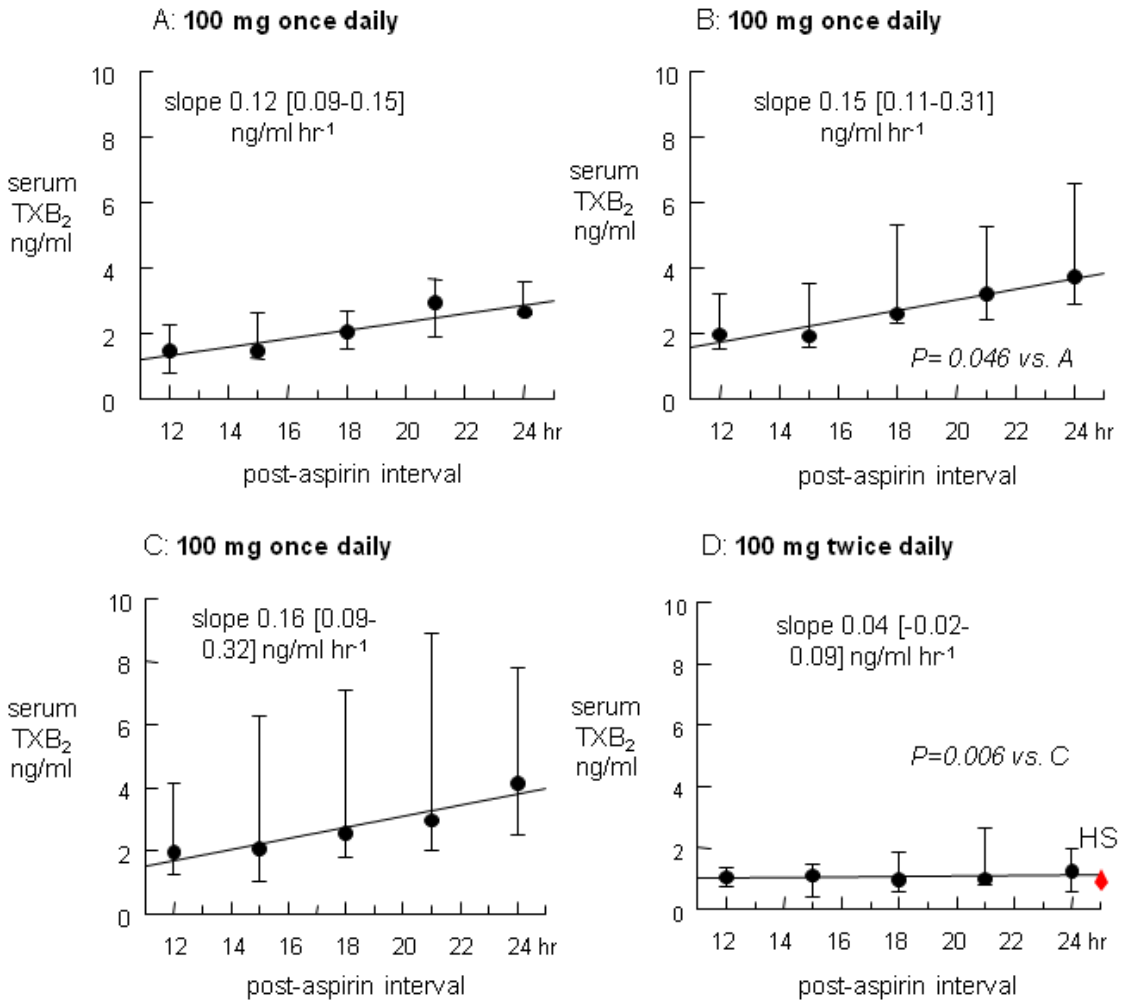


Figure 6