

# Patient-independent variables affecting the assessment of aspirin responsiveness by serum thromboxane measurement

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## Summary

The serum TXB<sub>2</sub> (sTXB<sub>2</sub>) assay reflects the pharmacodynamics of platelet inhibition by low-dose aspirin. However, different studies reported variable sTXB<sub>2</sub> values. sTXB<sub>2</sub> assay requires whole blood incubation at 37 °C as a condition for optimal thrombin generation, arachidonic acid release and its metabolism by platelet cyclooxygenase-1 to form TXA<sub>2</sub>. Access to 37 °C incubation may be variably delayed, and different methods to quantitate sTXB<sub>2</sub> may contribute to variable results between different Centers. We investigated whether delaying 37 °C incubation and/or analytical issues affect sTXB<sub>2</sub> concentrations, biasing the assessment of aspirin responsiveness. Sixty-eight samples from 54 volunteers, on- and off-aspirin, were incubated at 37 °C immediately after sampling (reference sample) or after 5, 10, 15, 20, 30 or 60 minutes at room temperature (RT); 8 samples remained at RT 60 minutes, without subsequent incubation; 314 sera were measured by enzyme immunoassay (EIA) and liquid chromatography-tandem mass-

spectrometry (LC/MS-MS) methods. sTXB<sub>2</sub> concentrations decreased exponentially as a function of the delay before 37 °C incubation, ranging from 94 ± 11 % at 5 minutes to 23 ± 22 % of the reference sample after 60 minutes at RT. There was high agreement between EIA and LC/MS-MS. Moreover, we simulated the influence of a 15- or 30-minute delayed incubation on 300 sTXB<sub>2</sub> measurements from previously-studied, aspirin-treated patients. Delayed incubation reduced the percentage of aspirin 'non-responders' by 22 % to 52 %, depending on the response threshold. In conclusion, a variable delay in the 37 °C incubation of blood samples may affect the assessment of platelet cyclooxygenase-1 inhibition by aspirin and confound the characterization of the determinants of aspirin responsiveness.

## Keywords

Thromboxane B<sub>2</sub>, aspirin, cyclooxygenase-1, liquid chromatography-tandem mass spectrometry, enzyme immunoassay

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## Introduction

The human pharmacology of aspirin as an inhibitor of platelet cyclooxygenase (COX)-1 has been characterised by the *ex vivo* measurement of thromboxane (TX)B<sub>2</sub> generated during whole blood clotting at 37 °C (1, 2). This assay relies on the physiological generation of endogenous thrombin during blood clotting, which triggers the release of arachidonic acid from platelet membranes (3), and its metabolism to TXA<sub>2</sub> via the sequential activity of COX-1 and TX-synthase (4). TXA<sub>2</sub> is rapidly hydrolysed to TXB<sub>2</sub> which can be directly measured in serum by specific immunometric methods (1). Thus, serum TXB<sub>2</sub> (sTXB<sub>2</sub>) reflects the maximal biosynthetic capacity of blood platelets to generate TXA<sub>2</sub> in a COX-1-dependent fashion. This assay allowed to characterise the essential features of platelet COX-1 inactivation by aspirin in humans, i.e. the cumulative nature of sTXB<sub>2</sub> inhibition upon re-

peated daily dosing and its saturability at low doses (5). The latter correctly predicted a ceiling antithrombotic effect of aspirin at daily doses of 75–150 mg, as demonstrated by the results of randomised controlled trials in high-risk patients (6, 7). Moreover, when combined with an analogous whole blood assay for COX-2 activity (8), sTXB<sub>2</sub> measurements *in vitro* and *ex vivo* enabled describing the variable COX-isozyme selectivity of different COX-2 inhibitors (9). The European Medicines Agency (EMA) has indicated sTXB<sub>2</sub> as a surrogate biomarker for assessing bioequivalence of new aspirin formulations (10). Furthermore, increased, residual sTXB<sub>2</sub> levels (>3.1 ng/ml) have been associated with adverse outcomes in a cohort of 700 aspirin-treated coronary artery disease patients (11). Moreover, repeated measurements of sTXB<sub>2</sub> during the 24-hour (h) dosing interval have characterised some of the determinants of reduced aspirin responsiveness in selected clinical settings, such as type 2 diabetes (12) and essential thrombocythemia

(13). However, a recent comparison of sTXB<sub>2</sub> levels in two large cohorts of aspirin-treated patients (11, 14) showed up to 10-fold difference in median sTXB<sub>2</sub> levels between the two study populations (7 and 0.6 ng/ml in the ADRIE (14) and BOSTON (11) studies, respectively) that could not be explained by patient characteristics or analytical biases (15). However, the time interval from blood sampling to 37°C incubation was not recorded in either study (15).

It should be emphasised that TXA<sub>2</sub> is not a circulating substance (max estimated plasma concentration: 1–2 pg/ml) (16), and its abundant presence in serum (300–400 ng/ml in the absence of aspirin) reflects its platelet biosynthesis during whole blood clotting, as the end-product of a chain of enzymatic reactions that are both time- and temperature-dependent (1). In order for this assay to reflect the maximal biosynthetic capacity of blood platelets and its inhibition by COX-1 inhibitors in a reproducible fashion, initiation of whole blood clotting at 37°C must follow peripheral blood sampling without delay. However, a uniform implementation of this procedure in large, multi-center studies might face practical hurdles such as delays between blood withdrawal from patients and access to a thermostatic bath due to logistics as well as to lack of appreciation of the time- and temperature-dependence of TXB<sub>2</sub> production during whole blood clotting. Nevertheless, to what extent a delay in starting 37°C incubation may affect sTXB<sub>2</sub> levels, and therefore may be responsible for variable assessment of aspirin responsiveness across studies, is currently unknown.

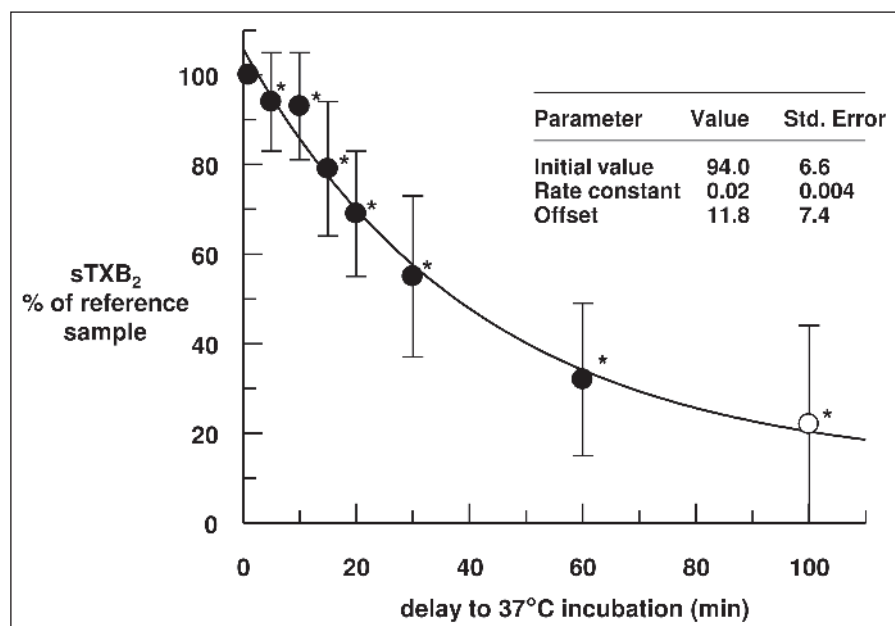
In the present study, we aimed to formally assess and quantitate the influence of this pre-analytical variable on sTXB<sub>2</sub> determinations in both aspirin-naïve and aspirin-treated subjects, and verify the potential contribution of immunological recognition of TXB<sub>2</sub> versus the golden standard of prostanoid measurements, i.e. liquid chromatography-tandem mass-spectrometry (LC/MS-MS), to sTXB<sub>2</sub> variability.

## Materials and methods

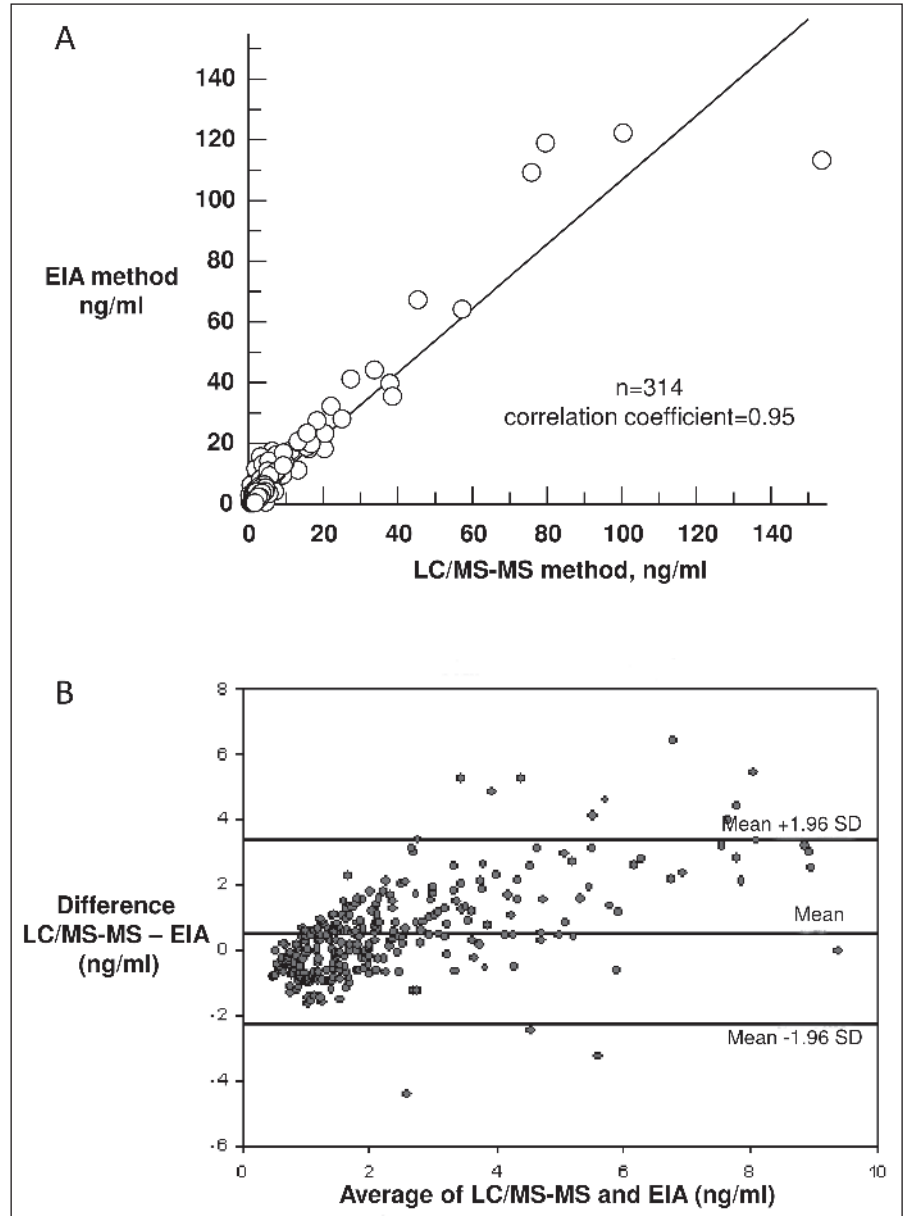
Sixty-eight samples were collected from 54 volunteers (23 females, 31 males, mean age 50 ± 15 years). Twenty-two samples were obtained from 22 aspirin-naïve subjects (3 healthy subjects and 19 diabetic subjects), while 46 samples were obtained from 32 subjects on aspirin (100 mg daily) (14 healthy subjects and 18 diabetic patients) and blood was withdrawn from these subjects between 4 and 72 h after the last aspirin intake. These healthy and diabetic subjects have been enrolled in recently-published (17) or ongoing observational studies (approved protocols: 32/A1668/CE/2009; P/852/CE/2012; PE/1019/CE/2012) on diabetic and healthy subjects. The inclusion of aspirin-treated and -naïve subjects was made on purpose to encompass a wide range of sTXB<sub>2</sub> concentrations. All protocols were conducted in accordance with the Declaration of Helsinki and received Ethics Committee's approvals.

Peripheral blood was withdrawn by a plastic syringe without anticoagulant and immediately dispensed into different glass tubes (1-ml aliquots). One tube was quickly (<3 minutes [min]) placed at 37°C for 1 h into a water bath located in the outpatient Unit and considered as the reference sample. The remaining 1-ml aliquots were kept at room temperature (RT, 21–25°C), from 5–60 min, and then incubated at 37°C for 1 h. After 37°C incubation, all samples were centrifuged at 1,200g for 10 min, the serum supernatant was collected and stored at –20°C until assayed. Moreover, some aliquots were allowed to clot for 1 h at RT without 37°C incubation, centrifuged and the serum was stored until assayed.

Serum TXB<sub>2</sub> was measured by enzyme immunoassay (EIA) as previously described (1, 12), with a specific polyclonal antibody raised against TXB<sub>2</sub> (18). Under our experimental conditions, the EIA had a limit of detection calculated as 80% B/B<sub>0</sub> of 3 ± 2 pg/ml, approximately 100 times below the lowest detectable value in



**Figure 1: Effect of the delay to 37°C incubation on sTXB<sub>2</sub> values.** The graph depicts the best fitting of sTXB<sub>2</sub> values (mean ± SD) measured after increasing delays to 37°C incubation, expressed as % of the matching reference sample (immediately incubated at 37°C) (n=20 at 5 min, n=27 at 10 min, n=19 at 15 min, n=18 at 20 min, n=26 at 30 min, n=24 at 60 min delay). Experimental data were best described by an exponential decay model, whose parameters are shown in the figure. The white dot indicates the mean ± SD of sTXB<sub>2</sub> measured in the eight samples kept at RT for 60 min without subsequent 37°C incubation. \* indicates p<0.05 vs reference sample.



**Figure 2: Comparison between sTXB<sub>2</sub> values measured by the EIA and LC/MS-MS assays.** The plots show the comparison between the sTXB<sub>2</sub> values measured by the EIA and LC/MS-MS methods in 314 serum samples from a biobank. A) Scatter plot between sTXB<sub>2</sub> concentrations measured by each method, and the correlation coefficient value is indicated. B) Bland-Altman analysis of the sTXB<sub>2</sub> values measured by the two methods; the horizontal lines indicate mean, upper and lower 1.96 standard deviations (SD).

aspirin-treated subjects (0.2 ng/ml) (19). Its intra-assay coefficient of variation (CV) was <3%, and the inter-assay CV was <5%.

We also measured 314 sera from our biobank with both the previously described EIA (13) and LC/MS-MS (20) methods. For LC/MS-MS, serum samples were added with deuterated TXB<sub>2</sub> (TXB<sub>2</sub>-d4) as internal standard, and the solid-phase extraction was performed before resolution onto a reversed-phase XBridge C18 column (Waters, Milan, Italy). Detection was made by a negative ion electrospray ionisation-tandem MS (TSQ, Thermo Fisher, San Jose, CA, USA). The inter- and intra-assay CV of LC/MS-MS were <3% and the lower limit of quantification, i.e. the lowest concentration that could be reliably and reproducibly measured, was 0.24 ng/ml (20).

In addition, we simulated the effect of a delayed access to 37°C incubation on the assessment of aspirin responsiveness, and con-

sidered sTXB<sub>2</sub> measurements obtained in 300 patients from our previously published studies (12, 13, 17, 21), all sampled 24 h after the last witnessed aspirin administration. The measurement of sTXB<sub>2</sub> from these patients was performed in the same Laboratory, following the same pre-analytical procedure, i.e. blood was immediately incubated at 37°C after sampling without delays, and using the same EIA method and reagents.

**Statistical analysis**

A sample size of 20 determinations was calculated in order to assess as statistically significant a sTXB<sub>2</sub> reduction ≥10% after the shortest 37°C delay, i.e. 5 min, with respect to the reference value, assuming a 15% standard deviation (SD), and 80% power and alpha=0.05. Data were compared by Friedman test for related

samples and by paired t-test, and reported as mean  $\pm$  SD or median and inter-quartile range [IQR] according to their distribution. Serum TXB<sub>2</sub> levels were analysed as absolute values or as a percentage of the reference sample, as indicated. Experimental measurements were fitted with the Grafit 7 (Erithacus Software, Staines,

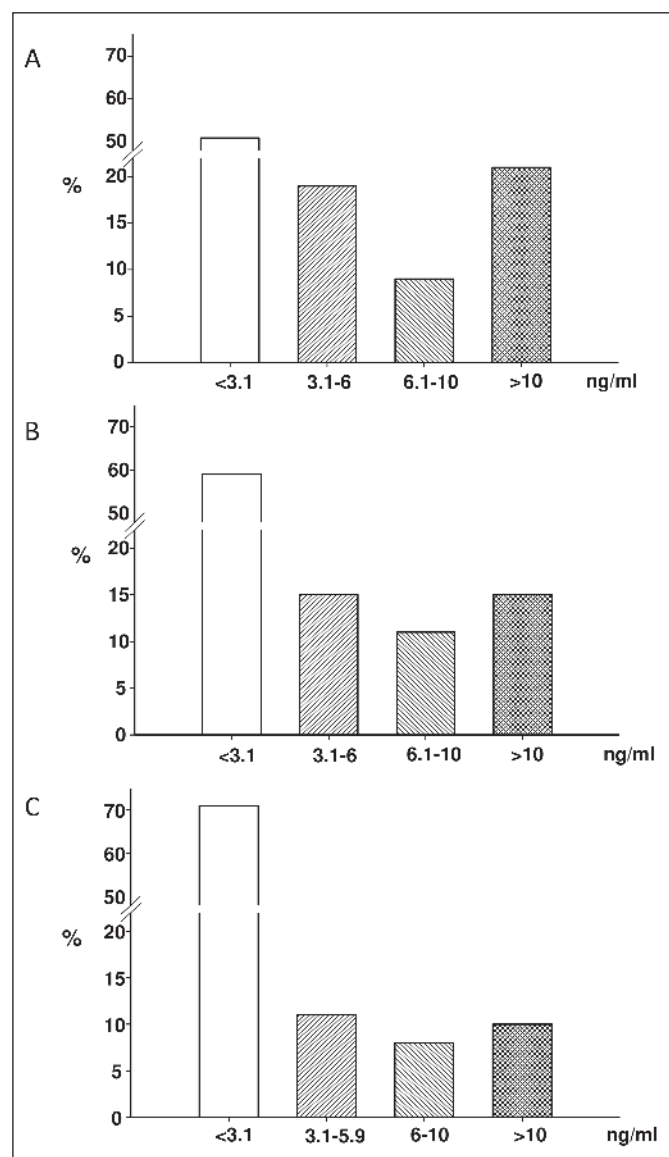
UK). The two analytical methods were compared by the Bland-Altman method (22). The concordance of the two methods in identifying potential „non-responder“ samples was assessed by contingency tables using different cut-offs, as indicated.

## Results

The mean sTXB<sub>2</sub> of reference samples (n=68), immediately incubated at 37°C was  $137 \pm 170$  ng/ml (mean  $\pm$  SD; min 0.99 ng/ml–max 585 ng/ml). The broad range of sTXB<sub>2</sub> values reflected the inclusion of samples from aspirin-naïve subjects ( $318 \pm 157$  ng/ml, n=22) and samples which were collected between 4 and 72 h after aspirin withdrawal ( $36 \pm 73$  ng/ml, n=46) to explore the effect of delayed 37°C incubation over a wide range of sTXB<sub>2</sub> concentrations. Serum TXB<sub>2</sub> values were compared by the Friedman test and included the reference samples and the corresponding samples kept for 5 (n=20), 10 (n=27), 15 (n=19), 20 (n=18), 30 (n=26) or 60 (n=24) min at RT before 37°C incubation. There was a statistically significant difference among groups (p<0.001), and between sTXB<sub>2</sub> of reference samples versus the corresponding samples at each time-interval delay. The slopes of the linear fittings of absolute sTXB<sub>2</sub> values measured in reference samples versus the corresponding values from samples at RT for different time intervals, decreased as a function of increasing delay to 37°C incubation, with slope values of 0.92, 0.58, 0.56 and 0.22 at 10, 20, 30 and 60 min delays, respectively. Thus, the dependence of the two sets of measurements significantly and time-dependently declined, with a substantial drop of concentration at time points >10 min.

To investigate the overall kinetics of the sTXB<sub>2</sub> decay independently of the absolute values of the reference sample, we analysed sTXB<sub>2</sub> data as percentage of the reference samples. As shown in ► Figure 1, the best fitting of the experimental data as a function of 37°C-incubation delay was an exponential rather than a linear decay. The same pattern of an exponential decay was also observed when aspirin-naïve and on-aspirin samples were considered separately. Notably, at 5 and 10 min, the sTXB<sub>2</sub> levels were  $94 \pm 11\%$  and  $93.7 \pm 12\%$  of the reference values (p<0.05), and by 30-min delay sTXB<sub>2</sub> values were approximately halved ( $55 \pm 17\%$ ) as compared to the concentration of the corresponding reference sample (► Figure 1). Moreover, samples kept for 1 h at RT had the lowest sTXB<sub>2</sub> values averaging  $32 \pm 17\%$  (n=24) and  $23 \pm 22\%$  (n=8) of reference samples (p<0.001 vs reference), in the presence or in the absence of subsequent 37°C incubation, respectively.

We also assayed 314 serum samples by both LC/MS-MS and EIA methods, and the results of the matched determinations are shown in ► Figure 2. The correlation between the two methods was highly significant (correlation coefficient: 0.95; n=314, p<0.0001, ► Figure 2A) without statistically significant differences between the two datasets (p=0.3 for paired comparisons). Based on the consideration that these assays might be used to investigate aspirin responsiveness (11, 14, 23, 24), the concordance between methods was assessed by the Bland-Altman analysis within a sTXB<sub>2</sub> range between 0.2 and 10 ng/ml (n=278 determinations). In this range we observed an absolute difference of 0.5 ng/ml between the two methods (► Figure 2B),



**Figure 3:** Effect of different delays in starting 37°C incubation on the estimate of aspirin responsiveness, according to different thresholds of sTXB<sub>2</sub>. A) Distribution (indicated as % of all determinations) of the sTXB<sub>2</sub> values measured in 300, aspirin-treated patients from previous studies (12, 13, 17, 21) (30 type 1 diabetes patients, 100 type 2 diabetes patients, 73 high-risk patients, 97 essential thrombocythaemia patients) where patient samples were immediately incubated at 37°C after blood withdrawal, by using different arbitrary thresholds: <3.1 ng/ml, from 3.1 to 6 ng/ml, from 6.1 to 10 ng/ml and >10 ng/ml. B) Distribution of sTXB<sub>2</sub> values according to the above-indicated thresholds, in the same samples, by simulating a 15-min delay in starting 37°C incubation. C) Distribution of sTXB<sub>2</sub> values in the same samples according to the above-indicated thresholds, by simulating a 30-min delay in starting 37°C incubation.

with 96% of the determinations (266 out of 278) comprised within the  $\pm 1.96$  SD, which indicates a high concordance between the two assays. We further analysed the concordance of the two methods in identifying values of poor responsiveness to aspirin by using two different sTXB<sub>2</sub> thresholds: >6 ng/ml or >10 ng/ml, which represent the upper limits (mean + 2SD) of values we measured in healthy subjects treated for three weeks with aspirin 100 mg daily, 12 h after the last witnessed intake ( $2.8 \pm 1.6$  ng/ml) (17), and at the end of the 24-h dosing interval ( $4.2 \pm 3$  ng/ml) (17), respectively. We observed a concordance of 91% and 95% between the two analytical methods in identifying samples above the 6 ng/ml or 10 ng/ml thresholds, respectively.

Finally, on the basis of the above results, we re-considered sTXB<sub>2</sub> measurements of a cohort of 300 patients from our own previously-published studies all on aspirin (100 mg once daily) (12, 13, 17, 21), from poorly-responsive clinical settings, and we simulated how sTXB<sub>2</sub> concentrations would have changed in these patients if samples had been subjected to a 15-min or to a 30-min delay prior to 37°C incubation. The 15- and 30-min delays would have likely caused a relative reduction of 22% and 45% of sTXB<sub>2</sub> values, respectively, as compared to samples immediately incubated at 37°C, and a delayed incubation of whole blood samples would have significantly dwarfed the estimate of the relative fraction of „poor-responder“ samples (and patients), regardless of the arbitrary sTXB<sub>2</sub> threshold used to define aspirin responsiveness, as shown in ► Figure 3.

## Discussion

Altogether, our data clearly show that pre-analytical processing of blood samples, namely a variable delay in starting the 37°C incubation, significantly influences sTXB<sub>2</sub> values, due to a time-dependent progressive decrease in the capacity of platelet COX-1 to maximally synthesize TXA<sub>2</sub> during whole blood clotting. A statistically-significant difference, albeit minimal (~6%), is already observed after a delay of 5 min, while beyond 10 min, sTXB<sub>2</sub> values decay exponentially, rather than linearly, as shown in ► Figure 1. This pre-analytical bias seems quite relevant and is independent of the sensitivity and specificity of the analytical assay used to measure sTXB<sub>2</sub>. Our data showing a good agreement between our EIA and LC/MS-MS determinations (► Figure 2) are consistent with a previous similar comparison between an immunoenzymatic assay different from the one we used which was also compared to LC/MS-MS (15).

Therefore, a delayed 37°C incubation will result in a variable, but potentially substantial, under-estimation of sTXB<sub>2</sub> values. This underestimate can indeed influence the assessment of aspirin responsiveness from patients, which is typically based on variable sTXB<sub>2</sub> thresholds (11, 14, 15, 23, 25) as shown by our simulation of progressive delays to 37°C in 300 aspirin-treated patients from our own previously-published studies (12, 13, 17, 21). These patients belong to poorly-responsive clinical settings, such as type 2 diabetes and essential thrombocythemia. We simulated the changes in sTXB<sub>2</sub> concentrations if samples had been subjected to a 15-min

(22% reduction) or to a 30-min (45% reduction) delay prior to 37°C incubation. As shown in ► Figure 3, a delayed incubation of whole blood samples would significantly affect the estimate of „poor-responder“ patient fractions, regardless of the arbitrary sTXB<sub>2</sub> thresholds. In fact, a simulated delay in starting 37°C incubation, reduced the relative percentage of aspirin ‘non-responders’ by 22% to 52%, depending on the response threshold (► Figure 3). This might have contributed to some large discrepancies of sTXB<sub>2</sub> observed among different studies (15), as well as to different clinical characteristics of poorly-responsive populations (11, 14).

An under-estimation of sTXB<sub>2</sub> is also relevant from a regulatory perspective, considering that the EMA indicates TXB<sub>2</sub> measured in serum (not in plasma) as a surrogate parameter required to demonstrate the pharmacodynamic equivalence and non-inferiority of new modified-release aspirin formulations, and this parameter is sufficient to grant the indication for secondary prevention of cardiovascular events (10). In particular a ‘close to 100% sTXB<sub>2</sub> inhibition’ is mandatory to demonstrate the non-inferiority. Furthermore, the assessment of the percentage of subjects defined as ‘responders’ is required as an additional secondary parameter for approval, according to the EMA (10). If we consider our simulation showed in ► Figure 3, the estimate of responders (<3.1 ng/ml) at 24 h from aspirin intake, increases by nearly 50% (from 50% to 72%) in samples with 30 min delay to 37°C as compared to reference measurements.

In conclusion, the pre-analytical handling of whole blood samples is a major determinant of the accuracy in assessing the maximal, platelet-derived TXA<sub>2</sub> production in response to endogenously formed thrombin and its inhibition by aspirin (and other COX-1 inhibitors). The use of sTXB<sub>2</sub> as a biomarker of aspirin responsiveness requires standardisation of the pre-analytical procedures as well as LC/MS-MS validation of its immunological recognition by commercially available anti-TXB<sub>2</sub> sera.

### What is known about this topic?

- The serum TXB<sub>2</sub> (sTXB<sub>2</sub>) assay reflects the pharmacodynamics of platelet inhibition by low-dose aspirin.
- sTXB<sub>2</sub> assay requires whole blood incubation at 37°C as condition for optimal thrombin generation, arachidonic acid release and its metabolism by platelet cyclooxygenase-1, to form TXA<sub>2</sub>.
- Rapid access to 37°C incubation might be not always feasible in large or multicentre studies.

### What does this paper add?

- sTXB<sub>2</sub> concentrations decrease exponentially as a function of the delay in starting 37°C incubation, already after 5 min.
- Delayed incubation might bias the estimate of the fraction of aspirin ‘non-responder’ patients.
- Pre-analytical, patient-unrelated variables may explain the heterogeneity among studies and confound the characterisation of the determinants of poor platelet inhibition.

## Abbreviations

COX: cyclooxygenase; EIA: enzyme immunoassay; EMA: European Medicines Agency; LC/MS-MS: liquid chromatography-tandem mass spectrometry; TX: thromboxane; RT: room temperature.

### Author contributions

BR, CP and ET contributed to study concept and design. DP, RP, AR, FZ coordinated the research on patients and clinical protocol. GP, VC, AH, FP acquired experimental data. BR, FZ and FV performed statistical analysis and interpretation of data. CP and ET critically revised the manuscript and provided relevant intellectual content. BR, CP, DP and FZ drafted the manuscript. All the Authors have approved the final version of the manuscript and vouch for the completeness and accuracy of the reported data.

### Conflicts of interest

GP, AR, DP, FZ, PR, VC, FV, AH, FP, ET declare no conflict of interest. BR received honoraria from MSD-Italia. CP received speaker fee from Astra-Zeneca, Institutional grant for Investigator-initiated research and speaker fees from Bayer.

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