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ORIGINAL ARTICLE

Stability of the thromboxane B_2 biomarker of low-dose aspirin pharmacodynamics in human whole blood and in long-term stored serum samples

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Abstract

Background: Serum thromboxane B_2 ($sTXB_2$) is a validated biomarker of low-dose aspirin pharmacodynamics. In the original method, nonanticoagulated blood samples must be incubated at 37 °C immediately after withdrawal, centrifuged and serum supernatant should be frozen until assayed. Timely completion of all preanalytical steps may affect the feasibility and quality of $sTXB_2$ measurements. The storage duration of frozen serum can also affect $sTXB_2$ stability.

Objectives: We assessed the stability of $sTXB_2$ in clotted blood samples stored at 4 °C before further processing and in sera stored at −40 °C for over a decade.

Methods: Venous whole blood withdrawn from individuals on chronic low-dose aspirin was dispensed in different tubes and immediately incubated at 37 °C for 1 hour. The reference tube was promptly processed following the original protocol; the remaining tubes were stored at 4 °C for 12 to 72 hours before further processing. Sera stored at a controlled −40 °C temperature for <1 to 15 years were reassayed. Values within the interassay variation limits (±9%) vs baseline were considered acceptable.

Results: Baseline $sTXB_2$ values (median, 5.4 ng/mL; IQR, 2.4-13.4 ng/mL; n = 40) were comparable with those in samples at 4 °C up to 48 hours (median, 97% [IQR, 86%-104%] of the reference; n = 26), but at 72 hours, the variability exceeded the interassay variation. Thromboxane B_2 levels were stable in frozen sera for up to 10 years (median, 101% [IQR, 87%-108%] of the reference; n = 32) but decreased significantly afterward (median, 87% [IQR, 74%-109%] at 15 years; P = .005; n = 32).

Conclusion: Thromboxane B_2 is stable in clotted blood samples stored at 4 °C for up to 48 hours before further processing and in serum samples stored at −40 °C over 10 years.

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KEYWORDS
aspirin, assay, biomarkers, study feasibility, platelet activation, stability, thromboxane A_2, serum thromboxane B_2

Essentials

- Preanalytical handling and storage length may alter thromboxane (TX) stability in collected human blood.
- TXB_2 level was stable in clotted blood samples kept at 4 °C for 2 days before further processing.
- TXB_2 level was stable for 10 years in sera stored at −40 °C and then markedly decreased.
- These data may improve feasibility and quality of TXB_2 measures in large, long-term studies.

1 | INTRODUCTION

Cyclooxygenase (COX) enzymes in activated platelets convert arachidonic acid (AA) into prostaglandin G/H_2, which is further transformed into thromboxane (TX) A_2 by the TX synthase [1]. TXA_2 is a short-lived potent platelet activator, acting as an autacoid, which is rapidly and nonenzymatically hydrated into TXB_2. TXB_2 is stable, biologically inactive, and can be measured in all aqueous biological fluids [2].

Low-dose (ie, 75-100 mg once-daily) aspirin exerts its antithrombotic action by inhibiting >98% COX-dependent TXA_2 released from activated platelets [3]. The measurement of serum TXB_2 ($sTXB_2$) generated during whole blood clotting *ex vivo* reflects the maximal enzymatic capacity of platelet's COX activity and therefore is the reference pharmacodynamic (PD) biomarker of low-dose aspirin [4]. During whole blood clotting *ex vivo*, thrombin is maximally generated and activates the platelet's phospholipase (PL) A_2 to release AA from the membrane's phospholipids. AA in platelets is rapidly converted via the sequential activities of COX and TX synthase into TXA_2 and then nonenzymatically into TXB_2 in the collecting tube [4]. The measurement of $sTXB_2$ has been instrumental in describing the clinical pharmacology of aspirin at low doses in cardiovascular prevention and treatment [2], and this PD biomarker is part of the data required by the major medicines agencies to approve new aspirin formulations [5,6] or to release boxed warnings [7], since $sTXB_2$ is considered a surrogate of clinical efficacy.

In the original method of the $sTXB_2$ assay described by Patrono et al. [4], whole blood collected without anticoagulants should be immediately incubated at 37 °C for 1 hour [8], then clotted blood samples must be centrifuged to separate the serum supernatant, which is usually frozen to be stored until measurement. The feasibility of this assay in large observational studies and trials is limited by the need to timely perform all the preanalytical steps after blood withdrawal to avoid preanalytical artifacts. However, the 24/7 feasibility can be limited in multicentric studies by the logistics (ambulatory, laboratory, and storage rooms in different places) and availability of different personnel, especially at night, on weekends, and on holidays. We had already demonstrated that blood should be incubated at 37 °C within <5 minutes after withdrawal and that the incubation temperature significantly affects the quality of the results in both aspirin-treated and -naïve subjects [8,9].

Thus, the feasibility of measuring $sTXB_2$ as a PD biomarker of low-dose aspirin in large studies could be improved by the possibility of storing clotted blood samples after incubation at 4 °C for several hours until transportation, centrifugation, separation, and storage are possible, as may happen during nonoperating hours of laboratories (nights, holidays, or weekends). Moreover, the long-term stability of TXB_2 in frozen serum samples is another critical preanalytical variable, especially in long studies or when measurements from multicenter studies are centralized, which may create artifacts affecting the results. However, the long-term stability of $sTXB_2$ in frozen samples has never been explored.

Thus, we investigated the stability of $sTXB_2$ in whole blood samples timely incubated at 37 °C and kept at 4 °C for 12 to 72 hours before subsequent processing, ie, centrifugation and storage. We also investigated the long-term stability of TXB_2 in frozen sera that were stored for up to 15 years.

2 | METHODS

2.1 | Stability of TXB_2 in clotted whole blood samples

Peripheral blood was dispensed in multiple tubes without anticoagulant (2 mL per tube, Vacuette Z Serum Clot Activator Tube, Greiner Bio-One) from 40 patients on chronic 100 mg daily aspirin (18 males and 22 females). Tubes were immediately incubated in a water bath at 37 °C for 1 hour, as in the original protocol [4], then 1 tube (reference sample) was immediately centrifuged (1200 × *g*, 10 minutes at room temperature; 5702 Centrifuge, Eppendorf), the serum supernatant was collected, aliquoted into 500 mL samples, frozen, and stored at −40 °C under controlled temperature (PDF 440W, EVERmed, Medical Refrigeration; MN and KBPF600 PP, KW Apparecchi Scientifici) until first measurement. The other clotted tubes (1 or 2) were kept in the capped tubes at 4 °C for 12, 24, 48, or 72 hours before further processing, ie,

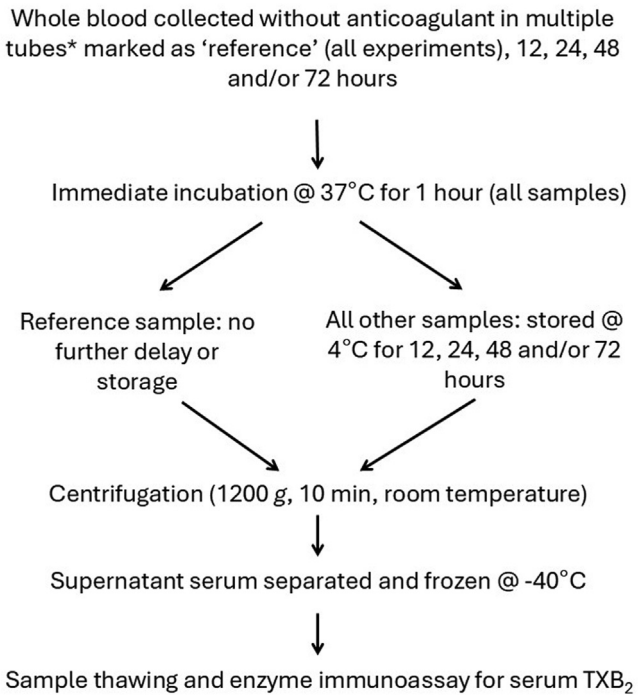

FIGURE 1 The figure shows the experimental design of the study of the short-term stability of serum thromboxane (TX) B_2 in samples incubated at 4 °C at different time intervals after whole blood clotting. *Collection tubes were Vacuette Z Serum Clot Activator Tube (Greiner Bio-One).

centrifugation for serum separation and storage (Figure 1). In the original method, venous blood was collected in a syringe with a straight needle technique and then dispensed from the syringe to the glass tubes [4]. However, nowadays, this 2-step technique of venous blood collection and dispensing has been replaced by the safer vacutainer systems. Thus, in preliminary experiments, we compared different vacutainer systems with tubes without anticoagulant with the traditional syringe-glass tube technique. The $sTXB_2$ values in blood collected with Vacuette Z Serum Clot Activator Tubes were highly correlated with those measured with blood collected and dispensed according to the original technique (n = 35; rho = 0.96; $P < .0001$).

$sTXB_2$ was measured by a standard enzyme-linked immunosorbent (ELISA) assay, as previously described [8], using a specific polyclonal antibody; the ELISA method was previously validated by liquid chromatography/tandem mass spectrometry [8]. The absolute lower limit of detection, calculated as 80% bound/maximum bound, was 5.5 pg/mL (5.34-5.88 pg/mL; n = 104 plates), which is similar to the limit of detection of commercial kits (5 ng/mL) [10]. Samples collected from the same individual (reference samples and those kept at 4 °C) were always measured in the same 96-well ELISA plate. The interassay variability was calculated as the interassay coefficient of variation (CV) of repeated measurements (n = 211) of the same commercial standard (Cayman Chemicals) and was 9%. Moreover, the CV of serum samples from an individual on low-dose (100 mg daily) aspirin was 9% (n = 273; absolute mean, 3 ± 0.27 ng/mL) and that from an individual off aspirin was 10.9% (n = 193; absolute mean, 628 ± 69 ng/mL).

2.2 | Stability of TXB_2 in stored frozen sera

One hundred twenty-eight serum samples (approximately 500 μL each) from healthy individuals or patients on low-dose aspirin were collected and measured in previously published studies [11–16] (baseline measure) and then immediately frozen and stored at −40 °C under controlled temperature (PDF 440W, EVERmed, Medical Refrigeration; MN and KBPF600 PP, KW Apparecchi Scientifici) from <1 to 15 years. Samples were quickly thawed for the second measurement.

Both the first and second measurements of $sTXB_2$ were performed using the same ELISA method and plate preparation. In particular, we always used the same plates (Thermo Scientific Nunc Microplate Immuno Maxisorp) that were coated with a primary commercial mouse anti-rabbit antibody (Bertin Bioreagent), and we always used the same polyclonal anti-TXB_2 antibodies and the same revealing acetylcholinesterase conjugate commercial system (always from Cayman Chemical) [8].

All previously published studies included in this work were approved by the local Ethics Committee and performed following the Helsinki Declaration.

2.3 | Statistical analysis

As the CV of repeated measurements of the same sample with the ELISA method (interassay variation) was ±9%, calculated both by using a commercial standard (28.5 ± 2.6 ng/mL; n = 210 plates) as well as an on-aspirin serum (7.02 ± 0.62 ng/mL; n = 273), the sample size per time point was computed to comply with the equivalence hypothesis considering an absolute change of 4% with an SD of 9% and an equivalence margin of 10%. Under the above hypotheses, the number of samples to be analyzed, with α = 0.05 and 90% power, was 29.

Data were reported as median with IQR according to their distribution, and comparisons were performed using Wilcoxon test for paired data. Correlations were analyzed by Spearman rank tests according to data distribution. The associations between measurements were also estimated by a linear regression model (second measurement = b × first measurement + a). The significance was set as $P < .05$.

3 | RESULTS

3.1 | Stability of TXB_2 in clotted whole blood samples

In samples kept at 4 °C for 12 hours before further processing (n = 28), $sTXB_2$ values were comparable with their reference samples (5.1 [2.4-13.4] ng/mL and 5.4 [2.4-12.7] ng/mL, respectively; P = .84; Figure 2A) and were highly correlated (rho = 0.99; $P < .001$; Figure 2B), with $sTXB_2$ levels being 94% (90%-103%) of their reference sample (b = 0.89; $P < .001$). Similarly, $sTXB_2$ values in samples stored at 4 °C for 24 hours (n = 27) were comparable with their reference samples (5.0 [2.3-12.4] ng/mL and 5.3 [2.4-12.2] ng/mL,

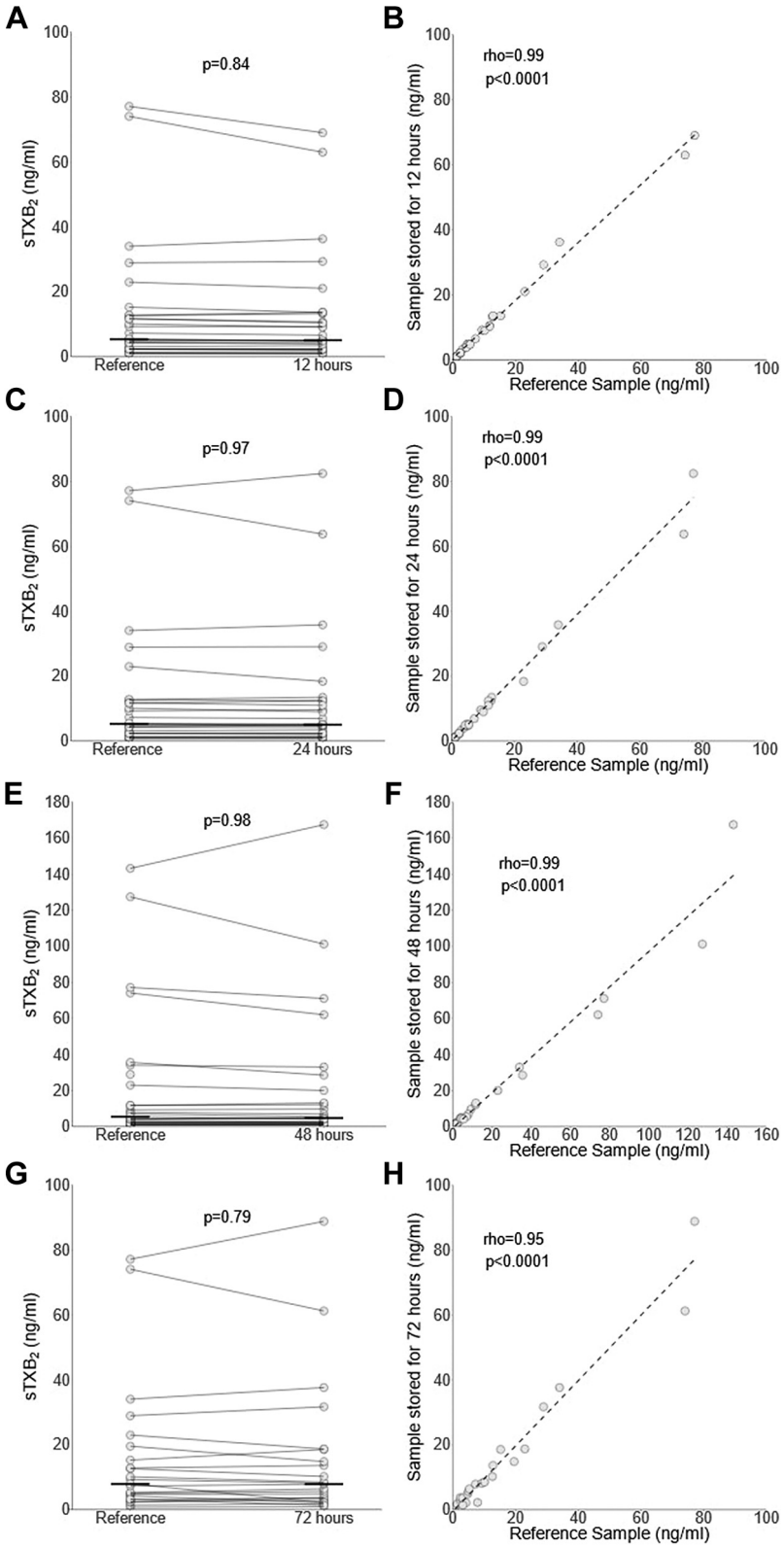

FIGURE 2 The figure shows the absolute values of serum thromboxane B_2 ($sTXB_2$) in the reference samples and in the corresponding samples stored at 4 °C for (A) 12 hours (n = 28), (C) 24 hours (n = 27), (E) 48 hours (n = 26), or (G) 72 hours (n = 23) and (B, D, F, H) their correlations. Horizontal bars indicate medians. Dotted line indicates regression line.

respectively; P = .97; Figure 2C), highly correlated (rho = 0.99; P < .001; Figure 2D), and were 100% (94%-105%) of their reference samples (P for equivalence < .01; b = 0.97; P < .001).

Similarly, samples stored for 48 hours (n = 26) were comparable with their reference samples (4.7 [2.2-18.3] ng/mL and 4.9 [2.4-20.2] ng/mL, respectively; P = .98), highly correlated (rho = 0.99; P < .001;

Figure 2E, F), and were 97% (86%-104%) of their corresponding reference samples (P for equivalence < .01; b = 0.98; P < .001).

Finally, in samples stored for 72 hours (n = 23), $sTXB_2$ values were overall similar to the reference sample (7.9 [3.3-16.7] ng/mL and 7.9 [3.7-17.4] ng/mL, respectively; P = .79), still correlated (rho = 0.95; P < .001; Figure 2G, H), but showed a high variability of 108% (82%-118%) vs their reference samples, with a nonsignificant P for equivalence (P = .08; b = 1.01; P < .001).

3.2 | Long-term stability of TXB_2 in frozen sera

In samples stored at −40 °C and assayed for a second time after <1 (5 ± 3 months, from 1 to 11 months; n = 32), 5 (n = 32), and 10 (n = 32) years, the first and second measurements were similar (<1 year, 32.0 [13.9-107.0] and 34.2 [14.3-108.0] ng/mL, respectively; 5 years, 35.5 [17.4-107.0] and 38.3 [20.2-97.2] ng/mL, respectively; 10 years, 182.0 [23.0-256.0] and 173.0 [20.5-262.0] ng/mL, respectively; all P > .7) and highly correlated (rho = 0.99, 0.99, and 0.97 for <1, 5, and 10 years, respectively; P < .001; Figure 3). The beta coefficients were 1.02, 1.06, and 0.85 for <1, 5, and 10 years, respectively; P < .001. $sTXB_2$ levels expressed as a percentage of the first measurement were 104% (95%-112%), 104% (95%-114%), and 101% (87%-108%) at <1, 5, and 10 years, respectively (P for equivalence, all P < .05; Figure 4).

At variance with the shorter time intervals, $sTXB_2$ levels in samples stored for 15 years were significantly lower than the first measurement (210.0 [126.0-276.0] and 165.0 [96.5-220.0] ng/mL, respectively; n = 32; P = .006; Figure 3G), although the 2 measurements remained significantly correlated (rho = 0.89; P < .001; Figure 3H). The beta coefficient was 0.76, P < .001. $sTXB_2$ levels expressed as a percentage of the first measurement were 87% (74%-109%), with a nonsignificant P for equivalence (P = .48; Figure 4).

4 | DISCUSSION

Our study investigated relevant preanalytical issues related to the *ex vivo* measurement of the TXB_2 in human samples. In particular, we investigated for the first time the short-term stability of TXB_2 in whole blood samples kept at 4 °C for different time intervals in capped tubes after clotting at 37 °C before further processing, mimicking logistics that can easily occur in clinical studies. Under these conditions, we showed that TXB_2 is stable for up to 2 days since the variability of those measurements reflected the interassay variability. Moreover, we investigated the effect of another preanalytical feature, ie, the long-term storage time at −40 °C controlled temperature for nearly 2 decades, and we showed the stability of this biomarker for a decade with deterioration afterward. To our knowledge, this is the longest storage time ever investigated.

Few studies investigated short-term stability (hours) of TXB_2 in plasma samples but not in sera [17–19]. Only one study reported substantial stability of TXB_2 in plasma samples stored over 6 months at −80 °C [17]. Importantly, TXA_2 is not a systemic hormone (maximum estimated plasma concentration is 1-2 pg/mL [20], while serum concentration is 300-400 ng/mL in aspirin-naïve subjects [3,4,9]), and therefore, plasma TXB_2 does not reflect the maximal enzymatic activity of COX in circulating platelets since thrombin is not generated in plasmas, and thus, platelet PLA_2 is not activated. Thus, we measured TXB_2 in serum, not in plasma, since this is the reference PD biomarker of low-dose aspirin [2]. Initially, this assay was instrumental in describing the capacity of aspirin at low doses to completely inhibit platelet-derived TXA_2 [4]; the cardiovascular efficacy of low doses was confirmed by several large phase 3 trials afterward [2], making low-dose aspirin still central in cardiovascular disease prevention and treatment [21]. Importantly, $sTXB_2$ is also considered by regulatory agencies in the applications to approve new aspirin formulations or to issue boxed warnings as a surrogate of clinical efficacy [5–7]. High residual $sTXB_2$ levels while on chronic treatment with low-dose aspirin have been associated with adverse outcomes in coronary artery disease patients [22], have helped to characterize the determinants of reduced aspirin responsiveness in human diseases such as type 2 diabetes [23] and essential thrombocythemia [15,24], and have been key to generate an *in silico* model of aspirin pharmacokinetics and PD [25,26]. Consistently, recent trials in rare diseases or studies in special populations used $sTXB_2$ as a surrogate of treatment efficacy and a tool for precision pharmacology [13,27,28]. Therefore, it is critical to identify and avoid preanalytical artifacts when measuring $sTXB_2$.

According to the original description of the $sTXB_2$ method, the blood should be almost immediately (eg, within <5 minutes) incubated at 37 °C [4,8,9,19] after withdrawal to allow the optimal temperature conditions for the enzymatic coagulation cascade to occur [29]. Consistently, when the incubation temperature is below 37 °C, there is an apparent reduction in $sTXB_2$ levels due to suboptimal efficiency of blood clotting and thrombin generation [4,8,29,30]. However, the effects and optimal preanalytical handling of delaying blood processing in clotted samples and of very long-term storage have never been investigated. The need to perform centrifugation, serum separation, and storage relatively soon after incubation can reduce the feasibility of the assay; central laboratories for blood processing and sample storage spaces or biobanks are often logistically far from patients' units or unavailable 24/7. Thus, a rapid transfer of blood samples to the central laboratory may not be feasible or continuously available in large clinical studies [31], while refrigerators are usually available both in out- and in-patient units. Thus, the substantial stability of $sTXB_2$ in clotted blood samples stored at 4 °C in the collecting tubes for up to 48 hours before further processing may increase the feasibility and the use of this PD biomarker in clinical studies as well as in daily practice. Starting from 72 hours of storage at 4 °C of clotted blood samples, the variability of the $sTXB_2$ values exceeded the variability associated with the method. Particularly, at 72 hours, some samples showed an apparent reduction, while others showed a paradoxical increase compared with their reference, which exceeded the lower and upper limits of the method's variability. While lower values may indicate degradation of the molecule, a slow release of TXA_2/TXB_2

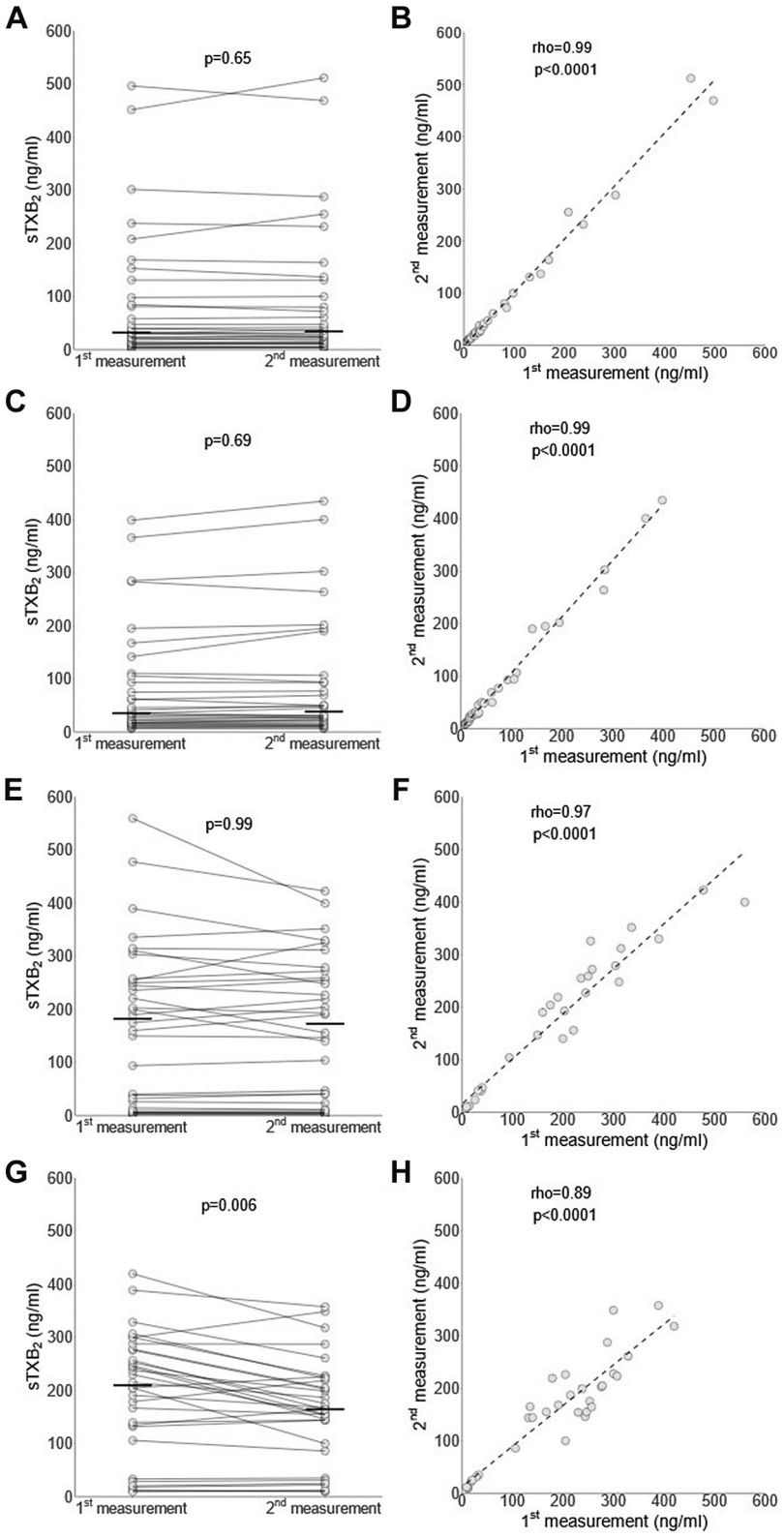

FIGURE 3 The figure shows the absolute values of serum thromboxane B_2 ($sTXB_2$) in the first (reference) and second measurements of serum samples stored at −40 °C for (A) <1 year (n = 32), (C) 5 years (n = 32), (E) 10 years (n = 32), and (G) 15 years (n = 32) and (B, D, F, H) their correlations. Horizontal bars indicate medians. Dotted line indicates regression line.

from damaged blood cells in sample tubes over 3 days of storage with mechanisms different from thrombin and platelet activation may explain the apparent TXB_2 increase, as reported for other blood cells [32], thus creating preanalytical artifacts not reflecting the PD of aspirin. Of note, in large studies of aspirin-naïve and -treated patients [4,22,28,33], $sTXB_2$ levels showed up to 20 times difference

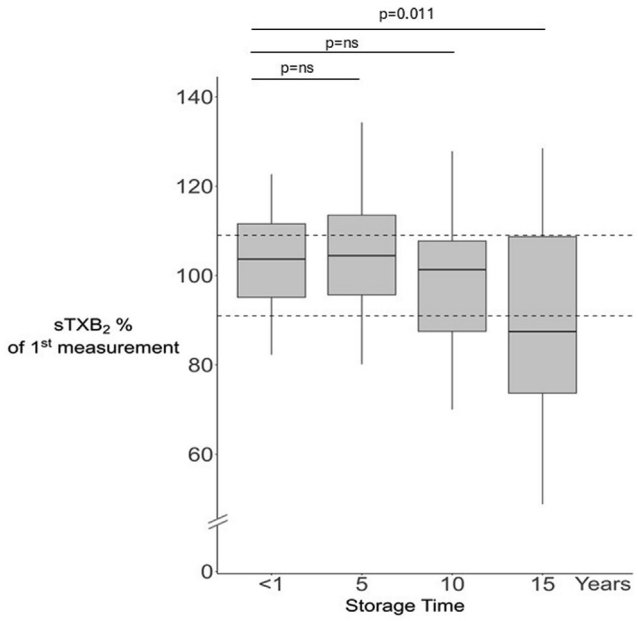

FIGURE 4 The figure shows the box plots of the serum thromboxane B_2 ($sTXB_2$) values in serum samples stored at −40 °C for <1 year (n = 32), 5 years (n = 32), 10 years (n = 32), and 15 years (n = 32), expressed as a percentage of the reference measurement. The dashed lines represent the lower and upper limits of the interassay coefficient of variation. ns, not significant.

in the reported average values (between 17 and 400 ng/mL in aspirin-naïve subjects; between 7 and <1 ng/mL in aspirin-treated subjects) that individual's characteristics or analytical methodologies could not explain [34], thus further highlighting the relevance of simple and reproducible preanalytical procedures in large studies. Moreover, our data show that TXB_2 in sera stored at −40 °C is stable for up to 10 years, while it apparently decreases afterward, which can be relevant for large studies with long durations or follow-ups. Notably, a study published in 2019 reported $sTXB_2$ values from 1002 pregnant women [28] who participated in a placebo-controlled randomized trial performed some decades before [35]. Levels of $sTXB_2$ in this trial, both off and on aspirin, are very different and rather lower than those reported in other studies and trials with less delay between blood collection and analysis [3,4,9,36].

A limitation of the current study is the lack of data under storage temperatures different from −40 °C. However, our study has the longest storage interval, and −40 °C storage temperature is more practicable and less expensive than −80 °C, for instance.

In conclusion, $sTXB_2$ is stable in human blood samples stored for up to 48 hours at 4 °C after incubation at 37 °C, indicating that further sample processing, ie, centrifugation and storage, can be delayed without generating relevant preanalytical artifacts. Moreover, TXB_2 in serum samples stored at −40 °C is stable for a decade but shows a significant degradation and artifactual reduction afterward. These data could inform and increase the feasibility of multicenter studies and the analysis of large databases when stored biological samples are used, as well as data interpretation.

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AUTHOR CONTRIBUTIONS

G.P. performed experiments and data analysis. A.R. analyzed the data. B.R. and FADOI designed the study; B.R. wrote the first draft. A.R., D.P., and P.R. participated in clinical studies. All authors discussed the results reviewed and approved the manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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