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LETTER TO THE EDITOR

Preanalytical Conditions Impact Fibrin Monomers but Not D-Dimer: A Study With Rigorous Comparisons of a Broad Range of Simulated Conditions

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Dear editors,
Fibrin monomers (FMs) and D-dimer are fibrin-related biomarkers of in vivo coagulation activation [1, 2].

Daily monitoring of FMs and D-dimer levels can be useful in the hospital setting for the detection of thrombotic events (TEs) in patients with severe COVID-19 [3, 4]. Indeed, FMs have the advantage of being at normal plasma levels before the occurrence of TEs and of rising suddenly and sharply, specifically capturing fibrin formation. However, FMs levels quickly return to normal (average half-life 2–6 h), resulting in transient plasma peaks [3, 4].

D-dimer has a longer half-life than FMs (> 8 h), making it easier to detect episodic boosts of coagulation activation during TEs. However, D-dimer levels are often elevated in hospitalized patients, and determining a diagnostic threshold for the detection of TEs is complicated, hence the importance of monitoring plasma levels over time [3, 4].

To the best of our knowledge, the effect of preanalytical conditions on FMs levels is unknown but may be significant, as non-optimal conditions may activate coagulation and promote thrombin and fibrin generation [5]. In contrast, the effect of preanalytical conditions has been well described for D-dimer [2].

The study aimed to evaluate the impact of nine simulated conditions, compared to the reference condition, on FMs and D-dimer assays and to compare the sensitivity of these two biomarkers to preanalytical artifacts.

The study was approved by the hospital's local Ethics Committee (NUB: B03920096633), and samples were collected after volunteers' written consent. Twenty healthy volunteers (10 men, 10 women, age range [21–65] years, mean age: 36 years) were selected using a screening questionnaire according to the following criteria: volunteers in good health, with no medication that could affect hemostasis (combined oral contraceptives, antiplatelet agents, and anticoagulants).

For this study, nine simulated conditions were selected and compared to the reference condition to assess their impact on the measurement of FMs and D-dimer.

As shown in Figure 1, nine tubes were filled with blood withdrawn from each volunteer. The second tube (reference

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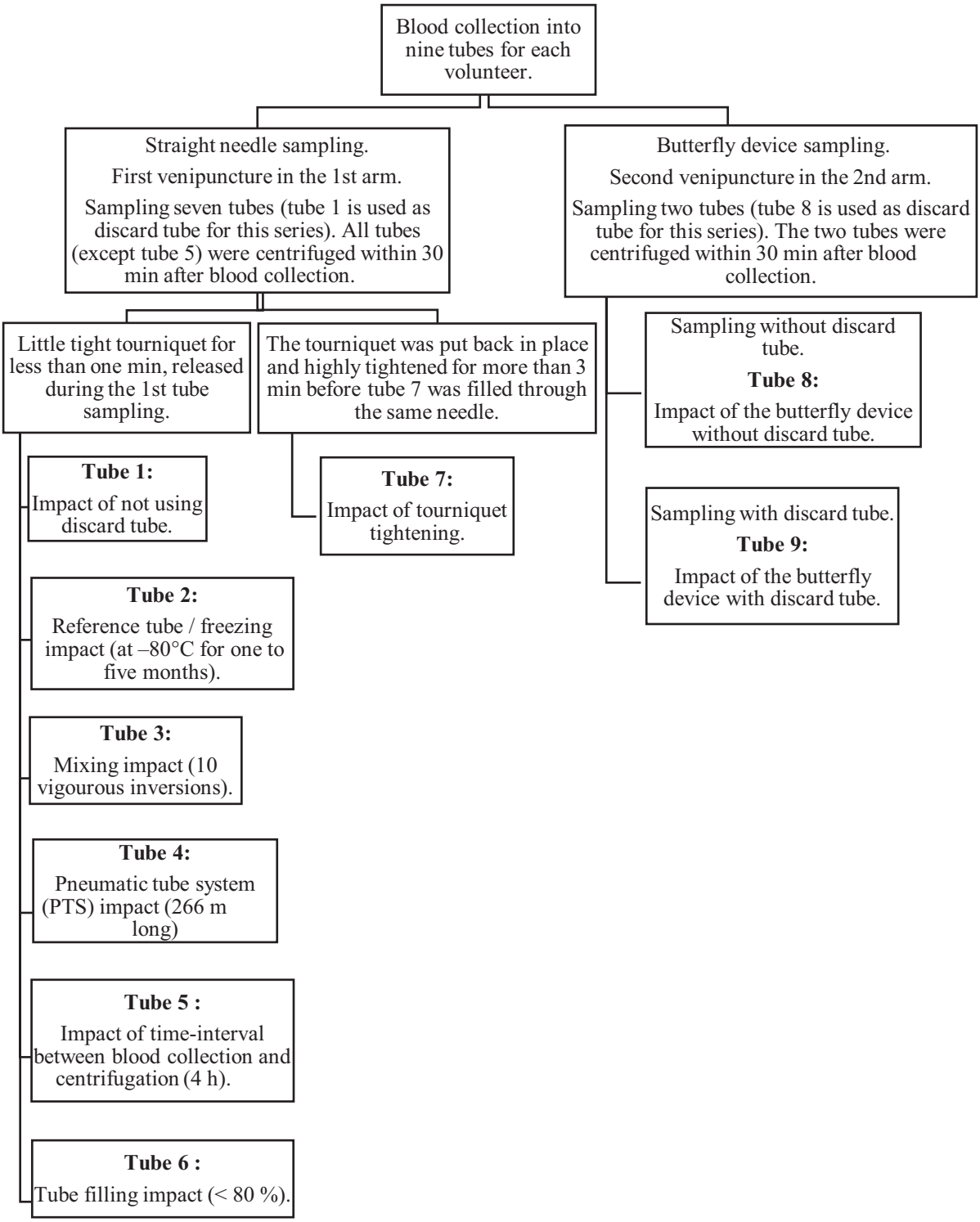

FIGURE 1 | Protocol used to collect blood into nine tubes from each of the 20 healthy volunteers. Tube 2 was considered the reference condition because it complied with EFLM recommendation [6, 7], which are: blood collection using a straight needle, use of a first discard tube, little tight tourniquet for less than 1 min and released during the collection of the discard tube, tube filling ≥ 90%, one tube inversion immediately after blood collection and four times after filling all tubes, manual transport to the lab and centrifuged within 1 h after blood collection. For Tube 2, plasma aliquots were frozen, after analysis, at −80°C for 1–5 months to assess the impact of freezing.

condition) was collected in accordance with the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) recommendations [6, 7]. For each other tube, one of the conditions was modified, and the impact of this modification was investigated.

All samples were collected in the morning from 20 healthy, fasted volunteers. Blood was collected after venipuncture of the median cubital or basilic vein, with a 21 G straight needle (Vacuette, Greiner Bio-One) and a 21 G butterfly needle (Vacutainer, Becton Dickinson). Sodium citrate 3.2% tubes

(PET, 109 mM, $V = 2.7$ mL, Vacutainer, Becton Dickinson) were used.

Tubes 3–6 were collected in alternating order for each volunteer following a pre-established scheme, while the order of the other tubes remained fixed to avoid altering the study conditions.

After centrifugation at 1500 g for 15 min at 20°C, plasma samples were analyzed with a STAR MAX 2 automated analyzer (STAGO, Asnières-sur-Seine, France) for FMs and D-dimer assays, using STA-LIATEST FM (lot 263 170 then lot 264 435) and STA-LIATEST D-DI Plus (lot 262 714 then lot 262 901), respectively. The lot change, for FMs and D-dimer, was made on Volunteer 6. The analytical methods were locally validated according to the ISO15189 accreditation. Lower and upper limits of quantification provided by the manufacturer were evaluated during the local assay validation. The locally determined limits were 3.35 and 150 μg/mL for FMs, and 0.27 and 4 μg/mL for D-dimer, respectively. The measurement uncertainty was 3 μg/mL for an FMs concentration of 14.22 μg/mL.

Activated partial thromboplastin time (aPTT), prothrombin time (PT), thrombin time (TT), and fibrinogen levels were also performed in Tube 2 to ensure that there was no significant coagulation defect.

The plasma from each tube was aliquoted into three microtubes and stored frozen for 1–5 months, at −80°C. One frozen aliquot (Tube 2) was thawed in a water bath at 37°C for 5 min and then analyzed to measure the effect of freezing.

Friedman tests were used as a general test to assess differences in FMs and D-dimer levels obtained from the same volunteer under different conditions.

When Friedman tests indicated statistically significant differences ($p < 0.05$), post hoc paired Wilcoxon tests were performed to make pairwise comparisons between each condition and the reference condition (p values were adjusted for multiplicity according to Bonferroni correction). Statistical analyses were performed in R [8].

Clinical relevance was assessed for conditions considered statistically significant by comparing the median of the differences (Md) in FMs or D-dimer values, between each condition and the reference condition, with the reference change value (RCV), using the following formula [2]:

$$\mathrm{RCV}\,(\%) = 2^{1/2} \times Z \times \left(\mathrm{CV_A}^2 + \mathrm{CV_I}^2\right)^{1/2}$$

While $\mathrm{CV_A}$ is the analytical variation (8.43% for FMs and 12.3% for D-dimer, locally calculated), $\mathrm{CV_I}$ is within-subject biological variation (set to zero, since all samples from one given individual were withdrawn during the same blood collection) and $Z = 1.96$ for the 95% confidence level. RCV was 1.4 and 0.17 μg/mL for FMs and D-dimer, respectively. A pre-analytical condition was associated with a clinically relevant deviation if Md was > 1.4 μg/mL for FMs and > 0.17 μg/mL for D-dimer.

The raw data for FMs and D-dimer are represented in Figure 2. The number of volunteers with elevated FMs and D-dimer levels, compared to the thresholds for clinical interpretation (< 6 μg/mL for FMs and < 0.5 μg/mL for D-dimer), is shown in Figure 3. The individual values for FMs, D-dimer, and standard coagulation tests are presented in Table S1, along with the median and interquartile range (IQR) of the FMs and D-dimer results, represented in Figure S1, all provided in the supporting information.

Standard coagulation tests for all 20 healthy volunteers were within locally validated reference values, reasonably ruling out a significant coagulation defect.

Preanalytical conditions had a significant influence on FMs levels ($p < 0.0001$, Freidman test). Compared to the reference condition where FMs levels were 5.82 μg/mL (IQR, 4.73–6.77 μg/mL), they significantly increased to 7.76 μg/mL (7.04–11.08 μg/mL) for Condition 3 ($p = 0.0002$, Md = 2.75 μg/mL); 9.02 μg/mL (6.29–11.71 μg/mL) for Condition 4 ($p < 0.0001$, Md = 3.34 μg/mL); 11.25 μg/mL (6.98–19.56 μg/mL) for Condition 5 ($p < 0.0001$, Md = 6.09 μg/mL); 13.29 μg/mL (8.25–30.34 μg/mL) for Condition 6 ($p = 0.0007$, Md = 8.48 μg/mL); and 16.34 μg/mL (12.16–35.67 μg/mL) for Condition 7 ($p < 0.0001$, Md = 11.31 μg/mL). Since all median differences with the reference condition were higher than the RCV(FMs), FMs increases under those conditions were considered clinically relevant.

For the other conditions, FMs levels were not significantly elevated compared to the reference condition ($p = 0.39$ for Condition 1, $p = 0.21$ for Condition 8, $p = 0.30$ for Condition 9, and $p = 0.85$ for Condition 10).

Unlike FMs, D-dimer levels were not significantly different among all conditions ($p = 0.18$, Friedman test). As shown in Figure 3, elevated levels were observed in only a few volunteers across all conditions.

Some healthy volunteers had increased levels of FMs and D-dimer, compared to the thresholds for clinical interpretation, under the reference condition (nine volunteers for FMs and one volunteer for D-dimer). However, only two volunteers had FMs levels higher than 9 μg/mL (i.e., 6 μg/mL + the measurement uncertainty). In addition, the cutoff of 6 μg/mL used in this study for FMs levels was provided by the manufacturer. Normal values have not been defined locally, which may be worth doing according to the results we obtained.

As each volunteer was compared to themselves, there is no reason to believe that the use of a different batch could have influenced the results.

In the literature, several studies dealt with the effect of the pre-analytical phase on hemostasis parameters and coagulation tests, pointing to the risk of artifactual coagulation activation.

Lippi et al. [9] showed that venous stasis promotes hemoconcentration and coagulation activation. Lima-Oliveira et al. [10] and Le Quellec et al. [11] reported that strong vibrations can cause hemolysis and ADP release. Loeffel et al. [5] demonstrated that prolonged whole blood storage can cause hemolysis and thrombin generation.

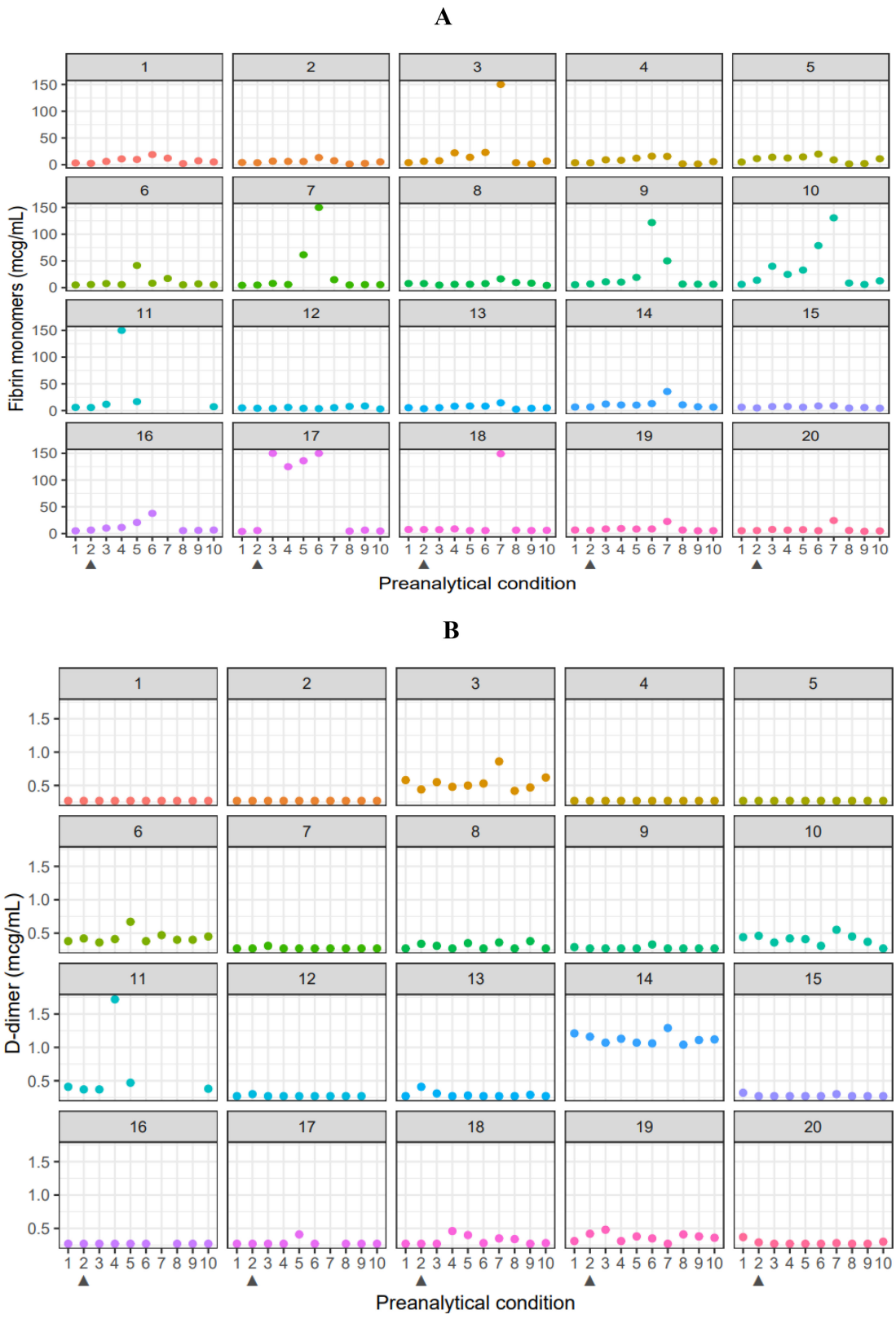

FIGURE 2 | Graphical representation of FMs (A) and D-dimer (B) raw data for the 20 healthy volunteers for each preanalytical condition. Numbers from 1 to 10 on the *x*-axis represent the nine simulated preanalytical conditions with the reference condition (marked with a black arrow): 1 (no discard tube), 2 (reference condition), 3 (tube mixing), 4 (PTS use), 5 (time interval between blood collection and centrifugation), 6 (tube filling), 7 (tourniquet tightening), 8 (butterfly device without discard tube), 9 (butterfly device with discard tube), and 10 (freezing). Missing values correspond to uncollected tubes for practical reasons: Volunteer 11 (Tubes 6/7/8/9), Volunteer 12 (freezing tube for D-dimer), Volunteer 16 (Tube 7), Volunteer 17 (Tube 7). Sampling notes: vein missing (new discard tube): V2T4, V2T5, V7T5, V7T6, four attempts to fill V3T6 and two attempts to fill V9T6, new venipuncture for VTT4 and V7T7. Abbreviations: V (volunteer) and T (tube).

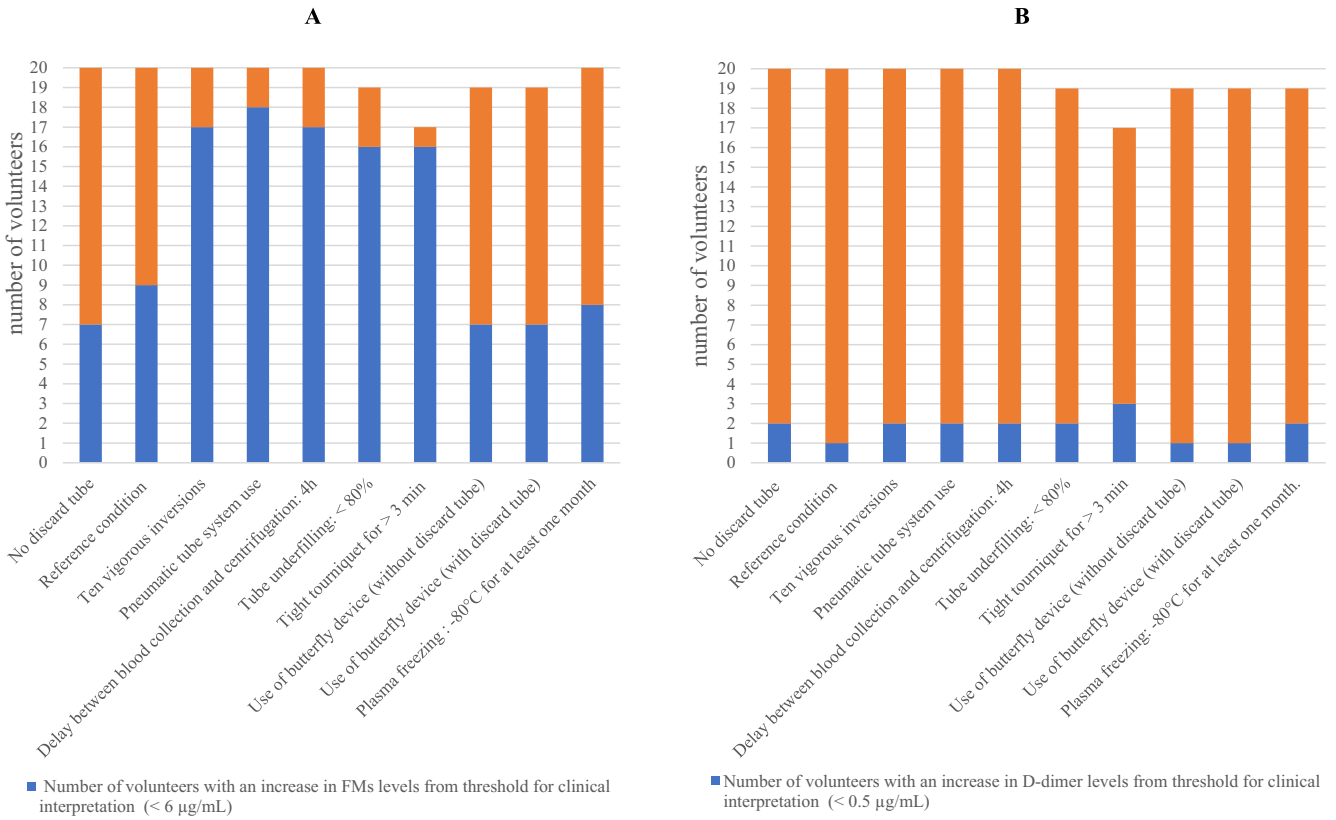

FIGURE 3 | Number of volunteers with an increase in FMs (A) and D-dimer (B) levels from the thresholds for clinical interpretation (< 6 µg/mL for FMs and < 0.5 µg/mL for D-dimer), for each preanalytical condition. Missing values correspond to volunteers with uncollected tubes: Tube underfilling (one volunteer), tight tourniquet for more than 3 min (three volunteers), use of butterfly device without discard tube (one volunteer), use of butterfly device with discard tube (one volunteer), and freezing (one volunteer for D-dimer).

According to the D-dimer [2] literature, the impact of the preanalytical phase has been well described and reported to be minimal which can reflect its robustness under inappropriate conditions. This can be explained by the low levels of circulating t-PA of normal plasma, and low fibrinolytic activity in the absence of a fibrin mesh [12, 13]. However, spurious elevations can occur.

A strength of our study resides in the careful, systematic, and controlled parallel evaluation of nine conditions according to a rigorous protocol. In addition, obtaining samples from a single bleed enables a direct comparison between each preanalytical variable, ensuring that differences observed were due to the variables being tested, rather than intraindividual variations, which remain unknown for FMs.

However, this study had some limitations. It was performed on a limited number of healthy volunteers, and the results should be confirmed on patient samples in different clinical settings to represent, as closely as possible, clinical use of FMs and D-dimer. Additionally, some volunteers were more affected than others by preanalytical conditions, for reasons that deserve further studies. Finally, some conditions such as centrifugation speed and temperature could not be evaluated for practical reasons.

In conclusion, the FMs assay is sensitive to several preanalytical artifacts: vigorous mixing, PTS use, time interval between blood collection and centrifugation (4 h), tube underfilling (< 80%), and tourniquet tightening (highly tight for more than 3 min). All those conditions were statistically significant and clinically relevant, potentially leading to different clinical management. Regarding D-dimer, none of the conditions studied was found to influence the results. We advocate for more stringent guidelines to minimize FMs assay artifactual overestimation, including using a lightly tightened tourniquet for less than 1 min and releasing it during the collection of the first tube, ensuring tube filling ≥ 90%, one tube inversion immediately after blood collection and four inversions after filling all tubes, manual transport to the laboratory, and centrifugation within 30 min after blood collection.

Author Contributions

H.Z.B., M.H., and F.M. designed study. T.L. provided critical feedback on the study. H.Z.B. performed the laboratory analyses and descriptive analysis, and wrote the initial draft of the manuscript. M.H. performed statistical analysis. M.H., T.L., and F.M. critically reviewed the draft.

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Disclosure

F.M. has received speaker fees from Fresenius, Technoclone, Stago, and Werfen, all outside the submitted work.

Ethics Statement

The study was approved by the hospital's local Ethics Committee (NUB: B03920096633).

Consent

Written informed consent was obtained from all participants before inclusion in the study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Michaël Hardy
Thomas Lecompte
François Mullier

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.