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ISTH SSC COMMUNICATIONS

Comparison of assays measuring extracellular vesicle tissue factor in plasma samples: communication from the ISTH SSC Subcommittee on Vascular Biology

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Abstract

Background: Scientific and clinical interest in extracellular vesicles (EVs) is growing. EVs that expose tissue factor (TF) bind factor VII/VIIa and can trigger coagulation. Highly procoagulant TF-exposing EVs are detectable in the circulation in various diseases, such as sepsis, COVID-19, or cancer. Many in-house and commercially available assays have been developed to measure EV-TF activity and antigen, but only a few studies have compared some of these assays.

Objectives: The International Society on Thrombosis and Haemostasis Scientific and Standardization Committee Subcommittee on Vascular Biology initiated a multicenter study to compare the sensitivity, specificity, and reproducibility of these assays.

Methods: Platelet-depleted plasma samples were prepared from blood of healthy donors. The plasma samples were spiked either with EVs from human milk or EVs from TF-positive and TF-negative cell lines. Plasma was also prepared from whole human blood with or without lipopolysaccharide stimulation. Twenty-one laboratories measured EV-TF activity and antigen in the prepared samples using their own assays representing 18 functional and 9 antigenic assays.

Results: There was a large variability in the absolute values for the different EV-TF activity and antigen assays. Activity assays had higher specificity and sensitivity compared with antigen assays. In addition, there was a large intra-assay and interassay variability. Functional assays that used a blocking anti-TF antibody or immunocapture were the most specific and sensitive. Activity assays that used immunocapture had a lower coefficient of variation compared with assays that isolated EVs by high-speed centrifugation.

Conclusion: Based on this multicenter study, we recommend measuring EV-TF using a functional assay in the presence of an anti-TF antibody.

KEYWORDS
blood coagulation, extracellular vesicles, flow cytometry, functional assays, tissue factor

1 | INTRODUCTION

Extracellular vesicles (EVs) are a heterogeneous population of submicron membrane vesicles released by cells. EVs are present in biological fluids, such as blood [1], saliva [2], and pleural fluid [3]. They carry proteins, lipids, and nucleic acids and are thought to be involved in intercellular communication and pathophysiological processes. Clinical and scientific interest in EVs is growing exponentially. Different subtypes of EVs, such as leukocyte-, endothelial-, or tumor-derived EVs, can trigger coagulation by exposing tissue factor (TF), which binds factor (F)VII/VIIa. Indeed, the TF/FVIIa complex is the main activator of the extrinsic coagulation pathway and, thus, leads to fibrin clot formation by activating FIX and FX in the presence of anionic phospholipids [4]. Although TF was initially thought to be exclusively present outside the vasculature ("envelope model"), there is increasing evidence that procoagulant EVs exposing TF (EV-TF) can be present in the circulation in different diseases, such as infectious disorders [5], cancer [6], and COVID-19 [7,8].

Many in-house and commercially available assays have been developed to measure EV-TF [9], but only a few studies have compared different assays. These assays have shown promising results for the prediction of venous thromboembolism in pancreatic cancer and COVID-19 patients, but further investigations are warranted to prove their clinical utility [6–8].

Therefore, the International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Committee (SSC) Subcommittee on Vascular Biology initiated a study to compare the analytical performance (sensitivity, specificity, and repeatability) of currently available assays to measure TF activity or TF antigen of EVs in human plasma samples.

2 | MATERIALS AND METHODS

2.1 | Participants

The collaborative project was proposed at the ISTH SSC Congress 2018 (64th annual SSC Congress). All interested laboratories that use 1 or several methods to measure EV-TF were included. Twenty-one laboratories from 13 different countries used 18 functional (activity) and 9 flow cytometry (FCM) assays.

2.2 | Tested samples

Three types of human plasma samples were prepared by the core laboratories (Supplementary Figure S1).

Three sources of EVs were used. First, EVs from the haploïd-1 cell line (HAP-1) cell line wild-type or knockout TF (TFKO) were employed as a pertinent model to investigate TF specificity of the assays [10]. Indeed, such a strategy allows the generation of EVs with the same composition except for TF, in particular regarding other molecules involved in the EVs' procoagulant capacity such as phosphatidylserine. Second, milk EVs, although not present in blood, represent a source of EVs with high level of TF [11]. Third, lipopolysaccharide (LPS)− and LPS+ EV samples were used as a source of EVs, which mimic healthy donors (LPS−) and inflammatory conditions, such as endotoxemia (LPS+), known to increase TF expression by monocytes and their EVs [12].

2.2.1 | Plasma samples spiked with EV-TF and EV-TFKO

EVs were produced from the same cell line, HAP-1 (haploid), and genetically modified by CRISPR associated protein 9 technology to generate a knockout TF (TFKO) cell line. Parental HAP-1 and TFKO HAP-1 cell lines were purchased from Thermo Fisher Scientific. Cells were maintained in Iscove's Modified Dulbecco Media (Thermo Fischer Scientific) supplemented with 10% EV-depleted fetal bovine serum, prepared as recommended by the minimal information for studies of extracellular vesicles guidelines [13], and 1% penicillin/streptavidin. The conditioned culture medium was collected at 48 hours to prepare EVs. EVs were purified by sequential centrifugation steps: 300 × *g* for 5 minutes followed by 2500 × *g* for 10 minutes to remove cells and debris. Then, ultracentrifugation at 100 000 × *g* for 90 minutes was performed to pellet the EVs, followed by size-exclusion chromatography using a qEV original 70 nm column (Izon Science Ltd) to remove soluble proteins. EVs were quantified by FCM. The gating strategy and acquisition protocol on the CytoFLEX LX (Beckman Coulter) instrument were previously described [14]. EVs were defined as annexin V+ and CD59+ events. Aliquots of 500 µL were stored at −80 °C. Purified EVs were spiked into EV-depleted plasma (prepared from plasma by centrifugation at 100 000 × *g* for 90 minutes at 20 °C [Blood Bank, Etablissement Français du Sang, Marseille]) to produce low (1×10^9/L) and high (5×10^9/L) levels of EV-TF and EV-TFKO.

2.2.2 | Plasma samples spiked with milk EVs

Human breast milk was collected with approval of the Ethics Committee of the Medical University of Vienna (#1721/2015); details on collection, handling, and storage have been described previously [11]. Human milk (stored at −80 °C) was thawed for 1 minute at 37 °C in a water bath. After thawing, milk was fractionated by size-exclusion chromatography using a Sepharose 2B (GE Healthcare) column as described earlier for plasma [15]. Briefly, 1 mL of milk was fractionated using a qEV original 70 nm column (Izon Science Ltd). Collected fractions were screened using the fibrin generation test (FGT) for their ability to shorten the plasma clotting time in the presence or absence of an anti-TF antibody (TF; clone HTF-1; Thermo Fisher Scientific). As described previously [11], Sepharose 2B fractions 8 and 9 contained the bulk of EVs, and these fractions also contained the highest TF activity. Fractions 8 and 9 were pooled, and from these pooled fractions, a dilution series was generated using the FGT to determine the dilutions that induced shortening of the plasma clotting time comparable with that induced by 142 and 353 fM Innovin (Siemens Healthcare Diagnostics GmbH). To prepare the test samples, the selected dilutions of pooled fractions 8 and 9 were diluted in citrate-anticoagulated EV-poor human pooled plasma. After collection, blood was centrifuged for 5 minutes at 4190 × *g*, followed by centrifugation of the supernatant for 15 minutes at 3000 × *g*. The plasma was used in agreement with the guidelines of the Medical Ethical Committee of the Amsterdam Medical Centre, University of Amsterdam (W19_271#19.421). EV-poor pooled plasma was prepared by centrifugation at 18 890 × *g* for 60 minutes at 20 °C. Test samples were thawed in the Amsterdam laboratory to validate the stability of EV-TF activity using the FGT before sending out the samples.

2.2.3 | Human whole blood samples stimulated by LPS

LPS− and LPS+ control plasma samples were prepared as previously described [16]. Briefly, LPS− plasma was prepared using whole blood from healthy volunteers immediately after collection. LPS+ plasma was prepared from whole blood stimulated with LPS (Sigma-Aldrich; 10 μg/mL) for 5 hours at 37 °C with agitation. Platelet-depleted plasma was prepared by centrifugation of whole blood at 2500 × *g* for 15 minutes at room temperature (RT), followed by a second centrifugation at 2500 × *g* for 15 minutes at RT as described by the ISTH SSC Collaborative Workshop [17]. Platelet-depleted plasma was aliquoted and stored at −80 °C.

2.3 | TF standard for generation of standard curves

TF calibrant (reference, 14/238) was kindly provided by the National Institute for Biological Standards and Control [18]. The TF calibrant is made of recombinant TF at the initial concentration of 100 U/mL. Participant laboratories were asked to dilute the calibrant 1:4 (25 U/mL) before using it to create an 8-point calibration curve resulting from 1:2 serial dilutions (from 25 to 0.2 U/mL). The purity of the standard was evaluated by Western blotting (anti-TF antibody, catalog #AF2339, R&D Systems; Supplementary Figure S2). The TF calibrant was used to calibrate functional assays, whatever the principle of TF measurement, to enable comparison using the same units (unit per milliliter).

2.4 | Reverse transcription quantitative polymerase chain reaction

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. A 2-step reverse transcription quantitative polymerase chain reaction was performed. Total RNA was reverse transcribed into complementary DNA using the PrimeScript RT Reagent Kit (TaKaRa catalog #RR037A). Twenty nanograms of complementary DNA were amplified in a 20 μL reaction in an MxP3000 instrument (Stratagene) using TaqMan Fast Advanced Master Mix (TaqMan, Applied Biosystems) with predesigned primers for *F3* (Hs00175225_m1) and *RPL13* (Hs00744303_1s; TaqMan, Applied Biosystems). Each sample was run in duplicate. The relative fold change was determined using the $2^{-\Delta\Delta cycle\ threshold}$ method and normalized to RPL13A expression. The absence of TF expression in HAP-1 TFKO cells was confirmed by reverse transcription quantitative polymerase chain reaction (Supplementary Figure S3A).

2.5 | Western blotting

Western blotting was performed on EVs that were lysed with radioimmunoprecipitation assay buffer. Proteins were separated on a 4% to 12% NuPAGE (Thermo Fisher Scientific) gel in the presence of sodium dodecyl sulfate and then transferred onto nitrocellulose membranes (Amersham Protran, Merck Sigma-Aldrich). Membranes were blocked with 3% bovine serum albumin/tris-buffered saline (ET220B, Euromedex) for 1 hour at RT. Next, the membranes were incubated overnight at 4 °C with antibodies against integrin β_3 (1:1000, catalog #4702, Cell Signaling Technology) or TF (1:1000, catalog #EPR22548-240, Abcam). Next, horseradish peroxidase–conjugated secondary polyclonal antibody (1:2000, Thermo Fisher Scientific, catalog #31460) was added for 1 hour at RT. Immunocomplexes were detected by enhanced chemiluminescence (substrate) according to the manufacturer's instructions (Pierce) and visualized using a G-BOX Imaging System (GeneSys). The absence of TF expression in HAP-1 TFKO cells and EVs was confirmed by Western blotting (Supplementary Figure S3B, C).

2.6 | Measurement of TF activity of wild-type and TFKO HAP-1 cells

A FXa generation assay was performed using HAP-1 wild-type and TFKO cell lines. Briefly, 0.1 × 10^6 cells in 70 μL were incubated in

hydroxyethyl-piperazineethane-sulfonic acid (HEPES) buffer for 30 minutes at 37 °C with either an inhibitory anti-TF monoclonal antibody (10 μg/mL final, clone SBTF-1, BioCytex) or a control antibody (10 μg/mL, clone a-DNP 2H11–2H12, BioCytex). Next, 7 μL of HEPES-Ca^{2+} buffer (150 mM NaCl, 20 mM HEPES and 0.1% NaN_3, 50 mM $CaCl_2$, pH 7.4, 0.22 μm filtrated) containing purified human FVII and FX (Stago BNL) was added to each sample (final concentrations of 10 nM, 190 nM, and 5 mM $CaCl_2$, respectively) and incubated for another 2 hours at 37 °C. FXa generation was stopped by the addition of 8 μL of EDTA buffer (150 mM NaCl, 20 mM HEPES and 0.1% NaN_3, 200 mM EDTA, pH 7.4, 0.22 μm filtrated), and a FXa chromogenic substrate (1 mM final, CBS 31.39, Stago) was added. Finally, the color at 390 nm (excitation) and 460 nm (emission) was measured for 15 minutes at 37 °C on a microplate reader (GloMax, Promega). The maximum reaction velocity was calculated and corrected by subtracting values generated in the presence of the anti-TF antibody SBTF-1 from those generated in the presence of the control antibody. Data were expressed as femtomolar per liter by comparison with a calibration curve generated using the TF standard. The absence of TF activity in HAP-1 TFKO cells was confirmed by a TF-dependent FXa generation assay (Supplementary Figure S3D).

2.7 | Study design

Each participating laboratory performed its own assay. A set of 3 aliquots of 8 samples were provided to the participant laboratories (high EV-TF, low EV-TF, high EV-TFKO, low EV-TFKO, high milk-EVs, low milk-EVs, LPS+ EVs, and LPS− EVs; Supplementary Figure S1). All the samples were blinded for the participants and were measured in triplicate using the 3 aliquots provided for each sample. These 8 samples allowed 5 different comparisons to be performed (high EV-TF/high EV-TFKO, low EV-TF/low EV-TFKO, high EV-TFKO/low EV-TFKO, high milk-EVs/low milk-EVs, and LPS+ EVs/LPS− EVs). TF calibrant was also provided for functional assays. Detailed protocols for storage and thawing of the samples were provided to the participating laboratories. Samples were stored at −80 °C. Before use, samples were quickly thawed at 37 °C in a water bath and then incubated at RT for 15 minutes. The participants measured EV-TF in the plasma samples with their own assays (functional and/or antigenic). Raw data and calibrated results were deposited on a secure server using a standardized format.

2.8 | Analyses of data

Data were analyzed for statistical relevance with GraphPad Prism 8 software (GraphPad Software). Comparisons between 2 groups were performed using a nonparametric test (Mann–Whitney U-test) for quantitative variables. The robust regression and outlier removal (ROUT) outlier test was used to identify outliers.

3 | RESULTS

3.1 | Method characteristics

Twenty-seven assays were included in the study: 18 functional assays and 9 FCM assays. Among the FCM assays, 8 assays were based on a classical principle where EVs are analyzed individually, and 1 assay analyzed EVs after immunocapture with beads using a commercial assay. The functional assays were divided into 3 assay types: assays measuring the generation of FXa, thrombin, or fibrin. In addition, some functional assays used an anti-TF antibody, and the value obtained for the anti-TF antibody condition was subtracted from the value for the isotype control antibody. The detailed characteristics of the functional and FCM assays are described in Supplementary Tables S1 and S2, respectively. These assays were evaluated with regard to their specificity, sensitivity, and repeatability.

3.2 | Specificity of the TF functional assays

To assess the specificity of the functional TF assays, we evaluated their capacity to discriminate between plasma spiked with low or high concentrations of EVs derived from HAP-1 wild-type or TFKO cells. An assay was considered specific when undetectable activity with EV-TFKO samples was observed. Moreover, in order to analyze the specificity among assays that detected an activity with EV-TFKO samples, we calculated the ratio between the high EV-TF and high EV-TFKO samples and the ratio between the low EV-TF and low EV-TFKO samples. We considered that a ratio above 1.2 indicated that the assay was specific for TF based on repeatability analysis (see below). The absolute values used to calculate the ratios of each functional assay are shown in Figure 1A, B.

Figure 1A shows that 4 of 18 (22%) assays had an undetectable activity with low EV-TFKO sample and that 12 of 18 (66%) assays had a ratio of above 1.2 for the low EV-TF/low EV-TFKO samples. Assay 7 had a ratio below 1.2, and assay 18 did not measure the EV-TFKO sample. The median ratio for the low EV-TF/low EV-TFKO samples was 10.9 (Table). Figure 1B shows that 3 of 18 (16%) assays had an undetectable activity with high EV-TFKO sample and that 14 of 18 (77%) assays had a ratio of above 1.2 using high EV-TF/high EV-TFKO samples. Assay 17 had a ratio below 1.2. The median ratio for the high EV-TF/high EV-TFKO samples was 10.1 (the ROUT outlier test excluded ratios from assays 4 and 13; Table).

Among the FXa generation assays, 9 of 14 assays used either an anti-TF antibody or immunocapture and 5 of 14 assays did not use an anti-TF antibody (Supplementary Table S1). The median ratio for the 6 assays that used an antibody for the low EV-TF/low EV-TFKO was 14.2 compared with 4.6 for the 4 assays that did not use an antibody (a 3.1-fold difference; P = .100; Table). The median ratio for the 5 assays (the ROUT outlier test excluded assay 4) that used an antibody for the high EV-TF/high EV-TFKO was 23.3 compared with 2.4 for the

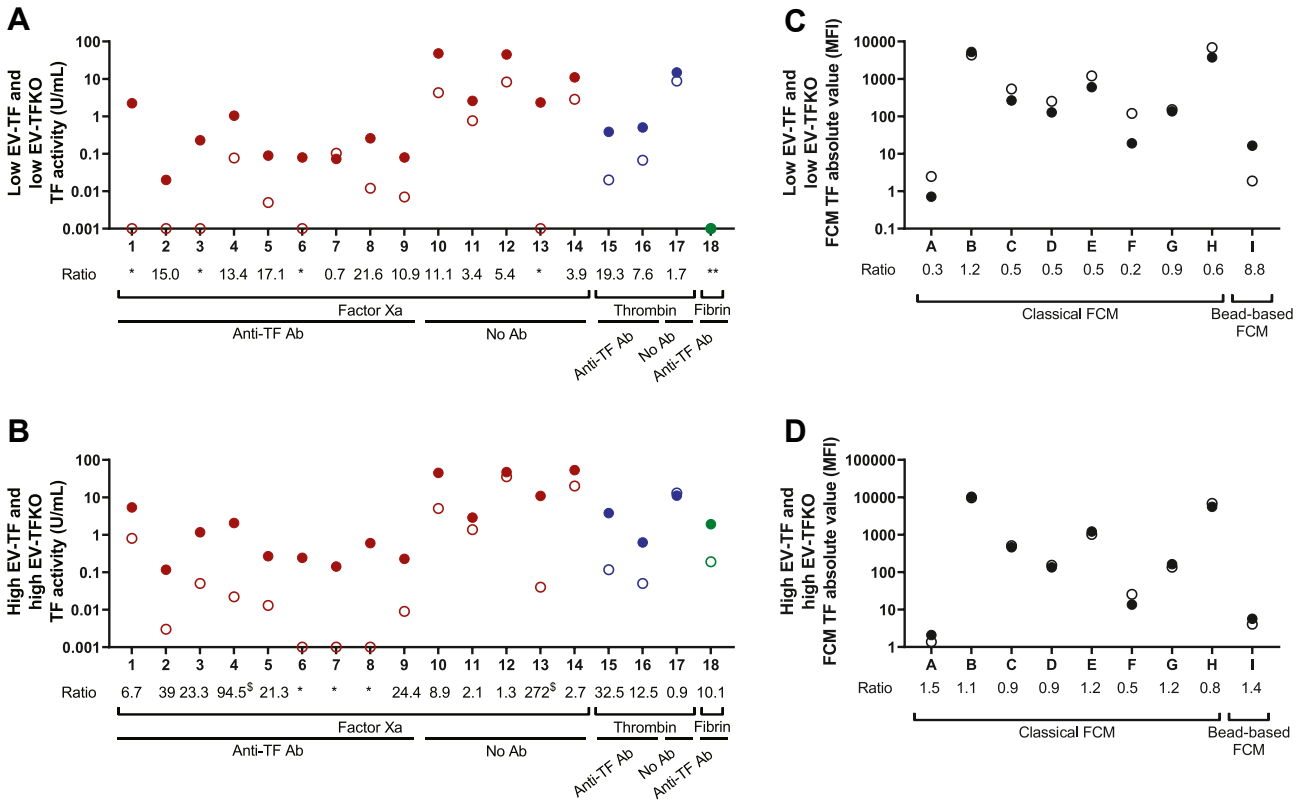

FIGURE 1 Evaluation of the specificity of the assays. Samples used to assess the specificity of the assays included 2 pairs of samples: low extracellular vesicle (EV)–tissue factor (TF)/low EV–knockout TF (TFKO) and high EV-TF/high EV-TFKO. (A, C) Absolute values of low EV-TF (filled dot) and low EV-TFKO (empty dot). (B, D) Absolute values of high EV-TF (filled dot) and high EV-TFKO (empty dot). (A, B) Evaluation of the specificity of the functional assays. For functional assay 18, this investigator did not measure activity in the low EV-TF sample. (C, D) Evaluation of the specificity of the flow cytometry (FCM) assays. Data from assay numbers 2, 5, 7, 9, and 16 contain extrapolated values (2, high and low EV-TF; 5, low EV-TF; 7, high and low EV-TF; 9, low EV-TF; and 16, high and low EV-TF). These data contain values lower than the lowest calibration point. Legend: red dots, factor Xa generation assays; blue dots, thrombin generation assays; green dots, fibrin generation assays; white and black dots, FCM assays. Ab, antibody; MFI, mean fluorescence intensity. *Ratio could not be determined because the denominator was equal to 0. **Ratio could not be determined because one of the samples was not analyzed. $Outlier. Robust regression and outlier removal test (q = 1) was used to determine outliers.

4 assays that did not use an antibody (the ROUT outlier test excluded assay 13; a 9.8-fold difference; P = .015; Table). For the thrombin generation assays, 2 of 3 assays used an anti-TF antibody (Supplementary Table S1). Similarly, the ratios for the low EV-TF/low EV-TFKO and high EV-TF/high EV-TFKO for the 2 assays that used an antibody were higher than the ratio for these samples for the assays that did not use an antibody (Figure 1).

Taken together, these results show that the use of a blocking antibody against TF or specific immunocapture allows a more specific detection of EV-TF activity using functional assays.

3.3 | Specificity of the TF antigen assays

To assess the specificity of the FCM TF assays, we evaluated their capacity to discriminate EV-depleted plasma spiked with low or high concentrations of EVs derived from HAP-1 wild-type or TFKO cells. Figure 1C, D shows the absolute values for the FCM assays. Only assay B of the 8 classical FCM groups had a ratio above 1 (1.2) for the low EV-TF/low EV-TFKO samples. The bead-based FCM used in assay I had a ratio of 8.8. For the high EV-TF/high EV-TFKO samples, assay A had a ratio of 1.5, 2 assays (E and G) had a ratio of 1.2 or above, 1 assay (B) had a ratio above 1, and 4 assays (C, D, F, and H) had a ratio below 1. The bead-based FCM had a ratio of 1.4 for the high EV-TF/high EV-TFKO sample. The high EV-TF/high EV-TFKO samples would be expected to give a greater ratio compared with the low EV-TF/low EV-TFKO samples. However, the bead-based FCM had a higher ratio for the low EV-TF/low EV-TFKO sample compared with the high EV-TF/high EV-TFKO sample. These data suggest that FCM has a low specificity for detecting TF-positive EVs in plasma.

3.4 | Comparison of the specificity of the TF functional and antigen assays

The median ratio of the TF functional assays for the low EV-TF/low EV-TFKO samples (n = 13) was 10.9 compared with 0.5 for the TF antigen assays (classical FCM only, n = 8; a 21.4-fold difference; P = .001; Table).

TABLE Ratios of functional assays with or without an anti–tissue factor antibody and antigen assays.

Assay	Ratio low EV-TF/low EV-TFKO	Ratio high EV-TF/high EV-TFKO	Ratio high milk-EV/low milk-EV	Ratio high EV-TF/low EV-TF	Ratio LPS+/LPS−
All functional assays	10.9 (3.6-16.1) *n* = 13	10.1 (2.4-23.8) *n* = 13	2.2 (1.1-3.2) *n* = 17	2.4 (1.1-4.2) *n* = 16	6.2 (2.8-14.1) *n* = 14
FXa assay + Ab	14.2 (8.4-18.2) *n* = 6	23.3 (14.0-31.7) *n* = 5	2.4 (1.6-3.3) *n* = 9	2.9 (2.2-4.0) *n* = 9	6.4 (5.9-8.6) *n* = 7
FXa assay − Ab	4.6 (3.5-9.7) *n* = 4	2.4 (1.5-7.3) *n* = 4	1.3 (1.1-2.8) *n* = 5	1.1 (1.0-4.8) *n* = 5	2.5 (1.7-25-4) *n* = 4
Fold change FXa assays ± Ab	3.1	9.8	1.9	2.6	2.6
All classical FCM assay	0.5 (0.3-0.8) *n* = 8	1.0 (0.8-1.2) *n* = 8	0.9 (0.6-1.0) *n* = 8	1.6 (1.1-2.0) *n* = 8	2.2 (1.3-3.3) *n* = 8
Fold change functional assays/classical FCM assays	21.4	10.2	2.4	1.5	2.8

Data are expressed as median (IQR). Outliers were not included in this analysis.

Ab, antibody; EV, extracellular vesicle; FCM, flow cytometry; FXa, factor Xa; LPS, lipopolysaccharide; TF, tissue factor; TFKO, knockout tissue factor.

The median ratio of the TF functional assays for the high EV-TF/high EV-TFKO samples (*n* = 13, the ROUT outlier test excluded laboratories 4 and 13) was 10.1 compared with 1.0 for the TF antigen assays (classical FCM only, *n* = 8; a 10.2-fold difference; *P* = .009; Table). This indicates that the functional assays are more specific than the antigen assays.

3.5 | Sensitivity of the TF functional assays

To assess the sensitivity of the TF functional assays, we evaluated the capacity of each assay to discriminate plasma spiked with different concentrations (high and low) of either milk EVs, EV-TF from a cell line, or plasma from healthy donors with or without LPS stimulation (LPS+ and LPS−, respectively). Three ratios were calculated for signals from 1) high and low milk-EV samples, 2) high and low cellular EV-TF samples, and 3) LPS+ and LPS− samples. We considered that a ratio above 1.2 indicates that the assay is sensitive for TF. The absolute values used to calculate the ratios of each functional assay are shown in Figure 2A–C.

The median ratios for the high/low milk-EV, high/low cellular EV-TF, and LPS+/LPS− samples were 2.2 (*n* = 17), 2.4 (*n* = 16, the ROUT outlier test excluded assay 15), and 6.2 (*n* = 14, the ROUT outlier test excluded assays 6 and 15), respectively (Table). For the milk-EV samples, 12 of 18 (66%) assays had a ratio greater than 1.2. Five of the assays had a ratio less than 1.2, and 1 had a value of 0 for the low milk-EV sample. For the cellular EV-TF samples, 13 of 18 (72%) assays had a ratio greater than 1.2. Four of the assays had a ratio less than 1.2, and 1 had a value of 0 for the low EV-TF sample. For the LPS+/LPS− samples, 15 of 18 (83%) assays had a ratio greater than 1.2 (Table). One of the assays had a ratio less than 1.2, and 2 had a value of 0 for the LPS− sample.

Among the FXa generation assays, 9 of 14 assays used either an anti-TF antibody or immunocapture and 5 of 14 did not (Supplementary Table S1). The median ratio for the 9 assays that used an antibody or immunocapture for the milk-EV samples was 2.4 compared with 1.3 for the 5 assays that did not use an antibody or immunocapture (a 1.9-fold difference; *P* = .393; Table). The median ratio for the 9 assays that used an antibody or immunocapture for the high EV-TF/low EV-TF samples was 2.9 compared with 1.1 for the 5 assays that did not use an antibody or immunocapture (a 2.6-fold difference; *P* = .282; Table). The median for the 7 assays that used an antibody or immunocapture for the LPS+/LPS− samples (the ROUT outlier test excluded assay 6) was 6.4 compared with 2.5 for the 4 assays that did not use an antibody or immunocapture (a 2.6-fold difference; *P* = .230; Table). For the thrombin generation assays, 2 of 3 assays used an anti-TF antibody (Supplementary Table S1). Similarly to the FXa generation assays, the ratios for the milk-EV, cellular EV-TF, and LPS+/LPS− samples for the 2 assays that used an antibody were higher than the ratios of the assay that did not use an antibody. These data indicate that the use of an anti-TF antibody or immunocapture increases the sensitivity of the functional assays.

3.6 | Sensitivity of the TF antigen assays

To assess the sensitivity of the TF antigen assays, we evaluated the capacity of each assay to discriminate plasma spiked with different concentrations (high and low) of either milk EVs, EV-TF from a cell line, or plasma from healthy donors with or without LPS stimulation (LPS+ and LPS−, respectively). The absolute values used to calculate the ratios of each antigen assay are shown in Figure 2D–F.

For the milk-EV samples, the ratio was below 1.2 for all the assays based on classical FCM. The variability of the triplicate measurements can be seen in the Supplementary Figure S4. Laboratories C and D had ratios of 1.10 and 1.05, respectively. The bead-based assay had a ratio of 2.0. For the cellular EV samples, 5 of 8 (62%) assays based on classical FCM had a ratio above 1.2. Two assays had a ratio of 1, and 1 assay had a ratio of <1. The bead-based assay had a ratio of <1. For the LPS+/LPS− samples, 7 of 8 (88%) assays based on classical FCM had a ratio above 1.2 and 1 had a ratio <1. The bead-based assay had a ratio of 1.5. These results indicate that some of the antigenic assays could discriminate between the paired samples.

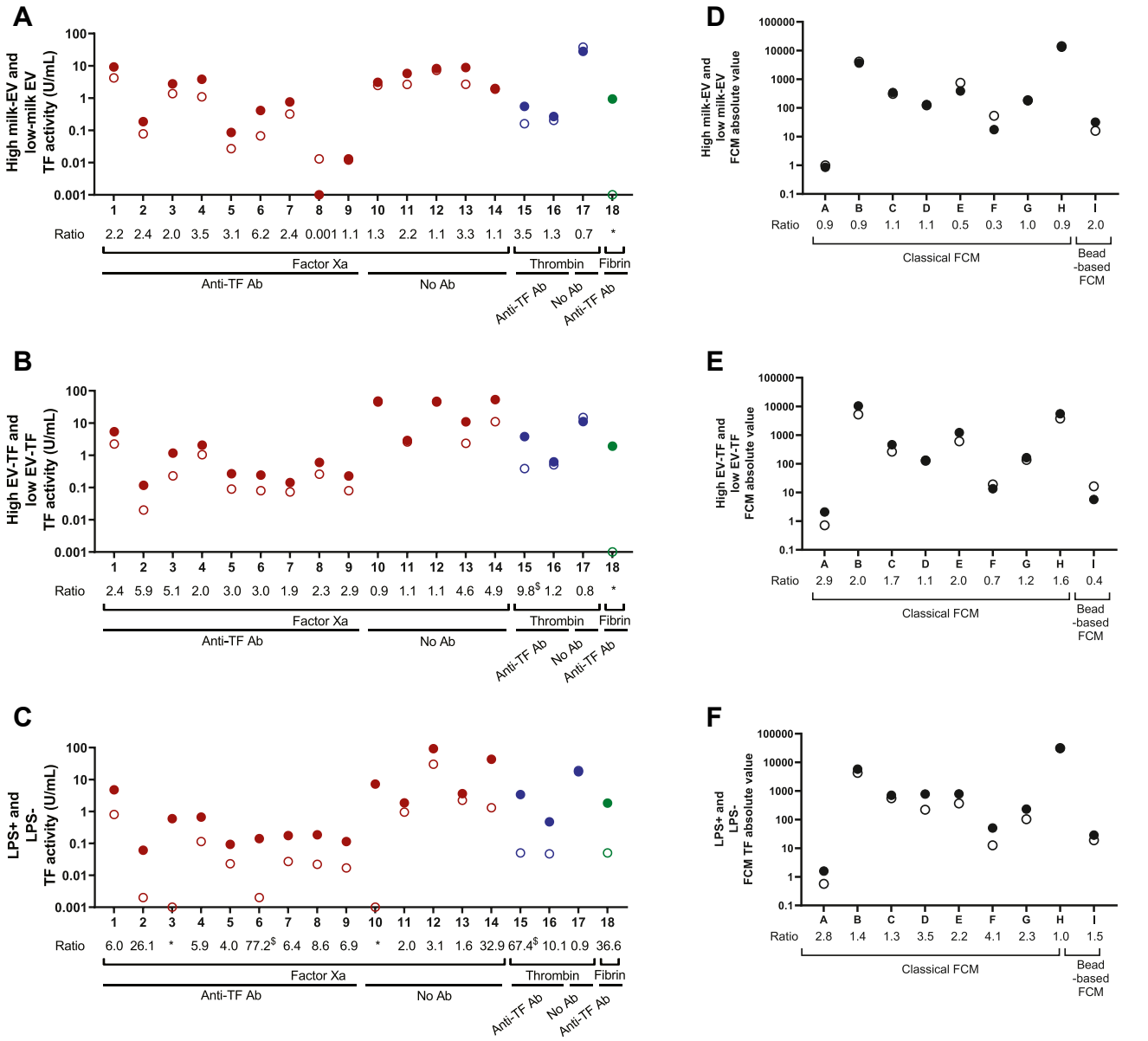

FIGURE 2 Evaluation of assay sensitivity. Samples used to assess the sensitivity of the assays included high and low levels of milk-extracellular vesicles (EVs), high and low levels of EV–tissue factor (TF), and platelet-depleted plasma from whole blood with or without lipopolysaccharide (LPS). Three ratios were calculated between high and low milk-EV, high and low EV-TF, and LPS+/LPS− samples to assess the sensitivity of the assays. (A, D) Absolute values of high milk-TF (filled dot) and low milk-EVs (empty dot). (B, E) Absolute values of high EV-TF (filled dot) and low EV-TF (empty dot). (C, F) Absolute values of LPS+ (filled dot) and LPS− (empty dot) samples. (A–C) Results for functional assays. (D–F) Results for flow cytometry (FCM) assays. Data from assays number 2, 5, 7, 8, 9, and 16 contain extrapolated values (2: high and low milk-EVs, high and low EV-TF, LPS+; 5: high and low milk-EVs, low EV-TF; 7: high and low milk-EVs, high and low EV-TF, LPS+; 8: high and low milk-EVs, low EV-TF, LPS+; 9: high and low milk-EVs, low EV-TF, LPS+; 16: high and low milk-EVs, high and low EV-TF, LPS+). These data contain values lower than the lowest calibration point. Legend: red dots, factor Xa generation assays; blue dots, thrombin generation assays; green dots, fibrin generation assays; white and black dots, FCM assays. Ab, antibody.

3.7 | Comparison of the sensitivity of the TF functional and antigen assays

The median ratio of the TF functional assays for the high milk-EV/low milk-EV samples was 2.2 compared with 0.9 for TF antigen assays (classical FCM only; a 2.4-fold difference; $P = .016$; Table). The median ratio of the TF functional assays for the high EV-TF/low EV-TF samples (the ROUT outlier test excluded assay 6) was 2.4 compared with 1.1 for the TF antigen assays (classical FCM only; a 2.6-fold difference; $P = .007$; Table). The median ratio of the TF functional assays for the LPS+/LPS− samples (the ROUT outlier test excluded assays 6 and 15) was 6.2 compared with 2.2 for TF antigen assays (a 2.80-fold difference; $P = .016$; Table). This indicates that the functional assays are more sensitive than the antigen assays.

3.8 | Intra-assay repeatability

To assess the repeatability of functional and FCM assays measuring EV-TF, all samples were measured in triplicate. The coefficient of variation (CV) was calculated for 5 samples: high and low milk-EV, high and low cellular EV-TF, and LPS+ samples.

As shown in Figure 3, a large variability was observed, with CVs ranging from 0.6% to 154% and a median CV of 16% for both functional and antigenic assays. Thus, it was established that a minimum increase of 20% between the EV-TFKO and the EV-TF samples is required to demonstrate TF specificity in each analysis (ratios above 1.2). Even for the same assay, the repeatability varied significantly between samples (eg, assay 1 varied from 12% to 55%). Interestingly, we observed a higher variability in the TF functional assays isolating EVs by high-speed centrifugation compared with those using immunocapture strategies (mean ± SD, 18.4% ± 15.9% [n = 15] vs 10.4% ± 37.2% [n = 3]; P = .037).

Taken together, these results indicate the large variability of both functional and antigenic assays measuring TF-positive EVs.

3.9 | Inter-assay reproducibility

To assess the interassay reproducibility, we calculated the interassay CV for the 6 functional assays (4 FXa assays, 1 thrombin assay, and the fibrin assay) with ratios of specificity and sensitivity above the median (assays 2, 3, 4, 6, 15, and 18) using 5 samples (high milk-EV, low milk-EV, high cellular EV-TF, low cellular EV-TF, and LPS+). The LPS− samples were not used because the activity measured was too low and close to the detection threshold of the assays. As shown in Figure 4, despite the use of a common calibrant, the interassay reproducibility ranged from 88% to 134% according to the sample, highlighting a lack of interassay reproducibility.

4 | DISCUSSION

Multiple assays are used in the literature to measure EV-TF [9]. Major concerns include the specificity, sensitivity, and repeatability of these assays to ensure robust and reproducible data between laboratories. This multicenter study compared the analytical performances of 27 EV-TF assays performed by expert laboratories. The main findings of the study are that functional assays using a blocking anti-TF antibody or specific immunocapture were most sensitive and specific compared with functional assays that did not use an anti-TF antibody, activity assays are more sensitive and specific compared with antigen assays, there was lower variability using immunocapture compared with isolation of EVs using centrifugation, and there was a high variability between the different assays.

Previous monocentric studies have compared functional assays. Two studies compared in-house FXa and thrombin generation assays with a TF immunocapture commercialized assay and reported a lower specificity and sensitivity of the TF immunocapture commercialized assay than of the in-house assays [19,20]. Another study comparing 2 FXa generation assays improved the sensitivity by using FVII instead of FVIIa and the clone SBTF1 as a TF-blocking antibody instead of HTF-1 [21]. Recently, a study compared the thrombin generation assay published by Østerud et al. [10] with the FXa generation assay

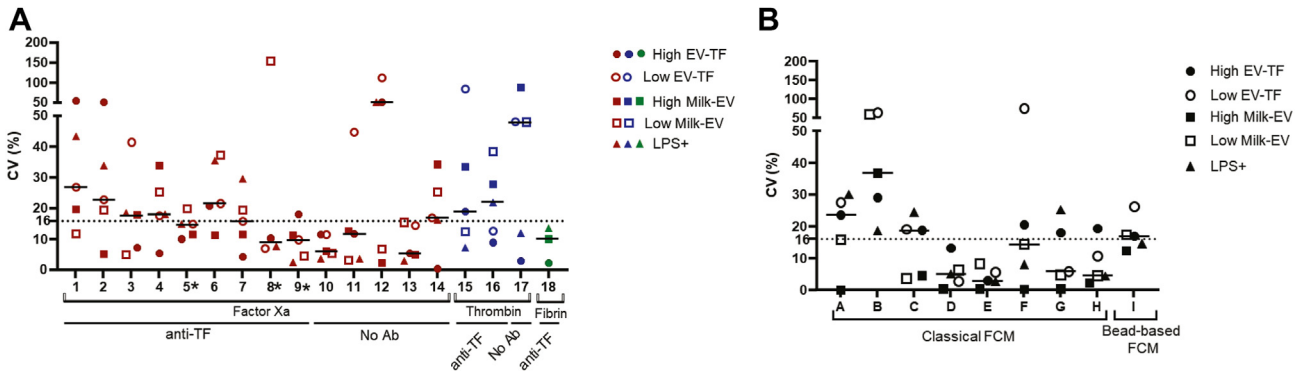

FIGURE 3 Evaluation of the repeatability of the assays. The mean and SD values for each sample and for each assay were obtained from the values of the measurements carried out on the 3 aliquots provided for each sample. All measurements were performed on the same day. (A) Intra-assay variability of the functional assays. (B) Intra-assay variability of the flow cytometry (FCM) assays. The dashed lines indicate the median for the functional and flow FCM assays. Data from assays number 2, 5, 7, 8, 9, and 16 contain extrapolated values (2: high and low milk-extracellular vesicles [EVs], high and low EV–tissue factor [TF], lipopolysaccharide [LPS+]; 5: high and low milk-EVs, low EV-TF; 7: high and low milk-EVs, high and low EV-TF, LPS+; 8: high and low milk-EVs, low EV-TF, LPS+; 9: high and low milk-EVs, low EV-TF, LPS+; 16: high and low milk-EVs, high and low EV-TF, LPS+). These data contain values lower than the lowest calibration point. Legend: red symbols: factor Xa generation assays; blue symbols: thrombin generation assays; green symbols: fibrin generation assays; white and black symbols: FCM assays; filled dots: coefficient of variation (CV) high EV-TF; empty dots: CV low EV-TF; filled squares: CV high milk-EVs; empty squares: CV low milk-EVs; filled triangle: CV LPS+ sample; dotted line: median CV. Ab, antibody. *Functional assays that used immunocapture to capture TF-positive EVs.

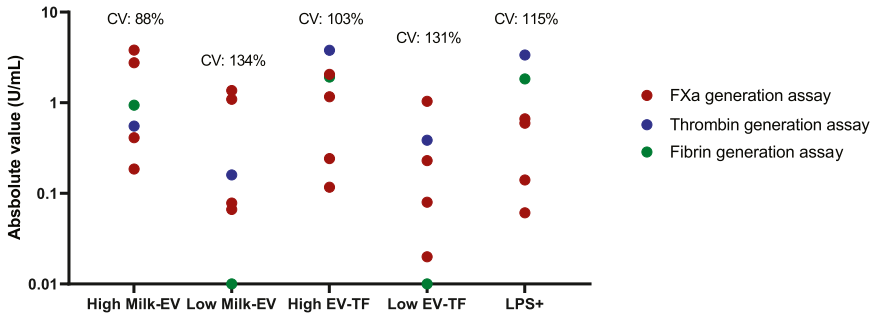

FIGURE 4 Interassay reproducibility. Mean values of the triplicate and respective coefficients of variation (CVs) from 6 specific and sensitive individual functional assays (assays 2, 3, 4, 6, 15, and 18) for 5 samples (high and low milk-extracellular vesicles [EVs], high and low EV–tissue factor [TF], and platelet-depleted plasma from lipopolysaccharide [LPS]-stimulated whole blood). Each dot represents the mean value of the activities obtained with 1 assay. FXa, factor Xa.

developed by Mackman et al. [9]. It showed that these 2 assays allow measurement of EV-TF in a specific and reproducible manner [22].

Our collaborative multicenter study is the first to compare 18 functional assays with different principles (FXa, FIIa, or fibrin generation assays) performed by 16 expert laboratories. We observed that functional assays displayed variable performances in terms of specificity and sensitivity. The main demonstration of this collaborative study is that the use of an antibody that inhibits TF activity increased the specificity and sensitivity of the assays. This conclusion strengthens the recommendation of expert opinion to use a TF-blocking antibody [18,23]. Moreover, it is consistent with the demonstration that TF-independent activity can be caused by 1) the presence of phospholipids in a concentration-dependent manner [21] and 2) the use of FVIIa instead of FVII [21] because FVIIa can activate FX in a concentration-dependent manner independent of TF [24]. The Zymuphen MP-TF immunocapture assay (Hyphen Biomed) (assays 8 and 9) displayed a lack of sensitivity to discriminate low and high milk-EV samples compared with the FXa generation assays that used a TF-blocking antibody. This could be explained, in part, by a failure of the immunocapture of these EVs purified from milk. Furthermore, in the current study, the sensitivity of the assays was evaluated as the discriminative capacity to differentiate 2 levels of EVs. Therefore, the study did not allow for determination of the limit of detection or the ability to detect an activity in healthy samples, which will require further evaluation.

The 8 classical FCM assays were less specific and sensitive compared with the functional assays. Only 1 of 8 assays had a ratio >1.2 for the low EV-TF/low EV-TFKO samples, whereas 50% of the assays had a ratio >1.0 (1.06-1.52) for the high EV-TF/high EV-TFKO samples. Additionally, 7 of 8 FCM assays show a ratio >1 for high EV-TF/low EV-TF and LPS+/− samples. The functional assays showed that the 3 different paired samples had a range of sensitivities between the high and low samples in the following order: LPS+/LPS− samples > cellular EV samples > milk-EV samples. Interestingly, the median ratios for the FCM assays for the LPS+/−, cellular EV, and milk-EV samples were 2.21, 1.61, and 0.91, respectively. Altogether, these data suggest a trend toward detecting TF+ EVs in plasma, which may be due to a variation of the TF antigen density on the surface of EVs between those produced after LPS stimulation of whole blood and those spiked into plasma.

These issues in terms of specificity and sensitivity of FCM are in line with a large range of values reported in healthy donors, from less than 10 to several thousand TF+ EVs per microliter in plasmatic samples [25–28]. The sensitivity of EV-TF measurement by FCM is reduced for several reasons. First, EVs are small (most have a diameter near 100-150 nm) and consequently have a low antigen density, exposing at best only a few TF molecules. Even when an antibody can label all TF molecules on a single EV, the fluorescence signal may be below the limit of detection of an FCM fluorescence detector. Here, the accurate identification of EV through FCM analysis primarily relies on the performances of the instrument used in the analysis. Second, TF could be masked for antibody labeling by FVII(a) binding to TF or by coverage of TF+ EVs by a fibrin cap [29]. This hypothesis is supported since 1) FVII/FVIIa has a very high affinity for TF and 2) EV-TF activity was compared with or without exogenous FVIIa, and significant levels were observed in the absence of exogenous FVIIa-TF activity after addition of a TF pathway inhibitor antibody was found, suggesting that TF pathway inhibitor is bound to the TF/FVII complex. However, it should be noted that the most used anti-TF monoclonal antibody (HTF-1 clone) used to block TF competes with FVII/FVIIa for binding to TF. This means that anti-TF antibodies used for FCM can displace bound FVII/FVIIa from TF. In addition to these issues impacting sensitivity, another major limitation is FCM specificity. The misuse of isotype control antibodies in FCM experiments may lead to the detection of false-positive events [30,31]. Consequently, the events thought to be TF+ EVs are sometimes correlated with the total number of EVs in the sample and therefore may just reflect the increase in total EVs in diseases [32,33]. This could explain some of the differences in EV-TF quantification between LPS− and LPS+ samples in our study. However, in a recent study, LPS stimulation of whole human blood did not increase the levels of EVs isolated using a 20 000 × *g* spin [36]. In sum, the current limitations of FCM analytic performances and associated reagents mean that it is difficult to reliably measure TF carried by EVs in biological samples using FCM. FCM has already benefited from tremendous developments over the past decade to improve its sensitivity to measure EVs with a positive impact in many applications [31]. It is likely that future developments may change the conclusion of the present study.

The ratios from the bead-based FCM from the different samples are difficult to interpret. The ratio for the high EV-TF/high EV-TFKO sample (1.41) is lower than the ratio for the low EV-TF/low EV-TFKO sample (8.76). The ratios for the milk-EV, cellular EV, and LPS+/LPS− samples were 2.01, 0.35, and 1.52, respectively. A recent study

concluded that the bead-based FCM kit did not have the sensitivity to detect TF-positive EVs in plasma [34].

Both intra-assay and interassay reproducibility show a huge heterogeneity of the results. First, most of the functional assays displayed a CV below 20%, but their repeatability was inconsistent and lacked robustness. Indeed, within the same assay, CVs ranging from 2% to 112% were observed (assay 12). Interassay reproducibility also showed a large variability of the absolute values between assays using a common calibrant.

Several reasons can explain this variability. The preanalytical step has been identified as one of the major sources of variability in the EV measurement. Previous studies have identified the delay before the first centrifugation, the agitation of the tubes during transportation, and the centrifugation step as the most critical parameters [17]. In this study, we can rule out the impact of the time delay and the transportation because similar aliquots already prepared by core labs were sent to the participant laboratories. Thus, the centrifugation remains the most probable cause of the intra-assay variability. Previous studies show that the recovery of the pellet depends on the rotor type, the centrifugation speed (g-force), the temperature, the use of brake [35], and the centrifugation time and limits the repeatability of EV measurement [21,36]. Another source of variability may be the detection step (ie, the equipment used, time, and agitation), but the use of a common calibrant in the study should have prevented a significant impact of these variables between assays. An additional cause of the interassay variability may be the manual aspect of the EV-TF assays, which may contain some steps with high risk of EV loss.

The future directions for improving the variability of results are based on 3 potential strategies, which are not exclusive. first, standardization of reagents and homogenization of protocols that emerge from the assays having presented the best analytical performances in terms of sensitivity and specificity; second, proposing a preanalytical step independent of centrifugation, which is the most important cause of variability, as was recently published with the preparation of EVs by magnetic immunoseparation [37]; and third, moving to an automated version of these assays.

This study represents a first step toward a better selection of the most appropriate methods to measure EV-TF, but cannot be considered a standardization study. Indeed, FCM assays do not benefit from a common standardization tool, and the calibrant used to compare functional assays (recombinant soluble TF) has by nature significant differences from EV-TF. Moreover, the initial choice of the calibration curve range resulted in some extrapolated values for 6 of 18 functional assays. However, further methodological and standardization efforts are mandatory before considering EV-TF as a biomarker in clinical practice to predict thrombosis in patients at high risk, such as those with cancer or thromboinflammatory diseases.

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AUTHOR CONTRIBUTIONS

F.D.-G., N.M., R.L., and R.N. conceived and designed the analysis. A.B. collected the data. A.B., A.T.A.S., F.D.-G., N.M., R.L., R.N., and Y.H. analyzed and interpreted the data and wrote the paper. F.D.-G. and R.L. supervised the work. All authors contributed data, critically contributed to the revised version of the manuscript for important intellectual content, and gave final approval for the version to be published.

DECLARATION OF COMPETING INTERESTS

F.D.-G. and R.L. filed a patent on microvesicle fibrinolytic activity licensed to Stago and obtained a common grant within the framework of the excellence program innovative tests to customize antiplatelet therapy in chronic kidney disease with acute coronary syndrome.

The remaining authors declare no competing financial interests.

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SUPPLEMENTARY MATERIAL

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