



HAL
open science

Increasing the sensitivity of the human microvesicle tissue factor activity assay

Loris Vallier, Tarik Bouriche, Amandine Bonifay, Coralie Judicone, Jeremy Bez, Corentin Franco, Christophe Guervilly, Yohei Hisada, Nigel Mackman, Reaves Houston, et al.

► **To cite this version:**

Loris Vallier, Tarik Bouriche, Amandine Bonifay, Coralie Judicone, Jeremy Bez, et al.. Increasing the sensitivity of the human microvesicle tissue factor activity assay. *Thrombosis Research*, 2019, 182, pp.64-74. 10.1016/j.thromres.2019.07.011 . hal-02571424

HAL Id: hal-02571424

<https://amu.hal.science/hal-02571424>

Submitted on 20 Dec 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Increasing the sensitivity of the human microvesicle tissue factor activity**
2 **assay**

3

4 Loris Vallier¹, Tarik Bouriche², Amandine Bonifay¹, Coralie Judicone^{2,3}, Jeremy
5 Bez², Corentin Franco^{1,2}, Christophe Guervilly⁴, Yohei Hisada⁵, Nigel
6 Mackman⁵, Reaves Houston⁵, Philippe Poncelet², Françoise Dignat-George^{1,3*},
7 and Romaric Lacroix^{1,3}

8 *1. Aix Marseille University, INSERM, INRA, C2VN, Marseille, France*

9 *2. Research and Technology Department, BioCytex, Marseille, France*

10 *3. Department of Hematology and Vascular Biology, CHU La Conception, APHM, Marseille,*
11 *France*

12 *4 Medical Intensive Care Unit, North Hospital, APHM, Marseille, France.*

13 *5. Division of Hematology and Oncology, Thrombosis and Hemostasis Program, Department*
14 *of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina*

15

16 *Corresponding author:

17 Françoise Dignat-George; C2VN, UMR-1263, INRA-1260, Faculté de Pharmacie, 27 Bd Jean
18 Moulin, 13010, Marseille, France; francoise.dignat-george@univ-amu.fr

19 Tel.: 0033(0)4 91 83 56 00; fax: 0033(0)4 91 83 56 02

20

21

22 **Abstract**

23 **Introduction:** The TF-FVIIa complex is the primary activator of coagulation. Elevated levels
24 of microvesicle (MV) bearing tissue factor (TF)-dependent procoagulant activity are
25 detectable in patients with an increased risk of thrombosis. Several methods have been
26 described to measure MV TF activity but they are hampered by limited sensitivity and
27 specificity. The aim of this work was to increase the sensitivity of the MV TF activity assay
28 (called Chapel Hill assay).

29 **Material and Methods:** Improvements of the MV TF activity assay included i/ speed and
30 time of centrifugation, ii/ use of a more potent inhibitory anti-TF antibody iii/ use of FVII and
31 a fluorogenic substrate to increase specificity

32 **Results:** The specificity of the MV TF activity assay was demonstrated by the absence of
33 activity on MV derived from a knock-out-TF cell line using an anti-human TF monoclonal
34 antibody called SBTF-1, which shows a higher TF inhibitory effect than the anti-human TF
35 monoclonal antibody called HTF-1. Experiments using blood from healthy individuals,
36 stimulated or not by LPS, or plasma spiked with 3 different levels of MV, demonstrated that
37 the new assay was more sensitive and this allowed detection of MV TF activity in platelet free
38 plasma (PFP) samples from healthy individuals. However, the assay was limited by an inter-
39 assay variability, mainly due to the centrifugation step.

40 **Conclusions:** We have improved the sensitivity of the MV TF activity assay without losing
41 specificity. This new assay could be used to evaluate levels of TF-positive MV as a potential
42 biomarker of thrombotic risk in patients.

43

44

45

46 **Highlights:**

- 47 • Current methods to measure MV TF activity have limited sensitivity
- 48 • An improved TF-dependent FXa generation assay was developed
- 49 • The updated MV TF activity assay includes a new anti-TF inhibitory antibody
50 (SBTF-1)
- 51 • The MV TF activity assay improve sensitivity as compared with previous test

52 **Keywords** : microvesicles; extracellular vesicles; tissue factor; procoagulant test

53 **Introduction**

54 Microvesicles (MV) are extracellular vesicles released from the cellular membrane
55 which have been described as procoagulant entities since their first report by Peter Wolf, 50
56 years ago [1]. This procoagulant phenotype relies on the exposure of anionic phospholipids,
57 especially the phosphatidylserine (PS) on the external leaflet of the membrane, allowing the
58 binding of coagulation factors at the MV surface by their carboxylglutamic acid-rich (GLA)-
59 domains [2]. In addition, the presence of the coagulation initiator tissue factor (TF) on subsets
60 of MV also significantly contributes to their procoagulant activity. Different studies that
61 infused MV into mouse models of venous or arterial thrombosis demonstrate the procoagulant
62 activity of MV *in vivo* [3–5]. Special attention has been given to cancer-associated thrombosis
63 and the underlying mechanisms linking MV and venous thromboembolism (VTE) [6,7]. Data
64 from animal models show that tumour-derived TF-positive MV are key players of thrombus
65 formation by activating both the coagulation system and platelets [8–12].

66 These mechanistical data in murine model unequivocally demonstrate the contribution
67 of MV TF in thrombus formation. Indeed, in humans, elevated plasma levels of MV TF have

68 been associated with an increased risk of developing VTE in cancer patients. [13–18].
69 However, the association between levels of MV TF activity and VTE has been shown in
70 patients with pancreatic cancer but no other types of cancer. This may be due to different
71 pathophysiological mechanisms involved in the VTE formation in cancer [12] but also limited
72 sensitivity of the MV TF activity.

73 Several methods have been described to measure MV TF in clinical samples using
74 either activity or antigen-based assays [6,19]. However, the specificity and sensitivity of these
75 assays is a concern. Among these assays, antigenic detection of TF on circulating MV
76 provides the advantage to detect both cryptic and decrypted TF but the measurement of TF by
77 flow cytometry remains very challenging because of the low levels of TF and concerns about
78 some anti-TF antibodies [20]. Currently, there are two non-commercial methods that have
79 been reported for MV TF activity that use either a kinetic monitoring of the specific substrate
80 (Leiden assay) or a end point (Chapel Hill assay) to measure factor Xa (FXa) generation
81 [6,21,22]. These assays use an antibody which inhibits TF activity. A good correlation was
82 found between these two versions of the FXa generation assay in 54 pancreatic cancer
83 patients [24] and they proved to be more sensitive than commercial assays [24]. A recent
84 paper described the Chapel Hill assay in detail [25].

85 The aim of this work was to improve the MV TF-dependent FXa generation assay
86 (MV TF activity assay) and evaluate its analytical performances in comparison with a
87 currently used test (Chapel Hill assay).

88 **Materials and Methods**

89 *Blood sample processing*

90 Blood samples from healthy donors, who signed an informed consent form, were
91 collected and processed according to the current International Society on Thrombosis and

92 Haemostasis guidelines [19,26]. Briefly, after a light tourniquet was applied, samples were
93 drawn from the antecubital vein using a butterfly device with a 21-gauge needle. Blood was
94 collected into 5 mL Vacutainer tubes containing 0.129 mol/L sodium citrate (BD Diagnostics,
95 Franklin Lakes, NJ, US), and the first few milliliters were discarded. The samples were
96 subjected to two successive centrifugations (2,500 g for 15 min at room temperature (RT)) to
97 prepare platelet-free plasma (PFP). The PFP was homogenized before being aliquoted and
98 stored at -80°C until use.

99 For specific experiments, whole blood was incubated with bacterial
100 lipopolysaccharide (LPS) (10 µg/mL, *Escherichia coli* O111: B4; Sigma Aldrich, St. Louis,
101 MO, USA) for 5h at 37°C. Then PFP were prepared with two successive centrifugations
102 (2,500g, 15 min, RT with a Multifuge X3R centrifuge, rotor TX-1000, k-factor : 9470,
103 Thermofisher, Courtaboeuf, France).

104 ***MV preparation***

105 Human myeloid leukemia HL60 cells (Sigma Aldrich, Lyon, France) and human
106 pancreatic BxPC3 cells (Sigma Aldrich, Lyon, France), regularly tested for mycoplasmas with
107 Mycoalert (Lonza Biosciences, Basel, Switzerland) and DAPI (Sigma Aldrich, Lyon, France)
108 were cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented
109 with 10% of fetal bovine serum (FBS) 1% of penicillin and 1% of streptomycin (GIBCO
110 BRL, Gaithersburg, MD, USA), in humidified atmosphere at 37 °C, 5% CO₂. Cell viability
111 was assessed by trypan blue dye exclusion. Haploid human cell line (HAP1) cells and its
112 derivative KO-TF-HAP1 made by CRISPR/Cas9 (Thermofisher, Courtaboeuf, France) (TF-
113 protein expression was tested by flow cytometry and TF-gene expression was tested by
114 qPCR) were grown at 37°C and 5% CO₂ in Iscove's Modified Dulbecco's Medium (IMDM)

115 (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% of FBS, 1% of penicillin
116 and 1% of streptomycin. All mediums were filtrated at 0.22 μm (Corning, New York, USA).

117 MV purified from culture supernatants : HL60-MV, BxPC3-MV and HAP1-MV were
118 purified from conditioned medium after cells and debris by two successive centrifugations at
119 300g, 5 min and an additional centrifugation at 2,500g centrifugation, 10 min.

120 MV purified from clinical samples : Platelet-derived MV (PMV) were generated from
121 PRPs as already described [27]. Erythrocyte-derived MV (Ery-MV) were purified generated
122 from purified red blood cells either by aging (48h) or sonication by VIBRA Cell 75186; Pulse
123 S9 (60%) 3 times for 60s. All MV subsets were pelleted at 70,000g, 90 min, 4°C (JA-30.50 Ti
124 fixed-angle rotor, k-factor: 280, Beckman Coulter, Villepinte, France) and washed twice in
125 PBS buffer (2x 70,000g, 90 min). Isolated MV were enumerated by flow cytometry (Gallios,
126 Beckman Coulter, Villepinte, France) standardized by Megamix strategy [28] by reference to
127 counting beads (MP-Count beads, BioCytex, Marseille, France) [29]. Finally, MV were
128 spiked in MV-free plasma (removing MV by high-speed centrifugation 3x70,000g, 90 min)
129 before performing TF-dependent procoagulant testings.

130

131 ***Optimized MV TF dependent FXa generation assay design***

132 An optimized TF-dependent FXa generation assay (MV TF activity assay) was
133 adapted from the Chapel Hill TF-dependent FXa generation assay [22,30] as described [31].
134 Briefly, MV were pelleted by centrifugation at 24,000 g for 60 min at RT from 500 μl of
135 plasma 1:2 diluted in HEPES buffer (150 mM NaCl, 20 mM HEPES and 0.1% NaN₃, pH 7.4,
136 0.22 μm filtrated), washed in HEPES buffer and resuspended in 140 μl of HEPES buffer.
137 Aliquots (70 μl) were pre-incubated for 30 min at 37°C with either an inhibitory anti-TF
138 monoclonal antibody (10 $\mu\text{g}/\text{ml}$ final, clone SBTF-1, BioCytex, Marseille, France) or a
139 control antibody (10 $\mu\text{g}/\text{ml}$, clone a-DNP 2H11-2H12, BioCytex, Marseille, France) (Figure

140 1A). Then, 7 μ l HEPES-Ca²⁺ buffer (150 mM NaCl, 20 mM HEPES and 0.1% NaN₃, 50mM
141 CaCl₂, pH 7.4, 0.22 μ m filtrated) containing purified human FVII and FX (Stago BNL, JV
142 Leiden, Netherland) was added to each 70 μ l sample, to produce final concentrations of 10
143 nM, 190 nM and 5 mM CaCl₂ respectively and incubated for another 2 h at 37°C (Figure 1B).
144 FXa generation was halted by the addition of 8 μ l of EDTA buffer (150 mM NaCl, 20 mM
145 HEPES and 0.1% NaN₃, 200 mM EDTA, pH 7.4, 0.22 μ m filtrated) and a FXa fluorogenic
146 substrate (1 mM final, BioCytex, Marseille, France) was added (Figure 1C). Finally, the
147 fluorescence at 390 nm (excitation) and 460 nm (emission) was monitored for 15 min at 37°C
148 on a microplate fluorescence reader (Fluoroskan, CAT instrument, Stago, Asnières-sur-Seine,
149 France) (Figure 1D). Maximum reaction velocity (V_{max}) was calculated with the associated
150 software (Ascent Software, Luqa, Malta). V_{max} were corrected by subtracting those
151 generated in the presence of SBTF-1 from those generated in the presence of the control
152 antibody. Data from plasma-purified MV were expressed as fmol/L by comparison to a
153 calibration curve generated using recombinant TF (Figure 1E).

154 For comparison experiments, different centrifuge rotors were tested (FA45-24-11, k-
155 factor: 321; FA45-24-11, k-factor: 321; F15-6x100y, k-factor: 1536, Thermofisher,
156 Courtaboeuf, France; JA-30.50, k-factor: 280; Beckman Coulter, Villepinte, France). SBTF-1
157 anti-TF inhibitory antibody was compared to HTF-1 (BD Biosciences, San Jose, CA) at
158 various concentration (0.3-20 μ g/ml) and incubation time (5-10 min). Purified human FVII
159 was compared to purified human FVIIa (Stago BNL, JV Leiden, Netherland).

160 *Chapel Hill assay*

161 This assay has already been described in details in Khorana et al. 2008 [21,25,30]. Briefly, the
162 measurement of MV TF activity in plasma is based on an end point FXa generation

163 chromogenic assay, the use of a monoclonal antibody to inhibit TF activity (clone HTF-1, BD
164 Biosciences, San Jose, CA) and the use of FVIIa as TF cofactor.

165 *Statistical analysis*

166 All statistical analyses were performed with GraphPad Prism software version 5.0
167 (GraphPad Software, San Diego, CA, US). Significant differences were determined using a
168 non-parametric Mann-Whitney test or a paired t-test. Analysis of variances (ANOVA) was
169 used to compare rotors. A p-value less than 0.05 was considered statistically significant.
170 Spearman's rank correlation was used as a measure of the correlations between assays.

171 **Results**

172 *Specificity of the MV TF activity assay*

173 A major modification of the new MV TF activity assay relies on using a novel
174 inhibitory anti-TF antibody (SBTF-1) which ensures the assay specificity. The SBTF-1 clone
175 was compared to the HTF-1 clone used in the Chapel Hill assay at different concentrations
176 and incubation times with all other parameters being the same. As demonstrated on figure 2A,
177 compared to HTF-1, SBTF-1 showed a significantly more potent inhibition of the TF-
178 dependent FXa generation ($95\pm 1\%$ vs $92\pm 0.4\%$, at $20\ \mu\text{g/mL}$, 5 min, $p=0.04$). The superiority
179 of SBTF-1 over HTF-1 was confirmed at lower concentration. Twenty-two percent of
180 inhibition were lost at $2.5\ \mu\text{g/mL}$ compared with $20\ \mu\text{g/mL}$ with HTF-1 while the inhibitory
181 effect of SBTF-1 was unchanged. At $10\ \mu\text{g/mL}$ with both antibodies, we show a significant
182 increase in the inhibition % with SBTF-1 compared to HTF-1 ($95\pm 0.7\%$ vs $90\pm 0.5\%$,
183 $p<0.001$). This difference is amplified at $5\ \mu\text{g/mL}$ ($94\pm 1\%$ vs $86\pm 1\%$, $p<0.001$) (Figure 2A).
184 Moreover, the inhibitory effect of SBTF-1 was maintained over a broad range of spiking
185 doses of HL60 MV ($0.6 - 5\times 10^5$) with a saturating concentration above $2.5\ \mu\text{g/mL}$ that

186 decrease significantly in a concentration dependent manner at 0.63 $\mu\text{g/mL}$ ($p=0.03$), 0.32
187 $\mu\text{g/mL}$ ($p=0.03$) and 0.16 $\mu\text{g/mL}$ ($p=0.03$) (Figure 2B) and dramatically decrease at 0.02
188 $\mu\text{g/mL}$ ($p=0.03$) and at 0.002 $\mu\text{g/mL}$ ($p=0.03$) (Figure 2B). Thus, these data demonstrated that
189 the SBTF-1 clone has a more potent inhibitory activity than HTF-1 clone, whatever the
190 concentration used. For further experiments, a concentration of 10 $\mu\text{g/ml}$ of SBTF-1 was
191 chosen.

192 The TF-dependent FXa generation of the MV TF activity assay was then calculated by
193 the difference between the total FXa activity and the residual FXa activity which is not
194 inhibited by the SBTF-1 antibody (non-specific activity). As illustrated on a range of spiked
195 TF-positive HAP1-MV (Figure 2C), a significant difference of TF activity was noted
196 between, total and non-specific activity. Interestingly, when the assay was performed in
197 presence of the same amount of parental MV which have been knocked-out for TF (KO-TF-
198 MV) no TF specific activity was measured in contrast to parental MV (Figure 2D). This result
199 demonstrates the TF specificity of the SBTF-1 antibody and therefore the specificity of the
200 MV TF activity assay for TF.

201 The impact of MV surface phospholipids (PLs) was also tested. As illustrated in figure
202 2E, the addition of a range of KO-TF-MV to MV of the same PLs nature (parental HAP1-
203 MV) results in a slight increase in the FXa activity ($+17\pm 17\%$ with 0.25×10^6 KO-TF-MV).
204 This increase of FXa activity was only due to an increase in the non-specific activity
205 ($+11\pm 7\%$ with 0.25×10^6 KO-TF-MV) while the TF specific activity remained unchanged
206 (Figure 2E). In contrast, a significant increase of the specific activity generated by HL60-MV
207 was observed after spiking of sonicated Ery-MV, old Ery-MV, KO-TF-MV and PMV. As
208 shown on Figure 2F, the extent of the increase varies according to the PL origins ranging from
209 $100\pm 20\%$ with 1×10^6 sonicated Ery-MV ($p < 0.001$), $50\pm 20\%$ with 0.5×10^6 old Ery-MV
210 ($p=0.01$), $40\pm 20\%$ with 0.5×10^6 KO-TF-MV ($p=0.02$) to no significant impact with PMV.

211 These results demonstrate that MV TF specific activity can be impacted according to the
212 origin of MV surface phospholipids.

213 *Optimizing sensitivity of the MV TF activity assay*

214 The MV TF activity assay was optimized in order to improve its sensitivity. First, the
215 impact of the centrifugation protocol was evaluated by comparing MV TF activity in MV-free
216 plasma spiked by three different levels of BxPC3-MV. As illustrated in figure 3A, the
217 experiment was performed at the same centrifuge force (24,000g) but with different rotors.
218 We observed a significant different activity in all concentrations (LL : 11 ± 3 ; 47 ± 6 and 33 ± 5
219 fM for rotor 1, 2 and 3 respectively, $p = 0.003$; ML : 35 ± 3 ; 100 ± 10 ; 70 ± 2 fM, $p = 0.002$;
220 HL: 120 ± 16 ; 300 ± 50 ; 230 ± 13 fM, $p = 0.002$). Because of this variation, all further
221 comparison was performed with a same rotor. (rotor 2). Regarding the centrifugation time, a
222 significantly increased activity was observed with 60 min compared to 15 or 30 min as
223 performed on the Chapel Hill assay (28 ± 10 fM vs 60 ± 10 fM, $p = 0.009$, at 15 and 60 min,
224 respectively) while no further increase was observed with 90 min (64 ± 6 fM) (Figure 3B).
225 Regarding the centrifugation speed, no significant difference was observed between the speed
226 used in the Chapel Hill assay protocol 20,000g and 24,000g. Surprisingly, further increase in
227 the centrifugation speed results in a significant decrease in the activity (60 ± 10 ; 38 ± 13 ; 32 ± 17
228 fM at 24,000g, 70,000g and 100,000g, respectively, $p = 0.02$) (Figure 3C). Therefore,
229 centrifugation of PFP at 24,000 g for 60 min at RT, using the same rotor were delineated as
230 optimized preanalytical conditions to measure TF activity in a controlled range.

231 The Chapel Hill assay includes as reaction mix FX, CaCl_2 and FVIIa to generate FXa.
232 First, we compare FVII with FVIIa. As a result, FVII generated significantly increased MV
233 TF activity compared to FVIIa (38 ± 3 fM vs 27 ± 2 fM, $p=0.02$) with significantly less non-
234 specific activity (6 ± 2 fM vs 14 ± 1 fM, $p=0.004$) (Figure 3D). Secondly, we determined the

235 optimal concentrations of the FX or FVII to be used in the reaction mix without increasing the
236 non-specific activity. We compared different concentration of FX and FVII. As shown in
237 figure 3E, a concentration of FVII above 10 nM does not improve the sensitivity. **In contrast,**
238 **increasing the FX concentration results in a significant gain in the MV TF activity without**
239 **increasing the non-specific activity (36±6, 52±4, and 72±10 fM, at 190, 380 and 760 nM (10**
240 **nM FVII), respectively, p=0.002).** Finally, a concentration of FX of 190 nM was chosen for
241 cost reasons. Regarding calcium concentration, an activity plateau was reached at 5 mM
242 regardless of the levels of MV TF activity (Figure 3F) showing that this concentration is
243 sufficient for the FXa generation assay. Thirdly, we determine the optimal incubation time
244 with the reaction mix. As observed in figure 3G, MV TF activity increased with incubation
245 time. A 2h of incubation was chosen for further experiments as a good sensitivity/time ratio
246 (Figure 3G). Therefore, the incubation time was the same as the Chapel Hill assay.

247 Taken together, these results established the optimal experimental conditions to
248 improve sensitivity of the MV TF activity assay without losing specificity: centrifugation at
249 24,000 g for 60 min; 10 nM FVII, 190 nM FX and 5 mM CaCl₂ incubated for 2 h instead of
250 centrifugation at 20,000g for 15 min; 10 nM FVIIa, 300 nM FX and 10 mM CaCl₂ incubated
251 for 2h in the Chapel Hill assay.

252 ***Linearity and reproducibility of the MV TF activity assay***

253 The impact of the MV TF activity assay optimizations was evaluated, on linearity and
254 reproducibility. First, to demonstrate the linearity of the assay, a dose-effect relationship of
255 the MV TF activity was measured on different volumes of plasma spiked with HL60-MV. A
256 significant linear relationship between plasma volume centrifugate and MV TF activity
257 measured (HL, $r^2=0.97$, $p<0.0001$; ML, $r^2=0.98$, $p<0.0001$; LL, $r^2=0.91$, $p<0.0001$) (Figure
258 3H).

259 The variability of the MV TF activity assay was evaluated either after MV purification
260 or on PFP with the aim to include the impact of the centrifugation procedure. As shown in
261 Table 1, the repeatability and reproducibility over a 12 month-period of three levels of
262 purified MV were low (4% and 4-13% respectively) while the repeatability and
263 reproducibility over time of the assay in PFP was above 20% (20%, 22-26%, respectively)
264 due to the centrifugation step. Because the quality of the recovery of the MV pellet may vary
265 between operators, the inter-operator reproducibility (n=4) was evaluated using samples
266 containing three different levels of MV TF activity. As a result, the variability gradually
267 decreases with the FXa activity, (LL, CV=38%, ML, CV=17%, HL, CV=3%, Table 1). While
268 the direct comparison was not made, no difference in variability is expected between the MV
269 TF activity assay and the Chapel Hill assay because both assays share a centrifugation step
270 which is the main cause of variability within the assays.

271 *Evaluation of the MV TF activity assay sensitivity*

272 The sensitivity of the MV TF activity assay was first compared to the Chapel Hill
273 assay evaluating the detection limit of the method which was defined as the linearity
274 breakpoint in a serial dilution of recombinant source of TF. As shown in the figure 4A, a
275 lower detection limit was found for the new assay compared to the Chapel Hill assay (3 ± 1 vs
276 12 ± 3 fM, $p=0.03$). This low detection limit permitted the detection of MV TF activity in
277 plasma samples from **healthy individuals** (26 ± 15 fM) which was specifically inhibited by the
278 anti-TF antibody SBTF-1 (Figure 4B). After adjusting for the enrichment factor of 3.6
279 between the sample of 500 μ L and the pellet recovered in 140 μ L of buffer, this value
280 represents 7 ± 4 fmol/mL of PFP. This result was obtained after discarding the first milliliters
281 of blood because of a potential release of subendothelial TF during the venipuncture [32].
282 Indeed, as shown in figure 4C, when measured on 6 successive 5 ml tubes from the same

283 donor, the activity was significantly higher in the first tube compared to the following (-
284 $20\pm 10\%$, $p= 0.05$). This result suggests that the first tube was contaminated by subendothelial
285 TF and therefore should be discarded from the analysis. Next, the ability of MV TF activity
286 assay to discriminate MV TF activity from unstimulated blood compared to the same blood
287 stimulated with LPS was measured. As illustrated in figure 4D, the activity was significantly
288 increased in LPS-stimulated compared to unstimulated conditions (190 ± 120 fM vs 26 ± 15 fM,
289 $p = 0.002$).

290 Finally, the sensitivity of the MV TF activity assay was directly compared to the Chapel
291 Hill assay. In healthy plasma the FXa generation specifically inhibited by the anti-TF
292 antibodies was significantly detected with the MV TF activity assay (26 ± 15 fM) while it
293 remains undetectable with the Chapel Hill assay (Figure 5A). In LPS-stimulated plasma, a
294 significant increase of $30\pm 40\%$ with the MV TF activity assay was measured ($p = 0.04$)
295 (Figure 5A) with a good correlation between assays ($r^2 = 0.952$; $p < 0.0001$, Figure 5B). Both
296 assays were performed with a different plasma volume ($500\mu\text{L}$ vs $200\mu\text{L}$). After
297 normalization of this volume ($200\mu\text{L}$ or $500\mu\text{L}$) the MV TF activity remains significantly
298 higher with the MV TF activity assay than the Chapel Hill assay (29 ± 8 vs 14 ± 4 fM; $p=0.03$;
299 99 ± 9 vs 35 ± 8 fM; $p=0.008$ for 200 and $500\mu\text{L}$, respectively, figure 5C). These results were
300 confirmed in plasma spiked with three different levels of TF+MV (BxPC3). As shown in
301 figure 5D, a significantly higher activity was found in the MV TF activity assay compared to
302 the Chapel Hill assay (57 ± 11 vs 20 ± 7 fM ; $p=0.003$, 129 ± 19 vs 30 ± 9 fM ; $p=0.002$ and
303 251 ± 21 vs 168 ± 16 fM ; $p=0.002$; for LL, ML and HL, respectively). Altogether these results
304 demonstrate that the MV TF activity assay is more sensitive than the Chapel Hill assay and
305 that the difference of initial plasma volume was not the key factor explaining this difference.

306 **Discussion**

307 Although many studies have suggested that MV TF activity may be a useful
308 biomarker to identify patients with an increased risk of thrombosis, the most convincing
309 results were published in patients with cancer [14,15,22,24,33,34]. In contrast, significant
310 increases in MV TF activities were not observed in cardiovascular disorders [6]. It is thought
311 that the major part of TF-positive MV are derived from tumor cells in cancer in particular for
312 pancreatic cancer displaying the highest MV TF activity [12,18], whereas they are derived
313 from hematopoietic cells in non-tumoral disease. But one can also hypothesized that the
314 current tests are hampered by a lack of sensitivity. In the present study, we showed that the
315 sensitivity of MV TF activity can be significantly improved by 1/ increasing the plasma
316 volume, the speed and time of centrifugation, the FX concentration, 2/using an anti-TF
317 antibody (Clone SBTF-1) with a more potent inhibitory and, 3/ using kinetic monitoring of a
318 fluorogenic substrate that measures Xa generation and the use FVII instead of FVIIa to reduce
319 TF-independent FXa generation.

320 We demonstrated that this new assay was able to measure MV TF activity with a high
321 specificity and an improved sensitivity, especially in the low concentration range of MV TF
322 activity. Indeed, we were able to detect MV TF activity in normal PFP samples. However, the
323 assay was still limited by an inter-assay variability, mainly due to the centrifugation step.

324 The MV TF activity assay developed in the present study combines an enzymatic
325 assay of generated FXa, with MV purification by centrifugation. A first step was to improve
326 some parameters that influence the preanalytical step, one of the most important issue, as
327 extensively discussed in previous reviews [35,36]. Centrifugation is frequently used to pellet
328 MV because it can be performed easily. However, as illustrated in our study, isolation of the
329 MV introduces some variability, as shown by the CV of MV TF activity. We found
330 significantly less variability with MV isolated using purified MV. Consistent with previous

331 studies showing that the recovery of the pellet depend on the rotor type, the centrifugation
332 speed (g-force) and the centrifugation time [37], we demonstrated that MV TF activity is
333 significantly affected by 1) type of rotor 2) speed of centrifugation, 3) centrifugation time.
334 According to our result, centrifugation of PFP at 24,000 g for 60 min at RT, using the same
335 rotor were delineated as optimized preanalytical conditions to measure TF activity in a
336 controlled range.

337 Another disadvantage of centrifugation is to cause the aggregation of MV and/or their
338 contaminations by unwanted elements, such as protein/lipid aggregates [38]. Accordingly, in
339 the future, an option to overcome the disadvantages of centrifugation would be to use anti-TF
340 antibody coated magnetic beads to specifically capture MV from larger volume of blood, thus
341 reducing the time to isolate MV and avoiding washing steps We recently used such a strategy
342 in a new assay for the measurement of plasmin activity of MV [29], with an improvement of
343 time, sensitivity, specificity and reproducibility.

344 We also focused on improving the analytical settings of the FXa generation assay and
345 showed that the sensitivity of the MV TF activity was optimized by 1) adding factor VII
346 instead of VIIa, 2) an optimized calcium concentration, and 3) an optimized incubation time
347 that allows a better recovery of activated FX and a better cleavage of the fluorescent substrate.

348 An important modification was provided by introducing a new inhibitory anti-TF
349 antibody with a high inhibitory potential (SBTF-1). Using MV generated from the cell line
350 HAP1 that has been KO for TF and MV generated from their derivative HAP1 parental cell,
351 no TF specific activity was generated in MV from KO-TF-MV, indicating that SBTF-1
352 confers a high specificity to the novel assay. In comparison with the commercially available
353 anti-TF antibody HTF-1 (the most widely used antibody in the previous studies
354 [22,25,30,39]), the SBTF-1 exhibited an increased inhibitory effect, as attested by the

355 difference of inhibition of the FXa generation at different time and concentration. Moreover,
356 the SBTF-1 inhibitory effect operated over a broad range of MV concentration.

357 Non TF elements that can modify TF activity in blood are widely described in the
358 previous studies, such notably negatively charged PLs [40–42]. Indeed, we showed that TF
359 activity can be influenced by MV generated by erythrocyte-derived MV (Ery-MV) probably
360 by increasing the rate of FX availability for the TF-FVIIa complex. We have shown that
361 hemolysis increase the non-specific FXa generation in plasma samples from dogs with
362 immune-mediated hemolytic anemia[43]. This emphasizes the importance of the pre-
363 analytical treatment of samples and the interpretation of data from hemolysis samples.

364 Having optimized the analytical settings of MV TF activity assay, we challenged its
365 sensitivity and specificity. We illustrate here the sensitivity by showing 1/ a lower detection
366 limit, 2/ the existence of a basal level of MV TF activity in blood plasma from healthy donors
367 and, 3/ a significant increase of activity for MV from LPS stimulated blood.

368 Finally, we demonstrated that MV TF activity assay presented a higher sensitivity than
369 the end point Xa generation assay compared with the Chapel Hill assay where methods
370 differences are summarized in table 2. Indeed, significantly more TF activity was always
371 measured by the optimized Xa generation assay 1/ using plasma from healthy controls,
372 untreated or LPS treated 2/ using plasma enriched with low, medium and high known
373 concentrations of MV from the pancreatic cancer cell line BxPC3, with a better correlation
374 between assays observed for higher TF activity plasma in contrast to low TF activity samples.

375 In conclusion, the MV TF activity assay presented here shows a higher sensitivity
376 without reducing specificity. Therefore, this modified assay could provide a significant
377 improvement to measure TF-positive MV as a potential biomarker of thrombotic risk in

378 patients.

379 SOURCES OF FUNDING

380 This work was partly funded by INSERM and Aix-Marseille University (France).
381 Financial support was also received from the Stago Company. This work was supported by
382 funds from the John C. Parker Endowed Professorship and the National Institute of Health
383 (T32HL007149).

384 DISCLOSURES

385 We disclose as a conflict of interest that a patent on this topic has been licensed by the
386 Stago Company, and P.Poncelet, T. Bouriche, J. Bez and C. Judicone are full-time employees
387 of Biocytex.

388 FIGURE LEGENDS

| | Sample nature | Number of samples | Number of operators | Coefficient of variation | |
|---------------------------------------|---------------|-------------------|---------------------|--------------------------|-----|
| Repeatability | Purified MVs | 4 | 4 | 4% | |
| Reproducibility over time | Purified MVs | 52 | 1 | LL | 13% |
| | | | | ML | 9% |
| | | | | HL | 4% |
| Repeatability | PFP | 4 | 1 | 20% | |
| Reproducibility over time | PFP | 25 | 1 | LL | 26% |
| | | | | ML | 22% |
| | | | | HL | 25% |
| Inter-operator reproducibility | PFP | 4 | 3 | LL | 38% |
| | | | | ML | 17% |
| | | | | HL | 3% |

389

390 **Table 1.** Reproducibility of the MV TF activity assay. Different reproducibility expressed
391 with the coefficient of variation. Type of sample, number of experiment and number of

392 operators are indicated. MV-free plasma spiked by three distinct levels of BxPC3-MV : HL =
 393 high level, ML = medium level, LL = low level.

| | MV TF activity assay | Mackman Assay |
|-----------------------------|--|--|
| Sample | 500 μ L plasma | 200 μ L plasma |
| Isolation of MVs | 1h, 24.000g centrifugation with one wash | 15 min, 20.000g centrifugation with two washes |
| Blocking antibody | SBTF1 (10 μ g/mL) | HTF1 (4 μ g/mL) |
| Reaction MIX | 10 nM VII, 190 nM X and 5 mM CaCl_2 | 10 nM FVIIa, 300 nM FX and 10 mM CaCl_2 |
| Substrate monitoring | Kinetic | End-point |
| Substrate | Fluorogenic | Chromogenic |

394

395 **Table 2.** Differences between the MV TF activity assay and the Chapel Hill assay

396 *Figure 1. Schematic sketch of the MV TF activity assay.*

397 **A.** Microvesicles (MV) were extracted from platelet-free plasma (PFP) using
 398 ultracentrifugation. Blocking anti-TF (or sham) antibody was reacted with MV for specificity
 399 evaluation. **B.** Reaction mix containing factor VII (FVII), factor X (FX) and calcium (CaCl_2)
 400 was added, FX cleavage into activate factor X (FXa) was induced by tissue factor (TF)/
 401 activated FVII (FVIIa) complex during incubation at 37°C. **C.** The reaction was stopped by
 402 ethylenediaminetetraacetic acid (EDTA) that captures the calcium. **D.** The generated FXa was
 403 quantified by fluorometry using a specific fluorogenic substrate, and the fluorescence
 404 (excitation 390nm/emission 460 nm) was monitored in RFU/min. **E.** Calibration range of
 405 liposome associated recombinant TF (PRP-reagent) allow us to convert the values in
 406 femtomolar of TF.

407 **Figure 2. Specificity of the MV TF activity assay.**

408 **A.** Comparison of blocking effect between SBTF-1 and HTF-1 blocking antibodies on HL60-
409 derived MV as function of antibody concentration with two times of incubation. The results
410 are expressed in inhibition percentage (n=3). **B.** Comparison of different antibody
411 concentrations (0,002-10 µg/mL) on a range of HL60-MV (0,6-5.10⁵ MV) . The results are
412 expressed in inhibition percentage (n=3). **C.** FXa generation measured on a range of parental
413 HAP1-MV. White histograms represent total activity and black histograms represent non-
414 specific activity. Results are expressed in arbitrary unit (AU) (n=3). **D.** FXa generation
415 measured on a range of KO-TF-HAP1 derivative MV (KO-TF-MV). White histograms
416 represent total activity and black histograms represent non-specific activity. Results are
417 expressed in AU (n=3). **E.** FXa generation measured on 10⁵ parental HAP1-MV added to a
418 range of KO-TF-MV. Curve with circles represent the total activity, curve with squared
419 represent non-specific activity and column bar represents the specific activity. Results are
420 expressed in percentage (n=4). **F.** MV TF activity measured on 10⁵ HL60-MV added to a
421 range of erythrocyte-derived MV (Ery-MV) obtain either by sonication or blood aging and
422 platelet-derived MV (PMV). Results are expressed in percentage compared with HL60-MV
423 alone (n=4).

424 **Figure 3. Increasing sensitivity of the MV TF activity assay.**

425 **A.** Comparison of MV TF activity in plasma spiked with BxPC3-MV between three different
426 rotors (n=6 with rotor 1 and 2, n=3 with rotor 3). Results are expressed in fM of TF. **B.**
427 Comparison of MV TF activity on PFP between four centrifugation times at 24.000g (15 min,
428 30 min, 60 min, 90 min) (n=6). Results are expressed in fM of TF. **C.** Comparison of MV TF
429 activity on PFP between four centrifugation speeds during 60 min (20.000g, 24.000g,
430 70.000g, 100.000g) (n=6). Results are expressed in fM of TF. **D.** Comparison between FVII

431 and FVIIa in the reaction mix on HL60-MV (n=3). Results are expressed in AU. **E.**
432 Comparison between different reaction mixes with different concentrations of FVII and FX
433 on HL60-MV. White histograms represent total activity and black histograms represent non-
434 specific activity. Results are expressed in AU. **F.** MV TF activity measured on HL60-MV
435 with a reaction mix that contain a range of concentrations of calcium. Results are expressed in
436 AU. **G.** MV TF activity measured on HL60-MV with different reaction mix incubation times.
437 **H.** Evaluation of linearity by measured MV TF on a range of volumes of the same plasma.
438 MV-free plasma spiked by three various levels of HL60-MV are used: high level (HL),
439 medium level (ML) and low level (LL) (HL = 7500 MV/ μ L, ML = 2500/ μ L, LL = 750
440 MV/ μ L). Results are expressed in AU (n=3).

441 ***Figure 4. Validation of MV TF assay sensitivity.***

442 **A.** Limit of linearity determined by a range of successive two-fold dilutions of PRP reagent.
443 Results are expressed in relative fluorescence unit per minute (RFU/min) with the MV TF
444 assay and in optical density at 405 nm (OD_{405nm}) with the Chapel Hill assay. **B.** MV TF
445 activity measured on **healthy individuals**. White histograms represent total activity and black
446 histograms represent non-specific activity. Results are expressed in AU (n=9). **C.**
447 Measurement of MV TF activity on 6 successive blood collection tubes. Results are expressed
448 in percentage compared to the first tube (N=5). **D.** MV TF activity compared between PFP
449 extracted from untreated blood and LPS-treated blood. Results are expressed in fM TF (n=9).

450 ***Figure 5. Comparison of the in-house MV TF activity method and pre-existing assays***

451 **A.** MV TF activity measured with the MV TF activity assay (In-house) and the Chapel Hill
452 assay described by Khorana et al. in 2008 were compared on the PFP of 9 **healthy individuals**
453 from untreated and LPS-treated blood. **B.** Correlation between in-house method and Chapel

454 Hill assay for LPS-treated samples, $r^2=0.952$, $p<0.0001$, significant. **C.** MV TF activity
455 measured with the MV TF activity assay (In-house) and the Chapel Hill assay describes by
456 Khorana et al. in 2008 were compared on spiked MV TF in plasma featuring three levels of
457 activity (HL = 7500 MV/ μ L, ML = 2500/ μ L, LL = 750 MV/ μ L). **D.** MV TF activity
458 measured with the MV TF activity assay and the Chapel Hill assay with the same PFP volume
459 200 μ L and 500 μ L. Results are expressed in fM.

460 **Bibliography**

- 461 [1] P. Wolf, The nature and significance of platelet products in human plasma, *Br. J.*
462 *Haematol.* 13 (1967) 269–288.
- 463 [2] V.C. Ridger, C.M. Boulanger, A. Angelillo-Scherrer, L. Badimon, O. Blanc-Brude, M.-
464 L. Bochaton-Piallat, E. Boilard, E.I. Buzas, A. Caporali, F. Dignat-George, P.C. Evans,
465 R. Lacroix, E. Lutgens, D.F.J. Ketelhuth, R. Nieuwland, F. Toti, J. Tunon, C. Weber,
466 I.E. Hoefer, Microvesicles in vascular homeostasis and diseases. Position Paper of the
467 European Society of Cardiology (ESC) Working Group on Atherosclerosis and Vascular
468 Biology, *Thromb. Haemost.* 117 (2017) 1296–1316. doi:10.1160/TH16-12-0943.
- 469 [3] E. Biró, K.N. Sturk-Maquelin, G.M.T. Vogel, D.G. Meuleman, M.J. Smit, C.E. Hack, A.
470 Sturk, R. Nieuwland, Human cell-derived microparticles promote thrombus formation in
471 vivo in a tissue factor-dependent manner, *J. Thromb. Haemost.* 1 (2003) 2561–2568.
- 472 [4] J. Chou, N. Mackman, G. Merrill-Skoloff, B. Pedersen, B.C. Furie, B. Furie,
473 Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation
474 during thrombus propagation, *Blood.* 104 (2004) 3190–3197. doi:10.1182/blood-2004-
475 03-0935.
- 476 [5] E. Ramacciotti, A.E. Hawley, D.M. Farris, N.E. Ballard, S.K. Wroblewski, D.D. Myers,
477 P.K. Henke, T.W. Wakefield, Leukocyte- and platelet-derived microparticles correlate

- 478 with thrombus weight and tissue factor activity in an experimental mouse model of
479 venous thrombosis, *Thromb. Haemost.* 101 (2009) 748–754.
- 480 [6] Y. Hisada, W. Alexander, R. Kasthuri, P. Voorhees, F. Mobarrez, A. Taylor, C.
481 McNamara, H. Wallen, M. Witkowski, N.S. Key, U. Rauch, N. Mackman, Measurement
482 of microparticle tissue factor activity in clinical samples: A summary of two tissue
483 factor-dependent FXa generation assays, *Thromb. Res.* 139 (2016) 90–97.
484 doi:10.1016/j.thromres.2016.01.011.
- 485 [7] D. Mege, S. Mezouar, F. Dignat-George, L. Panicot-Dubois, C. Dubois, Microparticles
486 and cancer thrombosis in animal models, *Thromb. Res.* 140 (2016) 21–26.
487 doi:10.1016/S0049-3848(16)30094-9.
- 488 [8] G.M. Thomas, L. Panicot-Dubois, R. Lacroix, F. Dignat-George, D. Lombardo, C.
489 Dubois, Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1
490 accelerate thrombus formation in vivo, *J. Exp. Med.* 206 (2009) 1913–1927.
491 doi:10.1084/jem.20082297.
- 492 [9] J.-G. Wang, J.E. Geddings, M.M. Aleman, J.C. Cardenas, P. Chantrathammachart, J.C.
493 Williams, D. Kirchhofer, V.Y. Bogdanov, R.R. Bach, J. Rak, F.C. Church, A.S.
494 Wolberg, R. Pawlinski, N.S. Key, J.J. Yeh, N. Mackman, Tumor-derived tissue factor
495 activates coagulation and enhances thrombosis in a mouse xenograft model of human
496 pancreatic cancer, *Blood.* 119 (2012) 5543–5552. doi:10.1182/blood-2012-01-402156.
- 497 [10] G.M. Thomas, A. Brill, S. Mezouar, L. Crescence, M. Gallant, C. Dubois, D.D. Wagner,
498 Tissue factor expressed by circulating cancer cell-derived microparticles drastically
499 increases the incidence of deep vein thrombosis in mice, *J. Thromb. Haemost.* 13 (2015)
500 1310–1319. doi:10.1111/jth.13002.
- 501 [11] J.E. Geddings, Y. Hisada, Y. Boulaftali, T.M. Getz, M. Whelihan, R. Fuentes, R. Dee,
502 B.C. Cooley, N.S. Key, A.S. Wolberg, W. Bergmeier, N. Mackman, Tissue factor-

- 503 positive tumor microvesicles activate platelets and enhance thrombosis in mice, *J.*
504 *Thromb. Haemost.* 14 (2016) 153–166. doi:10.1111/jth.13181.
- 505 [12] Y. Hisada, N. Mackman, Cancer-associated pathways and biomarkers of venous
506 thrombosis, *Blood*. 130 (2017) 1499–1506. doi:10.1182/blood-2017-03-743211.
- 507 [13] M.E.T. Tesselaar, F.P.H.T.M. Romijn, I.K. Van Der Linden, F.A. Prins, R.M. Bertina, S.
508 Osanto, Microparticle-associated tissue factor activity: a link between cancer and
509 thrombosis?, *J. Thromb. Haemost. JTH.* 5 (2007) 520–527. doi:10.1111/j.1538-
510 7836.2007.02369.x.
- 511 [14] J.I. Zwicker, H.A. Liebman, D. Neuberg, R. Lacroix, K.A. Bauer, B.C. Furie, B. Furie,
512 Tumor-derived tissue factor-bearing microparticles are associated with venous
513 thromboembolic events in malignancy, *Clin. Cancer Res. Off. J. Am. Assoc. Cancer*
514 *Res.* 15 (2009) 6830–6840. doi:10.1158/1078-0432.CCR-09-0371.
- 515 [15] A. Bharthuar, A.A. Khorana, A. Hutson, J.-G. Wang, N.S. Key, N. Mackman, R.V. Iyer,
516 Circulating microparticle tissue factor, thromboembolism and survival in
517 pancreaticobiliary cancers, *Thromb. Res.* 132 (2013) 180–184.
518 doi:10.1016/j.thromres.2013.06.026.
- 519 [16] F.J.S.H. Woei-A-Jin, M.E.T. Tesselaar, P. Garcia Rodriguez, F.P.H.T.M. Romijn, R.M.
520 Bertina, S. Osanto, Tissue factor-bearing microparticles and CA19.9: two players in
521 pancreatic cancer-associated thrombosis?, *Br. J. Cancer.* 115 (2016) 332–338.
522 doi:10.1038/bjc.2016.170.
- 523 [17] D. Faille, M.-C. Bourrienne, E. de Raucourt, L. de Chaisemartin, V. Granger, R.
524 Lacroix, L. Panicot-Dubois, P. Hammel, P. Lévy, P. Ruzniewski, N. Ajzenberg, V.
525 Rebours, Biomarkers for the risk of thrombosis in pancreatic adenocarcinoma are related
526 to cancer process, *Oncotarget.* 9 (2018) 26453–26465. doi:10.18632/oncotarget.25458.

- 527 [18] C.-J. Cui, G.-J. Wang, S. Yang, S.-K. Huang, R. Qiao, W. Cui, Tissue Factor-bearing
528 MPs and the risk of venous thrombosis in cancer patients: A meta-analysis, *Sci. Rep.* 8
529 (2018) 1675. doi:10.1038/s41598-018-19889-8.
- 530 [19] F.A.W. Coumans, A.R. Brisson, E.I. Buzas, F. Dignat-George, E.E.E. Drees, S. El-
531 Andaloussi, C. Emanuelli, A. Gasecka, A. Hendrix, A.F. Hill, R. Lacroix, Y. Lee, T.G.
532 van Leeuwen, N. Mackman, I. Mäger, J.P. Nolan, E. van der Pol, D.M. Pegtel, S. Sahoo,
533 P.R.M. Siljander, G. Sturk, O. de Wever, R. Nieuwland, Methodological Guidelines to
534 Study Extracellular Vesicles, *Circ. Res.* 120 (2017) 1632–1648.
535 doi:10.1161/CIRCRESAHA.117.309417.
- 536 [20] P. Poncelet, S. Robert, N. Bailly, F. Garnache-Ottou, T. Bouriche, B. Devalet, J.H.
537 Segatchian, P. Saas, F. Mullier, Tips and tricks for flow cytometry-based analysis and
538 counting of microparticles, *Transfus. Apher. Sci. Off. J. World Apher. Assoc. Off. J.*
539 *Eur. Soc. Haemapheresis.* 53 (2015) 110–126. doi:10.1016/j.transci.2015.10.008.
- 540 [21] A.A. Khorana, C.W. Francis, K.E. Menzies, J.-G. Wang, O. Hyrien, J. Hathcock, N.
541 Mackman, M.B. Taubman, Plasma tissue factor may be predictive of venous
542 thromboembolism in pancreatic cancer, *J. Thromb. Haemost.* 6 (2008) 1983–1985.
543 doi:10.1111/j.1538-7836.2008.03156.x.
- 544 [22] M.E.T. Tesselaar, F.P.H.T.M. Romijn, I.K. Van Der Linden, F.A. Prins, R.M. Bertina, S.
545 Osanto, Microparticle-associated tissue factor activity: a link between cancer and
546 thrombosis?, *J. Thromb. Haemost.* 5 (2007) 520–527. doi:10.1111/j.1538-
547 7836.2007.02369.x.
- 548 [23] J. Thaler, C. Ay, N. Mackman, R.M. Bertina, A. Kaider, C. Marosi, N.S. Key, D.A.
549 Barcel, W. Scheithauer, G. Kornek, C. Zielinski, I. Pabinger, Microparticle-associated
550 tissue factor activity, venous thromboembolism and mortality in pancreatic, gastric,

- 551 colorectal and brain cancer patients, *J. Thromb. Haemost.* 10 (2012) 1363–1370.
552 doi:10.1111/j.1538-7836.2012.04754.x.
- 553 [24] K. Tatsumi, S. Antoniak, D.M. Monroe, A.A. Khorana, N. Mackman, Subcommittee on
554 Hemostasis and Malignancy of the Scientific and Standardization Committee of the
555 International Society on Thrombosis and Hemostasis, Evaluation of a new commercial
556 assay to measure microparticle tissue factor activity in plasma: communication from the
557 SSC of the ISTH, *J. Thromb. Haemost.* 12 (2014) 1932–1934. doi:10.1111/jth.12718.
- 558 [25] Y. Hisada, N. Mackman, Measurement of tissue factor activity in extracellular vesicles
559 from human plasma samples, *Res Pr. Thromb Haemost.* 0 (2018).
560 doi:10.1002/rth2.12165.
- 561 [26] R. Lacroix, F. Dignat-George, Microparticles: new protagonists in pericellular and
562 intravascular proteolysis, *Semin. Thromb. Hemost.* 39 (2013) 33–39. doi:10.1055/s-
563 0032-1333310.
- 564 [27] R. Lacroix, L. Plawinski, S. Robert, L. Doeuvre, F. Sabatier, S. Martinez de Lizarrondo,
565 A. Mezzapesa, F. Anfosso, A.S. Leroyer, P. Poullin, N. Jourde, M.-S. Njock, C.M.
566 Boulanger, E. Anglés-Cano, F. Dignat-George, Leukocyte- and endothelial-derived
567 microparticles: a circulating source for fibrinolysis, *Haematologica.* 97 (2012) 1864–
568 1872. doi:10.3324/haematol.2012.066167.
- 569 [28] S. Robert, R. Lacroix, P. Poncelet, K. Harhour, T. Bouriche, C. Judicone, J.
570 Wischhusen, L. Arnaud, F. Dignat-George, High-sensitivity flow cytometry provides
571 access to standardized measurement of small-size microparticles--brief report,
572 *Arterioscler. Thromb. Vasc. Biol.* 32 (2012) 1054–1058.
573 doi:10.1161/ATVBAHA.111.244616.
- 574 [29] S. Cointe, K. Harti Souab, T. Bouriche, L. Vallier, A. Bonifay, C. Judicone, S. Robert,
575 R. Armand, P. Poncelet, J. Albanese, F. Dignat-George, R. Lacroix, A new assay to

576 evaluate microvesicle plasmin generation capacity: validation in disease with fibrinolysis
577 imbalance, *J. Extracell. Vesicles.* 7 (2018) 1494482.
578 doi:10.1080/20013078.2018.1494482.

579 [30] R.D. Lee, D.A. Barcel, J.C. Williams, J.G. Wang, J.C. Boles, D.A. Manly, N.S. Key, N.
580 Mackman, Pre-analytical and analytical variables affecting the measurement of plasma-
581 derived microparticle tissue factor activity, *Thromb. Res.* 129 (2012) 80–85.
582 doi:10.1016/j.thromres.2011.06.004.

583 [31] I. Agouti, S. Cointe, S. Robert, C. Judicone, A. Loundou, F. Driss, A. Brisson, D.
584 Steschenko, C. Rose, C. Pondarré, E. Bernit, C. Badens, F. Dignat-George, R. Lacroix, I.
585 Thuret, Platelet and not erythrocyte microparticles are procoagulant in transfused
586 thalassaemia major patients, *Br. J. Haematol.* 171 (2015) 615–624.
587 doi:10.1111/bjh.13609.

588 [32] N. Tekkeşin, O.B. Esen, C. Kiliç, O. Eviyaoğlu, Discard first tube for coagulation
589 testing, *Blood Coagul. Fibrinolysis Int. J. Haemost. Thromb.* 23 (2012) 299–303.
590 doi:10.1097/MBC.0b013e328351ebbf.

591 [33] F. van Doormaal, A. Kleinjan, R.J. Berckmans, N. Mackman, D. Manly, P.W.
592 Kamphuisen, D.J. Richel, H.R. Büller, A. Sturk, R. Nieuwland, Coagulation activation
593 and microparticle-associated coagulant activity in cancer patients. An exploratory
594 prospective study, *Thromb. Haemost.* 108 (2012) 160–165. doi:10.1160/TH12-02-0099.

595 [34] M.T. Sartori, A. Della Puppa, A. Ballin, E. Campello, C.M. Radu, G. Saggiorato, D.
596 d’Avella, R. Scienza, G. Cella, P. Simioni, Circulating microparticles of glial origin and
597 tissue factor bearing in high-grade glioma: a potential prothrombotic role, *Thromb.*
598 *Haemost.* 110 (2013) 378–385. doi:10.1160/TH12-12-0957.

- 599 [35] N.S. Key, N. Mackman, Tissue factor and its measurement in whole blood, plasma, and
600 microparticles, *Semin. Thromb. Hemost.* 36 (2010) 865–875. doi:10.1055/s-0030-
601 1267040.
- 602 [36] R. Lacroix, S. Robert, P. Poncelet, R.S. Kasthuri, N.S. Key, F. Dignat-George, ISTH
603 SSC Workshop, Standardization of platelet-derived microparticle enumeration by flow
604 cytometry with calibrated beads: results of the International Society on Thrombosis and
605 Haemostasis SSC Collaborative workshop, *J. Thromb. Haemost.* 8 (2010) 2571–2574.
606 doi:10.1111/j.1538-7836.2010.04047.x.
- 607 [37] A. Cvjetkovic, J. Lötvall, C. Lässer, The influence of rotor type and centrifugation time
608 on the yield and purity of extracellular vesicles, *J. Extracell. Vesicles.* 3 (2014) 23111.
609 doi:10.3402/jev.v3.23111.
- 610 [38] R. Linares, S. Tan, C. Gounou, N. Arraud, A.R. Brisson, High-speed centrifugation
611 induces aggregation of extracellular vesicles, *J. Extracell. Vesicles.* 4 (2015) 29509.
612 doi:10.3402/jev.v4.29509.
- 613 [39] T. Nielsen, A.F. Kristensen, S. Pedersen, G. Christiansen, S.R. Kristensen, Investigation
614 of procoagulant activity in extracellular vesicles isolated by differential
615 ultracentrifugation, *J. Extracell. Vesicles.* 7 (2018) 1454777.
616 doi:10.1080/20013078.2018.1454777.
- 617 [40] Y. Fu, J. Zhou, H. Li, F. Cao, Y. Su, S. Fan, Y. Li, S. Wang, L. Li, G.E. Gilbert, J. Shi,
618 Daunorubicin induces procoagulant activity of cultured endothelial cells through
619 phosphatidylserine exposure and microparticles release, *Thromb. Haemost.* 104 (2010)
620 1235–1241. doi:10.1160/TH10-02-0102.
- 621 [41] H. Kothari, R.C. Nayak, L.V.M. Rao, U.R. Pendurthi, Cystine 186-cystine 209 disulfide
622 bond is not essential for the procoagulant activity of tissue factor or for its de-encryption,
623 *Blood.* 115 (2010) 4273–4283. doi:10.1182/blood-2009-09-241356.

- 624 [42] J. Zhou, J. Shi, J. Hou, F. Cao, Y. Zhang, J.T. Rasmussen, C.W. Heegaard, G.E. Gilbert,
625 Phosphatidylserine exposure and procoagulant activity in acute promyelocytic leukemia,
626 *J. Thromb. Haemost.* 8 (2010) 773–782. doi:10.1111/j.1538-7836.2010.03763.x.
- 627 [43] L. Kidd, N. Mackman, Prothrombotic mechanisms and anticoagulant therapy in dogs
628 with immune-mediated hemolytic anemia, *J. Vet. Emerg. Crit. Care San Antonio Tex*
629 2001. 23 (2013) 3–13. doi:10.1111/j.1476-4431.2012.00824.x.
- 630

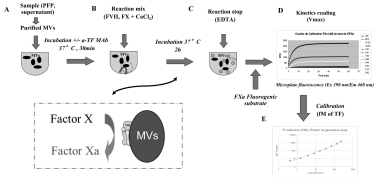
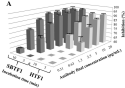
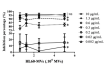
FIGURE 1

FIGURE 2

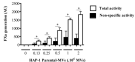
A



B



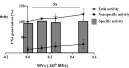
C



D



E



F

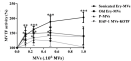


FIGURE 3

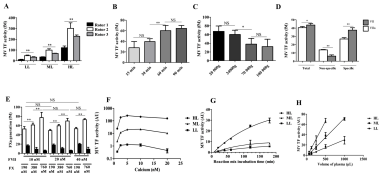


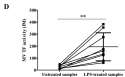
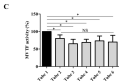
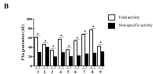
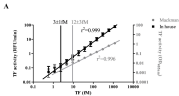
FIGURE 4

FIGURE 5

