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Extracellular vesicles and coagulation in blood from healthy humans revisited

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ABSTRACT

Background: In 2001, we studied the presence and coagulant properties of "microparticles" in the blood of healthy humans. Since then, multiple improvements in detection, isolation and functional characterization of the now called "extracellular vesicles" (EVs) have been made, and shortcomings were identified. **Aim**: To revisit the presence and function of EVs in blood from healthy humans. **Methods**: Blood was collected from 20 healthy donors. EV-containing plasma was prepared according to new guidelines, and plasma was diluted to prevent swarm detection. Single EVs were measured by flow cytometry with known sensitivity of fluorescence and light scatter. The haemostatic properties of EVs were measured by thrombin-, fibrin-, and plasmin generation. Plasma concentrations of thrombin-antithrombin complexes and prothrombin fragment 1 + 2 were measured to assess the coagulation status *in vivo*. **Results**: Compared to 2001, the total concentrations of detected EVs increased from 190- to 264-fold. In contrast to 2001, however, EVs are non-coagulant which we show can be attributed to improvements in blood collection and plasma preparation. No relation is present between the plasma concentrations of EVs and either TAT or F1 + 2. Finally, we show that EVs support plasmin generation. **Discussion**: Improvements in blood collection, plasma preparation and detection of EVs reveal that results from earlier studies have to be interpreted with care. Compared to 2001, higher concentrations of EVs are detected in blood of healthy humans which promote fibrinolysis rather than coagulation.

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Introduction

At present, the number of publications on extracellular vesicles (EVs) is growing exponentially. In 2001, we published a manuscript entitled "*Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation*". At that time, "microparticles" were measured by flow cytometry [1]. Although we and others were aware that we were using an instrument developed to measure single cells and not "microparticles", to which extent the sensitivity of our instrument hampered our EV measurements was completely "terra incognita". Although our old manuscript is still being cited, in total 438 citations in Web of Science and 638 citations in Google Scholar since 2001 (9 May 2019), the greatly improved understanding of detection of single EVs by flow cytometry, combined with the development of dedicated protocols for blood collection and handling, made us to reconsider our earlier findings.

In 2001, the term "microparticles" was used for "larger" extracellular vesicles (EVs), supposedly released directly from the plasma membrane as "ectosomes". These larger EVs were considered to be the "tip of the iceberg" because the true size of the iceberg, that is all EVs including those *below* the detection limit, was unknown. In 2014, Brisson and co-workers reported the first size distribution of all EVs in normal human plasma, and thus for the very first time they showed the true size of the iceberg [2]. Importantly, they showed that the size distribution of EVs is a continuum, and that most EVs have a diameter of less than 300 nm.

In 2001, the sensitivity of flow cytometers to detect EVs was unknown. In the latest standardization study on the concentration of EVs measured by flow cytometry, still one in three flow cytometers failed to detect any EVs, and most detected <1% of all EVs present. In fact, only a very few dedicated flow cytometers are capable of detecting a substantial fraction of all EVs present [3]. Thus, with hindsight, most if not all of the earlier flow cytometry results should be interpreted with caution [4].

Finally, blood collection and handling procedures have been a major confounder in the earlier studies on the presence and functional activity of EVs. For example, in 2001, platelet-*poor* plasma was used to study the coagulation properties of EVs. The current ISTH guidelines on

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plasma EVs, however, recommend the use of platelet-*depleted* plasma because platelet-*poor* plasma still contains a considerable number of residual platelets which may affect both the detection of "platelet-derived EVs" as well as their supposed coagulant properties.

Taken together, we revisited the presence and haemostatic function of EVs in plasma of healthy humans, and explain the discrepancies between our earlier and present findings.

Materials and methods

Reagents and assays

CD14-APC (clone 61D3) was obtained from eBioscience (San Diego, CA), CD41-FITC (clone HIP8) and CD45-APC (clone HI30) from BioLegend (San Diego, CA), CD61-APC (clone Y2/51) and CD235a-FITC (clone JC159) from Dako (Glostrup, Denmark), CD66b-FITC (clone 80H3) from Beckman Coulter Inc. (Marseille, France), CD62E-PE (clone 68-5H11), CD142-PE (clone HTF-1), IgG_1-PE (clone X40), IgG_1-FITC (clone X40) and IgG_1-APC (clone MOPC-21) from BD Biosciences (San Jose, CA). Bovine Lactadherin-FITC is from Haematologic Technologies Inc. (Essex Junction, VT). Reptilase was obtained from Roche (Mannheim, Germany) and the chromogenic substrate S2238 from Chromogenix AB (Mölndal, Sweden). The plasma concentrations of prothrombin fragment 1 + 2 (Enzygnost F1 + 2 monoclonal kit) and thrombin-antithrombin complexes (Enzygnost TAT micro kit) were determined by ELISA as described by the manufacturer Siemens (Marburg, Germany). Dade Innovin (contained recombinant tissue factor) was from Siemens (Newark, NJ), and Synthasil (contained synthetic phospholipids) from Instrument Laboratory (Bedford, MA).

Blood collection

Venous blood (blood/citrate ratio 9:1) was collected from 20 healthy individuals (10 males) in 0.109 mol/L trisodium-citrate (Becton Dickinson, San Jose, CA) with informed consent and as approved by the medical ethical committee of the Amsterdam UMC (W18-289-18335). Platelet-depleted plasma was prepared by two consecutive centrifugation steps for 20 min at 1560*g* at room temperature, first the blood and then the plasma layer, to prepare platelet-*depleted* plasma. The plasma was aliquoted, snap-frozen with liquid nitrogen for 15 min and stored at −80°C until use. Our procedure to prepare platelet-depleted plasma differs slightly from the protocol of Lacroix et al. [5], which will be discussed in the Discussion section.

Flow cytometry analysis

Plasma was thawed for 1 min at 37°C. To avoid swarm detection, the plasma was diluted 4 to 200-fold to 5000 events/s with PBS/citrate buffer (1.45 mmol/L phosphate, 154 mmol/L sodium chloride and 10.9 mmol/L trisodium-citrate; pH 7.4, 0.05 µm filtered). The dilution was donor dependent. Before labelling, the antibodies were centrifuged at 18,900*g* for 5 min at 4°C to remove antibody aggregates. To establish the cellular origin or exposure of phosphatidylserine (PS) of EVs, 20 µL diluted plasma was incubated with 2.5 µL antibodies of interest or PS binding agent. After addition of antibodies, EV-containing samples were incubated in the dark for 2 h at 20°C. Subsequently, the labelling was stopped by the addition of 200 µL citrate-PBS buffer followed by detection of EVs using an Apogee A60 Micro Flow Cytometer (Apogee Flow Systems, Hertfordshire, UK). Samples were analysed for 2 min with a flow rate of 3.01 µL/minute. The trigger was set on Large Angle Light Scattering (LALS) at 14 and the voltages for 405-Small Angle Light Scatter (SALS), 405-LALS, 638-D Red, 488-Green, 488-Orange were set at 380, 375, 510, 520 and 520, respectively. Because we demonstrated recently that light scatters triggering outperforms triggering on generic dye fluorescence of EVs in plasma on our current flow cytometer, we used light scatter rather than fluorescence triggering [6]. Data were analysed with software FlowJo (Version 10, FlowJo LLC, Ashland, OR). The fluorescence gate was set on IgG control samples. To estimate the number of EVs/L plasma, the number of vesicles (N) found in the upper and lower right quadrant of the flow cytometry analysis was used in the formula: Number/L = N x [60/time measurement] x [total volume/flowrate] x [10^6/volume sample] x dilution sample.

Coagulation

We used two assays to measure the ability of EVs to promote coagulation. The first assay is the thrombin generation test (TGT), which was also used in 2001 [1]. In this assay, thrombin generation is monitored in reptilase-treated and thus defibrinated "EV-depleted" pooled plasma using a chromogenic substrate. In brief, 20 µL of isolated EVs (10-fold concentrated) was reconstituted in defibrinated, pooled plasma from healthy volunteers ("EV-depleted"; 30 min centrifuged by 18,890 x g at 20°C). It should be mentioned that due to the limited sensitivity of flow cytometry in 2001, we called this "EV-depleted plasma". However, as shown recently for foetal calf serum [7,8], most small EVs cannot be removed from plasma or serum by (ultra) centrifugation. To this plasma (120 µL), either EVs, Innovin (50-fold diluted; 6.1 pmol/mL final concentration) or Synthasil (fivefold diluted before use) were added, the last two to initiate the extrinsic or intrinsic coagulation, respectively (20 µL). At t = 0, thrombin

generation was triggered by addition of 30 µL $CaCl_2$ (16.7 mmol/L final concentration). At fixed intervals after t = 0, 3 µL aliquots were removed from this mixture and added to 147 µL prewarmed (37°C) chromogenic substrate S2238. After 3 min, the conversion of S2238 was stopped by the addition of 90 µL citric acid (1 mol/L) and the generated amount of p-nitroaniline was determined at λ = 405 nm.

The second coagulation assay is the fibrin generation test (FGT), which was performed as described earlier [9]. In brief, 90 µL platelet-depleted plasma from healthy volunteers with 6 µL saline was incubated for 5 min at 37°C. After recalcification (15 µL), the half time to reach maximal fibrin formation (½OD max) was recorded by measuring the optical density (λ = 405 nm; SPECTRAmax microplate reader, Molecular Devices, San Jose, CA). In control experiments, 3 µL Innovin (50-fold diluted) or Synthasil (fivefold diluted) were added to 90 µL of EV-depleted normal plasma.

Fibrinolysis

The plasmin generation capacity of the EVs was assessed by isolating EVs and measuring their ability to initiate plasmin generation using a chromogenic assay [10,11]. In brief, 200 µL citrate-anticoagulated plasma was diluted (1:2) in PBS/BSA (phosphate buffer saline, 0.1% bovine serum albumin and 0.1% NaN_3) and incubated for 1 h at room temperature under mild rotation with magnetic beads (M450 Dynabeads; InVitrogen/Dynal, Oslo, Norway) coated with specific antibodies (anti-CD15). Plasmin was generated by incubating plasminogen (4 µmol/L, Stago, Asnières, France) with the EVs in the presence of ε-aminocaproic acid (5 mmol/L, Sigma-Aldrich, Saint-Louis, MO) and was detected at 405 nm after incubation with a plasmin-selective chromogenic substrate ([methyl-malonyl]-hydroxypropylarginine-p-nitroaniline, CBS0065, Stago, 2 mmol/L). The kinetics were followed for 2 h at 37°C, and V_{max} was measured in the linear part of each curve. The results were expressed in units of A_{405} nm × 10^{-3}/minute.

Results

Concentration of extracellular vesicles in plasma of healthy humans

The concentration and cellular origin of EVs were measured in plasma of healthy humans as described (Table 1). In 2001, flow cytometry results were corrected for isotype control. Since then, it has been questioned whether this isotype control correction is the right choice [12,13]. Therefore, in Table 1, our old (2001) and new (2018) data are shown with and without correction of isotype control.

When corrected for isotype control, the "total" concentration of plasma EVs was 506 × 10^6 EVs/L in 2001, defined as the sum of platelet EVs (82%), erythrocyte EVs (15%) and leukocyte EVs (3%). Without correction, this concentration increased to 524 × 10^6 EVs/L, without affecting the cellular origin of EVs. Using our current procedures and infrastructure, and without isotype control correction, this "total" concentration of plasma EVs increases to 138,300 × 10^6 EVs/L, a 264-fold increase, of which 52% are platelet EVs, 29% erythrocyte EVs and 20% leucocyte EVs. As shown in Table 1, also a low concentration of EVs from (activated) endothelial cells (E-selectin, CD62E) can be detected, suggesting that venous blood from healthy subjects also contains EVs originating from the vessel wall. Taken together, substantially higher concentrations of plasma EVs are detected compared to 2001.

Extracellular vesicles and coagulation

In 2001, we demonstrated that EVs from plasma of healthy humans promote thrombin generation [1]. When the old (2001) thrombin generation test (TGT) results (Figure 1(a)) are compared to the results of our "new" (2018) fibrin generation test (FGT) [9], it is clear that no fibrin formation is observed in healthy control plasma containing EVs (Figure 1(b)). This is unexpected, because thrombin is the enzyme that converts soluble fibrinogen into insoluble fibrin, and thus thrombin generation should also result in fibrin formation. In a control experiment, we confirmed that all (four) donors tested showed thrombin generation under conditions identical to 2001 with regard to blood collection and sample preparation (data not shown). To exclude that this apparent discrepancy is due to a difference in sensitivity between both assays, we activated in a control experiment both the extrinsic and intrinsic coagulation pathways by addition of increasing concentrations of Innovin and Synthasil. As shown in Figure 1(c–f), the FGT is more sensitive than the TGT for both Innovin and Synthasil. Thus, the observed discrepancy that EVs generate thrombin but no fibrin is not explained by the fact that the FGT is less sensitive than the TGT. Despite the fact that our new coagulation assay is more sensitive than our old assay, we were unable to confirm our earlier finding that plasma EVs promote coagulation.

Blood collection and EV-mediated coagulation

Trying to explain why EVs triggered coagulation in 2001 (TGT, Figure 1(a)) and not in 2018 (FGT, Figure 1(b)), we compared the procedures of blood collection and handling

Table 1. Concentration and cellular origin of extracellular vesicles in human plasma.

Cellular origin	Biomarker	Concentration x 10^6/L (correction with IgG control) range		Fold difference	Concentration x 10^6/L (no correction with IgG control) range		Fold difference
		2001	2018		2001	2018	
Erythrocyte	CD235a	74 (39–123)	28,300 (14,000–120,000)	382	80 (43–128)	39,500 (25,600–186,000)	494
		15%	**29%**		**15%**	**29%**	
Granulocytes	CD66e	46 (16–94)	ND		49 (22–103)	ND	
	CD66b	ND	4400 (0–24,600)		ND	18,600 (4900–84,100)	
Leukocyte	CD45	16 (7–31)	13,800 (6300–82,200)	862	22 (11–36)	27,400 (9700–151,000)	1245
		3%	**14%**		**4%**	**20%**	
Monocyte	CD14	0 (0–10)	1870 (0–10,800)		4 (2–14)	12,000 (2500–79,600)	3000
Endothelial	CD62E	88 (22–172)	1000 (0–75,600)		92 (27–179)	12,600 (1800–89,000)	
Platelet	CD61	416 (148–839)	54,100 (24,400–162,000)	130	422 (152–843)	71,400 (33,500–228,000)	169
		82%	**56%**		**81%**	**52%**	
	PS (annexin V)	NA	ND		319 (146–623)	ND	
	PS (lactadherin)	ND	NA		ND	257,000 (145,000–1,310,000)	
Total platelet, erythrocyte, leucocyte EVs		506	96,200	190	524	138,300	264

Concentration and cellular origin of extracellular vesicles (EVs), with (left) or without (right) correction for isotype control, as reported in 2001 and 2018. Major differences between 2001 and 2018 occur not only from the improved sensitivity (lower limit of detection) of a new generation of flow cytometers, but also from differences in blood collection, plasma preparation, labelling of EVs, and the prevention of "swarm" detection. Data are presented as median and range. ND: Not determined; NA: Not applicable.

[3]. A comparison between the protocols showed two main differences (Table 2).

Firstly, blood was collected in siliconized glass tubes in 2001, whereas now plastic collection tubes are used (please see Methods for details). Secondly, to remove platelets a single centrifugation step was used to produce platelet-*poor* plasma in 2001, whereas now two centrifugation steps are used to prepare platelet-*depleted* plasma [3,14].

To determine whether these two before mentioned differences explain the observed (in)ability of EVs to promote coagulation, we compared the effects of blood collection tubes and plasma preparation. Blood was collected in siliconized glass tubes and platelet-*poor* plasma was prepared as in 2001. For comparison, blood from the same donors (n = 6) was collected in plastic collection tubes to prepare both platelet-*poor* and platelet-*depleted* plasma.

Figure 2 shows representative fibrin generation (Figure 2, left) and thrombin generation curves (Figure 2, right). Recalcification of platelet-*poor* plasma from blood collected in siliconized glass tubes triggers fibrin formation (Figure 2 left, curve A). Also, "EVs" isolated from this plasma trigger thrombin generation upon reconstitution in normal plasma (Figure 2 right, curve A). When blood is collected in a plastic tube and platelet-*poor* plasma is prepared, the plasma clots in the FGT albeit slower than when collected in a siliconized glass tube (Figure 2 left, curve B), whereas the EVs isolated from this blood trigger no detectable thrombin generation (Figure 2 right, curve B). When platelet-*depleted* plasma is prepared from blood collected in plastic tubes, neither fibrin formation (Figure 2 left, curve C) nor thrombin generation (Figure 2 right, curve C) is observed. Thus, the observed ability of EVs to trigger coagulation depends strongly on the blood collection tube used and on the plasma preparation conditions. Taken together, and our earlier reported "coagulant activity" of EVs from healthy donors is likely explained by contact activation due to blood collection in glass tubes combined with insufficient removal of platelets.

Figure 3 confirms that platelet-*poor* plasma contains residual platelets (upper right "cloud"), irrespective of whether the blood was collected in glass or plastic tubes (Figure 3(a,b), respectively). In contrast, platelets are virtually undetectable when platelet-*depleted* plasma is prepared (Figure 3(c)), showing that "double centrifugation" is essential to remove platelets adequately. Taken together, changes in procedures of blood collection and handling markedly affect the plasma composition and the downstream functional measurements of EVs.

Coagulation activation *in vivo*

As in 2001, we measured the concentrations of F1 + 2 and TAT as markers of the *in vivo* coagulation activation status

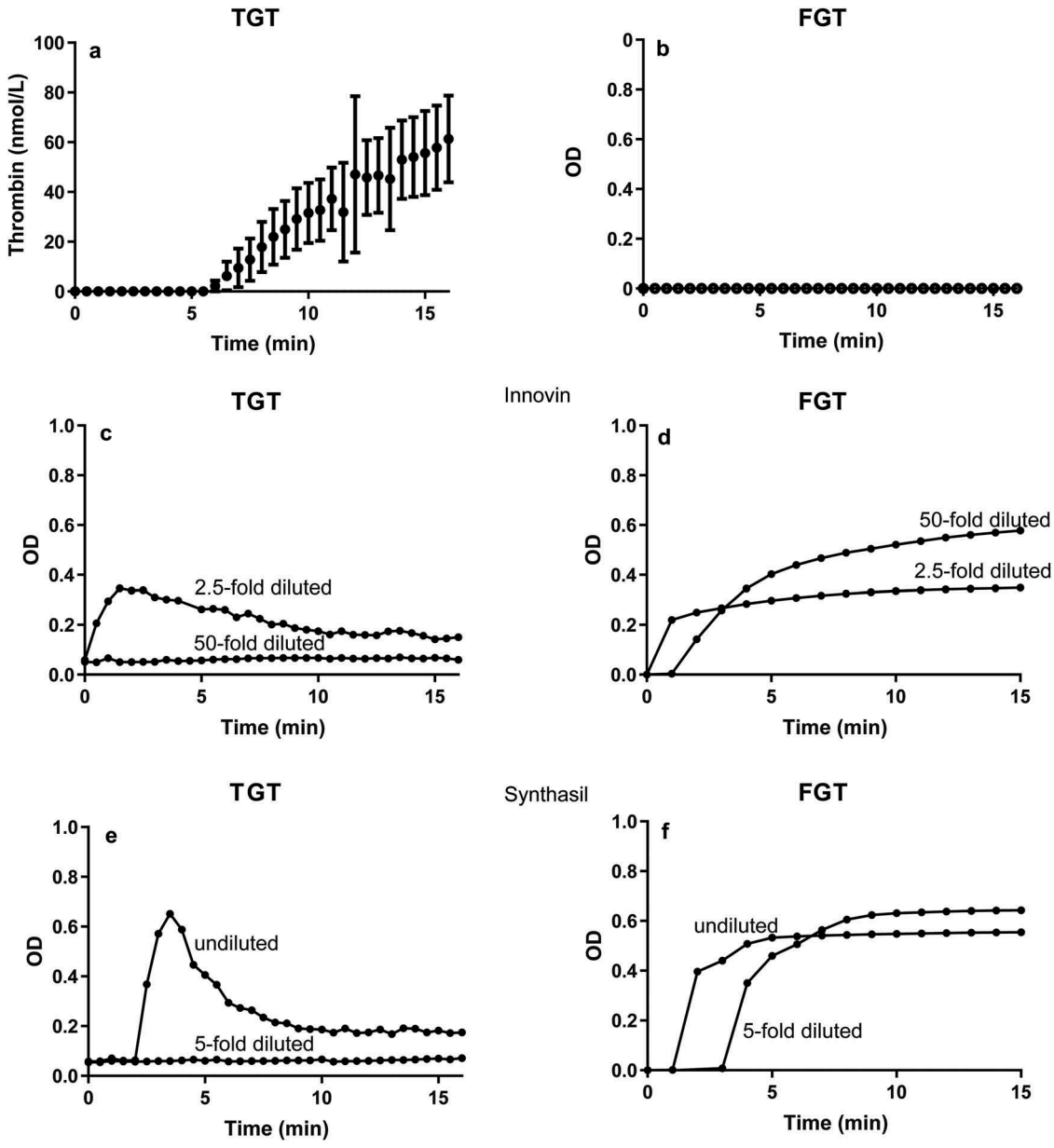

Figure 1. Comparison of the thrombin and fibrin generation tests in human plasma. Thrombin generation (TGT; thrombin generation test) 2001 (a) versus fibrin formation (FGT; fibrin generation test) 2018 (b) by extracellular vesicles, Innovin (c, d) or Synthasil (e, f). In both assays, calcium chloride is added at t = 0.

(Figure 4(a,b)). All measured concentrations except two are within the reported reference ranges of the assays, 181.5 ± 76.0 pmol/L and 2.1 ± 0.42 µg/L (mean ± standard deviation), respectively, and no correlations were present with the measured concentration of EVs (data not shown), indicating that there is no direct relationship between coagulation activation *in vivo* and measured concentrations of plasma EVs *ex vivo*.

Plasmin generation by the EVs

Plasmin activity generated by the EVs was detectable (11.5 ± 10.2 mOD/minute, n = 20, Figure 4(c)), with two values outside the expected normal range. Our results are about two-fold higher than those reported earlier, which may be due to difference in pre-analytics, such as the lower centrifugation speed used to prepare plasma [10,11]. Nevertheless, under normal (physiological) conditions, plasma EVs support fibrinolysis rather than coagulation.

Discussion

The two main findings of this study are that (i) the total concentration of EVs in human plasma has increased 190 to 264-fold compared to 2001, and that

Table 2. Protocols to detect extracellular vesicles and their coagulant activity in 2001 and 2018.

	2001	2018	Ref
Blood collection			
Collection tube	Glass	Plastic	[30]
Plasma preparation (1550*g*)	Once	Twice	[3,5]
Flow cytometry			
Incubation time (labelling)	15 minutes	2 hours*	
Centrifugation of EVs after labelling before measurement by flow cytometry	Yes	No	[3]
Prevention of "swarm"	No	Yes	[20]
Estimated scatter detector sensitivity to detect single EVs (lower limit of detection)	~500 nm	~170 nm	[17]
Coagulation			
Concentration of EVs by centrifugation before measurement	Yes	No	[9]
EVs reconstituted in defibrinated pool plasma or coagulant activity measured directly in plasma	Reconstituted	Directly in plasma	[1]
Read-out	Thrombin activity	Clotting	
Read-out	Chromogenic substrate	Fibrin formation	[9]
Coagulant activity	Likely contact activation	Not detectable	

*The final concentration of antibodies now being used is 5- to 20-fold higher compared to 2001.

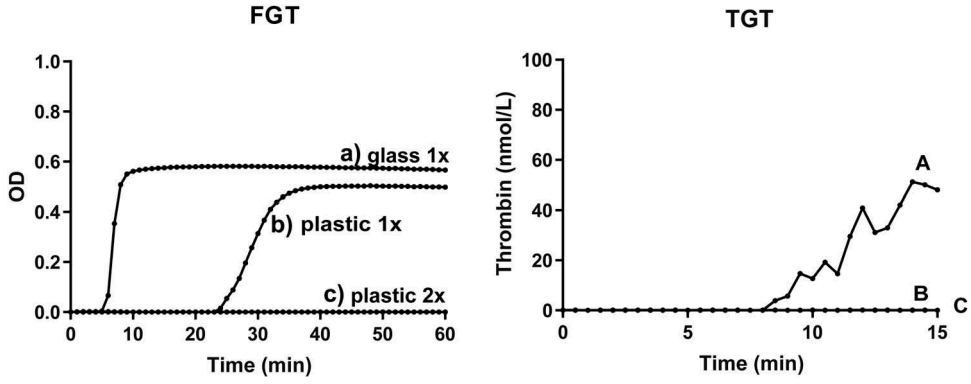

Figure 2. Effect of blood collection and handling on the coagulant properties of human plasma. Platelet-*poor* plasma (single centrifugation to remove platelets) collected in a siliconized glass tube (a) or plastic tube (b); (c) platelet-*depleted* plasma (double centrifugation) prepared from blood collected in a plastic tube. For the fibrin generation test (FGT, left), the prepared plasma samples (containing endogenous EVs) were recalcified at t = 0 as described in Methods. For the thrombin generation test (TGT, right), EVs were isolated from the prepared plasma samples and reconstituted in EV-depleted normal plasma as described in Methods, and thrombin generation was initiated by recalcification at t = 0. Please notice that the time scale (X-axis) differs for FGT and TGT. Representative data from one volunteer are shown. In total, six independent experiments were performed.

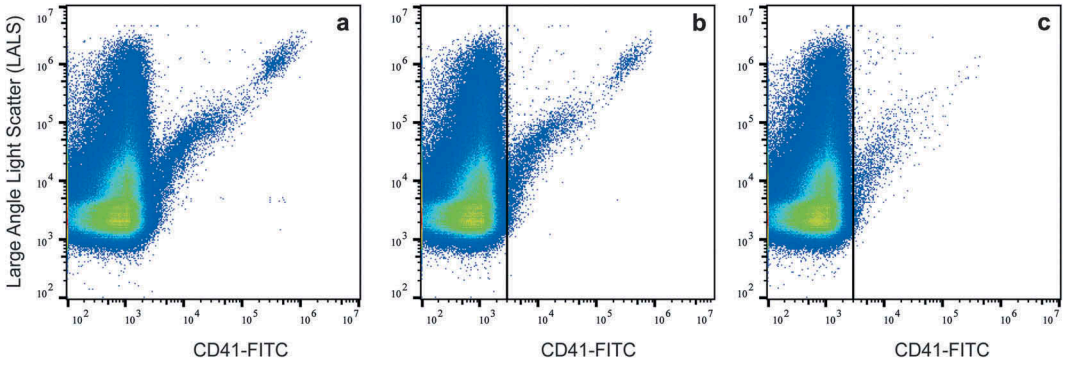

Figure 3. Presence of residual platelets in platelet-poor and platelet-depleted plasma. Platelet-*poor* plasma was prepared from blood collected in a glass tube (a) or plastic tube (b); (c) platelet-*depleted* plasma was prepared by double centrifugation from blood collected in a plastic tube.

(ii) the reported coagulant activity of EVs could not be reproduced.

There are several reasons why a higher concentration of EVs is now being measured (Table 2). Firstly, by optimizing the concentration of antibodies and by prolonging the labelling time, a substantial increase in detected concentrations of EVs has been achieved [2,3]. Moreover, a previous centrifugation step aimed to

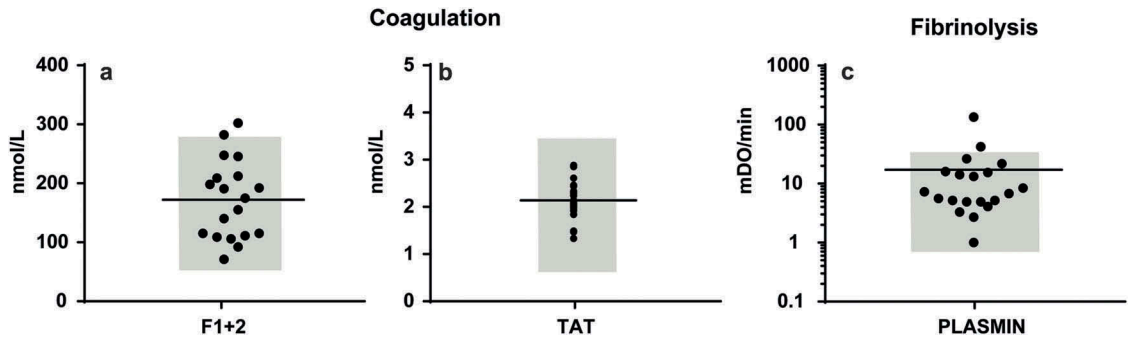

Figure 4. Concentrations of plasma coagulation and fibrinolysis activation markers (a) Concentration of coagulation activation markers prothrombin fragment (F) 1 + 2 and thrombin-antithrombin complexes (TAT) in human plasma; (b) Plasmin generation by EVs in human plasma. Reference ranges are shown in grey.

concentrate EVs prior to measuring and to remove excess of unbound antibodies resulting in clumping of platelet EVs and/or residual platelets was omitted. In fact, the formation of these clumps or aggregates probably explains why previously, on relative insensitive flow cytometers, mainly "EVs" of platelet origin were measured [2,15]. Given the fact that recent evidence suggests that the bulk of small EVs cannot be pelleted from plasma or serum by (ultra) centrifugation supports the idea that residual platelets rather than platelet-derived EVs explained these earlier observations [8].

Secondly, recently we showed that lactadherin outperforms other "generic" fluorescent markers for detection of plasma EVs by flow cytometry [16]. Both lactadherin and annexin V bind to phosphatidylserine (PS) exposed on plasma EVs, but the term "generic markers" is misleading here because not all plasma EVs expose detectable levels of PS [17]. In contrast to annexin V, however, lactadherin does not require calcium ions for binding to PS, and therefore lactadherin is easier to use in a "plasma environment" because there is no risk of clotting. When comparing the total concentration of EVs stained by annexin V (2001) and by lactadherin (2018; Table 1), the increase in the total concentration of detected EVs is 805-fold. Although this increase is substantial, two remarks should be made here. Firstly, when looking at our 2018 results (Table 1), an almost two-fold higher concentration of plasma EVs stain for lactadherin than with fluorescently labelled antibodies. Thus, either part of these lactadherin-positive EVs are false-positive events, and/or there is still a large population of EVs being undetected by flow cytometry. The latter seems likely because we recently showed that a sensitive method that detects *multiple* EVs by antibody capturing to a surface, surface plasmon resonance imaging (SPRi), clearly outperforms detection of *single* EVs using the same antibodies [18]. Therefore, the difference in sensitivity between SPRi and flow cytometry is likely explained by the fact that in SPRi *multiple* EVs are captured which expose with numbers of antigens, whereas *single* EVs generate scatter and fluorescence signals below the detection limit of detectors used in even the newest generation of flow cytometers [3,19].

Thirdly, because the concentration of EVs in human body fluids is high, and because the volume of the flow cell on flow cytometers has dimensions developed to measure single cells and thus are relatively large for EVs, multiple EVs are simultaneously present within the laser beam, and together these EVs generate a single trigger "event" above the threshold, the lower limit of detection [20]. Due to "swarm detection" the concentration of EVs was underestimated, and this underestimation can be easily circumvented by diluting the sample before measuring. Moreover, dilution is also necessary because flow cytometers can analyse only a maximum number of events/s, for example, about 5000 events/s for the Apogee A60 [21]. Obviously, correction for "swarm detection" strongly contributes to the higher concentrations of EVs now being measured.

Fourthly, light scattered by a particle depends on both its diameter and refractive index (RI). Until recently, light scatter-based size gates for EVs were set on flow cytometers using polystyrene reference particles [22,23]. Because polystyrene particles have a higher RI than EVs [24,25], such size gates overestimate the diameter of measured EVs [20]. After correction for the difference in RI between EVs and reference materials, much less EVs are being measured than previously anticipated [4]. Thus, most flow cytometers are still insufficiently sensitive and measure only the larger EVs, and even the most dedicated instruments are still unable to measure single EVs with a diameter below 150–200 nm. Scatter and fluorescence detectors differ in sensitivity between instruments. By developing mathematical (Mie) models, it is has become possible to correct for

differences in optical configuration, i.e. sensitivity, of commercially available flow cytometers. Combined with the fact that in such models also the difference in RI between reference particles and EVs is taken into account, these models provide a solid basis and a realistic insight into the ability of a flow cytometer to detect EVs. In fact, these models offer the opportunity to set absolute size gates in nm for EVs on different types of flow cytometers [4]. Based on this knowledge, we now know that in 2001 the (old) BD FACS Calibur system measured single EVs >500 nm, whereas the (new) Apogee A60 detects single EVs >170 – 180 nm. Because the size distribution of EVs follows a power-law distribution [2,26], the Apogee A60 detects a higher concentration of EVs than the BD FACS Calibur in the same sample.

Taken together, although the detection of single EVs by flow cytometry is still far from perfect, at least we know what we are measuring and which limitations need to be overcome the coming years. Given the fact that also flow cytometry companies are becoming interested in detection of EVs, one may anticipate that new instruments will have improved sensitivity with regard to the detection of smaller EVs. Despite all improvements, however, one still may wonder whether all detected "EVs" are truly EVs. Apart from the fact that EVs are detected based on their binding of fluorescently labelled antibodies and/or lactadherin, we recently validated that >90% of the CD61-positive events with a diameter >200 nm, as detected as by our (Apogee) flow cytometer, have low RI of 1.39, which is close to the reported RI of platelets of 1.40 and which differs significantly from the RI of the lipoprotein population, which is 1.47 on our flow cytometer (Leonie de Rond, personal communication).

As mentioned, we were unable to reproduce the earlier reported coagulation activation activity of EVs in healthy plasma despite the fact that the FGT is more sensitive than the TGT. Of note, the earlier observed thrombin generation in plasma of healthy subjects is absent when blood is collected in the presence of corn trypsin inhibitor, an inhibitor of (activated) coagulation factor XII(a), confirming that the observed thrombin generation was due to contact activation (data not shown). This discrepancy seems to be explained by major changes in blood collection conditions and plasma preparation protocols, although it would be interesting to validate this finding using other currently available coagulation assays. Table 3 provides an overview of publications in which the coagulant properties of EVs have been reported in plasma of healthy humans, often as a control group in a patient study. To which extent these findings, including our own, are caused by blood collection conditions and plasma preparation protocols needs to be confirmed but contact activation is likely to play a major role in most if not all earlier studies. In fact, in recalcified fresh platelet-*depleted* plasma from 20 healthy subjects, spontaneous fibrin formation (clotting) was observed in five samples (20%), with clotting times between 1379–2585 s. Upon addition of polybrene, a contact activation inhibitor, before recalcification, none of the samples clotted for at least 1 h (data not shown). Thus, a role for contact activation seems likely, and despite the fact that using platelet-depleted instead of platelet-poor plasma suppressed contact activation in most plasma samples efficiently, the addition of an inhibitor of the contact activation system seems to rule out this cofounder completely.

Our present study shows that EVs in plasma from healthy humans support fibrinolysis. Although it would be interesting to determine whether this fibrinolytic activity is limited to a particular (sub) population of EVs, we consider this beyond the scope of our present study.

Table 3. Publications presenting information on the coagulant properties of plasma EVs in healthy humans.

Assay	Plasma preparation	Detectable coagulant activity	References
Thrombin[1,2]	1550*g*	Yes	[1]
	2800*g* → 10,000*g*	Yes	[31]
	2500*g* (2x)	Yes	[14]
	2000*g* (2x)	Yes	[32]
	1500*g* → 13,000*g*	Yes	[33]
	2500*g* (2x)	Yes	[34]
	3000*g* (2x)	Yes	[35]
	2500*g* (2x)	Yes	[36]
Thrombin[3]	1500*g* → 12,000*g*	Yes	[33,37]
Fibrin[4]	1550*g*	Yes	[38]
	1550*g* → 13,000*g*	Yes	[39]
	1550*g* (2x)	No	This manuscript
Factor Xa	1550*g*	Yes	[38]
	1550*g* → 13,000*g*	Yes	[39]
	2500*g* (2x)	Yes	[34]
PPL-dependent[5]	1550*g*	Yes	[14,32,35,36,38,40,41]

[1]TGT: thrombin generation test; [2]TGA: thrombin generation assay (Calibrated Automated Thrombogram); [3]Capture ELISA (enzyme-linked immunosorbent assay); [4]FGT: fibrin generation test; [5]PPL: phospholipid-dependent clotting assay.

Recently, we summarized artefacts, flaws and limitations made by us and other EV researchers in detection and characterization of EVs during the last 15–20 years [3]. By learning from our earlier shortcomings, and by understanding recent developments being made in EV detection and characterization, the overall progress in this still novel research field is tremendous, and at present we are in the process of developing "minimal requirements" [27–29], which are currently also being developed for detection of EVs by flow cytometry.

Authorship addendum

RB designed, analyzed and interpreted the results. RB and CH performed most experiments. RL performed and analyzed the plasmin generation assay. RB wrote the manuscript. AS and RN helped designing the experiments and writing the manuscript. All authors approved the final version of the manuscript.

Declaration of interest disclosures

Romaric Lacroix has a collaboration contract with Stago (licensed patent). The other authors state that they have no conflict of interest.

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