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RECOMMENDATIONS AND GUIDELINES

Toward standardization of assays measuring extracellular vesicle-associated tissue factor activity

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1 | INTRODUCTION

The clinical and scientific interest in extracellular vesicles (EVs) is growing exponentially. The term EVs is an umbrella term for various types of vesicles that are present in body fluids and other (bio) fluids. This umbrella term is used because clear hallmarks to distinguish different types of EVs from each other are lacking. Thus, the term "EVs" encompasses earlier "microparticles" or "microvesicles" and exosomes, which are vesicles released directly from the plasma membrane or by secretion of intraluminal vesicles stored in multivesicular endosomes, respectively.[1,2] There is evidence that EVs play a role in intercellular communication and contribute to coagulation and likely inflammation.[3–6]

The oldest-known function of "platelet dust," now known as platelet-derived EVs, is their ability to support coagulation by exposing negatively charged phospholipids, such as phosphatidylserine (PS). Such PS exposing EVs facilitates formation of tenase and prothrombinase complexes.

Furthermore, different subtypes of EVs, such as leukocyte, endothelial, or tumor-derived EVs, can also trigger coagulation by exposing tissue factor (TF).[7] Tissue factor-exposing EVs (TF-EVs) are present in body fluids, such as saliva and urine, under physiological conditions. The presence of TF-EVs in saliva may explain the reflex to lick a wound, thereby exposing blood to extravascular TF and accelerating hemostasis and reducing the risk of infection.[8] Although Tissue factor was initially thought to be exclusively present outside the vasculature ("envelope model"); there is increasing evidence that during medical intervention and in various clinical conditions, such as surgery, or in patients suffering from sepsis or cancer, the presence of coagulant TF-EVs is associated with disseminated intravascular coagulation and venous thrombosis.[9,10]

There are two reasons why a proposed standardization is timely and relevant. First, there is a growing interest to improve the reproducibility of results in science in general, and this also holds true for the new field of EV research. During the last few years, "minimal requirements" have been published by the International Society of Extracellular Vesicles (ISEV) regarding the reporting on studies involving EVs,[11–13] as well as a structure to record and score reporting of preanalytical variables [14–17] In addition, guidelines and position papers have been published[18,19] and an increasing number of standardization studies have been and are being performed involving various aspects of EV detection and characterization.[2,14,20,21] At present, various in-house and commercially available assays have been developed to measure the EV-associated TF (EV-TF) activity, but hitherto the

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results of these methods have not been easily compared and required standardization.

Second, to identify cancer patients at risk of developing venous thromboembolism, an EV-TF-based factor Xa generation assay and an EV-TF-based plasma clotting test have been developed and applied in clinical trials and have shown promising results for the prediction of VTE in pancreatic cancer patients. This underscores the relevance of studying TF-EVs as a potential clinically relevant biomarker.[22,23]

Taken together, we provide a summary of the outcomes of the questionnaire and discussion with the goal to improve future standardization of studies measuring the TF activity of EVs.

2 | QUESTIONNAIRE AND ROUND TABLE DISCUSSION OF ASSAYS MEASURING EXTRACELLULAR VESICLE-ASSOCIATED TISSUE FACTOR ACTIVITY

2.1 | Relevance of preanalytical variables

A number of variables were mentioned, including (a) anticoagulant, (b) time between blood collection and plasma preparation, (c) the use of platelet-*poor* versus platelet-*depleted* plasma, and (d) the use of fresh or frozen/thawed samples. Although these variables have been studied in single and multicenter studies within the frame of the ISTH, there is a scarcity of data on the effects of preanalytical variables on the EV-TF activity. All participants regarded 'preanalytics' and development of 'minimal requirements' as relevant.

2.1.1 | Considerations and recommendations

- There is a need to develop minimal requirements for preanalytics to standardize assays measuring the EV-TF activity in plasmas. Currently available methodological guidelines can give orientation[14,18]
- There is a need for "easy" protocols in clinics ("*complex protocols are also more likely to lead to mistakes*")

2.2 | Assays used to measure the extracellular vesicle-associated tissue factor activity

At present, different assays are being used to measure the EV-TF activity. The main differences are the use of (a) in-house or commercially available assays; (b) assays sensitive to PS but not TF, TF, or both PS and TF; (c) assays measuring factor Xa, thrombin activity, or fibrin formation; (d) assays measuring the procoagulant activity of (endogenous) EVs directly in plasma, or, indirectly, by reconstituting isolated EVs in either pooled plasma or incubating isolated EVs with purified coagulation factors; and (e) kinetic or "end-point" assays.

2.2.1 | Considerations and recommendations

- The duration of assays varies from <20 minutes to 2 hours. There is no consensual position on the impact of duration on the analytical performances of these assays.
- Presence or absence of tissue factor pathway inhibitor should be taken into account
- Results from different assays may provide additional information and may be combined, but direct comparison is not recommended
- Kinetic determination of the EV-TF-dependent factor Xa generation rate may be more reproducible than a single end point measurement

2.3 | Specificity of assays for tissue factor and phosphatidylserine

To demonstrate the specificity for TF, participants use (a) an antibody against TF (clone HTF-1), (b) active site-inhibited factor VIIa (FVIIai), (c) an antibody against factor VIIa, or (d) TF-deficient EVs. The participant using FVIIai recommended FVIIai because of low cost and consistency. Whereas the main interest is focused on detection of TF-EVs, most participants were less certain about the sensitivity of their assays for PS.

2.3.1 | Considerations and recommendations

- Anti-TF is preferred to anti-FVIIa antibody, because factor VIIa can activate factor X to Xa in the absence of TF[24]
- Clone HTF-1 (anti-TF) is recommended to inhibit TF coagulant activity
- The EV-TF activity might be increased by freeze thawing[25]

2.4 | Problem of contact activation?

There was no consensus whether contact activation should be considered a preanalytical problem for the investigation of procoagulant EVs.

2.4.1 | Considerations for future recommendations

- Preparation of platelet-depleted plasma by double-centrifugation reduces the risk of platelet contamination
- Make assays as simple as possible
- Use available blood collection tubes
- Routine use of the factor XIIa inhibitor corn trypsin inhibitor or equivalents is not recommended

2.5 | Need for a tissue factor standard?

All participants confirmed the need for a TF standard to standardize procoagulant activity measurements. "Innovin" is currently used but suffers from an unknown concentration of TF, batch-to-batch variation, and shipment/storage effects. A possible standard could be the use of TF-EVs from cultured cells.

2.6 | Measurement of EV-TF activity and antigen?

While one participant was in favor of combining assays for the quantification of TF-exposing EVs, other participants had objections. The major objection is the lack of convincing results demonstrating the presence of TF on EVs by flow cytometry. This lack is likely due to a limited number of TF epitopes per EV, quality of available antibodies, blockade of TF with factor VII and tissue factor pathway inhibitor, and the lack of sensitivity of current flow cytometers to detect dim (low fluorescent) EVs.

2.7 | Need for standard operating procedures and multicenter studies?

All participants agreed that standard operating procedures need to be developed and tested. A methodological interlaboratory comparison study seems timely. Performing multicener studies is considered relevant by all participants.

3 | WORKSHOP PROPOSAL TO COMPARE SENSITIVITY AND SPECIFICITY OF ASSAYS TO MEASURE TISSUE FACTOR COAGULANT ACTIVITY ASSOCIATED WITH EXTRACELLULAR VESICLES IN HUMAN PLASMA

Françoise Dignat-George suggests organizing two workshops to compare the sensitivity and specificity of assays that measure the coagulant activity of TF-exposing EVs in human plasma.

In the first-year core laboratories will prepare plasma samples that will serve as TF-negative and TF-positive standards. Aliquots of 5 to 10 different platelet-depleted plasma samples from healthy donors will serve as TF-negative standards, as these samples will be from non-stimulated blood, and therefore are expected to contain no detectable EV-TF activity. Tissue factor-positive standards will be generated form blood samples that will either be stimulated with lipopolysaccharide to trigger TF expression by monocytes followed by the release of TF-EVs, or, alternatively, the blood or plasma samples will be spiked with TF-EVs from various sources. The core laboratories will characterize the prepared plasma samples for stability and homogeneity during storage for particle size distribution, cellular origin, and coagulant activity of EVs by nanoparticle tracking analysis and/or tunable resistive pulse sensing, flow cytometry, and PS-dependent and/or TF-dependent coagulation assays. The characterized samples will be distributed to participating laboratories, which will describe their methods to determine TF antigen and activity, and in the second year, will analyze the provided samples. Data will be sent to core laboratories. Sensitivity is evaluated by measuring the ability of the various assays to discriminate platelet-depleted plasma (TF-deficient) from blood stimulated with lipopolysaccharide or spiked with TF-EVs. Specificity will be evaluated by measuring the signal of platelet-depleted plasma spiked (a) with similar concentrations of TF-EVs or "knockout" TF-EVs and (b) with activators or inhibitors of contact activation. Regular assay performance, including reproducibility and linearity, will be recorded. The final outcome will be reported to the SSC on Vascular Biology of the ISTH and will be submitted for publication to the Journal of Thrombosis and Haemostasis.

CONFLICT OF INTERESTS

The authors state that they have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

R. Niewland drafted the manuscript, which was reviewed, edited, and approved by all authors. The manuscript is based on (a) a questionnaire, which was drafted by R. Nieuwland and J. Thaler, and edited by all authors, and (b) a round table discussion, which all authors attended at the 64th Annual SSC Meeting of the ISTH (Dublin, Ireland).

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