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Elevated Plasma Levels of P-Selectin Glycoprotein Ligand-1 Positive Microvesicles in Patients with Unprovoked Venous Thromboembolism

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Essentials

- PSGL-1^+ microvesicles (MVs) may be important in venous thromboembolism (VTE).
- We measured plasma levels and parental origin of PSGL-1^+ MVs in patients with unprovoked VTE.
- VTE patients had higher plasma levels of PSGL-1^+ MVs levels than healthy controls.
- The PSGL-1^+ MVs originated mainly from monocytes and endothelial cells.

Abstract

Background: Microvesicles (MVs) express antigens from their parental cells and have a highly procoagulant surface. Animal studies suggest that P-Selectin Glycoprotein Ligand-1 positive (PSGL-1^+) MVs play a role in the pathogenesis of venous thromboembolism (VTE).

Objective: The aim of this study was to determine plasma levels, the cellular origin and the morphological characteristics of PSGL-1^+ MVs in patients with unprovoked VTE.

Methods: We conducted a population based case-control study in 20 patients with a history of unprovoked VTE and 20 age- and sex-matched healthy controls recruited from the general population. Plasma levels, the cellular origin and the morphological characteristics of PSGL-1^+ MVs was evaluated using flow cytometry, electron microscopy and confocal microscopy.

Results: Plasma levels of PSGL-1^+ MVs were associated with increased risk of VTE. The odds ratio (OR) per one standard deviation (SD) increase in PSGL-1^+ MVs was 3.11 (95% CI: 1.41-6.88) after adjustment for age and sex, and 2.88 (95% CI: 1.29-6.41) after further adjustment for BMI. The PSGL-1^+ MVs originated mainly from monocytes and endothelial cells determined by double-staining with markers of parental cells using flow cytometry and transmission electron microscopy (TEM). Scanning electron microscopy (SEM) of PSGL-1 labeled plasma-derived MVs displayed dominantly spherical vesicles that varied between 50 nm and 300 nm in diameter.

Conclusions: Increased plasma levels of PSGL-1^+ MVs are associated with the risk of unprovoked VTE. Large population-based prospective studies are required to validate our findings.

Key words: Cell-Derived Microparticles, P-Selectin, Venous Thromboembolism, Platelets, Case-Control Studies

Introduction

Venous thromboembolism (VTE), a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE), is the third most common cardiovascular disease with an annual incidence of 1-2 per 1000 inhabitants [1]. Venous imaging and autopsy studies have revealed that most venous thrombi originate from the valvular sinuses with intact endothelium [2, 3]. P-selectin is an adhesion molecule stored in the membranes of alpha granules in platelets and in Weibel-Palade bodies in endothelial cells, that can be mobilized rapidly to the plasma membranes upon stimulation [4, 5]. Activated endothelial cells expressing P-selectin may mediate tethering and rolling of leukocytes in inflamed vascular beds by interaction with its physiological ligand P-selectin glycoprotein ligand-1 (PSGL-1) expressed at the surface of leukocytes [6, 7]. This multistep cellular adhesion process is known to play a pivotal role in host-defense, inflammation and hemostasis.

Microvesicles (MVs) are small circulating membrane vesicles budded off from activated or apoptotic cells [8]. They are highly procoagulant due to large amounts of surface-exposed negatively charged phospholipids (e.g. phosphatidylserine), facilitating the assembly of protease complexes of the clotting cascade, and the presence of tissue factor (TF) [9]. MVs are defined by their size (0.1-1.0 μm) and expression of surface antigens specific to parental cells [9]. PSGL-1 is expressed in monocytes, neutrophils [10], endothelial cells [11],

and to a lesser extent in platelets [12]. Growing evidence from observational [13-15] and experimental studies [16-19] suggests that P-selectin-PSGL-1 interaction between MVs and target cells play a role in the pathogenesis of VTE. Potentially, PSGL-1 positive ($PSGL\text{-}1^+$) MVs could play a role in the pathogenesis of VTE by providing targeted accumulation of procoagulant MVs on the surfaces of activated endothelial cells and platelets. It is, however, not clear whether high plasma levels of $PSGL\text{-}1^+$ MVs is associated with unprovoked VTE. Therefore, we conducted a case-control study of patients with unprovoked VTE and age- and sex-matched healthy controls recruited from the general population to determine plasma levels, the cellular origin and the morphological characteristics of $PSGL\text{-}1^+$ MVs using flow cytometry, electron microscopy and confocal microscopy

Material and methods

Study population

A population based case-control study was performed in 20 patients with a history of unprovoked VTE and 20 age- and sex-matched healthy controls recruited from the general population. Patients with unprovoked VTE were recruited from the registry of VTE patients in the municipality of Tromsø [20]. A VTE case in this registry had all of the following criteria fulfilled; (1) objectively confirmed by diagnostic procedures, (2) the medical record indicated that a physician had made a diagnosis of DVT and/or PE, (3) signs and symptoms consistent with DVT or PE were present, and (4) therapy with anticoagulants (heparin, warfarin, or a similar agent), thrombolytics, or vascular surgery were required. Unprovoked VTE was defined as complete absence of provoking factors at the time of diagnosis. Recent surgery or trauma (within 8 weeks before the event), an acute medical condition (MI, ischemic stroke, major infectious disease), cancer, marked immobilization (bed rest >3 days, patients in wheelchairs, long-distance travel ≥4 hours within the last 14 days), pregnancy or

puerperium, estrogen supplementation, or another likely provoking factor specifically described by a physician in the medical record (e.g, intravascular catheter) were considered provoking factors. Subjects were eligible for the VTE group if they were aged 20-80 years, had an unprovoked VTE event 1-6 years prior to the investigation (and no recurrence), and had stopped anticoagulant treatment at least 3 months prior to the investigation. The timing between the VTE event and blood sampling is displayed in Table S1. For each VTE patient, one apparently healthy person matched for age and sex was recruited from a health survey (the sixth Tromsø study), and underwent the same screening visit as the VTE patients. Informed written consent was obtained from the participants, and the regional ethical committee approved the study.

Baseline measurements

Cases and controls were screened and included in the study between April and September 2008. At the screening visit, height and weight were measured with the participants wearing light clothing and no shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters (kg/m^2). Waist circumference was measured in centimeters at the umbilical line.

Plasma levels of antithrombin amidolytic activity (STA-STACHROM AT III), protein S cofactor activity (STA-STACLOT PROTEIN S) and protein C amidolytic activity (STA-STACHROM PROTEIN C) were determined by commercially available assays obtained from Diagnostica Stago (Asnieres-Sur-Seine, France) on an automatic analyzer (STA-R Evolution, Diagnostica Stago, France). Deficiency of antithrombin, protein S and total protein C was defined as plasma levels ≤65% of normal plasma levels. Assessment of factor V Leiden and factor II G20210A mutations was carried out on genomic DNA as previously described [21, 22].

Blood collection and storage

Blood was drawn from an antecubital vein using a 19-gauge needle at 7:45 am, after 12 hours overnight fasting and 48 hours refrain of exhaustive physical exercise and alcohol consumption. Blood for plasma preparation was collected into 4.5 mL vacutainers (Becton Dickinson, Meylan Cedex, France) with minimal stasis containing 0.129 M sodium citrate (1 vol. anticoagulant and 9 vol. whole blood) and 6-mL K2E-EDTA tubes as anticoagulant. Immediately after blood sampling, citrate plasma was prepared by centrifugation at 2000 x g for 15 minutes at 22ºC, transferred into cryovials (Greiner laboratechnik, Nürtringen, Germany) in aliquots of 1 ml, and stored at -70ºC until further analysis.

Cell counting and measurement of plasma P-selectin

Cell counts were recorded in EDTA-anticoagulated whole blood and in suspensions of peripheral blood mononuclear cells (PBMCs) using ABX micros 60-CT cell counter (ABX Diagnostics, Montpellier, France). Plasma levels of P-selectin were measured using a commercially available quantitative sandwich immunoassay purchased from R&D systems (Abingdon, UK, Cat:BBE6).

Plasma concentrations and cellular origin of PSGL-1$^+$ microvesicles by flow cytometry

Citrated plasma samples were thawed in room temperature and centrifuged at 13.500 x g for 2 minutes to get rid of remaining platelets and cellular debris. MVs were then pelleted by centrifugation (20 000 x g for 30 minutes at room temperature) and resuspended in phosphate-buffered saline (PBS). The plasma concentrations of Annexin V$^+$ MVs and PSGL-1$^+$ MVs were measured using FITC-Annexin V (Cat: 556419, BD Bioscience, Phamingen, San Jose, CA) and PE-CD162 (Cat: 328806, Biolegend, San Diego, CA) monoclonal antibodies, respectively. The cellular origins of PSGL-1$^+$ MVs were determined by

immunostaining of the total MV fraction with PerCP-Cy5.5 conjugated (PerCP-CY5.5 – antibody conjugation kit, Cat: LNK141PERCPCY5.5, AbD Serotec) anti-PSGL-1 antibody (Cat: 556052, BD Bioscience), and PE-conjugated monoclonal antibodies specific to parental cells: CD14 (Cat: 345785, BD Biosciences) for monocytes, CD62E (Cat: 336008, Biolegend) for endothelial cells and CD41a (Cat: 555467, BD Biosciences) for platelets. Mouse-IgG1k-PE (Cat: 555749, BD Bioscience) and mouse IgG1k (Cat: 400124, Biolegend) conjugated with PerCP-Cy5.5 were used as isotype controls. 50µl of isolated MVs in PBS were incubated with antibodies (2.5 µg/mL) at room temperature for 30 minutes. After incubation, 450 µl of PBS was added and the samples were analyzed with a FACSAria I flow cytometer (BD Bioscience, San Jose, USA). A size specific gate for MVs (<1 µm) was set by using megamix beads (mix of 0.5, 0.9, and 3 µm beads) (Biocytex, Marseille, France). Matched pairs of VTE patients and control subjects were run in parallel, but their identity was unknown for the technician running the analyses. Data were collected in logarithmic mode, and a minimum of 10,000 MVs gate events were recorded. The data was analyzed with FlowJo version 7.5 software (Tree Star, Inc., Ashland, OR).

Immunoelectron microscopy (IEM)

Double-labelled immunoelectron microscopy analysis was performed on isolated MVs from plasma of VTE patients and controls. Samples were immunolabelled according to standard procedure [23, 24] by mouse anti human-CD162 (PSGL-1 Cat: ab3986) as first primary antibody and either rabbit anti-human-CD14 (Cat: ab133335), rabbit anti human-CD41/Integrin alpha 2b (Cat: ab134131) or rabbit anti human-CD62E (Cat: ab18981) as second primary antibody (all from Abcam). In brief, the pelleted MVs (centrifuged 20 000 xg for 30 minutes at room temperature) from citrated plasma were resuspended in 100 µl HHBS buffer and fixed in 4% paraformaldehyde overnight. Formvar coated copper grids were

floated on sample drops for 20 minutes. After a blocking step with 0.8% BSA and 0.1% Fish Skin Gelatin (Sigma-Aldrich, Cat: G7756) the samples were incubated with the first primary antibody, and labeled with a Protein A gold (PAG) 5 nm particles probe. To avoid cross-reactions with the next primary antibody, sections were treated with 1% glutaraldehyde, and quenched in 0.12% glycine. Samples were incubated with the second primary antibody, and labeled with 5-nm PAG. After washing with PBS and ddH_2O, the grids were stained/dried with the addition of 1.8% Methyl Cellulose and 0.3 % Uranyl acetate according to the Tokuyasu method [25]. All antibodies and PAG particles were diluted in the blocking buffer. As control experiments, single labeling was performed for each antibody. In addition, crossover tests were performed to ensure that no change occurred in the labeling patterns. The images were obtained by Jeol JEM-1010 transmission electron microscope (Tokyo, Japan) and processed with Morada Soft Imaging System (Olympus, Germany) program.

Scanning Electron Microscopy (SEM)

To verify the presence, size, shape and purity of intact MVs, the grids were labeled by mouse anti human-CD162 (PSGL-1, Cat: ab3986) with similar protocol steps as for IEM. Finally, the grids were dehydrated through 30-100% ethanol and dried with Hexamethyldisilazane (HMDS). The images were obtained using a Zeiss Merlin VP Compact microscope (Carl Zeiss Microscopy GmBH, Jena, Germany).

Fluorescence confocal microscopy

PBMCs were isolated from EDTA anticoagulated whole blood immediately after blood collection by centrifugation (800 x g for 20 minutes) using lymphoprep (Axis-Shield PoC AS, Oslo, Norway) as density gradient. The PBMC interface was washed with endotoxin-free

PBS (BioWhittaker, Cambrex, Verviers, Belgium) three times at 232 x g for 10 minutes. All centrifugation steps were conducted at room temperature.

The pellet of PBMCs was resuspended and plated onto an 8-well chambered coverglass (Thermo Scientific, Nun, Lab-Tek, Chambered Coverglass) in 250µL RPMI-1640 media. Plated PBMCs were incubated without and with 50 µL isolated MVs (1500 MVs/ µL) at room temperature for 15 minutes. PBMCs were fixed with 4% paraformaldehyde for 10 minutes. All buffers, blocking buffers and primary and secondary antibody solutions were filtered with acrodisc syringe filter 0.2µm pore size (Pall life science). Cells were immunostained with purified mouse anti-human CD162 (BD Bioscience PSGL-1-Cat: 556053, BD Phamingen), conjugated with Alexa Fluor 555-secondary antibody (Cat: A-21422, Invitrogen Molecular probes), and CD42a (Cat: MCA1227F, AbD Serotec). The nuclei of the cells were visualized by using DRAQ5 dye (Biostatus Ltd, UK). The pictures were taken by Leica TCS SP5 confocal microscope (Leica Microsystems GmBH, Wetzlar, Germany) equipped with a 63X 1.2NA water immersion lens.

Statistical analysis

Statistical analyses was conducted using STATA version 14 (Stata Corporation, College Station, TX, USA). All variables were checked for frequency distribution, and the mean and standard deviation (SD), as well as medians and interquartile range of each variable were reported. PSGL-1 levels in cases and controls were displayed using a box-plot. Unconditional logistic regression was used to calculate odds ratios (ORs) with 95% confidence intervals (CI) per one SD increase in exposure level. The SD in the control group was used as the basis for these ORs. Crude ORs (model 1) as well as ORs adjusted for age and sex (model 2) and age, sex and BMI (model 3) were calculated.

Results

Characteristics of VTE patients and matched controls with regard to age, sex, smoking, obesity measures and inherited thrombophilia are shown in Table 1. VTE patients had higher BMI (29.3±4.4 kg/m^2 versus 26.3±3.9 kg/m^2) and greater waist circumference (98.2±12.5 cm versus 93.4±13.4 cm) than age and sex- matched controls. Although FV-Leiden was more frequent among the VTE patients, no significant differences in inherited thrombophilia (VTE group: 2 protein C deficiencies and 1 protein S deficiency; Control group: 2 protein S deficiencies) or family history of VTE were observed between the groups. No individual was found to have lupus anticoagulant (data not shown). Moreover, neither cases nor controls had any history of cardiovascular co-morbidities (myocardial infarction, ischemic stroke, hemorrhagic stroke, TIA, angina pectoris) (data not shown).

Plasma concentrations of MVs were measured by flow cytometry. Patients with a previous history of unprovoked VTE had higher plasma levels of total MVs, Annexin$^+$ MVs, and PSGL-1$^+$ MVs compared to healthy controls (Table 2 and Figure 1). The age and sex-adjusted OR per one standard deviation (3529 per µL) increase in the total number of MVs was 1.65 (95% CI: 1.00-2.71) and was slightly attenuated after adjustment for BMI (OR 1.58, 95% CI: 0.95-2.61). Similar results were seen for Annexin-V$^+$ MVs, where the age- and sex-adjusted OR per one SD (80 per µL) increase was 1.68 (95% 0.99-2.82). PSGL-1$^+$ MVs were associated with a significantly increased risk of VTE. The OR per one SD increase in PSGL-1$^+$ MVs was 3.11 (95% CI: 1.41-6.88) after adjustment for age and sex, and 2.88 (95% CI: 1.29-6.41) after further adjustment for BMI. The OR per one SD increase in P-selectin was 1.36 (0.69-2.69) in age- and sex-adjusted analyses, and 1.22 (0.60-2.46) after further adjustment for BMI (Table 2). Plasma levels of soluble P-selectin was neither correlated to PSGL-1$^+$ MVs in VTE patients (r= -0.26, p=0.3) nor in controls (r=0.10, p=0.7).

To determine the parental origin of PSGL-1$^+$ MVs, we double-labelled MVs for PSGL-1 and for potential parent cell markers. MVs derived from monocytes (Figure 2a) and endothelial cells (Figure 2b) were positive for PSGL-1, whereas platelet-derived MVs were only slightly positive for PSGL-1 (Figure 2c).

To visualize the morphology and cellular origin of PSGL-1$^+$ MVs, we pelleted MVs from plasma by high-speed centrifugation and prepared them for SEM and IEM. SEM images of PSGL-1 labeled MVs derived from plasma displayed dominantly round-shaped particles that mostly varied between 50 nm and 300 nm in diameter (Figure 3). IEM was then applied to further explore the morphology, cellular origin and expression of PSGL-1 at the surface of MVs isolated from plasma. MVs were labeled with specific antibodies and colloidal gold probes using small gold nanoparticles for PSGL-1 (5 nm) and large gold nanoparticles for parental cells (10 nm). IEM images confirmed our double-staining results from the flow cytometry, demonstrating high and evenly distributed label of PSGL-1 on MVs derived from monocytes, double labelled with anti CD14/CD162 (Figure 4a), and MVs derived from endothelial cell, double labelled with anti CD62E/CD162 (Figure 4b). Platelet derived MVs were mostly negative for PSGL-1, but rarely PSGL-1$^+$ platelet MVs were seen (Figure 4c).

To trace the cellular origin of PSGL-1$^+$ MVs in peripheral blood, we subjected PBMC preparations, enriched with mononuclear cells and platelets, to confocal microscopy imaging (Figure 5). Monocytes expressed evenly distributed amounts of PSGL-1 on their surfaces, while platelets displayed weak and patchy staining for anti-PSGL-1 labeling.

The total number of PSGL-1+ MVs did not vary according to the time between VTE and blood sampling in the VTE cases, thus indicating a low risk of reverse causation (Figure S1).

Discussion

Experimental studies in animal models suggest that thrombus formation is dependent on the interaction between PSGL-1^+ MVs and cellular expression of P-selectin [16-19]. Therefore, we wanted to explore the role of PSGL-1^+ MVs in VTE, and investigated plasma concentrations, cellular origin, and morphological characteristics of PSGL-1^+ MVs in patients with unprovoked VTE and in age-and sex-matched healthy controls. Patients with unprovoked VTE had higher plasma concentrations of PSGL-1^+ MVs than control subjects. PSGL-1^+ MVs originated mainly from monocytes and endothelial cells, and displayed a round-shaped morphology with a diameter between 50 and 300 nm. Our findings support the notion that PSGL-1^+ MVs may play an important role in the pathogenesis of VTE.

While several studies have investigated the impact of MVs, particularly TF^+ MVs, on VTE risk and patient survival [26], few studies have explored the association between the plasma concentration of MVs and risk of VTE in subjects with unprovoked VTE. In a case-control study including 186 unprovoked VTE patients and 418 healthy controls, subjects with plasma concentrations of Annexin V^+ MVs above the 95% percentile had a 2.2-fold higher risk of VTE than those with MVs concentration below the 95% percentile [27]. Accordingly, we found that the risk of unprovoked VTE increased with 1.6-fold per one standard deviation increase in plasma concentration of Annexin V^+ MVs. In addition, several studies have reported joint effects of plasma concentrations of Annexin V^+ MVs and inherited thrombophilias [27-30]. The latter findings may suggest that elevated levels of circulating MVs can play a role in thrombus formation in subjects with mild and severe inherited thrombophilia.

Our case-control study is, to the best of our knowledge, the first to explore plasma concentrations and cellular origin of PSGL-1^+ MVs in unprovoked VTE. We found that patients with unprovoked VTE had higher plasma concentration of PSGL-1^+ MVs than age-

and sex-matched healthy controls. Higher plasma levels of PSGL-1^+ MVs may imply targeted and augmented accumulation of MVs at sites of activated endothelial cells and platelets expressing P-selectin [31]. MVs enriched with phosphatidylserine on their surface will facilitate the assembly of protease complexes of the clotting cascade by electrostatic interactions between positively charged ɤ-carboxyglutamic acid in many clotting proteins and phosphatidylserine on the MV membrane, ultimately enhancing fibrin clot formation [32]. Phosphatidylserine is known to increase the catalytic efficiency of both the tenase (FVIIIa/FIXa) and prothrombinase (FVa/FXa) complexes by 200- and 1000-fold, respectively [33]. Furthermore, results from animal studies support the concept that PSGL-1^+ MVs may play an important role in the pathogenesis of VTE. In mice, it has been shown that accumulation of TF^+ MVs in the developing thrombus is dependent on interaction between PSGL-1^+ MVs and cellular (e.g. platelets and endothelium) expression of P-selectin in vivo [16]. Genetic overexpression of P-selectin in a mouse model caused increased levels of TF^+ MVs associated with shortened clotting times, which were abolished by infusion of inhibitory anti-PSGL-1 antibodies [17, 18]. Furthermore, P-selectin antagonism had similar efficacy as enoxaparin to reopen venous occlusion in animal models [19].

A previous study reported that PSGL-1 expression varied substantially between leukocytes, with highest expression on monocytes, followed by T and natural killer lymphocytes, then neutrophils, and finally precursors cells [34]. In platelets, the PSGL-1 expression is up to 100-fold lower than in leukocytes [12]. In addition, endothelial cells are known to express functional PSGL-1 which is able to mediate tethering and firm addition of monocytes and platelets to inflamed endothelium [11]. Further, the PSGL-1 expression is expected to be enriched on released MVs because it is associated with the lipid-raft sections within the cell membranes [35]. Accordingly, several lines of evidence support that monocytes and endothelial cells, but to a lesser extent platelets, were the main parental origin

of PSGL-1^+ MVs in our study. First, double staining of MVs for PSGL-1 and specific labels for potential parental blood cells using flow cytometry revealed that monocytes and endothelial cells, but to a lesser extent platelets, were positive for PSGL-1. Second, IEM images confirmed our double-staining results from flow cytometry, as they demonstrated high and evenly distributed labels of PSGL-1 on MVs derived from monocytes and endothelial cells, but only patchy and weak PSGL-1^+ platelet-derived MVs. Third, confocal microscopy revealed that only monocytes expressed substantial amounts of PSGL-1 on their surfaces, while platelets displayed weak positivity for anti-PSGL-1 labelling in peripheral blood mononuclear cell (PBMC) preparations enriched with mononuclear cells and platelets.

Strengths of our study include the recruitment of VTE patients from a population-based cohort and age- and sex-matched apparently healthy controls from the same source population. In order to minimize the effect of the acute VTE on the measurements of interest (reverse causation), all samples were collected more than one year after the acute VTE event. Although MVs are known to have a short half-life in the circulation [36], elevated plasma levels of MVs may reflect prolonged activation of parental cells of PSGL-1^+ MVs in patients with unprovoked VTE. Therefore, the fact that blood samples were collected more than a year after unprovoked VTE makes it uncertain to affirm the direction of the association between exposure (PSGL-1^+ MVs) and outcome (VTE). However, the total number of PSGL-1^+ MVs did not vary according to time between the VTE and blood sampling, thus indicating a low risk of reverse causation. For assessment of protein C deficiency we used the amidolytic assay. This assay may not detect type II deficiencies. However, given the small sample size of our study, and the rarity of this condition, it is very unlikely that misclassification of protein C deficiency have occurred. Another limitation of this study was the low number of participants included in our study, resulting in a considerable risk of

statistical type 1 and 2 errors. Thus, a larger, prospective study should be conducted to validate our findings.

In conclusion, results from our case-control study imply that plasma concentrations of PSGL-1^+ MVs are associated with risk of unprovoked VTE. Large population-based prospective studies are required to validate our findings.

Author contributions

S. Jamaly was responsible for laboratory analyses, microscopy imaging, and drafting the manuscript. M. G. Basavaraj and I. Starikova performed laboratory analyses. R. Olsen performed electron microscopy imaging. S. K. Brækkan was responsible for statistical analysis. J-B. Hansen conceived and designed the study, performed data collection, and wrote the manuscript. All authors revised the manuscript and approved the final submitted version.

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Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

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Figure legends:

Figure 1: Plasma concentrations of P-Selectin Glycoprotein Ligand-1 positive (PSGL-1$^+$) positive microvesicles (MVs) in patients with unprovoked VTE and controls. Plasma concentration of PSGL-1$^+$ MVs in VTE patients were significantly higher compared with the control group ($p<0.001$). Plasma levels of MVs are expressed as the number of elements per microliters (µL).

Figure 2: Cellular origin of P-Selectin Glycoprotein Ligand-1 positive (PSGL-1$^+$) positive microvesicles (MVs): The figure shows representative quadrants derived from flow cytometry quantitation of microvesicles (MVs) isolated from platelet free plasma of unprovoked VTE patients. MV gate was set using megamix fluorescent beads with diameters of 0.5, 0.9, and 3 µm. Cellular origin of PSGL-1$^+$ MVs was identified by dual staining (Events in Q2) with monoclonal antibodies specific to parental cells (CD14-PE for monocytes, CD62E-PE for endothelial cells and CD41a-PE for platelets), and CD162-PerCP-Cy5.5 for detecting PSGL-1$^+$ MVs. A distinct and significant population in Q2 can be recognized in (a) CD14$^+$/ CD162$^+$ and (b) CD62E$^+$/CD162$^+$ quadrants but not in (c) CD41a$^+$/CD162$^+$. (MMV: Monocyte derived MVs, EMV: Endothelial cell derived MVs, PMV: Platelet derived MVs)

Figure 3: Scanning electron microscopy (SEM) of PSGL-1 labeled plasma microvesicles (MVs). The particle attached to carbon coated formaver films on a copper grid, and labeled with anti-PSGL-1.The MVs were dominated by spherical morphology with a diameter between 50 and 300 nm. Scale bar=100 nm.

Figure 4: Visualization of MVs by immunoelectron microscopy (IEM). Detection of immunogold labeled MVs. MVs were incubated with mouse monoclonal CD162 (PSGL-1) – conjugated 5 nm gold nanoparticles and rabbit monoclonal Abs-conjugated 10 nm gold nanoparticles for parental cells. The majority of the MVs were positive for PSGL-1 and its specific gold particles are randomly distributed all around the MVs. (A) MVs derived from monocytes, scale bar = 500 nm. (B) MVs derived from endothelial cells, scale bar = 100 nm. (C) MVs derived from platelets, scale bar = 200 nm.

Figure 5: Confocal microscopy images of peripheral blood mononuclear cells (PMNCs). Platelets and PSGL-1^+ cells were identified using a platelet monoclonal antibody (CD42a), a PSGL-1(CD162) primary antibody, and a fluorescent secondary antibody (Alexa Fluor 555). Nuclei was stained by DRAQ5. The monocytes displayed disperse labeling of PSGL-1^+ proteins at the surface of the cell membranes. Panel A: Green signal from anti-CD42a; Panel B: Red signal from anti-PSGL-1; Panel C: differential interference contrast; Panel D: merged micrograph. (Scale bar = 10 μm).

Table 1. Characteristics of patients with a previous history of unprovoked VTE and healthy age- and sex matched controls. Values are means±1 SD or percentage with numbers in brackets.

Variables	**VTE patients (n=20)**	**Controls (n=20)**
Age (years)	56.4 ± 14.8	56.3 ± 14.4
Gender (% women)	50 (10)	50 (10)
BMI (kg/m^2)	29.3 ± 4.2	26.8 ± 3.5
Waist circumference (cm)	98.2±12.5	93.4±13.4
Smoking (%)	55 (11)	50 (10)
Family history of VTE (%)	20 (4)	10 (2)
Inherited thrombophilia		
FV-Leiden, heterozygous (%)	10 (2)	5 (1)
Antithrombin (%)	101 ± 9	103 ± 9
FII G20210A, heterozygous (%)	5 (1)	0 (0)
Protein C (%)	109 ± 24	94 ± 22
Protein S (%)	103 ± 19	95 ± 19

Table 2. Odds ratios (OR) of venous thromboembolism (VTE) with 95% confidence intervals (CI) according to one standard deviation (SD) increase in total number of microvesicles (MVs), Annexin-V positive (Annexin V^+) MVs, P-Selectin Glycoprotein Ligand-1 positive ($PSGL\text{-}1^+$) MVs, and plasma P-selectin.

	Cases Mean ± SD Median (ICR)	**Controls** Mean ± SD Median (ICR)	**Model 1** **OR (95% CI)***	**Model 2** **OR (95% CI)***	**Model 3** **OR (95% CI)***
Total number of MVs	11442 ± 8478 10813 (4975-14455)	6940 ± 3529 6415 (4852-9012)	1.63 (0.99-2.67)	1.65 (1.00-2.71)	1.58 (0.95-2.61)
$AnnexinV^+$ MVs	263 ± 146 248 (145-321)	185 ± 80 160 (140-209)	1.64 (0.98-2.72)	1.68 (0.99-2.82)	1.57 (0.93-2.67)
$PSGL\text{-}1^+$ MVs	108.2 ± 45.7 98.2 (74.9-124.3)	63.1 ± 20.0 64.1 (52.3-69.8)	2.75 (1.35-5.57)	3.11 (1.41-6.88)	2.88 (1.29-6.41)
P-selectin (ng/mL)	27.2 ± 8.4 25.9 (21.8-31.8)	24.8 ± 9.06 26.7 (19.9-29.5)	1.35 (0.69-2.65)	1.36 (0.69-2.69)	1.22 (0.60-2.46)

*OR per standard deviation increase (the standard deviation among controls was used to calculated the OR)
Model 1: Crude
Model 2: Adjusted for age and sex
Model 3: Adjusted for age, sex and body mass index (BMI)