1 **Plasma levels of platelet-derived microvesicles are associated with risk of future**

2 **venous thromboembolism**

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Essentials:

1. Platelet-derived microvesicles (PDMVs) may play a role in venous thromboembolism (VTE).

2. We investigated the association between proportion of PDMVs in plasma (PDMVs%) and risk of VTE.

3. High PDMVs% was associated with increased risk of future VTE, particularly provoked events.

4. The association was stronger when the time from blood sampling to VTE was shorter.

Abstract

Background

Microvesicles (MVs) are small double-membrane encapsulated particles shed from cells. Case-control studies have reported elevated plasma levels of platelet-derived MVs (PDMVs) in patients with venous thromboembolism (VTE). However, it is not known whether high PDMV-levels is a risk factor or a consequence of the acute VTE event.

Objectives

To investigate the association between PDMVs in plasma and risk of future incident VTE.

Methods

We performed a population-based nested case-control study with 314 VTE cases and 705 age- and sex-matched controls (from the Tromsø study) to investigate the association between the proportion of PDMVs (PDMVs%) in plasma and risk of future incident VTE. MVs isolated from plasma sampled at baseline (i.e. before VTE) were stained for platelet markers and analyzed by flow cytometry. PDMVs% were defined as the number of PDMVs divided by the total number of MVs. Odds ratios (ORs) with 95% confidence intervals (CI) for VTE risk were estimated across quartiles of PDMVs%.

Results

Subjects with PDMVs% in the highest quartile had an OR for VTE of 1.78 (95% CI: 1.21-2.64) and 1.99 (95% CI: 1.24-3.26) for provoked VTE, compared to those in the lowest quartile. The association was moderately affected by multivariable adjustment for age, sex, BMI, CRP, platelet count and cancer. The OR for VTE was higher when the time between blood sampling and event was shorter.

Conclusions

Our results show that high proportions of PDMVs are associated with future risk of incident VTE and imply a role of platelet activation in the pathogenesis of VTE.

58

59   **Introduction**

60   Venous thromboembolism (VTE) is a collective term for deep vein thrombosis (DVT) and

61   pulmonary embolism (PE). VTE is a frequent and complex disease with serious short- and

62   long-term complications, including recurrence, post-thrombotic syndrome, PE syndrome, and

63   death [1-4]. As the incidence of VTE is steadily increasing [5, 6] and the pathophysiological

64   mechanisms are not fully understood, discovery of molecular mechanisms and predictive

65   biomarkers of future VTE is needed to improve risk stratification and provide targeted

66   prevention.

67   Microvesicles (MVs) are extracellular vesicles of a defined size range (100-1000 nm in

68   diameter) that are budded off from the cytoplasmic membrane of activated cells and can be

69   traced to their cellular origin by the expression of cell-specific markers [7, 8]. MVs are highly

70   procoagulant due to the expression of negatively charged phospholipids (e.g.

71   phosphatidylserine (PS)), which facilitate the assembly of protease complexes of the clotting

72   cascade. Under certain pathological conditions some MVs also express tissue factor, the main

73   initiator of the coagulation system [7, 8]. The major proportion of MVs in circulating blood is

74   derived from platelets [9-11], and the procoagulant activity of plasma MVs is mediated mainly

75   by platelet-derived MVs (PDMVs) [12-14]. Ramacciotti et al. reported that plasma levels of

76   platelet-derived, but not leukocyte-derived, MVs were associated with thrombus weight in an

77   inferior vena cava (IVC) ligation model of venous thrombosis in mice, further supporting a

78   differential procoagulant effect by MVs of different cellular origin [15].

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79   Several [16, 17], but not all [18], studies have shown elevated plasma levels of MVs,

80   and of PDMVs in particular, in the acute phase of VTE and in traditional case-control studies

81   where plasma samples were collected at least 3 months after the VTE event [19, 20]. Of note,

82   these studies are susceptible to bias due to reverse causation, as augmented release of MVs

83   occur under pathological conditions [21]. Thus, regular case-control studies are not designed

84   to distinguish whether increased plasma levels of MVs are a consequence or an actual risk

85   factor for VTE. To determine whether plasma PDMV level is a risk factor for VTE, it is a

86   prerequisite that the exposure (plasma PDMV level) is measured before the outcome (VTE) to

87   avoid bias by reverse causation. In a nested case-control study, cases and controls are derived

88   from a cohort in which exposure parameters and blood is sampled before the outcome of

89   interest occurs.

90   No previous study has, to the best of our knowledge, explored the association between

91   plasma PDMV levels and future risk of VTE. Therefore, we set out to investigate whether

92   plasma PDMV levels were associated with future risk of VTE in a nested case-control study

93   with individuals recruited from a population-based cohort. We hypothesized that individuals

94   with higher plasma levels of PMDVs, determined as the absolute number of lactadherin-

95   positive MVs expressing platelets markers, or as the proportion of such MVs of the total

96   number of MVs would be at higher risk of VTE.

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98

99   **Methods**

100   *Study population*

101   Plasma samples from VTE cases and controls were retrieved from the fourth survey of The

102   Tromsø Study, a single-center, population-based cohort, with repeated health surveys of

103   inhabitants of Tromsø, Norway [22]. All inhabitants aged ≥25 years living in the municipality of

104   Tromsø were invited to participate in the fourth survey, conducted in 1994-1995. A total of

105   27,158 individuals participated, corresponding to 77% of those who were invited. Participants

106   were followed from the inclusion date until the date of an incident VTE, migration, death, or

107   end of follow-up (September 1, 2007) [23]. All first lifetime events of VTE occurring among the

108   participants were identified using the hospital discharge diagnosis registry, the autopsy

109   registry, and the radiology procedure registry from University Hospital of North Norway (UNN),

110   which is the sole provider of diagnostic radiology and treatment of VTE in the Tromsø area.

111   Participants with a history of VTE before baseline were excluded. Trained personnel performed

112   extensive review of medical records to adjudicate each VTE event. An episode of VTE was

113   confirmed if there were signs and symptoms consistent with DVT or PE in combination with

114   objective confirmation by radiological methods, leading to initiation of treatment, as previously

115   described [23]. A VTE event was classified as provoked when occurring in the presence of one

116   or more of the following provoking factors: active cancer, trauma or surgery (within 8 weeks

117   prior to the event), acute medical conditions (acute ischemic stroke, acute myocardial infarction

118   (MI) or acute infection), immobilization (bed rest for longer than 3 days or confinement to a

119   wheelchair within the past 8 weeks, or long distance travel of 4 hours or longer within the past

120   14 days), or other factors specifically described as being provoked by a physician in the

121   medical record (e.g. intravascular catheter).

122   Four hundred and sixty-two individuals experienced a VTE event during the follow-up

123   period (1994-2007). For each case, two age- and sex-matched controls, who were alive at the

124   index date of the corresponding VTE patient, were randomly sampled from the source cohort

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125 (n=924). Sufficient amount of plasma samples was available from 314 cases and 705 controls

126 (Figure 1), and these were included in our study. The missing plasma samples were likely at

127 random. To check this, we compared the baseline characteristics of the total and included

128 population. The regional committee for medical and health research ethics approved the study,

129 and all participants provided written consent.

130

131 *Blood collection and storage of blood products*

132 At inclusion in Tromsø 4 (1994-1995), non-fasting blood was collected from an antecubital vein

133 into 5-mL vacutainers (Becton Dickinson, Le Pont de Claix, France) containing EDTA (K3-

134 EDTA 40 μL, 0.37 mol/L per tube) as anticoagulant. Platelet poor plasma (PPP) was prepared

135 by centrifugation at 3000 g for 10 min at room temperature. PPP was then transferred to

136 cryovials (Greiner Labortechnik, Nürtingen, Germany) in 1-mL aliquots and stored at −80°C.

137 Plasma samples were thawed and centrifuged for 2 min at 13,500 g to obtain platelet-free

138 plasma (PFP).

139

140 *Baseline measurements*

141 Measurement of platelet count was performed within 12 h following blood collection on an

142 automated blood cell counter (Coulter Counter®, Coulter Electronics, Luton, UK), as previously

143 described [24]. Height (to the nearest centimeter) and weight (to the nearest 0.5 kg) were

144 measured in participants wearing light clothing and no shoes. Body mass index (BMI) was

145 calculated as weight divided by the square of height in meters (kg/m2). A self-administered

146 questionnaire was used to collect a detailed history of cardiovascular disease (CVD) events

147 (stroke, angina pectoris and MI) and cancer.

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149 *Measurement of C-reactive protein*

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150 Commercially available reagents by enzyme-immunoassay (R&D Systems, Minneapolis, MN,

151 USA) in a 384 format were used with the combination of a SELMA (Jena, Germany) pipetting

152 robot and a BioTek (Winooki, VT, USA) dispenser/washer (EL406) to measure high-sensitivity

153 C-reactive protein (CRP). Absorption was read at 450 nm using an EIA plate reader (Synergy

154 H1 Hybrid, BioTek, Vinooski, VT, USA) with a wavelength correction set to 540 nm. The intra-

155 and inter-assay coefficients of variation were 2.6% and 9.1%, respectively.

156

157 *Analysis of PDMVs in plasma*

158 Hundred microliter of PFP was diluted 20x in pre-filtered (100 kDa Amicon Ultra Filters)

159 Dulbecco's phosphate-buffered saline (DPBS) which was free of Ca^{2+}/Mg^{2+} (Thermo Fisher

160 Scientific). Samples were centrifuged at 20,000 g for 30 min at 4°C. Supernatants were

161 carefully aspirated and MV pellets were resuspended in DPBS and stained with (i) PS and

162 platelet markers (i.e. CD41a and CD62P), or (ii) PS and the respective isotype controls. All

163 samples were stained with FITC-labeled bovine lactadherin that is highly specific for PS

164 (Haematologic Technologies, Vermont, USA) and anti-CD41a-phycoerythrin clone HIP8

165 (Biolegend, San Diego, USA). A large portion of samples, 238 VTE cases and 543 controls,

166 were also stained with anti-CD62P-BV785 clone AKT (both from Biolegend, San Diego, USA)

167 for detection of MVs that were derived from activated platelets (hereafter aPDMVs). Matched

168 phycoerythrin- and BV785-labeled isotype controls (Biolegend) were used at the same

169 concentrations to detect non-specific staining. All antibodies and isotype controls were filtered

170 using 0.22 μm Ultrafree®-MC centrifugal filter (Merck, Millipore, Carrigtwohill, Ireland) before

171 use. MV pellets were re-suspended in a cocktail of antibodies or their respective isotype

172 controls for 20 min at 4°C in the dark. Samples were washed with 1 mL pre-filtered DPBS and

173 centrifuged at 20,000 g for 30 min at 4°C. Pellets were re-suspended in 200 μL pre-filtered

174 DPBS and samples were analyzed using CytoFLEX (Beckman Coulter, Indianapolis, USA) at

175   the lowest possible rate, 10 µL/min. Data analysis was performed using CytExpert 2.0

176   (Beckman Coulter, Indianapolis, USA).

177   Staining of MVs with CD9 PerCP-Cy5.5 (clone HI9a, Biolegend) was used to confirm

178   the expression of a MV-marker using flow cytometer. Specific detection of CD9 and CD41a by

179   flow cytometry is demonstrated in Supplementary Figure 1.

180

181   *Transmission electron microscopy*

182   Transmission electron microscopy (TEM) was used to confirm the presence of MVs in samples.

183   MVs were isolated from plasma as described above and re-suspended in DPBS. Five

184   microliters were applied on a clean surface glass covered with Parafilm and absorbed on

185   Formvar coated 75 mesh copper grids (Electron Microscopy Science, USA) for 5 min. Grids

186   were washed four times using double distilled water. Negative staining was performed using

187   3% uranyl acetate and 2% methylcellulose 1/10 vol/vol for 2 min on ice. Excess of stain was

188   removed and samples were dried at room temperature and analyzed on Hitachi HT7800

189   transmission electron microscope. Representative images of plasma MVs are shown in

190   Supplementary Figure 2.

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192   *Statistical analysis*

193   Statistical analyses were carried out using R version 4.0.2. The PDMVs levels were normalized

194   to have mean of zero and a standard deviation of one. The proportions of PDMVs in plasma

195   (i.e. PDMV%) were defined as the number of PDMVs divided by the total number of MVs in

196   plasma times 100, and categorized according to quartile cutoffs in the control population

197   (≤10.7, >10.7-17.7, >17.7-28.1, >28.1%). Similarly, the proportions of aPDMVs in plasma (i.e.

198   aPDMV%) were defined as the number of aPDMVs divided by the total number of MVs in

199   plasma times 100, and categorized according to quartile cutoffs in the control population (≤4.5,

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200 >4.5-9.3, >9.3-16.5, >16.5%). Means and proportions of baseline characteristics across

201 categories of PDMV% or aPDMV% were calculated using descriptive statistics. Logistic

202 regression models were used to estimate odds ratios (OR) for VTE with 95% confidence

203 intervals (CI) according to quartiles of PDMV% or aPDMV% adjusted for (i) age and sex; (ii)

204 age, sex and body mass index (BMI); (iii) age, sex, BMI and CRP; and in some cases also for

205 (iv) age, sex, BMI, CRP and platelet count. The lowest quartile of PDMV% or aPDMV% were

206 used as the reference group in the respective analysis. Subgroup analyses were performed

207 with unprovoked and provoked VTE as outcomes.

208 Because the follow-up time in the source cohort was long (≥12 years for several

209 individuals), estimates based on baseline measurement of PDMV% could be influenced by

210 regression dilution bias [25]. To investigate this, we performed analyses in which we restricted

211 the maximum time from blood sampling to the VTE events, while keeping all controls in the

212 analyses. The logistic regression analyses on time restrictions were set to require at least 10

213 VTE events, and each time a VTE occurred during follow-up, ORs for Q2-Q4 vs. Q1 were

214 estimated and plotted as a function of follow-up time.

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Results

The distribution of baseline characteristics according to quartiles of PDMV% are summarized in Table 1. The mean age and the presence of CVD were equally distributed across quartiles, whereas mean platelet count, mean total number of MVs per microliter (μL) plasma, mean body mass index (BMI), as well as the proportion of women and the proportion of individuals with a history of cancer increased across quartiles of PDMV%.

The baseline characteristics for all VTE cases and controls who were originally collected (n=1386) and those with available plasma samples who were included in this study (n=1019) are shown in Supplementary Table 1. All characteristics showed an essentially similar distribution in the two populations, except for the prevalence of cancer which was slightly higher in the included population (6.1% vs. 3.5%).

The characteristics of VTE patients at diagnosis are shown in Table 2. The mean age at the time of VTE was 67.4 years, and 61.5% of the events were classified as provoked. Moreover, 41.1% of the events were PE with or without concurrent symptomatic DVT.

Association between platelet-derived microvesicles and future risk of VTE

Odds ratios (ORs) for risk of future VTE, as well as DVT and PE across quartiles of PDMV% are shown in Table 3. The OR for VTE increased in quartiles of higher PDMV% (quartiles 2-4). Participants with PDMV% in the highest (fourth) quartile had an OR of VTE of 1.78 (95% CI: 1.21-2.64). The OR was unaffected by adjustment for age, sex, BMI and CRP, but additional adjustment for platelet count slightly attenuated the estimate (OR: 1.62; 95% CI: 1.08-2.44). Similar ORs were found for DVT (OR: 1.85, 95% CI: 1.15-3.04) and PE (OR: 1.68, 95% CI: 0.98-2.92) when subjects with PDMV% in the highest quartile were compared to those with PDMV% in the lowest quartile. Overall, a stronger association was observed for provoked than for unprovoked VTE (Supplementary Table 2).

243   To consider the possibility of underestimating ORs because of regression dilution bias,

244   we estimated ORs for VTE among subjects with lowest (lowest tertile) versus three highest

245   quartile of plasma levels of PDMV% as a function of time between blood sampling and VTE

246   events (Figure 2). The OR for VTE by high (quartiles 2-4) versus low PDMV% was substantially

247   higher with shortened time between blood sampling and event (Figure 2, left panel).

248   In 781 individuals (238 VTE cases and 543 controls) from the nested-control study,

249   isolated MVs from plasma were also analyzed for surface expression of P-selectin (CD62P), a

250   marker of platelet activation. MVs that were positive for lactadherin, CD41a and CD62P were

251   considered to be derived from activated platelets (aPDMVs). Importantly, very few MVs were

252   found to be positive for CD62P only. The characteristics at baseline and at VTE diagnosis of

253   subjects with measured aPDMV% are shown in Supplementary Tables 3 and 4, respectively.

254   A high correlation was found between PDMV% and aPDMV% (Pearson correlation coefficient,

255   r=0.92). ORs for the risk of future development of VTE, as well as DVT and PE, across quartiles

256   of aPDMV% are shown in Supplementary Table 5. Apparently, there was a threshold effect for

257   the risk of VTE between quartiles 1 (lowest aPDMV%) and quartile 2, without further increase

258   in OR from quartile 2 to 4 (Supplementary Table 5).

259   ORs for the risk of future VTE, DVT, and PE across quartiles of the absolute number

260   (number/μL) of PDMVs are shown in Table 4. There was an apparent threshold effect, where

261   the main increase in ORs for VTE occurred when individuals with absolute numbers of PDMVs

262   in quartile 2 were compared to those with PDMVs in quartile 1. There were no further increase

263   in OR from quartile 2 to quartile 4. Participants with an absolute number of PDMVs in the

264   highest (fourth) quartile had an OR of VTE of 1.41 (95% CI: 0.96-2.08) compared to those with

265   PDMVs in the lowest quartile. The OR was unaffected after adjustments in the multivariable

266   model. The OR for VTE by high (quartiles 2-4) versus low absolute number of PDMVs was

267   substantially higher with shortened time between blood sampling and event (Figure 2, right

268   panel).

Discussion

We found that elevated levels of PDMVs in plasma, either determined as the proportion of total lactadherin-positive MVs or absolute numbers, were associated with future risk of VTE, and DVT in particular, in a nested case-control study derived from the general population. In addition, the ORs for VTE by plasma PDMVs increased substantially with shortened time between blood sampling and the VTE events. Our findings suggest that elevated plasma levels of PDMVs are not only a consequence of an acute VTE event, but rather that increased platelet reactivity and subsequent release of PDMVs are associated with future risk of VTE.

Several case-control studies have reported elevated plasma MV levels, and PDMVs in particular, in patients with VTE compared to controls [16, 17,19, 20]. However, traditional retrospective case-control studies are not designed to determine whether elevated plasma levels of a biomarker is a consequence of the disease (reverse causation) rather than an actual risk factor. In prospective studies, however, plasma levels of biomarkers are usually measured at the time of inclusion and related to an outcome that occur several years later. As plasma levels of modifiable biomarkers are expected to change over time, fluctuations during follow-up will lead to an underestimation of the true association between exposure (e.g. plasma PDMV levels) and outcome (e.g. VTE events), a phenomenon called regression dilution bias [25]. Accordingly, we found that plasma PDMV levels were associated with future risk of VTE, independent of potential confounders, and that the risk of VTE by plasma PDMVs declined substantially with time between blood sampling and the VTE event. Therefore, our findings suggest that plasma PDMVs is a risk marker of VTE. As our findings are unchallenged, replications in other prospective cohorts are warranted.

PDMVs may play a role in the pathogenesis of VTE. Plasma MV levels, and PDMV levels in particular, are strongly related to procoagulant phospholipid levels and thrombin generation in plasma [26, 27]. Negatively charged phospholipids, and phosphatidylserine (PS) in particular, are located on the surface of activated platelets and MVs, and are vital to

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295   coagulation by facilitating the assembly of coagulation factors in blood [28], augmenting the

296   activity of the extrinsic tenase complex, TF-FVIIa, by several orders of magnitude [29].

297   Importantly, PDMVs have a 50- to 100-fold higher procoagulant activity compared with the

298   normal platelet surface [30]. Furthermore, parameters of TF-induced thrombin generation in

299   plasma, such as the endogenous thrombin potential (ETP) and thrombin are associated with

300   incident [31-34] and recurrent [35-37] VTE. Thus, it is likely that elevated plasma levels of

301   PDMVs may facilitate coagulation activation and promote thrombus formation under

302   pathological conditions.

303   Growing evidence supports the concept that platelet reactivity plays an important role

304   in the pathogenesis of VTE. First, experimental animal studies have shown that platelets are

305   involved in the formation of venous thrombi [38, 39]. Second, in a large cohort including

306   subjects recruited from the general population, Brækkan et. al. [24] demonstrated that subjects

307   with high mean platelet volume, a phenotype associated with increased platelet reactivity [40-

308   43], had increased risk for VTE. Third, subjects with high levels (highest quartile) of von

309   Willebrand factor in plasma, a factor instrumental for adhesion of platelets to the vascular wall,

310   are at elevated risk of future VTE [44, 45]. Fourth, carriers of the G allele(s) at GP6, a single

311   nucleotide polymorphism known to attenuate platelet function [46-49], has lowered risk of

312   incident VTE [50]. Fifth, randomized clinical trials have shown that treatment with the platelet

313   inhibitor aspirin is associated with 20-30% reduced risk of recurrent VTE [51, 52]. Our findings

314   that increased plasma levels of PDMVs, a marker of platelet activation [53], were associated

315   with future VTE risk, provide further evidence that platelet function is involved in the

316   mechanism of venous thrombus formation.

317   The association between PDMVs% and VTE was apparently stronger for provoked

318   than for unprovoked events. This could be due to an interaction between PDMVs and

319   provoking factors (e.g. surgery, trauma or acute medical conditions), leading to excess VTE

320   risk in these high-risk situations. Alternatively, the association could be confounded by

321 underlying conditions leading to both higher PDMVs and provoking factors. The latter is

322 considered less likely since the plasma samples to assess PDMVs were taken several years

323 before the provoking factors and the VTE events occurred.

324 The strength of our observational study includes the nested case-control design, in

325 which the VTE cases and age- and sex-matched controls were selected from the same

326 population-based source cohort. The prospective design of the study, with measurement of

327 the exposure prior to the outcome of interest, allowed for speculations concerning risk factors

328 and potential causal relationships. The analysis of PDMVs was performed using a sensitive

329 flow cytometer (CytoFlex). Our samples were analyzed using a comprehensive staining

330 protocol, which included: (i) Daily quality and alignment checkup of the flow cytometer, (ii)

331 careful gating strategy according to beads size and (iii) cellular markers, and (iv) a panel of

332 isotype control antibodies for each sample to detect and subtract non-specific staining.

333 Some limitations of the study also need to be mentioned. The non-fasting PPP used for

334 measurement of PDMVs were collected in 1994-95 and stored at -80°C until thawed, and

335 subjected to a second centrifugation step (13,500 g for 2 min) to remove remaining platelets

336 and cell debris (platelet free plasma, PFP) before isolation of MVs from PFP by

337 ultracentrifugation. Even though international guidelines recommend isolation of MVs from

338 PFP spun twice at 2,500xg for 15 min [54], we recently reported similar plasma concentrations

339 and size distribution of MVs isolated from plasma prepared as PPP with a subsequent

340 centrifugation step after thawing when compared to plasma spun twice before freezing and

341 storage [55]. In addition, the long storage time could potentially affect the plasma levels of

342 plasma MVs levels, but unlikely change the results since the storage effect would be similar in

343 VTE cases and controls. However, concerns related to these pre-analytical conditions may

344 lead to some degree of non-differential misclassification of plasma MV levels [56, 57], and

345 cause an underestimation of the true association between plasma PDMVs and VTE risk. This

346 phenomenon may also explain why the proportion, rather than the absolute number, of PDMVs

347 displayed the strongest association to the VTE risk as the pre-analytical conditions is more

348 likely to affect the absolute number of MVs than a selected population of MVs in plasma.

349   In conclusion, plasma levels of PDMVs were associated with future risk of VTE. Our

350 findings suggest that a high proportion of PDMVs in plasma is a risk marker of future VTE and

351 that platelet activation with subsequent PDMV formation is involved in the pathogenesis of

352 VTE. Future studies should be performed to replicate our findings.

353

354 **Author contributions:** O. Snir designed and participated in the laboratory analysis, analyzed

355 the data, drafted and revised the manuscript. L. Wilsgård and N. Latysheva performed the

356 laboratory analysis and revised the manuscript. C.J.E. Wahlund participated in the study

357 design and data analysis and revised the manuscript. K. Hindberg and S.K. Brækkan analyzed

358 data and participated in the revision of the manuscript. J-B. Hansen designed the study and

359 participated in the writing and revision of the manuscript. All the authors read and approved

360 the final manuscript.

363 **Disclosures:** The authors have no conflict of interest.

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561 **Table 1.** Characteristics of participants at study inclusion across quartiles of platelet-derived
562 MVs in percent of total number of MVs in plasma (PDMV%). Values are means ± 1 standard
563 deviation (SD) or percentages with numbers in brackets.

	Q1	Q2	Q3	Q4
PDMV%	≤10.7	>10.7-17.7	>17.7-28.1	>28.1
Number of individuals	233	256	256	274
Age, years	59.2±14.1	59.4±13.5	61.4±13.3	60.2±14.4
Sex, % men (n)	51.9 (121)	49.2 (126)	44.5 (114)	40.9 (112)
BMI, kg/m^2	25.8±3.8	26.3±3.7	26.7±4.5	27.0±4.8
hsCRP, mg/L	1.49±1.38	1.46±1.10	1.44±1.07	1.72±1.43
Platelet count, 10^9/L	229.8±50.1	235.8±47.6	244.0±52.1	265.3±55.6
Average MV/µL plasma	193.3±258.8	185.9±142.0	208.5±157.0	312.6±237.6
CVD[a]	13.3 (31)	16.8 (43)	16.8 (43)	14.6 (40)
Cancer[b]	2.6 (5)	4.0 (8)	8.5 (17)	8.9 (19)

564

565 [a] Self-reported history of cardiovascular disease (myocardial infarction, angina, stroke).

566 [b] History of cancer before baseline.

567

568

569 **Table 2.** Characteristics of VTE events at diagnosis (n=314). Values are means ± 1 standard
570 deviation (SD) or percentages with numbers in brackets.

571

Characteristic	
Age at VTE (years), mean ± SD	67.4±14.0
Sex (males), % (n)	47.1 (148)
Deep vein thrombosis, % (n)	58.9 (185)
Pulmonary embolism, % (n)	41.1 (129)
Unprovoked VTE, % (n)	38.5 (121)
Provoked VTE, % (n)	61.5 (193)
Active cancer, % (n)	27.3 (86)

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573

Table 3. Odds ratios (OR) with 95% confidence intervals (CI) for venous thromboembolism (VTE), deep vein thrombosis (DVT) and pulmonary embolism (PE) according to quartiles of platelet-derived MVs in percent of total number of MVs in plasma (PDMV%).

Category of PDMV%	Cases (n)	Controls (n)	Model 1 OR (95%) CI	Model 2 OR (95%) CI	Model 3 OR (95%) CI	Model 4 OR (95%) CI	Model 5 OR (95%) CI
VTE							
Q1 (0.0-10.7%)	56	177	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
Q2 (10.7-17.7%)	80	176	1.44 (0.97-2.16)	1.44 (0.97-2.17)	1.44 (0.97-2.17)	1.41 (0.94-2.12)	1.43 (0.91-2.26)
Q3 (17.7-28.1%)	80	176	1.45 (0.97-2.18)	1.43 (0.96-2.16)	1.44 (0.96-2.17)	1.25 (0.82-1.89)	1.35 (0.85-2.15)
Q4 (28.1-85.2%)	98	176	1.78 (1.21-2.64)	1.72 (1.16-2.57)	1.72 (1.16-2.57)	1.62 (1.08-2.44)	1.68 (1.07-2.67)
Per 1 SD	314	705	1.18 (1.04-1.34)	1.16 (1.02-1.33)	1.16 (1.02-1.33)	1.14 (1.00-1.31)	1.14 (0.98-1.33)
DVT							
Q1 (0.0-10.7%)	31	177	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
Q2 (10.7-17.7%)	51	176	1.66 (1.02-2.74)	1.69 (1.03-2.81)	1.69 (1.03-2.81)	1.67 (1.02-2.77)	1.79 (1.03-3.17)
Q3 (17.7-28.1%)	46	176	1.52 (0.92-2.53)	1.54 (0.93-2.58)	1.55 (0.93-2.59)	1.28 (0.76-2.18)	1.36 (0.75-2.47)
Q4 (28.1-85.2%)	57	176	1.85 (1.15-3.04)	1.85 (1.14-3.06)	1.86 (1.14-3.07)	1.82 (1.10-3.05)	1.82 (1.03-3.26)
Per 1 SD	185	705	1.19 (1.01-1.38)	1.18 (1.01-1.38)	1.18 (1.01-1.38)	1.18 (1.00-1.39)	1.17 (0.97-1.40)
PE							
Q1 (0.0-10.7%)	25	177	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
Q2 (10.7-17.7%)	29	176	1.18 (0.66-2.11)	1.14 (0.64-2.04)	1.14 (0.64-2.04)	1.10 (0.61-1.97)	1.01 (0.52-1.96)
Q3 (17.7-28.1%)	34	176	1.39 (0.79-2.44)	1.30 (0.74-2.30)	1.31 (0.74-2.32)	1.19 (0.67-2.13)	1.31 (0.70-2.48)
Q4 (28.1-85.2%)	41	176	1.68 (0.98-2.92)	1.52 (0.88-2.65)	1.51 (0.87-2.64)	1.34 (0.76-2.38)	1.52 (0.82-2.87)
Per 1 SD	129	705	1.17 (0.97-1.39)	1.12 (0.93-1.34)	1.12 (0.93-1.34)	1.08 (0.89-1.30)	1.09 (0.88-1.34)

Model 1: adjusted for age and sex; model 2: adjusted for age, sex and body mass index; model 3: adjusted for age, sex and body mass index and CRP; model 4: adjusted for age, sex and body mass index, CRP and platelet count; model 5: adjusted for age, sex and body mass index, CRP, platelet count and cancer

Table 4. Odds ratios (OR) with 95% confidence intervals (CI) for venous thromboembolism (VTE), deep vein thrombosis (DVT) and pulmonary embolism (PE) according to quartiles of absolute number of platelet-derived MVs (PDMVs) per microliter (µL) plasma.

Category of PDMV	Cases (n)	Controls (n)	Model 1 OR (95%) CI	Model 2 OR (95%) CI	Model 3 OR (95%) CI
VTE					
Q1 (0.40-15.32)	62	177	1 (ref)	1 (ref)	1 (ref)
Q2 (15.32-37.13)	86	176	1.39 (0.95-2.06)	1.37 (0.92-2.03)	1.37 (0.93-2.03)
Q3 (37.13-76.94)	79	176	1.28 (0.87-1.90)	1.23 (0.83-1.83)	1.24 (0.83-1.84)
Q4 (76.94-734.75)	87	176	1.41 (0.96-2.08)	1.39 (0.94-2.06)	1.39 (0.94-2.07)
Per SD	314	705	1.12 (0.98-1.29)	1.12 (0.97-1.29)	1.12 (0.97-1.29)
DVT					
Q1 (0.40-15.32)	41	177	1 (ref)	1.00 (ref)	1 (ref)
Q2 (15.32-37.13)	50	176	1.23 (0.77-1.95)	1.23 (0.77-1.97)	1.23 (0.77-1.97)
Q3 (37.13-76.94)	41	176	1.01 (0.62-1.63)	0.99 (0.61-1.62)	1.00 (0.61-1.62)
Q4 (76.94-734.75)	53	176	1.30 (0.82-2.06)	1.31 (0.82-2.09)	1.32 (0.83-2.11)
Per SD	185	705	1.09 (0.93-1.29)	1.10 (0.93-1.31)	1.11 (0.94-1.31)
PE					
Q1 (0.40-15.32)	21	177	1 (ref)	1 (ref)	1 (ref)
Q2 (15.32-37.13)	36	176	1.72 (0.98-3.11)	1.67 (0.94-3.03)	1.66 (0.93-3.02)
Q3 (37.13-76.94)	38	176	1.82 (1.04-3.27)	1.64 (0.92-2.97)	1.65 (0.93-2.98)
Q4 (76.94-734.75)	34	176	1.63 (0.92-2.95)	1.52 (0.85-2.77)	1.51 (0.84-2.76)
Per SD	129	705	1.16 (0.96-1.42)	1.13 (0.92-1.38)	1.12 (0.92-1.38)

Model 1: adjusted for age and sex; model 2: adjusted for age, sex and body mass index; model 3: adjusted for age, sex and body mass index and CRP
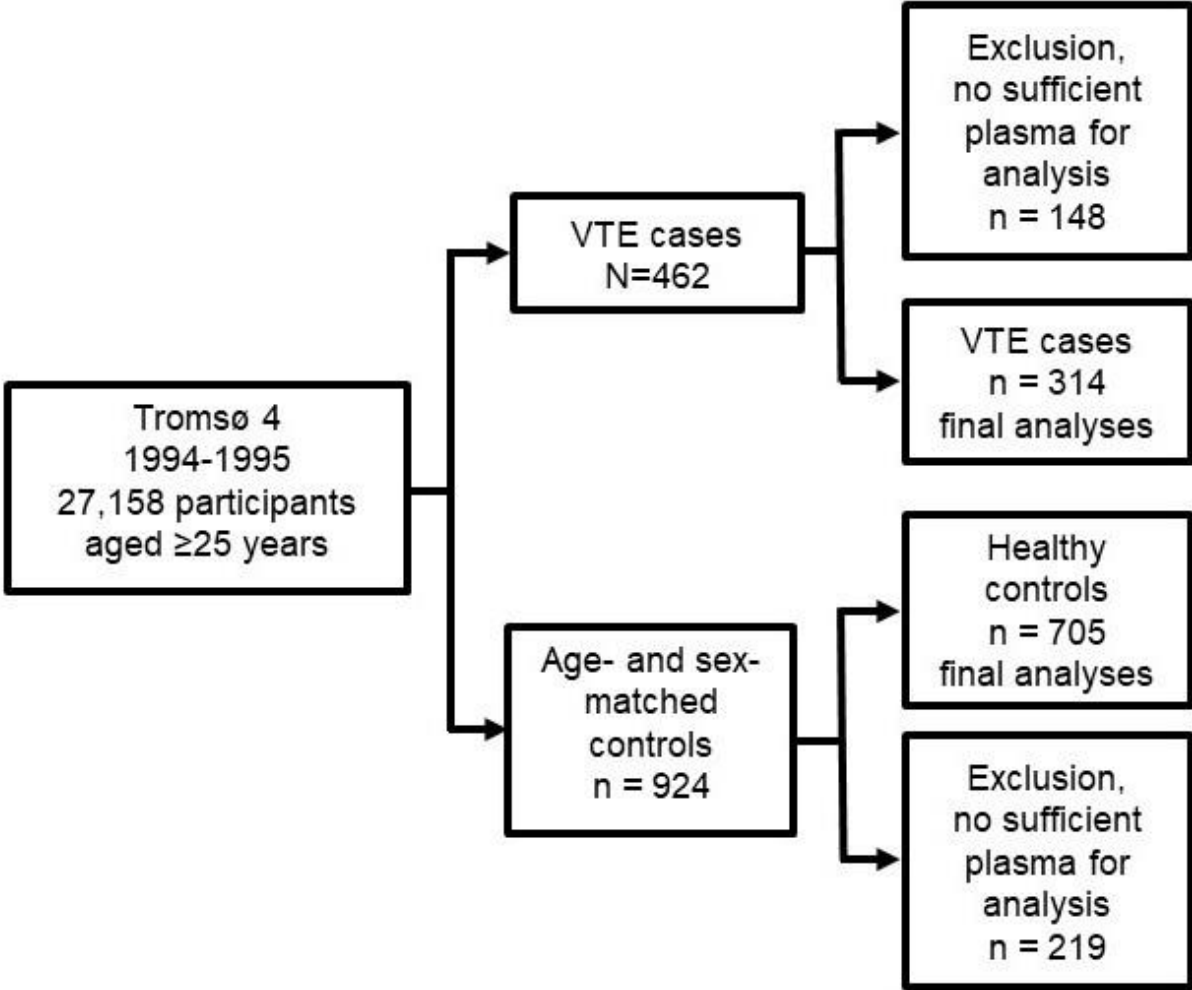
Figure 1

Figure 1. Flow chart of study participants. A flowchart illustrating the fourth survey of Tromsø Study (1994-1995), and the current nested case-control study of platelet-derived microvesicles in plasma of VTE patients and age- and sex-matched healthy controls.
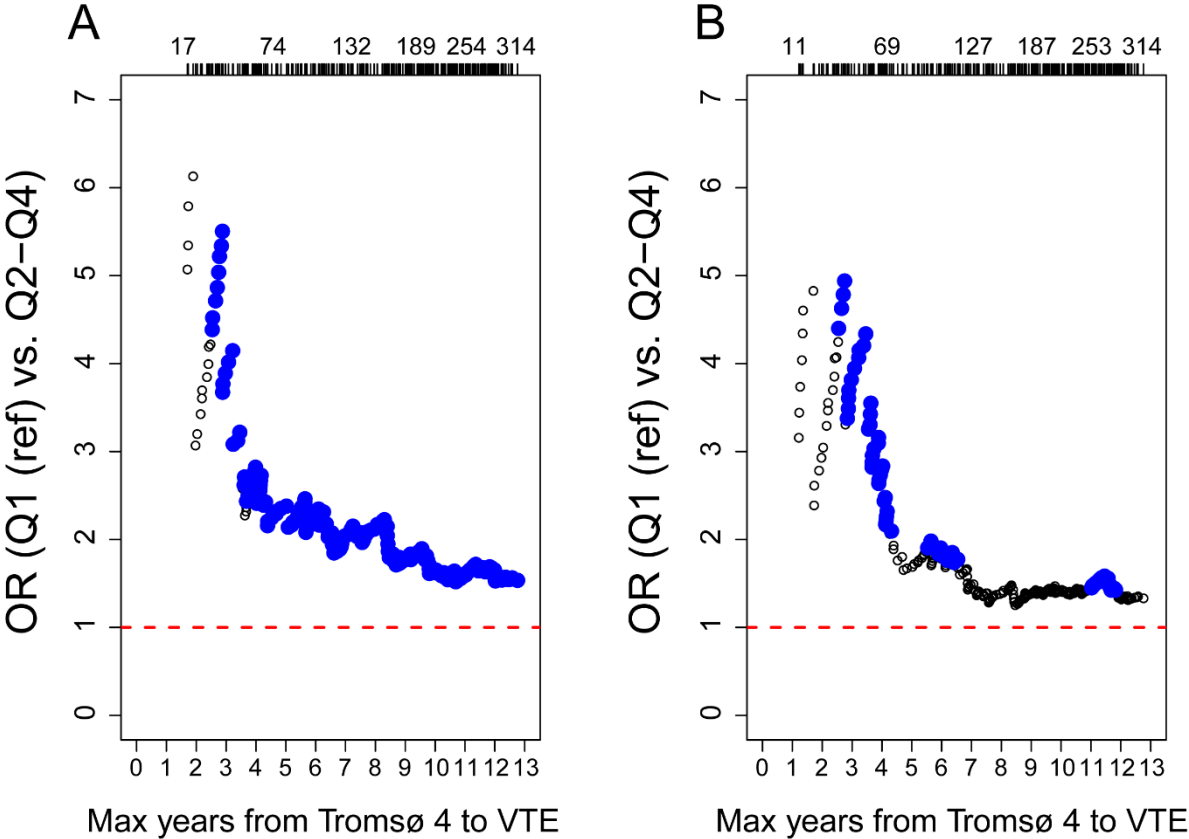
Figure 2

Figure 2. Plots of estimated odds ratios (ORs) for the association of PDMVs with VTE as a function of time from blood sampling in Tromsø 4 (1994-1995) to VTE events. Plots of estimated ORs for the association of the (**A**) proportion of platelet-derived MVs (PDMV%) or the (**B**) absolute number of PDMVs per microliter (µL) plasma and risk of future venous thromboembolism (VTE), as a function of time from blood sampling in the fourth survey of Tromsø 4 (1994–1995). Large, solid blue circles indicate ORs with P values <0.05.

Supplementary material

Supplementary Table 1. Baseline characteristics of all VTE cases and controls who were originally collected (n=1386) and those with available plasma samples analyzed in this study (n=1019)

	All individuals, n=1386	**Individual analyzed for PDMVs, n=1019**
Age, years	60.2	60.1
Women, %	52.8	53.6
Men, %	47.2	46.4
BMI, kg/m^2	26.4	26.5
CVD, %	15.8	15.4
Cancer, %	3.5	6.1
WBC, %	7.0	7.0
Platelet count, 10^9/L	245	245
Hematocrit, %	41.6	41.6

Supplementary Table 2. Odds ratios (OR) with 95% confidence intervals (CI) for provoked and unprovoked venous thromboembolism (VTE) according to quartiles of platelet-derived MVs in percent of total number of MVs in plasma (PDMV%).

Category of PDMV%	Cases (n)	Controls (n)	Model 1 OR (95%) CI	Model 2 OR (95%) CI	Model 3 OR (95%) CI	Model 4 OR (95%) CI	Model 5 OR (95%) CI
VTE provoked							
Q1 (0.0-10.7%)	31	177	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
Q2 (10.7-17.7%)	51	176	1.65 (1.02-2.73)	1.68 (1.02-2.78)	1.67 (1.02-2.78)	1.63 (1.00-2.71)	1.71 (0.98-3.03)
Q3 (17.7-28.1%)	50	176	1.62 (0.99-2.68)	1.63 (0.99-2.72)	1.64 (1.00-2.73)	1.42 (0.85-2.39)	1.56 (0.88-2.79)
Q4 (28.1-85.2%)	61	176	1.99 (1.24-3.26)	1.97 (1.22-3.25)	1.98 (1.22-3.26)	1.88 (1.14-3.13)	2.03 (1.17-3.58)
Per 1 SD	193	705	1.21 (1.04-1.40)	1.20 (1.03-1.40)	1.20 (1.03-1.40)	1.19 (1.01-1.39)	1.20 (1.01-1.44)
VTE unprovoked							
Q1 (0.0-10.7%)	25	177	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
Q2 (10.7-17.7%)	29	176	1.17 (0.66-2.09)	1.14 (0.64-2.04)	1.14 (0.64-2.05)	1.12 (0.63-2.01)	1.09 (0.57-2.09)
Q3 (17.7-28.1%)	30	176	1.24 (0.70-2.22)	1.18 (0.66-2.12)	1.19 (0.67-2.14)	1.03 (0.57-1.88)	1.11 (0.58-2.15)
Q4 (28.1-85.2%)	37	176	1.51 (0.88-2.65)	1.38 (0.79-2.44)	1.38 (0.79-2.44)	1.27 (0.71-2.29)	1.27 (0.66-2.44)
Per 1 SD	121	705	1.13 (0.94-1.36)	1.10 (0.91-1.32)	1.10 (0.90-1.32)	1.07 (0.87-1.30)	1.02 (0.81-1.28)

Model 1: adjusted for age and sex; model 2: adjusted for age, sex and body mass index; model 3: adjusted for age, sex and body mass index and CRP; model 4: adjusted for age, sex and body mass index, CRP and platelet count; model 5: adjusted for age, sex and body mass index, CRP, platelet count and cancer

Supplementary Table 3. Characteristics of participants at study inclusion across quartiles of plasma levels of MVs derived from activated platelets (aPDMVs%). Values are means ± 1 standard deviation (SD) or percentages with numbers in brackets.

	Q1	Q2	Q3	Q4
aPDMV%	≤4.5	>4.5-9.3	>9. 3-16.5	>16.5
Number of individuals	n=180 (44)	n=206 (70)	n=197 (62)	n=198 (62)
Age, years	58.1±14.8	58.4±15.2	58.0±14.9	60.3±15.5
Sex, % men	52.8 (95)	44.7 (92)	49.2 (97)	35.4 (70)
BMI, kg/m^2	26.2±4.0	26.4±4.2	26.3±3.9	26.9±5.2
hsCRP, mg/L	1.46±1.35	1.55±1.18	1.57±1.25	1.86±1.52
Platelet count, 10^9/L	224.1±48.4	243.0±51.2	245.7±52.8	267.9±55.9
Average EV/µL plasma	196.1±211.1	189.8±147.1	246.2±230.9	384.4±245.6
CVD^a (n)	12.8 (23)	11.7 (24)	17.8 (35)	15.2 (30)
Cancer^b (n)	2.2 (3)	7.3 (12)	6.0 (9)	10.2 (15)

[a] Self-reported history of cardiovascular disease (myocardial infarction, angina, stroke).

[b] History of cancer before baseline.

Supplementary Table 4. Characteristics of events at diagnosis (n=238 VTE). Values are means ± 1 standard deviation (SD) or percentages with numbers in brackets.

Characteristic	
Age at VTE (years), mean ± SD	66.2±15.2
Sex (males), % (n)	46.6 (111)
Deep Vein Thrombosis, % (n)	61.8 (147)
Pulmonary embolism, % (n)	38.2 (91)
Unprovoked VTE, % (n)	35.3 (84)
Provoked VTE, % (n)	64.7 (154)

Supplementary Table 5. Odds ratios (OR) with 95% confidence intervals (CI) for deep vein thrombosis (DVT) and pulmonary embolism (PE) according to quartiles of plasma levels MVs that are derived from activated platelets in percent of total number of MVs in plasma (aPDMV%)

| Category of aPDMV%	Cases (n)	Controls (n)	Model 1 OR (95%) CI	Model 2 OR (95%) CI	Model 3 OR (95%) CI
VTE					
Q1 (0.0-4.5%)	44	136	1 (ref)	1 (ref)	1 (ref)
Q2 (4.5-9.3%)	70	136	1.59 (1.02-2.50)	1.66 (1.06-2.62)	1.67 (1.07-2.64)
Q3 (9.3-16.5%)	62	135	1.42 (0.90-2.24)	1.46 (0.92-2.31)	1.47 (0.93-2.33)
Q4 (16.5-60.0%)	62	136	1.41 (0.90-2.23)	1.44 (0.91-2.30)	1.47 (0.93-2.35)
Per SD	238	543	1.10 (0.94-1.30)	1.10 (0.94-1.30)	1.11 (0.94-1.30)
DVT					
Q1 (0.0-4.5%)	25	136	1 (ref)	1 (ref)	1 (ref)
Q2 (4.5-9.3%)	44	136	1.76 (1.03-3.07)	1.87 (1.08-3.29)	1.89 (1.09-3.32)
Q3 (9.3-16.5%)	37	135	1.49 (0.86-2.64)	1.55 (0.89-2.77)	1.56 (0.89-2.79)
Q4 (16.5-60.0%)	41	136	1.64 (0.95-2.88)	1.74 (1.00-3.09)	1.78 (1.01-3.17)
Per SD	147	543	1.13 (0.94-1.38)	1.14 (0.95-1.39)	1.14 (0.95-1.40)
PE					
Q1 (0.0-4.5%)	19	136	1 (ref)	1 (ref)	1 (ref)
Q2 (4.5-9.3%)	26	136	1.37 (0.73-2.62)	1.41 (0.74-2.72)	1.42 (0.75-2.74)
Q3 (9.3-16.5%)	25	135	1.33 (0.70-2.55)	1.31 (0.69-2.54)	1.32 (0.69-2.55)
Q4 (16.5-60.0%)	21	136	1.11 (0.57-2.16)	1.06 (0.54-2.11)	1.08 (0.54-2.15)
Per SD	91	543	1.06 (0.85-1.35)	1.03 (0.83-1.32)	1.04 (0.83-1.33)

Model 1: adjusted for age and sex; model 2: adjusted for age, sex and body mass index; model 3: adjusted for age, sex and body mass index and CRP.

Supplementary Figure 1
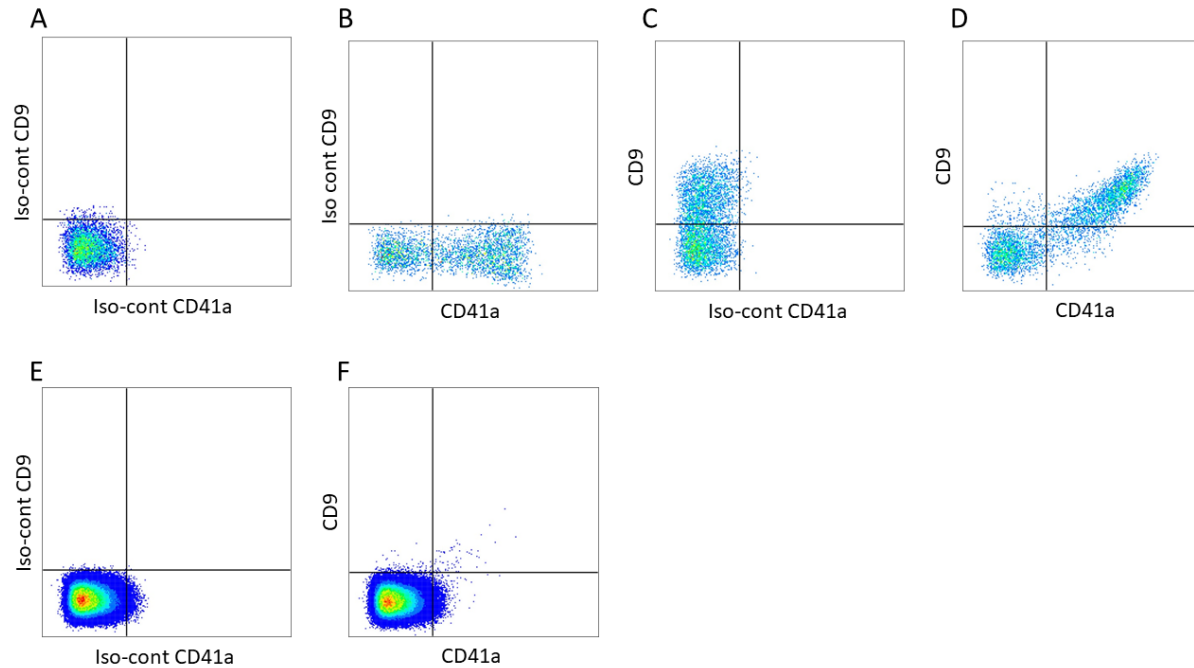
Supplementary Figure 1. Specificity of the detection of PDMVs using flow cytometry. Plasma MVs were stained with lactadherin-FITC to detect phosphatidylserine (PS) on the surface of MVs in combination with anti-CD41a and anti-CD9. The respective isotype controls for anti-CD9 and anti-CD41a antibodies were used to detect non-specific staining. MVs positively stained with lactadherin-FITC (i.e. PS-positive) are shown in plots **A-D**; Events that were not stained with lactadherin-FITC are depicted in **E** and **F**. (**A**) PS-positive MVs stained with isotype controls for anti-CD9 and anti-CD41a antibodies. (**B**) PS-positive MVs stained for CD41a and isotype control for anti-CD9 antibody. (**C**) PS-positive MVs stained for CD9 and isotype control for anti-CD41a antibody. (**D**) PS-positive MVs stained with for CD9 and CD41a. No staining was detected in PS-negative events using either (**E**) isotype controls or (**F**) anti-CD9 and anti-CD41a antibodies.

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Supplementary Figure 2
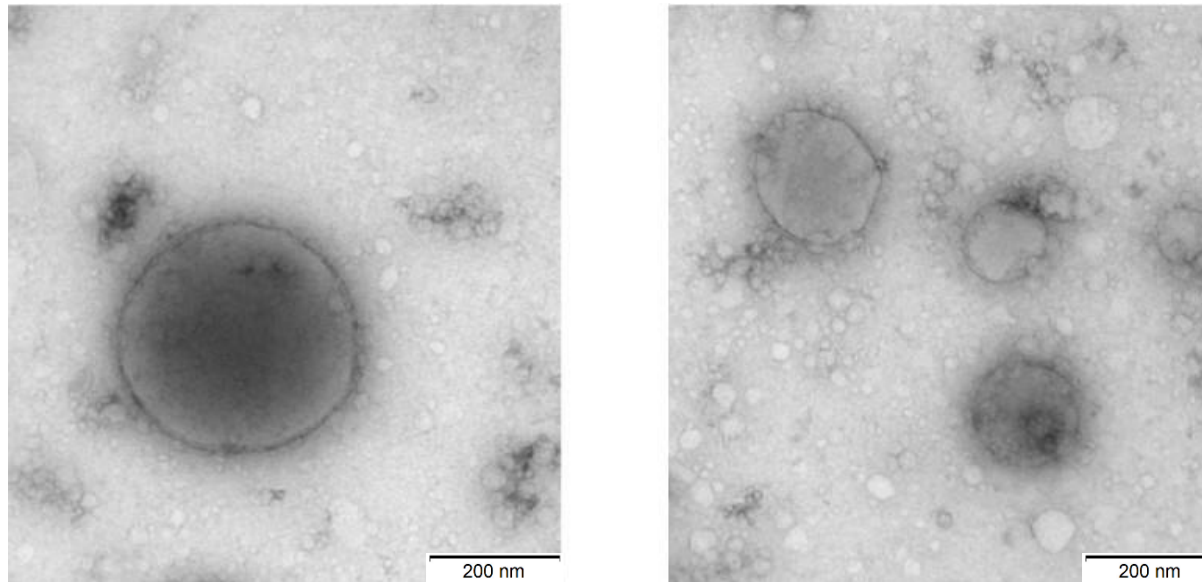
Supplementary Figure 2. Transmission electron microscopy images of plasma microvesicles. MVs were isolated from plasma using ultracentrifugation at 20,000 g and images were taken by transmission electron microscopy (25,000 x)