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Association of platelet-derived microvesicles and their phenotypes with carotid atherosclerosis and recurrent vascular events in patients after ischemic stroke

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ABSTRACT

Introduction: Platelet-derived microvesicles (pMVs) exhibit procoagulant and proinflammatory properties and play a role in the development and progression of atherosclerosis. The study examined the association between the total number of pMVs and their phenotypes with carotid atherosclerosis and recurrent vascular events (VEs) in patients in the convalescent phase of ischemic stroke (IS).

Materials and methods: The study group consisted of 72 patients with IS secondary to large artery atherosclerosis (LAA) (n = 40) and small arteries occlusion (SAO) (n = 32) and 69 matched cardiovascular disease risk-factor (RF) controls. Total pMV number, defined as CD61+ microvesicles (MVs), and their phenotypes, defined as the surface expression of proinflammatory (CD40L, CD62P, CD31) and procoagulant (PS, PAC-1) markers, were characterized and quantified using flow cytometry. The mean common carotid intima-media thickness (CCA mean IMT), maximal common carotid IMT (CCA max IMT) and maximal bifurcation IMT (BIF max IMT) were measured bilaterally using B-mode, color Doppler ultrasonography. All study subjects were observed for one-year to establish the occurrence of VEs.

Results: No differences in pMV parameters between LAA and SAO stroke subjects and between stroke subgroups and controls were found. Stroke patients with carotid atherosclerosis exhibited higher concentration of CD62P+/CD61+ and PAC-1+/CD61+ MVs compared to patients without the atherosclerosis. Positive associations between total number of pMVs, AnV+ MVs and AnV+/CD61+ MVs and atherosclerotic thickening of carotid intima-media in stroke patients were found. Elevated concentration of AnV+/CD61+, PAC-1+/CD61+, CD61P+/CD61+ and CD31+/CD61+ MVs, were revealed in stroke patients who suffered from recurrent VE in one-year follow-up period. Negative correlation of pMVs and CD62P+/CD61+ MVs concentration as well as percentage of total CD61+ in AnV+ population of MVs and time elapsed from IS in convalescent stroke subjects was revealed.

Conclusion: Our results confirm positive correlations between total pMV number, the number of PAC-1+/CD61+ and CD62+/CD61+ MVs and carotid atherosclerosis in stroke subjects. Some pMV parameters may exhibit a predictive value for the next VE in groups with a history of stroke. pMVs and some of their phenotypes decline over time elapsed from stroke in convalescent stroke subjects.

Abbreviations: pMVs, platelet-derived microvesicles; ECs, endothelial cells; PECAM-1, platelet/endothelial cell adhesion molecule; ICAM-1, intercellular adhesive molecular-1; PSGL-1, P-selectin glycoprotein ligand-1; IL, interleukin; TNF, tumor necrosis factor; TIA, transient ischemic attack; IS, ischemic stroke; VEs, vascular events; RF, risk-factor; IMT, intima-media thickness; LAA, large artery atherosclerosis; SAO, small arteries occlusion; CT, computed tomography; MR, magnetic resonance; *LDL*, low-density lipoprotein; BMI, body mass index; ACS, acute coronary syndrome; ASA, acetylsalicylic acid; CS, control subjects; mRS, modified Rankin scale; CCA, common carotid artery; BIF, bifurcation; PFP, platelet-free plasma; RT, room temperature; MVs, microvesicles; Ab, antibodies; PBS, phosphate-buffered saline solution; PE, phycoerythrin; APC, allophycocyanin; PS, phosphatidylserine; FITC, fluorescein isothiocyanate; FC, flow cytometry; SALS, small-angle light scatter; MALS, medium-angle light scatter; RI, refractive index; PMT, photomultiplier tube; FMO, fluorescence-minus-one; CV, variability coefficients; ADP, adenosine diphosphate; TRAP, thrombin receptor activating peptide; IQR, interquartile range; rS, Spearman rank; HDL, high-density lipoprotein; ACE-I, angiotensin-converting enzyme inhibitor; CCA mean IMT, carotid intima-media thickness; CCA max IMT, maximal common carotid IMT; BIF max IMT, maximal bifurcation IMT

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1. Introduction

Platelet-derived microvesicles (pMVs) exhibit proinflammatory and procoagulant properties and play a role in the development and progression of atherosclerosis and subsequent vascular complications [1]. These pMVs promote the adhesion of platelets and leukocytes, especially monocytes, to endothelial cells (ECs) at endothelial lesion sites via platelet/endothelial cell adhesion molecule (PECAM-1) and intercellular adhesive molecular-1 (ICAM-1) [2]. pMVs also express P-selectin and interact with monocytes via P-selectin glycoprotein ligand-1 (PSGL-1) [3]. pMVs carry CD40L, which is responsible for activating ECs, and recruit activated platelets to endothelium damage [4]. pMVs transfer the proatherogenic cytokine RANTES to ECs and induce monocytes and ECs to release many proinflammatory cytokines, such as interleukin (IL)-8, IL-1β, tumor necrosis factor (TNF)-α, and IL-6 [5]. pMVs stimulate the proliferation and migration of smooth muscle cells and participate in neovascularization in the atherosclerotic plaques. It should be emphasized that the majority of these pMV functions have been studied in animal models or healthy individuals. Increased pMV formation was observed in transient ischemic attack (TIA), multi-infarct dementia and the acute and chronic phases of ischemic stroke (IS) [6–12].

The main aim of this study was to evaluate the association between the total number of pMVs and pMV surface expression of studied molecules such as CD62P, active form of GPIIb/IIIa, CD40L and CD31 with carotid atherosclerosis and recurrent vascular events (VEs) in patients in the convalescent phase of IS and matched cardiovascular disease risk-factor (RF) controls. We hypothesized that phenotypic differences in pMV depended on stroke etiology, and the association between the concentration of circulating pMVs and recurrent VE in after stroke patients and the severity of carotid atherosclerosis defined as presence of atherosclerotic carotid plaques and increased carotid intima-media thickness (IMT).

2. Materials and methods

Recruitment to our study was conducted from January 2015 to December 2016. Based on the medical data at the Department of Neurology in Poznan, Poland we have reviewed the available medical records of 246 subjects who had been suffered from IS in the period from 3 to 12 months before the medical data collecting. Then, using the specified inclusion and exclusion criteria, we found a group of 110 subjects potentially suitable for inclusion in our study. Of these patients, 80 gave their informed consent and agreed to participate in our study, 9 were excluded due to a recurrent VE before study enrolment, 13 did not agree to participate in the study and 8 could not take a part in the study because of disability. Finally, based on patient's medical history and the results of the supplementary examinations, 72 patients with a history of IS within the period of 3 to 12 months prior to study enrolment who had not shown any additional exclusion criteria were included to the study. Based on results of the previous studies, the platelet hyperactivation normalizes up to 3 months after the stroke [13,14] thus we evaluate pMV parameters in stroke subjects 3–12 months after IS to exclude artefactual MVs release related to the acute ischaemic incident.

The following inclusion criteria were used: age over 45 years; IS as a results of large artery atherosclerosis (LAA) or small arteries occlusion (SAO) based on the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification criteria [15] and confirmed on computed tomography (CT) or magnetic resonance (MR) examination; and at least two concomitant vascular disease RF, including hypertension (systolic blood pressure ≥ 140 mmHg and diastolic pressure ≥ 90 mmHg or hypertensive treatment) and/or hypercholesterolemia (blood low-density lipoprotein (*LDL*) *cholesterol* level ≥ 115 mg/dl on a diet or treatment with statins) and/or current smoking and/or diabetes (managed with diet and/or hypoglycemic treatment) and/or obesity (defined as body mass index (BMI) ≥ 30 kg/m^2). Exclusion criteria included stroke of potential cardioembolic or undetermined cause, stroke subjects treated with thrombolysis, hemorrhagic stroke, VE in the period between stroke and blood sampling, malignancies, autoimmune diseases, inflammatory disorders, infections, heart diseases (acute coronary syndrome (ACS), atrial fibrillation, valvular heart diseases), liver and renal failure, hematological disorders, abnormal platelet count, and alcohol or drug abuse. The control group consisted of 69 age, sex- and vascular disease RF-matched individuals (concurrent control group) who were recruited from the Department of Neurology in Poznan, Poland and had never experienced any VEs (a TIA/stroke/myocardial infarction). They met the same inclusion (excepting the cerebral ischaemic event) and exclusion criteria as stroke patients and were examined simultaneously and according to the same protocol as the stroke subjects.

All stroke subjects were treated chronically with acetylsalicylic acid (ASA) as the secondary stroke prevention, and all control subjects (CS) received ASA for at least 7 days prior to blood sampling. None of the included subjects had been treated with antithrombotic or antiplatelet drugs other than ASA or non-steroid anti-inflammatory drugs during the previous 3 months. The study protocol was approved by the local ethics committee and informed consent was obtained from all study subjects.

2.1. Clinical assessment

Stroke was verified based on inpatient history and discharge files and confirmed based on the clinical examination and angio-CT or angio-MR scans. All studied subjects were given their medical history to collect data on vascular RF and medication. The same examiner (JR) clinically performed physical and neurological examinations. The standard diagnostic measures included blood pressure and height and weight assessments to calculate body mass index. The clinical evaluation was supplemented with routine laboratory investigations, including blood count, biochemical tests and electrocardiogram. The disability of stroke subjects was assessed with a modified Rankin scale (mRS). Study subjects were followed up every 3 months for 1 year using telephone interviews to establish the occurrence of adverse VEs and vascular deaths.

2.2. Ultrasound investigation

Color Doppler duplex ultrasonography was performed in all study subjects using high-resolution ultrasound equipment (Aloka Prosound Alpha 7, Japan) with a 7.5–15 MHz linear transducer. The far wall IMT was defined as the distance between the lumen-intima interface and media-adventitia interface, which was measured within the distal common carotid artery (CCA) segment and within the bifurcation (BIF). Measurements (mean and max IMT) were performed semi-automatically using the software provided with ultrasound machine. Atherosclerotic carotid plaques were also defined according to the updated Mannheim consensus as a focal structure that encroached into the arterial lumen at least 0.5 mm or 50% of the surrounding IMT value or demonstrated a thickness > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface [16]. Carotid plaques were classified as high-risk (hypoechogenic, irregular surface, soft) and low-risk (hyperechogenic, regular surface, calcified) of rupture based on morphology. One experienced examiner (WA) performed all ultrasound procedures. The intra-observer variability in measurements of IMT expressed as the coefficient of variance was 3.9%.

2.3. Blood samples

Fasting blood samples were obtained between 7.00 a.m. and 9.00 a.m. to avoid the influence of circadian variations. Samples were withdrawn using an 18-gauge needle via direct, single venipuncture of

the antecubital vein without applying venostasis to avoid artificial aggregation while the subject was in a sitting position. Sample were collected into a tube with 0.105 M buffered sodium citrate anticoagulant (Becton Dickinson, Plymouth, UK), and anticoagulant was immediately mixed with blood via gentle inversion. Citrated platelet-free plasma (PFP) was separated using low-speed centrifugation at 1500 ×*g* for 20 min at room temperature (RT). The plasma was transferred to Eppendorf tubes and centrifuged at 13000 ×*g* for 2 min to obtain PFP samples, which were immediately snap-frozen in liquid nitrogen and stored at −80 °C for analysis. An additional 2.6-ml aliquot of blood was drawn into a tube with EDTA potassium salt (Sarstedt Monovette, Sarstedt, Germany) to perform a blood count. An additional 10 ml of blood was drawn into a tube (Sarstedt Monovette) and allowed to clot for biochemical tests. Blood samples to the laboratory were carefully transported to avoid unnecessary platelet stimulation. Technicians coded the samples for blinded -analysis. All samples were processed identically and within 30 min after extraction.

2.4. Platelet-derived microvesicle preparation and labeling

Previously frozen PFP samples were thawed at RT, and the microvesicle (MV) fraction was isolated from PFP using two-step high-speed centrifugation for 30 min at 18890 ×*g* and washed twice with a 0.22-μm filtered citrate-phosphate-buffered saline solution (PBS) containing 1.4 mmol/l phosphate, 154 mmol/l NaCl, and 10.9 mM trisodium citrate (pH 7.4) after each centrifugation. The MV suspension (7.5 μl) was stained according to the three-color protocol and incubated (30 min, RT, in the dark) with 3.75 μl of the following antibodies (Ab): phycoerythrin (PE)-conjugated anti-CD61 (platelet-gating Ab) (Becton Dickinson), allophycocyanin (APC)-conjugated Annexin V (Ab against superficial phosphatidylserine (PS) (BioLegend), and fluorescein isothiocyanate (FITC)-conjugated PAC-1 (Ab against active form of GPIIb/IIIa) (BioLegend), FITC-conjugated CD62P (Ab against P selectin, BioLegend), CD154/FITC (Ab against CD40L, BioLegend) or CD31/FITC (Ab against PECAM-1, BioLegend) and 15 μl of 25 mM $CaCl_2$. Samples were diluted with 450 μl of 0.22-μm filtered 0.9% NaCl at the end of incubation prior to flow cytometry (FC) analysis.

2.5. Flow cytometric analysis

Analyses of labeled samples were performed using an Apogee A-50 Micro FC (Apogee Flow Systems, Hemel Hempsteadm, UK) equipped with 488-nm (blue) and 638-nm (red) lasers dedicated to the detection of microvesicles. We used two of three available light scatters: the small-angle light scatter (SALS) and medium-angle light scatter (MALS) as optimal for MV quantification and sizing. The reference bead mix (*ApogeeMix*, Apogee Flow Systems) composed of a mixture of non-fluorescent silica beads with diameters of 180 nm, 240 nm, 300 nm, 590 nm, 880 nm, and 1300 nm with a refractive index (RI) of 1.42, and 110-nm and 500-nm green fluorescent (excited by blue laser) latex spheres with an RI of 1.59 was used to set up photomultiplier tube (PMT) voltages and the thresholds for light scattering. Moreover reference bead mix were used on each day of measurements to verify FC performance. The FC tubing was washed after each calibration and sample acquisition to remove any noise-making particles using 0.22-μm filtered 10% bleach followed by a wash with 0.22-μm filtered 0.9% NaCl. Acquisition was performed in duplicate at a slow sample flow rate of 0.75 μl/min using a 150-μl sample volume for 180 s. The sample of 0.22-μm filtered 0.9% NaCl was acquired after the MV sample of each subject to ensure that unwanted noise did not interfere with measurements. An additional tubing wash was performed with bleach if the plot suggested sample contamination.

The pMV concentration (number of pMV per μl of prepared purified MVs) was measured automatically based on the sample volume, cytometer flow rate, and the number of fluorescence-positive events (n). pMVs were gated using MALS and a fluorescence triggering channel and defined as CD61-positive events in color fluorescence plots after staining with CD61-PE antibodies. Each phenotype of circulating pMVs was identified based on reactivity to specific monoclonal antibodies, as mentioned above. The gating procedures were presented in the Fig. 1.

Compensation procedures were performed to avoid any potential overlap between the emission spectra of two or more fluorochromes, and fluorescence-minus-one (FMO) controls were performed to ensure that any observed fluorescent signal arose only from the antibody of interest. Intra-sample and inter-sample variations were determined, and the following Pearson's variability coefficients (CV) were obtained: 2.3% and 5.3% for the percentages of CD61- and AnV-positive MVs, respectively, and 3.2% and 7.3% for the CD62P-, PAC-1-, CD40L-, CD31-positive events.

To ensure that analyzed dot plots represent the platelet derived particles we performed analyses of MVs isolated from the unstimulated and stimulated with platelet agonists' whole blood (Fig. 2).

2.6. Statistical analysis

All data were analyzed using PQStat software v.1.6.6. A normal distribution of the tested parameters was verified using the Shapiro-Wilk test. Categorical data were compared using the chi-square test. Data are expressed as medians and interquartile range (IQR). The parametric Student's *t*-test, ANOVA and post-hoc Tukey test were used for multiple comparisons of normally distributed data. Data manifesting a distribution other than normal were analyzed by non-parametric methods. Significance of intergroup differences was evaluated using the Mann–Whitney *U* test and multiple comparison Kruskal-Wallis test followed by Bonferroni's correction method. Possible relationships between studied parameters were evaluated using the Spearman rank (rS) correlation test. Multiple regression analysis was performed to assess the independent effects of stroke and vascular disease risk factors on the studied pMV parameters. The differences were assumed significant at $p < 0,05$ and $p < 0.01$ for multiple comparisons. A p-value < 0,05 was considered statistically significant for correlations.

3. Results

Table 1 shows the basic demographic and clinical characteristics of the studied subjects. Seventy-two stroke patients secondary to LAA ($n = 40$) and SAO ($n = 32$) were assessed in the convalescent phase of IS [9 IQR (6–11) months], and the results were compared to the control group ($n = 69$). A significant difference in the percentage of male sex was observed between the studied groups, but gender was not significant in the assumed regression models. There were no overall significant differences between stroke patients and controls in the main clinical data, except for β-blocker intake, IMT and the occurrence of atherosclerotic plaques between LAA stroke and controls. There were no significant differences in the number of total pMVs, defined as CD61+ MVs, total AnV+ MVs and pMV surface expression of studied molecules, between stroke subjects and controls and LAA and SAO stroke subjects (Table 2).

Higher concentrations of CD62P+/CD61+ and PAC-1+/CD61+ MVs were observed in stroke patients with carotid atherosclerotic plaques ($n = 61$) and increased carotid IMT ≥ 0.9 mm ($n = 60$) compared to patients without carotid atherosclerotic plaques ($n = 11$) (Fig. 3a) and patients with normal IMT < 0.9 mm ($n = 12$) (Fig. 3b). There were no differences in the concentrations of total pMVs or AnV+ MVs. Notably, no differences in the studied pMV parameters were observed between CS with ($n = 38$) and without carotid plaques ($n = 31$) and CS with increased ($n = 28$) and normal ($n = 41$) carotid IMT. No significant differences in the total pMVs and their phenotypes were found between subjects with high-risk and low-risk carotid atherosclerotic plaques in stroke group and controls.

The one-year follow-up period was completed in 69 (95.8%) stroke patients and 66 (95.6%) controls. A total of 26.1% of stroke subjects

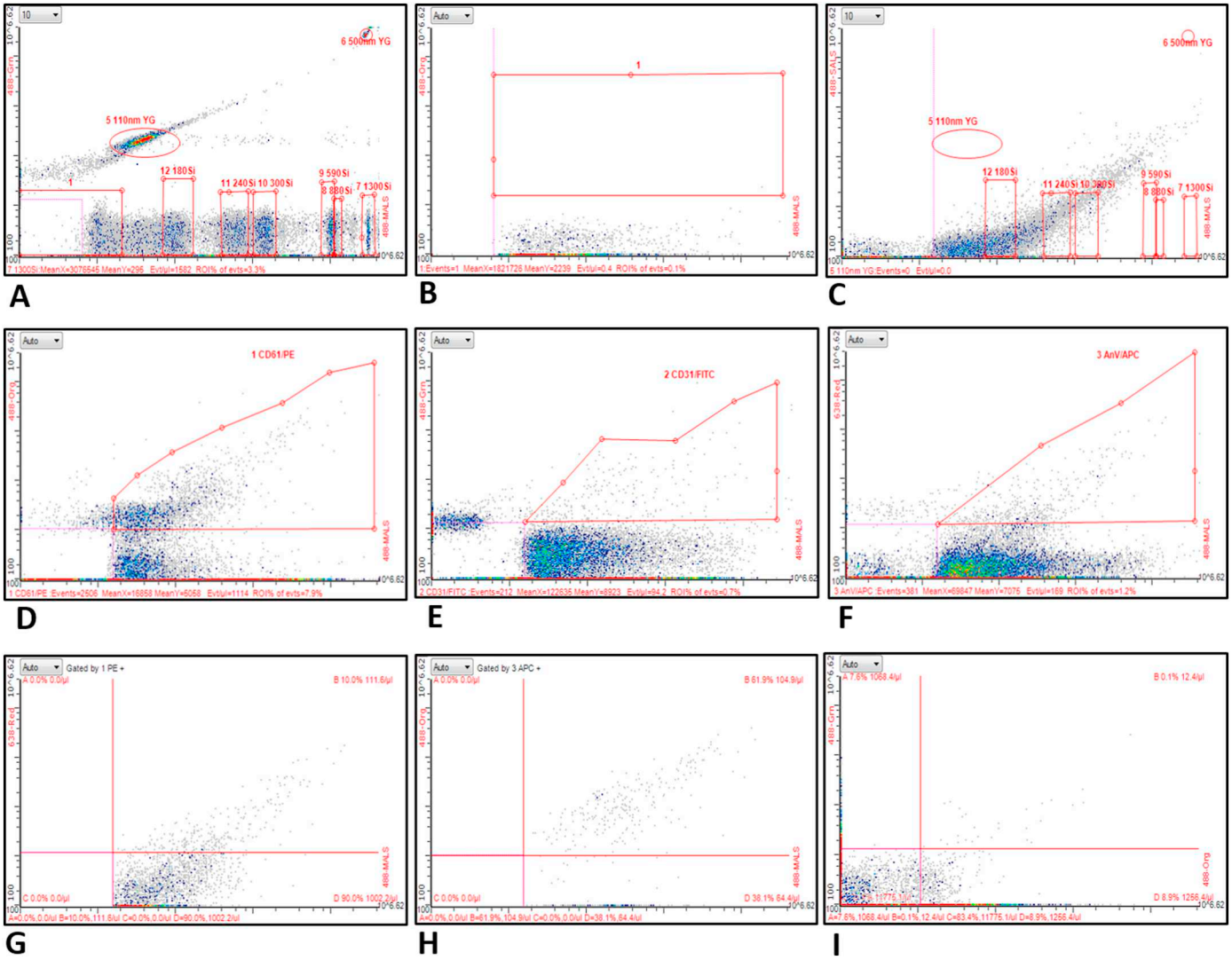

Fig. 1. Setting up the dedicated flow cytometer (FC) Apogee A-50 micro and analysis of microvesicles (MV); **A**. the reference bead mix dot plot at default settings: the gate 1 represents the instrument noise, gates 7–12 depicted the silica beads with refractive index (RI) 1.42 and diameters of respectively 1300 nm, 880 nm, 590 nm, 300 nm, 240 nm and 180 nm, and gates 5 and 6 represent green populations of latex microspheres (110 nm and 500 nm, RI 1.59); **B**. the gate 1 with no objects represents the gate for 0.22-μm filtered 0.9% NaCl as medium for isolated MVs; **C**. the small-angle light scatter (SALS) vs medium-angle light scatter (MALS) cytogram of isolated MV with projected areas of reference bead mix are plotted; **D**. the cytogram of CD61/PE (orange fluorescence, laser 488 nm) vs MALS with gated CD61-positive events (platelet-derived MV); **E**. the cytogram of CD31/FITC (green fluorescence, 488-nm laser) vs MALS with gated CD31-positive events; **F**. the cytogram of AnV/APC (red fluorescence, 638-nm laser) vs MALS with gated AnV-positive events. In cytograms D-F statistics below the OX axis show calculated automatically concentrations of MVs based on the gated area; **G**. the cytogram of AnV/APC vs MALS gated by CD61/PE positive events; in quadrant B the CD61/AnV-positive events are visible and the quadrant B statistics represent the concentration of MVs in studied sample and the percentage of AnV-positive MVs in population of platelet-derived MVs; **H**. the cytogram of CD61/PE vs MALS gated by AnV/APC-positive events; in quadrant B the CD61/AnV-positive events are visible and the quadrant B statistics represent the concentration of CD61/AnV-positive MVs in studied sample and the percentage of platelet-derived MVs in population of AnV-positive MVs; **I**. the cytogram of CD31/FITC vs CD61/PE; the statistics in quadrant B represent the concentration of CD61/CD31-positive MVs in studied sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(n = 18) experienced recurrent VEs, which were defined as recurrent IS (n = 7), TIA (n = 4) or ACS (n = 7). A total of 15.2% (n = 10) of CS experienced VEs, which were defined as IS (n = 3), TIA (n = 3) or ACS (n = 4). The mean time elapsed from stroke to the recurrent VE was 14 ± 4 months. No statistically significant differences in the total number of CD61 + MVs were found between stroke subjects with and without recurrent VEs. However, significantly higher concentrations of total AnV+ MVs and AnV+/CD61+, CD62P+/CD61+, PAC-1+/CD61+ and CD31+/CD61+ MVs were observed in stroke patients who experienced recurrent VEs (Table 3). Notably, no significant differences in pMV parameters were observed in CS who suffered their first vascular incident.

We have divided stroke subjects into two subgroups according to the time elapsed from IS: the first one including patients recruited 3–9 months after stroke and the second one – 9 to 12 months from stroke. We have found the higher total pMVs [1468 (989–1871) vs 1037 (680–1427) n/μl; p = 0.012], percentage of total CD61+ in AnV+ population of MVs [53.1 (46.2–58.1) vs 47 (38.9–51.9), %; p = 0.019] and CD62P+/CD61+ MVs [10 (7–16) vs 5 (4–12) n/μl; p = 0.027] in patients with shorter time elapsed from stroke. Moreover we have observed negative correlations between pMVs (rS = −0.37; p < 0.01), percentage of total CD61+ in AnV+ population of MVs (rS = −0.36; p < 0.01), CD62P+/CD61+ MVs (rS = −0.29; p = 0.04) and time elapsed from IS as well.

We found a positive association between CCA mean IMT and CCA max IMT and the concentration of CD61 + MVs (rS = 0.46; p < 0.001

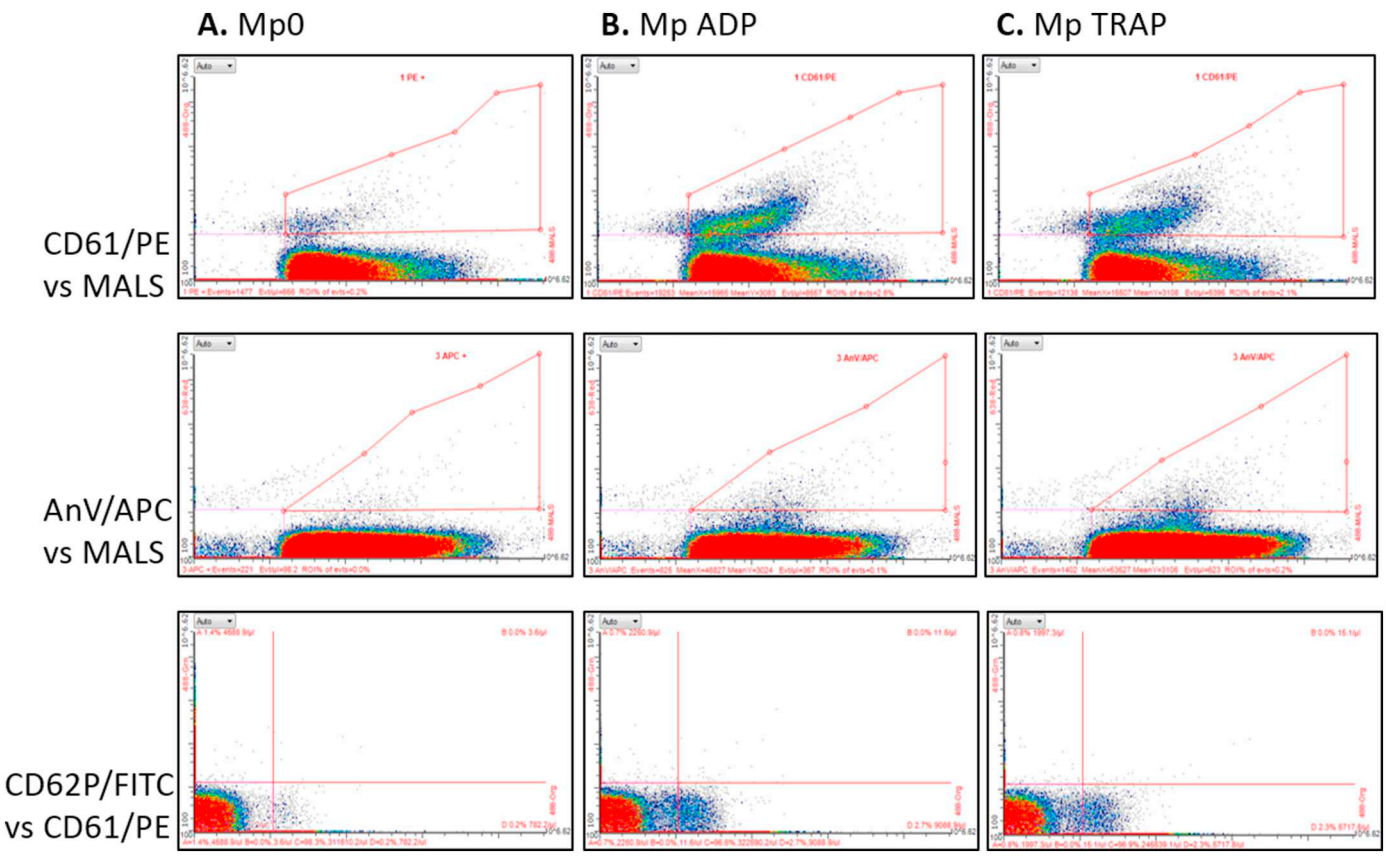

Fig. 2. The cytograms represent the dot plots of platelet-derived MVs and AnV-positive MVs (gated areas) as well as the dot plot of double positive (CD61/CD62P) MVs isolated from the healthy donor PFP obtained from the: unstimulated whole blood (column **A**), the whole blood stimulated with 5 μM adenosine diphosphate (ADP), 30 s (column **B**), and the whole blood stimulated with 8 μM thrombin receptor activating peptide (TRAP)-14, 4 min. (column **C**). The prominent increase in concentration of platelet-derived MVs (CD61-positive) and AnV-positive MVs as well as the concentration of double positive (CD61/CD62P) MVs was observed.

and rS = 0.4; $p < 0.01$ respectively) and percentage of CD61+ MV in the AnV+ population (rS = 0.36; $p < 0.001$ and rS = 0.38; $p < 0.01$ respectively) in stroke subjects. Positive correlations were observed between CCA mean IMT and AnV+ ($r = 0.36$; $p < 0.01$) and AnV +/CD61+ ($r = 0.36$; $p < 0.01$) MVs in this group. No correlations between other pMV phenotypes were found in stroke patients. No correlations between the studied MVs and IMT parameters were found in controls.

We performed multiple regression analysis to establish the effect of stroke vascular RF and carotid atherosclerosis on the pMV parameters. The total number of pMV and AnV+/CD61+ MVs positively correlated with CCA mean IMT and negatively correlated with stroke status. AnV +/CD61+ MVs also positively correlated with age (Table 4).

The regression models included total pMVs or AnV+/CD61+ MVs number as the dependent variable and age, gender, stroke status, cardiovascular RFs (hypertension, diabetes) and mean carotid IMT as an independent variable. Standardized regression coefficient B values are shown; R2, the corrected determination coefficient, which reflects how far a given set of independent variables explains the variability of the dependent variable.

4. Discussion

To the best of our knowledge, this report is the first to explore the association between not only the total pMVs or AnV+ MVs but also pMV phenotypes with carotid atherosclerotic plaques and VE recurrence in convalescent stroke patients as well as matched controls using dedicated flow cytometry.

Our data revealed no differences in total pMV concentration between stroke subjects and controls, which is inconsistent with the conclusions of most researchers suggesting that number of circulating pMV after stroke is higher than in controls. These discrepancies may result from the choice of studied phase of stroke - most of studies were conducted in the acute phase of IS [6,8,11,12,17], and only a few of them focused on the chronic phase [7–9], mostly up to 6 months after an ischaemic incident. In our study, the time elapsed from the stroke was longer, with a median time of 9 months, which may be a key factor, especially the more we confirmed significant negative correlation between the time elapsed from stroke and total pMV concentration, CD62P+/CD61concentration as well as the percentage of CD61+ in AnV+ MV population. Moreover, the concentration of pMVs is related to the severity and the outcome of the IS [18] thus, our findings may have resulted from the relatively good clinical conditions of the included convalescent stroke subjects [mRS 1, IQR (1–2)]. Furthermore, the discrepancies may result from the demographic characteristic of the control group. It did not consist of healthy subjects but was very carefully matched to stroke patients according to the age, sex and vascular disease RF as a concurrent group. Nonetheless, no differences between patients and CS in the number of pMV were described in subjects with severe symptomatic cerebral small vessel disease [19] or prosthetic heart valves patients with cerebrovascular events [20].

We demonstrated no significant differences in the formation of AnV + MVs or pMVs as well as in the pMV phenotypes between both studied subtypes of stroke, LAA and SAO. These results are consistent with a previous studies of CD61 + MVs in acute IS that included the LAA and SAO subtypes [8,15]. However, our results conflict with another study where significantly higher pMV concentrations were found in VEs secondary to SAO compared to secondary to LAA [6].

We demonstrated significantly increased carotid IMT in convalescent stroke subjects due to LAA compared to CS and SAO stroke

Table 1
Baseline characteristics of the studied population.

Parameters	LAA (n = 40)	SAO (n = 32)	CS (n = 69)	p
Age, years	66 (63–77)	66 (60–74)	65 (60–72)	0.4
Male sex, n (%)	31 (78)	12 (38)	34 (49)	**< 0.01**
				LAA vs CS 0.004
				SAO vs CS 0.27
				LAA vs SAO 0.0006
BMI, kg/m^2	26.8 (24.1–29.9)	28.3 (25.5–31.4)	27 (24.2–31)	0.17
Platelets, T/μl	225 (217–249)	246 (231–293)	228 (210–253)	**0.04**
				LAA vs CS 1
				SAO vs CS 0.06
				LAA vs SAO 0.08
Total cholesterol, mmol/l	166 (145–203)	183 (161–211)	193 (154–223)	0.22
LDL, mmol/l	82 (65–123)	98 (87–129)	104(74–144)	0.07
HDL, mmol/l	51 (45–65)	58 (48–64)	51(45–64)	0.34
Triglycerides, mmol/l	102 (84–145)	110(85–131)	110 (84–170)	0.64
Glucose, mg/dl	113 (100 − 130)	102 (91–121)	101 (93–110)	**0.02**
				LAA vs CS 0.015
				SAO vs CS 1
				LAA vs SAO 0.34
HbA1C	6.3 (5.9–7)	6 (5.6–6.6)	6.1 (5.6–6.9)	0.6
Hypertension, n (%)	38 (95)	30 (93.8)	65 (94)	0.97
Diabetes, n (%)	15 (37.5)	10 (31.3)	21 (30.5)	0.73
Dyslipidemia, n (%)	38 (95)	28 (87.5)	62 (89.9)	0.51
Smoking, n (%)	15 (37.5)	10 (31.3)	25 (36.2)	0.84
Obesity, n (%)	13 (32.5)	11 (34.4)	23 (33.3)	0.99
Medications, n (%)				
ACE-I	26 (65)	23 (71.8)	40 (58)	0.38
Sartans	5 (12.5)	6 (18.8)	18 (26)	0.23
Diuretics	18 (45)	11 (34.4)	27 (39.1)	0.65
Ca-channel blockers	15 (37.5)	14 (43.8)	20 (29)	0.32
β-Blockers	11 (27.5)	13 (40.6)	37 (53.6)	**0.03**
				LAA vs CS 0.008
				SAO vs CS 0.22
				LAA vs SAO 0.24
Statins	33 (82.5)	22 (68.8)	44 (63.8)	0.12
Oral hypoglycemic	14 (35)	8 (25)	16 (23.2)	0.39
Insulin	3 (7.5)	3 (9.4)	7 (10.1)	0.9

LAA, large artery atherosclerosis; SAO, small arteries occlusion; CS, control subjects; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BMI, body mass index; ACE-I, angiotensin-converting enzyme inhibitor.
Statistically significant differences ($p < 0.05$ and $p < 0.01$ for multiple comparisons) have been bolded in the table.

subjects, which is consistent with previous findings [21,22]. The elevated concentrations of CD62P+/CD61+ and PAC-1+/CD61+ MVs in stroke subjects with carotid atherosclerotic plaques compared to those without carotid plaque are consistent with previous research, which indicated positive correlations between enhanced pMV release and carotid IMT or carotid plaque in patients after IS [9,11] and with intracranial vessel stenosis [11]. An increased pMV proportion was also associated with carotid atherosclerosis in the general population with subclinical atheromatic lesions [23] and in obese patients [21]. No significant differences in studied pMV parameters between subjects with high- and low-risk carotid plaques were found in our study, but in previous research the increased number of circulating pMV correlated with lipid-rich atherosclerotic plaques and plaque burden [24,25]. However, the concentration of large pMVs are not associated with plaque echogenicity [23], and pMVs were not found in the atherosclerotic plaque itself [26].

Moreover, we found a positive association between carotid IMT and the concentration of pMV, percentage of CD61+ MV in AnV+ population of MVs, AnV+ and AnV+/CD61+ MVs only in post-stroke patients. These results suggest a significant role of pMVs in the development of atherosclerotic lesions and affecting the release of pMV in the presence of an atherosclerotic carotid plaque. Furthermore IS may be a trigger for these mechanisms because no similar relationship was found in subjects with carotid atherosclerosis without a history of stroke. The phenotype, but not the total amount, of pMVs may be important in this situation. The negative correlation of pMV and stroke with a positive correlation between total pMV and the number of PAC-1+/CD61+ and CD62P+/CD61+ MVs and carotid atherosclerosis only in the group after stroke suggests that the interaction of pMVs with the arterial wall is more dependent on the pMV phenotype than their total number in after-stroke patients. Multiple regression analysis revealed that the total number of pMV and AnV+/CD61+ MVs independently and positively correlated with CCA mean IMT and negatively correlated with stroke status. AnV+/CD61+ MVs was also positively correlated with age, which is consistent with previous studies [27]. Thus some pMV parameters may exhibit a predictive value for the next VE, but only in a group with a history of stroke, which creates an irrelevant in primary prediction.

Significantly higher AnV+, AnV+/CD61+, CD62P+/CD61+, PAC-1+/CD61+, CD31+/CD61+ MVs concentrations and percentage of AnV+ MVs in CD61+ MV population were found in stroke patients who experienced recurrent VE during the one-year follow-up period. These results may partially explain the progression of atherosclerosis in after-stroke subjects and the increased risk of recurrent stroke because a previous cerebral ischemic event is a major RF for a future stroke. Approximately 8–12% of patients suffer another stroke within 3 months after an ischemic incidence despite the introduction of secondary prevention [28], and the recurrence rate reaches 40% in a period of 5 years [29]. Therefore, the detection of a factor that is at least partially responsible for this phenomenon is a current unmet challenge. Ineffective prevention of cardiovascular diseases may be at least partially the result of pMV activity and may be associated with the triggering of recurrent VE, which was confirmed in our study.

Moreover, lower concentration of MVs with surface expression of

Table 2
Platelet-derived microvesicle data and parameters of intima-media thickness in subjects after stroke caused by LAA and SAO and control subjects (CS).

MV parameters	LAA (n = 40)	SAO (n = 32)	CS (n = 69)	p
CD61+, [n/μl]	1258 (768–1746)	1141 (899–1757)	1624 (990–2148)	0.12
AnV+, [n/μl]	388 (277–521)	373 (285–550)	396 (271–585)	0.81
AnV+/CD61+, [n/μl]	167 (110–252)	174 (122−313)	221 (139–327)	0.42
AnV+ % in CD61+ MV population [%]	15.7(13.9–17.4)	16.2 (14–19)	14.5 (13–18.2)	0.46
CD61+ % in AnV+ MV population [%]	50.5 (46.4–56.4)	49.1 (43–54.4)	54.3 (46–60.7)	0.07
CD62P+/CD61+, [n/μl]	8 (5–16)	8 (5–12)	15 (8–25)	**0.008** LAA vs CS 0.07 SAO vs CS 0.017 LAA vs SAO 1
PAC-1+/CD61+, [n/μl]	10 (5–16)	8 (3−12)	11 (7–19)	**0.046** LAA vs CS 1 SAO vs CS 0.04 LAA vs SAO 0.47
CD40L+/CD61+, [n/μl]	6 (5–10)	6 (4–12)	10 (6–14)	0.059
CD31+/CD61+, [n/μl]	23 (15–34)	23 (12–40)	24 (16–39)	0.82
Intima-media thickness parameters				
CCA mean IMT, [mm]	1 (0.86–1.17)	0.95 (0.71–1.14)	0.78 (0.68–0.89)	**< 0.001** **LAA vs CS < 0.001** SAO vs CS 0.05 LAA vs SAO 0.73
CCA max IMT, [mm]	1.21 (1.05–1.3)	1.1 (0.87–1.3)	0.88 (0.8–1.13)	**< 0.001** **LAA vs CS < 0.001** SAO vs CS 0.047 LAA vs SAO 0.5
BIF max IMT, [mm]	2.1 (1.68–2.6)	1.5 (1.29–1.9)	1.2 (0.85–1.7)	**< 0.001** **LAA vs CS < 0.01** SAO vs CS 0.04 LAA vs SAO 0.064
Carotid artery plaques, n (%)	35 (87.5)	26 (81.25)	38 (63)	**0.015** **LAA vs CS 0.008** SAO vs CS 0.08 LAA vs SAO 0.46
High-risk plaques, n (%)	16 (40)	11 (34.4)	14 (20.3)	0.2

LAA, large artery atherosclerosis; SAO, small arteries occlusion; CS, control subjects; MV, CCA IMT, common carotid intima-media thickness; BIF, common carotid artery bifurcation.
Statistically significant differences ($p < 0.05$ and $p < 0.01$ for multiple comparisons) have been bolded in the table.

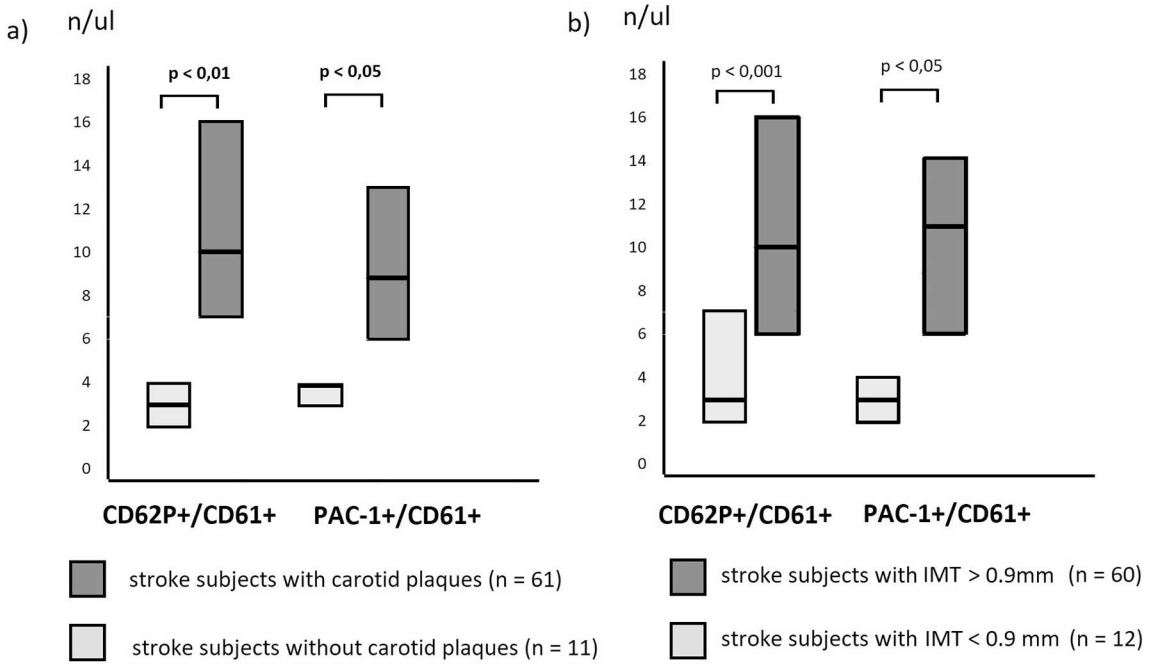

Fig. 3. Differences in concentrations of CD62P+/CD61+ and PAC-1+/CD61+ MVs in stroke patients with and without carotid atherosclerotic plaques (a) and with increased and normal IMT (b). The data are presented as medians with the upper and lower percentiles.

Table 3
Platelet-derived microvesicle data in all study subjects with and without vascular events in a one-year follow-up period.

MV parameters	Stroke subjects with recurrent VE (n = 18)	Stroke subjects without recurrent VE (n = 51)	CS with VE (n = 10)	CS without VE (n = 56)
CD61+, [n/μl]	1665 (966–1817)	1118 (763–1573)	1736 (1424–2142)	1624 (906–2114)
AnV+, [n/μl]	521 (310–702)[a,*]	346 (253–458)[a,*]	400 (374–475)	386 (269–631)
AnV+/CD61+, [n/μl]	308 (170–344)[a,*]	164 (108–220)[a,*]	243 (196–273)	207 (136–351)
AnV+ % in CD61+ MV population, [%]	17.5 (17–19)[a,**,b,*]	15.4 (13.3–16.5)[a,*]	13 (12–16)[b,*]	15 (13.6–18.4)
CD61+ % in AnV+ MV population, [%]	48.7 (44.5–55.7)	50.9 (42.7–56.1)	54 (46–60)	55.5 (46.4–60.7)
CD62P+/CD61+, [n/μl]	13 (9–20)[a,**]	8 (4–12)[a,**,c,**]	10 (8–14)	16 (8–25)[c,**]
PAC-1+/CD61+, [n/μl]	13 (8–21)[a,**]	7 (3−11)[a,**,c,**]	9 (8–9)	13 (7–20)[c,**]
CD40L+/CD61+, [n/μl]	10 (5–14)	6 (4–9)[c,**]	13 (8–14)	10 (5–14)[c,**]
CD31+/CD61+, [n/μl]	38 (23–56)[a,**]	20 (9–33)[a,**,c,*]	22 (14–25)	25 (17–41)[c,*]

CS, control subjects; VE, vascular event; data are expressed as median (IQR).
* $p < 0.05$.
** $p < 0.01$.
[a] Comparison of stroke subjects with recurrent VE vs stroke subjects without recurrent VE.
[b] Comparison of stroke subjects with recurrent VE vs controls with VE.
[c] Comparison of stroke subjects without VE vs controls without VE.

Table 4
Total pMV and AnV+/CD61+ MV number associated with mean carotid intima-media thickness, cardiovascular risk factors and stroke status.

Variables	Total pMV number		AnV+/CD61+ MVs	
	B	p-Value	B	p-Value
Model A	p = 0.012 R2 = 0.1		p = 0.038 R2 = 0.12	
Age	0.13	0.24	0.22	0.04
Gender	−0.05	0.64	0.04	0.69
HA	−0.13	0.19	−0.11	0.32
Stroke	−0.28	0.008	−0.24	0.02
CCA mean IMT	0.28	0.013	0.23	0.04
Model B	p = 0.02 R2 = 0.09		p = 0.01 R2 = 0.1	
Age	0.09	0.41	0.08	0.1
Gender	−0.04	0.68	0.05	0.61
DM	0.06	0.56	−0.09	0.4
Stroke	−0.27	0.01	−0.23	0.03
CCA mean IMT	0.27	0.02	0.25	0.03
Model C	p = 0.016 R2 = 0.1		p = 0.012 R2 = 0.1	
Age	0.09	0.41	0.2	0.08
Gender	−0.06	0.58	0.04	0.68
BMI	0.001	0.98	−0.04	0.68
Stroke	−0.3	0.006	−0.25	0.02
CCA mean IMT	0.3	0.009	0.24	0.04

CD62P or the active form of GPIIb/IIIa or CD40L or CD31have been found in stroke subjects without recurrent VE during follow-up period compared to adequate controls. This is in line with our previous observation that in convalescent phase of stroke a lower surface expression of CD62P on circulating platelets is present and a smaller increase in the surface expression of CD62P and active form of GPIIb/IIIa receptor after ex vivo stimulation with thrombin receptor activating peptide (TRAP) or adenosine diphosphate (ADP) takes place probably due to the chronic exhaustion of platelets [30]. One cannot exclude that, in subjects with recurrent VE in follow-up period persistent overexpression of procoagulant and pro-atheromatic surface molecules on MVs is present while in patients without recurrent VE these molecules are depressed.

There are some limitations of our study that must be addressed. It is difficult to relate our results to previous studies because of the many methodological differences in term of blood collection, sample processing and methods used for MVs identification and quantification. However, unified methodology is one of the challenge in the field of MVs researches. Furthermore, we did not study all IS subtypes and enrolled patients were in relatively good clinical condition. Therefore, a prospective study with larger samples, all IS etiological subtypes, and participation of convalescent stroke subjects with poorer functional outcome and with the subsequent determination of pMV concentrations during the follow-up period would be extremely valuable.

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Declarations of interest

The authors declare that they have no conflicts of interest.

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