

Original Research

Circulating Microvesicles in Convalescent Ischemic Stroke Patients: A Contributor to High-On-Treatment Residual Platelet Reactivity?

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Abstract

Introduction: Exploration of novel and effective antiplatelet strategies for the secondary prevention of ischemic stroke is utmost. Some platelet derived microparticles (PMVs) in convalescent stroke subjects were found to be predictive for the next vascular event. Patients with high-on-treatment platelet reactivity (HTPR) had a significantly higher risk for ischemic stroke. Here, we aimed to explore associations among circulating microparticles and responsiveness to antiplatelet (clopidogrel) therapy. **Methods:** A total of 18 patients on clopidogrel therapy due to secondary stroke prevention were respectively recruited into this study. Twenty age-matched healthy subjects served as controls. Flow cytometric measurements of microparticles (MPs) and data analysis were performed on Beckman-Coulter FC-500 cytometer with CXP software. Besides, platelet aggregometry data were revealed. Both measurements were performed in whole blood and from the lower and upper blood fractions separated after 1-hour gravity sedimentation by the analogy with erythrocyte sedimentation rate. **Results:** The total number of circulating MPs, and particularly the platelet derived CD42⁺ and PAC-1⁺ were significantly higher in post-stroke patients ($p < 0.001$). The platelet aggregation in the whole blood (area under the curve, AUC) showed a significant negative correlation with the total number of MPs in the lower blood sample after 1-hour gravity sedimentation ($r = -0.650$, $p = 0.005$). Next, we analyzed associations among MPs and aggregometry data obtained from clopidogrel responders and non-responders. Both, area under the curve (AUC) and velocity in the whole blood showed opposite correlation with the total number of MPs in the lower blood sample after 1-hour gravity sedimentation. Importantly, a significant negative correlation was observed for the velocity ($r = -0.801$, $p = 0.005$), but not for the AUC in responders. Platelet derived CD42⁺ and PAC-1⁺ MPs showed positive correlations with neutrophils in the lower blood sample ($p = 0.008$ and $p = 0.006$ respectively). **Conclusions:** Circulating MPs may allow to monitor the response to antiplatelet therapy in post-stroke patients. In addition, the link between platelet derived MPs and neutrophil granulocytes might become therapeutic targets in the future.

Keywords: microvesicles; platelet; neutrophil; ischemic stroke; antiplatelet therapy; clopidogrel

1. Introduction

Stroke is a highly prevalent condition that puts a significant burden on most societies. It is the leading cause of adult disability, the second leading cause of dementia and the fourth leading cause of death worldwide [1]. The prevalence of stroke and stroke-related costs will undoubtedly rise as the ratio of aging population increases worldwide, making prevention and identification of early signs of recurrent ischemic episodes a priority [2]. Peripheral circulating microvesicles (MPs) are small particles (0.1–1.0 micrometer in diameter) derived from the membrane blebs of activated cells, it can be found in synovial fluid, tear, liquor, saliva, urine, breastmilk and in bronchoalveolar lavage as well due to peripheral microcirculation [3]. Circulating platelet MPs (PMVs) are the most abundant type of MPs found in human circulation and they express vari-

ous platelet surface markers such as CD42a, CD42b, CD61, CD62P [4,5]. Microvesicles have multiple biological functions: (i) antigen presentation; (ii) intercellular communication; (iii) immune reaction and (iv) RNA and protein delivery. Thus, they enable intercellular communication by delivering lipids, proteins and genetic material to nearby or distant cells and modulating the functions of these cellular targets [6,7]. There is growing evidence that MPs are playing a pivotal role in regulation of hemostasis, inflammation and angiogenesis [8]. Previous studies suggest that a large increase in circulating platelet derived microvesicles (PMVs) have been observed in patients with cardiovascular disease [9]. Therefore, PMVs might be important biomarkers and tools in the identification of the risk of various recurrent cerebrovascular diseases [10]. Platelet anti-sedimentation rate (PAR) reflects the percentage of platelets



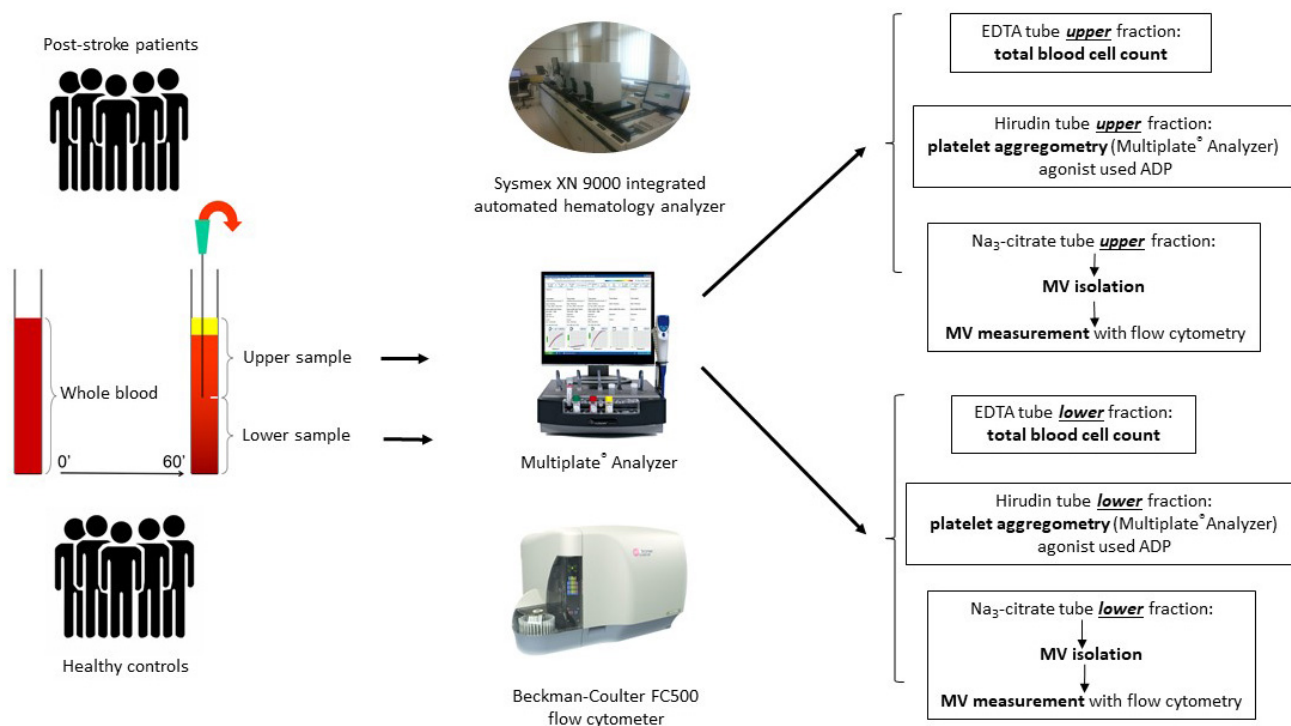


Fig. 1. Chart of sample preparation, separation of the upper and lower blood sample after 1-hour gravity sedimentation and further analytical process.

crossing the midline of the blood column upwards during 1-hour gravity sedimentation [11]. One of our previous studies concluded that the PAR value was able to discriminate clopidogrel non-responders from responders [11]. Activation of neutrophils reflected by neutrophil antisedimentation rate (NAR) proved to be a sensitive predictor of recurrent ischemic cerebral episodes in post-stroke patients on clopidogrel [12].

The aim of this prospective pilot-study was to explore: (i) differences of circulating MVs of different origin, comparing convalescent ischemic stroke patients vs healthy controls; (ii) whether the function of platelets correlate with certain MVs in patients on antiplatelet (clopidogrel) therapy; (iii) which PMVs show association with the high-on-treatment residual platelet reactivity (non-responder state).

2. Methods

2.1 Subjects

The study protocol was approved by the University of Pécs Clinical Centre Regional and Institutional Research Ethics Committee (Ref. number: 6735, Clinical Trial No: NTC03679858). All procedures were performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from both patients and healthy volunteers. A total of 18 patients (median age at 66 years, 12 males) on antiplatelet therapy (75 mg clopidogrel once daily) due to secondary stroke prevention were prospectively recruited into this study. The selected patients with previous anterior circulation large

artery atherothrombosis were on regular medical check-ups at the Outpatient Clinic of the Department of Neurology at the University of Pécs.

2.2 Blood Sampling

Venous blood samples were drawn with 21G needle after short strangulation from an antecubital vein into closed blood collection system tubes with 3.2% (0.109 M) Na₃-citrate, K₃-EDTA (Becton Dickinson, Diagon LTD Hungary) and hirudin (Sarstedt S-Monovette® 1.6 mL Hirudin) as anticoagulant. Upon blood collection the first 3 mL blood was discarded. The blood samples for measurements were transported immediately to laboratory and were processed within 1 hour.

2.3 Neutrophil Count (%), Electric Impedance Aggregometry (Multiplate® Analyzer)

The modified whole blood gravity sedimentation technique was developed for studying platelet and neutrophil sedimentation properties [11]. After a 1-hour gravity sedimentation, the upper and lower half of the venous blood column were separately removed from the EDTA and hirudin containing tubes and transferred into another EDTA and hirudin tube for further analysis (Fig. 1). The total blood cell count and the neutrophil (%) count were measured from the whole blood and after 1-hour gravity sedimentation from the upper and lower part of the blood on a Sysmex XN 9000 integrated automated haematology analyser (Sysmex Co., Kobe, Japan, 2017).

Table 1. The selected CD markers for MV measurement, cellular origin, fluorescent dye and the manufacturer specification.

CD marker	Cellular origin	Fluorescent dye	Manufacturer
CD62P (P selectin)	Platelet	PE	Beckman-Coulter
CD41 (GPIIb/IIIa)	Platelet	Cy5	Beckman-Coulter
CD42a (GPIb/V/IX)	Platelet	FITC	Becton-Dickinson
PAC1 (GPIIb/IIIa, near fibrinogen binding site)	Platelet	FITC	Becton-Dickinson
CD31 (PECAM-1)	Endothelial cell	PE	Becton-Dickinson
Annexin V	Recognize phosphatidyl-serine	FITC, Cy5	Becton-Dickinson
Mouse IgG1	Isotype control	FITC, PE, Cy5	Becton-Dickinson

Abbreviations: Cy5, Cychrome5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; Ig, immunoglobulin.

Platelet function test was performed in the whole blood and after 1 hour of sedimentation from the upper and lower part of the hirudin anticoagulated blood with a Multiplate® Analyzer (Roche Diagnostics, Mannheim, Germany). Platelet aggregometry was uniformly carried out 60 minutes after blood sampling using adenosine diphosphate (ADP; 6.5 M) as agonist [12]. Aggregation level was expressed as the area under the curve (AUC). AUC was calculated by a Multiplate® Analyzer using the product of aggregation unit (AU) × time (minutes). After ADP stimulation the normal aggregation range was expected as AUC: 53-220 — according to the manufacturer. Based on the whole blood AUC, patients on clopidogrel were categorized as responders with AUC <53 and non-responders with AUC ≥53.

2.4 Microvesicles (MVs) Measurement and Sample Preparation

After a one-hour sedimentation the upper and lower part of the citrated blood were centrifuged at $2500 \times g$ for 20 minutes at room temperature. The supernatant was transferred into a new test tube and centrifuged at $2500 \times g$ for further 20 minutes to obtain cell-free plasma. The top of the cell-free plasma was transferred into an Eppendorf tube and was immediately frozen on liquid nitrogen and stored at -80°C until further use. MVs measurement was described in our previous article [13]. Briefly, the samples for measurement were thawed on melting ice and pelleted at $18000 \times g$ for 10 minutes. The supernatant was carefully removed, leaving 25 μL of MV rich plasma at the bottom of the Eppendorf tube. MVs were suspended with gentle vortexing for 20 seconds in 1.0 mL Apo-binding buffer (10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L MgCl_2 , 136 mmol/L NaCl, pH = 7.4 HEPES was obtained from Sigma-Aldrich Ltd., Budapest, Hungary, other analytical grade reagents were obtained from Reanal Ltd., Budapest, Hungary) without CaCl_2 . The selected CD markers, their cellular origin, the fluorescent dye used for labelling and the manufacturer specification for our MV measurements are summarized in Table 1. For sample labelling 10 μL MV in Ca^{2+} free buffer was incubated in 100 μL Apo-binding buffer supplemented with 2.5 mmol/L CaCl_2 with a total of 10 μL antibody, previously diluted to optimal labelling concentration. Staining

was incubated for 30 minutes at room temperature in a dark chamber. All buffers were filtered through 0.2 μm membrane filters.

Flow cytometric measurements and data analysis were performed on a Beckman-Coulter FC-500 cytometer with CXP software (Version 2.3. Beckman Coulter Life Sciences, Indianapolis). The MV's reference gate was defined with Megamix beads (Biocytex). Side scatter, forward scatter and fluorescence channels were set in a logarithmic scale. MV size gate was determined between 0.5 μm and 1.0 μm size range. Events in the MV gate were further discriminated by labelling with Annexin [14]. MVs were defined as Annexin V positive events in the MV gate with fluorescence intensity above the isotype control. For determination of the MV number, known concentration ($1 \times 10^6/\text{mL}$) of 3 μm diameter microbeads (Becton Dickinson) were used. To determine the optimal labelling concentrations all antibodies and Annexin V were titrated. Labelling concentrations were defined by antibody staining of samples and sample-free buffers in the presence or absence of CaCl_2 . Labelling was considered optimal if CaCl_2 labelled sample measurement events were clearly distinguishable from background, CaCl_2 free staining, as well as from isotype controls.

2.5 Statistical Analysis

Statistical analysis was performed using SPSS version 23.0 (IBM Corporation, Armonk, NY, USA). Summary statistics of the participants were constructed using frequencies and proportions for categorical data and as mean and standard deviation (SD) for continuous variables. Conformity of data to normal distribution was determined by histogram and Kolmogorov-Smirnov test. The between-group difference was calculated with χ^2 , Fisher's exact and Mann-Whitney U tests in line with suitability. Data with nonparametric distribution were presented as median and interquartile range (IQR). Correlations of microvesicle counts with aggregometry data (area under the curve, AUC; velocity of the slope of aggregation) were tested by linear regression using Spearman correlation coefficient (Rho). The significance level was considered as $p < 0.05$.

Table 2. Demography and baseline laboratory parameters.

Observed parameters	Post-stroke patients (n = 18)			Healthy controls (n = 20)	p-value
Age	66 (60–70)			57 (49–63)	0.078
Male/Female	2/6			10/10	0.298
MVs ($\times 10^5/\text{mL}$)	whole blood	upper sample	lower sample	whole blood	
Total MVs	3.43 (2.34–4.70)	1.79 (1.37–2.82)	1.53 (0.96–1.89)	0.22 (0.13–0.37)	<0.001
CD31 ⁺	0.43 (0.12–0.55)	0.25 (0.10–0.46)	0.07 (0.03–0.155)	0.08 (0.04–0.16)	0.016
CD42a ⁺	0.21 (0.09–0.48)	0.13 (0.07–0.32)	0.05 (0.02–0.10)	0.02 (0.01–0.03)	<0.001
CD41 ⁺	0.25 (0.13–0.56)	0.15 (0.10–0.53)	0.04 (0.03–0.10)	0.15 (0.09–0.25)	0.251
CD62P ⁺	0.48 (0.09–0.85)	0.26 (0.07–0.59)	0.15 (0.03–0.24)	0.17 (0.09–0.33)	0.105
PAC-1 ⁺	0.009 (0.009–0.03)	0.008 (0.007–0.02)	0.003 (0.002–0.007)	0.01 (0.009–0.02)	0.515

MVs, microvesicles ($\times 10^5/\text{mL}$); CD31, endothel derived microvesicle; CD42a, glycoprotein Ib-V-IX complex on platelets; CD41, glycoprotein IIb/IIIa integrin on platelets; PAC-1, fibrinogen binding site after activation of glycoprotein IIb-IIIa complex on platelets. Data are presented as absolute values or median (25th–75th percentiles), p-values indicate comparison of MVs in the whole blood.

3. Results

3.1 Demography and Baseline MV Values

A total of 18 convalescent ischemic stroke patients on clopidogrel (all patients suffered from large vessel occlusion) and 20 age-matched healthy subjects were recruited into this study prospectively. Demography of patients and healthy controls and baseline laboratory parameters of the study population are summarized in Table 2. There was no significant difference in regard to age and gender. The total number of circulating microvesicles ($p < 0.001$), and particularly the endothelial-derived CD31⁺ ($p = 0.016$) and platelet derived CD42a⁺ ($p < 0.001$) MVs measured in the whole blood were significantly higher in post-stroke patients compared to healthy subjects. Interestingly, CD62P⁺ (P-selectin) positive MVs showed no significant difference between groups. All significant MV data from Table 2 are presented as Fig. 2.

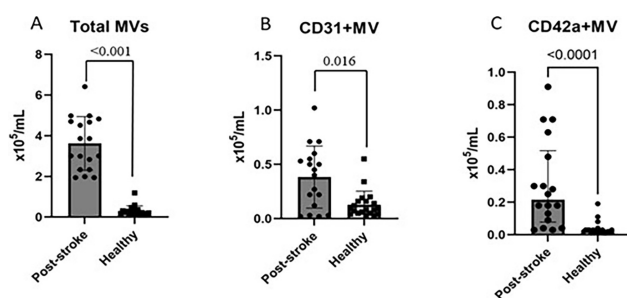


Fig. 2. Comparison of the total count of MVs (2A), the CD31⁺ MVs (2B) and the CD42a⁺ MVs (2C) in the whole blood of post-stroke patients and healthy subjects (Mann-Whitney U test).

3.2 Associations between MVs and Aggregometry Data

We analysed the correlation between MVs and aggregometry data. The platelet aggregation in the whole blood

(area under the curve, AUC) measured by Multiplate® in patients taking clopidogrel, but not in age-matched healthy controls, showed a significant negative correlation with the total number of MVs ($\times 10^5/\text{mL}$) in the lower blood sample after 1-hour gravity sedimentation ($r = -0.650$, $p = 0.005$; Fig. 3). Nevertheless, we did not observe any correlation between the AUC and the total number of MVs in the whole blood.

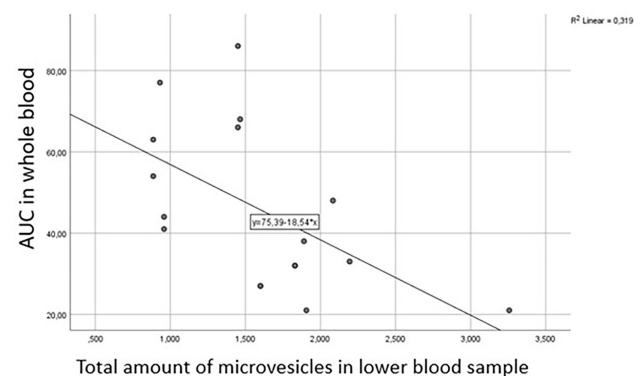


Fig. 3. Correlation between the area under the curve (AUC) in whole blood measured by Multiplate® and total number of microvesicles ($\times 10^5/\text{mL}$) in the lower blood sample after 1-hour gravity sedimentation in patients (Spearman correlation, $p = 0.005$).

3.3 Associations between MVs and Clopidogrel Responsiveness in Patients

Next, we explored the potential associations between MVs and aggregometry data (area under the curve, AUC and velocity respectively) obtained from clopidogrel responders and non-responders based on the previously defined cut-off value (AUC: 53). Both the AUC and the velocity in the whole blood showed negative correlation with the total number of MVs ($\times 10^5/\text{mL}$) in the lower blood sample

after 1-hour gravity sedimentation. Importantly, a significant negative correlation was observed for the velocity ($r = -0.801$, $p = 0.005$), but not for the AUC in responders ($n = 11$) (Fig. 4).

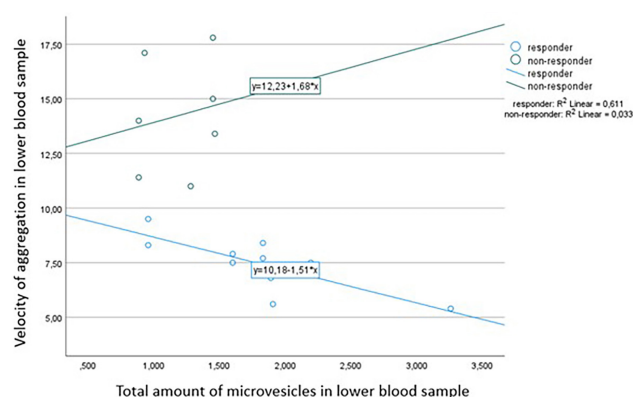


Fig. 4. Correlation between velocity measured by Multiplate® aggregometry and total number of microvesicles ($\times 10^5/\text{mL}$) in the lower blood sample in clopidogrel responders and non-responders respectively. Blue line indicates a negative correlation in responders (Spearman correlation, $r = -0.801$, $p = 0.005$).

3.4 Associations between MVs and Neutrophils in Patients

Activation-induced conformational epitope on CD41/CD61 complex positive (PAC-1⁺) MVs in the lower blood sample showed a significant positive correlation with the percentage of neutrophil granulocytes in the lower blood sample after 1-hour gravity sedimentation ($r = 0.634$, $p = 0.008$; Fig. 5). In contrast, this positive correlation disappeared when whole blood indices were analysed. Furthermore, the constitutive platelet marker (CD42a⁺) positive MVs measured in the upper blood fraction showed a significant correlation with the percentage of the neutrophils in the lower blood sample ($r = 0.652$, $p = 0.006$; Fig. 6). Nevertheless, no significant correlation was found in the whole blood samples.

4. Discussion

In accordance with a recently published meta-analysis [15], we found that the total number of circulating microvesicles, endothelial-derived CD31⁺ and platelet derived CD42a⁺ microvesicles were significantly higher in convalescent post-stroke patients when compared to age-matched healthy controls. Wang *et al.* [15] observed that pooled concentrations of total MVs (TMVs), endothelial-derived MVs (EMPs), platelet-derived MVs (PMVs), leukocyte-derived MVs (LMVs) and monocyte-derived MVs (MMVs) were significantly increased in ischemic stroke patients compared to the non-cerebrovascular disease controls [15].

We presumed that the quality (origin) and number of circulating microvesicles might affect the response to clopi-

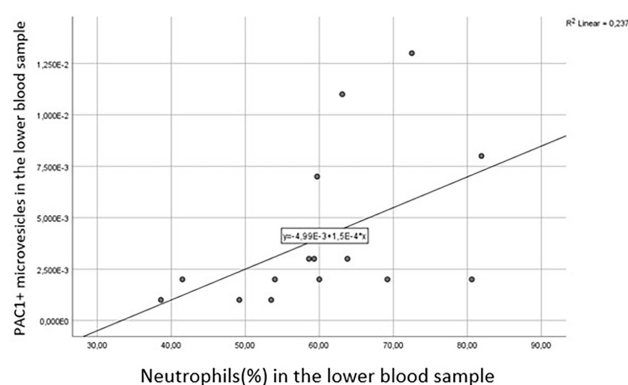


Fig. 5. Correlation between platelet derived PAC1⁺ MVs ($\times 10^5/\text{mL}$) and neutrophils (%) in the lower blood sample (Spearman correlation, $p = 0.008$).

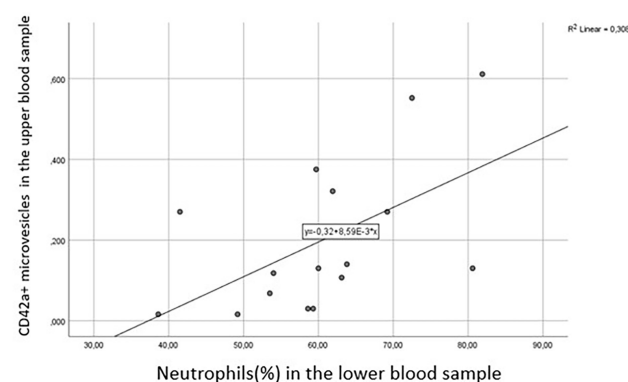


Fig. 6. Correlation between platelet derived CD42a⁺ MV number ($\times 10^5/\text{mL}$) measured in the upper blood sample and neutrophils (%) in the lower blood sample (Spearman correlation, $p = 0.006$).

dogrel in post-stroke patients. Although we did not observe any correlation between the platelet aggregometry reflected by AUC and the total number of MVs in the whole blood of post-stroke patients, we discovered a negative correlation between AUC_{whole blood} and the total number of MVs in the lower blood sample after 1-hour gravity sedimentation. Considering that the majority of the total MVs derived from platelets (PMVs) and the differently activated platelet clusters were previously identified based on their motion during 1-hour gravity sedimentation by the analogy of ESR, it seems plausible that sinking platelets and their ‘dust’ (PMVs) could affect the clopidogrel responsiveness. Supporting our hypothesis, Kafian *et al.* [16] described elevated levels of circulating PMVs in patients with HTPR during clopidogrel treatment, indicating ongoing platelet activation despite the antiplatelet therapy.

Interestingly, Rosinska *et al.* [17] revealed no relationship between circulating microvesicle number and platelet aggregation in post-stroke patients on aspirin (ASA), suggesting that residual platelet reactivity is not af-

ected by MVs in the presence of ASA. Nevertheless, elevated concentrations of PAC-1⁺/CD61⁺, CD62P⁺/CD61⁺ and CD31⁺/CD61⁺ microvesicles were found in acute stroke patients with treatment failure [17]. Accordingly, we observed negative correlation between the velocity of platelet aggregation and total MV count measured in the lower blood sample after 1-hour gravity sedimentation, suggesting that this sample separation technique might be suitable for discrimination of clopidogrel responders from non-responders. Moreover, in a recent study high levels of MVs with different origins were found to be linked to stroke severity and prognosis [18]. It should be noticed that patients with acute ischemic stroke were not investigated in our study. However, based on these preliminary results the advantage of the blood sedimentation technique may provide deeper insights into the behaviour of blood cell components in the acute phase of ischemic stroke. Considering the poor patient outcome despite successful recanalization, besides other factors (e.g., optimal perfusion of the ischemic penumbra), a personalized antiplatelet treatment strategy is vital. Therefore, extensive translational research will be needed in this field.

Another important aspect that arises in connection with platelets and PMVs is their role in immune processes. Importantly, we observed positive correlations between PAC1⁺, CD42a⁺ PMVs respectively and the percentage of neutrophil granulocytes in the lower blood sample after 1-hour gravity sedimentation indicating the relationship between the procoagulant potential of platelet derived MVs and the thromboinflammatory cascade [19]. Our data was supported by Michelson *et al.* [20], who identified platelet–neutrophil complexes as markers for platelet activation [20]. An increasing number of animal and human studies [21–24] recognise that neutrophils can contribute to venous and arterial thrombosis or ‘immunothrombosis’ due to hypercoagulability via the release of neutrophil extracellular traps (NETs) as a procoagulant surface in a septic state as well as in chronic vascular disorders.

Limitations of our study are the following: (i) small sample size; (ii) variance in time elapsed between the index event and the blood sampling; (iii) only patients taking clopidogrel were recruited, but other antiplatelet agents would be worth investigating in the future.

5. Conclusions

Based on our findings, the identification and quantification of circulating MVs may allow us to monitor the response to antiplatelet therapy with P2Y₁₂ antagonists (e.g., clopidogrel), providing a novel opportunity to identify non-responder patients thus allowing an individually tailored antiplatelet strategy. Besides, links among platelet derived MVs, particularly CD42a⁺ and PAC-1⁺, and other players in the thrombotic cascade, such as neutrophil granulocytes, might become therapeutic targets in the future.

Author Contributions

DS, EE and TM conceived, designed and coordinated the study, participated in acquisition, and interpretation of data. DS drafted the manuscript. MT-F performed the laboratory measurements. MT-F and TM participated in the statistical analysis. AM, TM took part in manuscript revision. All authors read and approved the manuscript.

Ethics Approval and Consent to Participate

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by Local Ethics Committee of the University of Pécs (Ref. number: 6735, Clinical Trial No: NTC03679858). Informed consent was obtained from all subjects involved in the study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2705158>.

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