Quantitative and qualitative evaluation of platelets-derived microvesicles

MARIA WĄSIK[1], EWELINA KAWKA[1], ELŻBIETA GÓRSKA[1], BEATA WALASZKIEWICZ-MAJEWSKA[2]

[1]Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Warsaw, Poland
[2]Public Children' Clinical Hospital, Warsaw, Poland

Abstract

Upon activation platelets shed membrane components containing certain membrane receptors and other proteins inherited from parental cells. These components are called platelets-derived microvesicles (PMVs). We present flow cytometric assay for immunophenotyping of PMVs in platelet rich plasma concentration of patients without signs of inflammation and with significant abnormalities of blood clottinig and in increased C-reactive protein (CRP) concentration. The assay has been used to study PMVs counts in non-stimulated samples of isolated platelets and short time after thrombin stimulation. In both studied groups the number of PMVs was decreased after thrombin stimulation. This phenomenon was probably dependent on procoagulative thrombin properties and was clearly seen in control group. In both studied group thrombin-stimulated platelets delivered more PMVs expressing activation marker CD62P but only in control group under influence of stimulation the number of CD41+ CD62P+ An+ PMVs increased. Obtained results suggest that increased CRP concentration accompanying inflammation decreased free PMVs deliveration as well as changed platelets reaction to thrombin stimulation.

Key words: *platelets microvesicles, activation, CD41, CD62P, phosphatidyloserine.*

(Centr Eur J Immunol 2011; 36 (3): 163-169)

Introduction

Platelets are terminally differentiated cells unable to undergo cellular division. After they are released from cytoplasm of megakariocytes to blood-stream it is possible to identify them in peripheral blood by assessing membrane glycoprotein receptors such as GPIIb (CD41), GPIbα (CD42), GPIIIa (CD61) and others [1, 2]. These platelet receptors recognize proteins expressed on the surface of monocytes, lymphocytes, neutrophils as well as activated endothelial cells [3-5]. Platelets are able to adhere to the vessel wall at the site of its injury where they become activated. The main mechanism of their activation include bridging of GpIb platelet receptor with circulating von Willebrand factor (vWF) connected to the collagen at the site of subendothelium lesion. Specific platelets receptors GPIIb (CD41)/GPIIIa (CD61) recognize fibrinogen and in consequence induce platelets aggregation and thrombus formation [6, 7]. Activation process also induces translocation of molecules from intracellular compartments to the membrane surface. P-selectin (CD62P) found in α-granules of resting platelets is expressed on the surface of activated cells. α-granule mambrane protein – GMP-33, CD63 (lysosomal protein) and CD41L (protein involved in the interaction between platelets and leukocytes) may also serve as indicators of platelets activation [3]. One of the most important changes induced by the platelet activation is a translocation of negatively charged phospholipids from the inner to the outer surface of the cell membrane [6-9].

Externalization of phosphatidylserine (PS) is associated with many physiologic and pathologic phenomena. It was demonstrated that membrane expression of PS is one of the first indicators of apoptosis [10]. Recently, it was documented that exposure of PS on cell the surface amplificate thrombus formation because PS serve as a receptor for X(a), XI(a) and VIII(a) factors [11]. Reorganization of membrane lipid asymmetry is accompanied by blebbing and shedding of microvesicles (MV) from the cell surface. Microvesicles exhibit cell surface markers that indicate their cellular origin. Microvesicles released from platelets (platelets-derived microvesicles – PMVs) promote clot formation, mediate pro-inflammatory processes, enable cell-to-cell interactions and cell signaling by transfering proteins and mRNA from one cell to another. In addition, they

Correspondence: Maria Wąsik, Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Marszałkowska 24, 00-576 Warsaw, Poland, phone number: 608 583 240, e-mail: mariawasik@aster.pl

may also express of cellular activation markers [12,13]. Elevated levels of circulating PMVs are associated with various vascular pathologies and their pathogenic potential has been widely documented. PMVs have been analyzed in plasma and cell cultures by means of flow cytometry or solid phase assays [10,11].

Here we present flow cytometric assay for immunophenotyping of PMVs in platelet rich plasma concentration of patients without signs of inflammation and with significant abnormalities of blood clottinig and in increased C-reactive protein (CRP) concentration. The assay has been used to study PMVs counts in non-stimulated samples of isolated platelets and short time after thrombin stimulation. We suggest that our version of this assay can be used for MVs analysis in experimental cell culture and as a diagnostic test to resolve some clinical problems.

Material and methods

The study was performed in a group of 20 patients treated in Clinical Children's Hospital. Routine blood coagulation tests, blood count, CRP and other basic biochemical tests ordered by a physician were performed in the group of children included to the study. Biochemical examinations were performed in the blood serum. For coagulation tests samples of venous blood were taken into sodium citrate (final concentration 0, 105 M). After examination of clotting parameters the remaining blood was used for the assessment of PMV release. Blood samples obtained from 10 children with normal results of all tests served as a control (group I).

Ten children (6 with nephritic syndrome and 4 after cALL treatment) with at least one abnormal parameters of coagulation system or increased CRP concentration were recognized as group of children with hallmark of inflammation and/or disturbances in coagulation system (group II). These children were study at the day of hospital admission. All children included to the study non-received anticoagulant and thrombolytic therapy or other anti-platelet medication. Age distribution in the both groups was comparable: 1/12 to 17 years with median of 5 years (age range 3-10) in the group I and median of 5 years (age range 4-6) in the group II. Each group included 5 boys and 5 girls. Platelet counts and mean platelet volume were measured with automated blood analyzer (LH 750 Beckman & Coulter). All determinations of blood clotting parameters were performed with fully automated coagulometer BCS-XP (Muenchen, Germany). Flow cytometry (Cytomix FC500, Beckman & Coulter Co, USA) was used for determination of PMVs after staining with AnnexineV and monoclonal antibodies against CD41 and CD62P conjugated with PC-7 and PE, respectively. All reagents used for flow cytometry were manufactures by Beckman & Coulter.

Platelet-rich plasma was collected as described earlier [14]. Number of platelets was adjusted to the concentration of 10×10^6/ml in the buffered medium. Aliquots of platelet suspension were added into 3 small tubes. One tube served as a non-stimulated control while the next tubes were supplemented with bovine thrombine (Thrombinum bovine, Biomed, Poland) as a stimulator (1 U and 2 U respectively). After 1 hour incubation in 37°C the tubes were centrifugated at 3200 g for 20 min. Supernatans were centrifugated once again for 2 min at 13 000 g. After the last centrifugation supernatants were cell-free as was proven by microscopic examination. To each 30 µl of samples of the supernatants 2 µl of monoclonal antibodies and 2 µl of annexin V were added. For each experiment isotypic control was set by the addition of 2 µl of mouse IgG1-FITC, IgG1-PE and IgG1-PC7 to the platelet samples. Next, 26 µl of buffer (Pharmingen) was added and samples were incubated for 20 min in room temperature without access to the light. After the incubation 1000 µl of the buffer

Table 1. Results of laboratory tests in two groups of children at the day of platelets-derived microvesicles examination

Test	Group I		Group II		Average of reference values
	Mean ±SD *n* = 10	Min.-Max. result	Mean ±SD *n* = 10	Min.-Max. result	
CRP	0.54 ±0.07	0.5-0.7	4.5 ±6.52	0.5-17.6	> 1
Prothrombin (INR)	1.1 ±0.11	0.9-1.24	1.03 ±1.16	0.8-1.17	0.9-1.25
Activated partial thromboplastn time (aPTT) (s)	34.4 ±2.88	27.3-35.95	34.5 ±9.71	20.8-45	28-40
Fibrinogen (g/l)	3.1 ±1.12	1.18-3.38	3.7 ±2.36	1.5-6.22	2-5
D-Dimer Ug/l FEU	n.d.		2118 ±1042	559-3142	170-550
Anti-thrombin III (ATIII)%	n.d.		92.8 ±46.1	46.1-92.8	60-125

Group I – children without inflammation and clotting disturbances, group II – children with inflammatory reaction and increased concentration of D-dimer and other clotting disorders
n.d. – not done; results presented as the mean ±SD

diluted 1 : 10 was added and 30 µl FluorosferFlow-Set (Beckman & Coulter Co.) were added. Every tube was mixed using minishaker and cytometric analysis was performed. Platelets-derived microvesicle were gated according to their size compared with 3 µm fluorescent beads.

Statistical analysis was performed using Statistica PL software. All data without gaussian distribution are presented as median. For assessing statistically differences between compared groups Mann-Whitney U-test was used. Differences were considered as statistically significant when *p*-value was equal or lower than 0.05. For analysis dependency between CRP, D-dimer blood concentration and number of PMVs delivered non-parametric tau-Kendall's test was used.

Results

Results of the performed blood tests that were basis of patients' categorization to group I and group II are shown in Table 1. In platelets rich plasma PMVs were identified by their characteristic light scatter (Fig. 1). As shown on Fig. 1, the molecules 10-times smaller than fluorospheres (gate S, channel 200 vs. 20), expressed antigens characteristic for platelets (Fig. 2) what give evidence that they are released from platelets. Moreover, a few CD62P positive PMVs demonstrate changes of surface membrane asymmetry (Fig. 3). The numbers of PMVs in plasma of group I and group II without additional treatment and after 1 hour of incubation in medium supplemented with 1 U and 2 U of bovine thrombin are presented in Table 2. In children with normal blood clotting and normal range of CRP (group I) numbers of PMVs significantly decreased after 1 hour of thrombin administration. In contrast with group I this thrombin effect was dose independent and was not observed in group II. In group II decreased of PMVs was observed only in medium supplemented with 1 U of thrombin. In medium supplemented with 2 U of thrombin the mean number of PMVs returned to the benchmark (Table 2). Only in group II between number of PMVs CD41+ and CRP

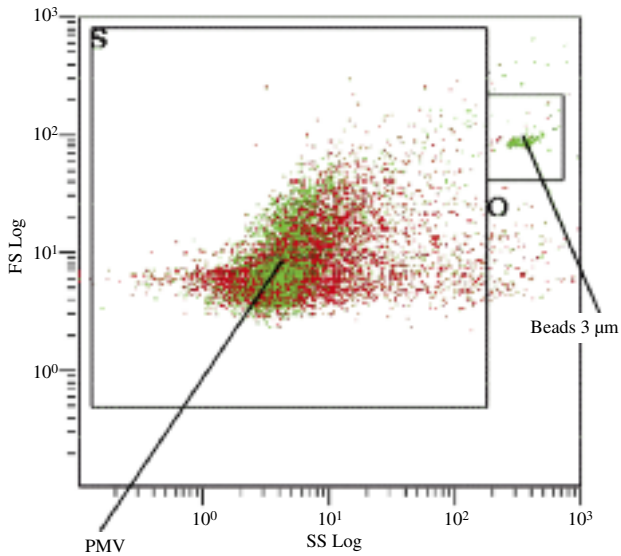

Fig. 1. Platelets microvesicles (PMVs) size distribution. Cytogram forward scater vs. side scater. In gate 0 with the mean channel 200 are located beads (3 µm). Gate S is the region with PMVs

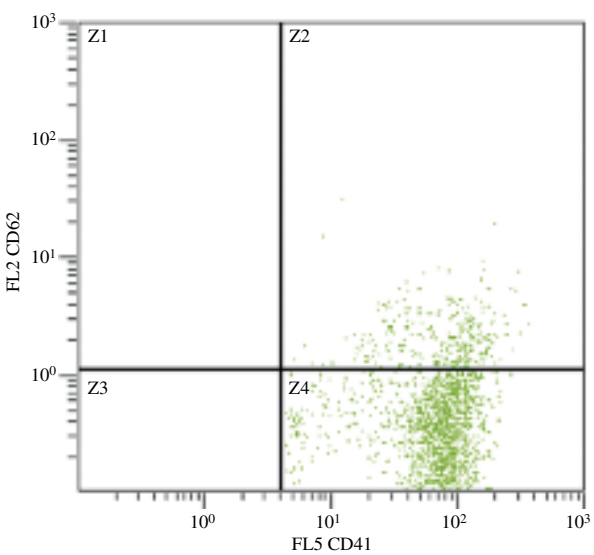

Fig. 2. Dual fluorescence analysis of PMVs (from gate S – Fig. 1) stained with monoclonal antibodies anti-CD41 and anti-CD62P. In the area Z2 are double positive PMVs

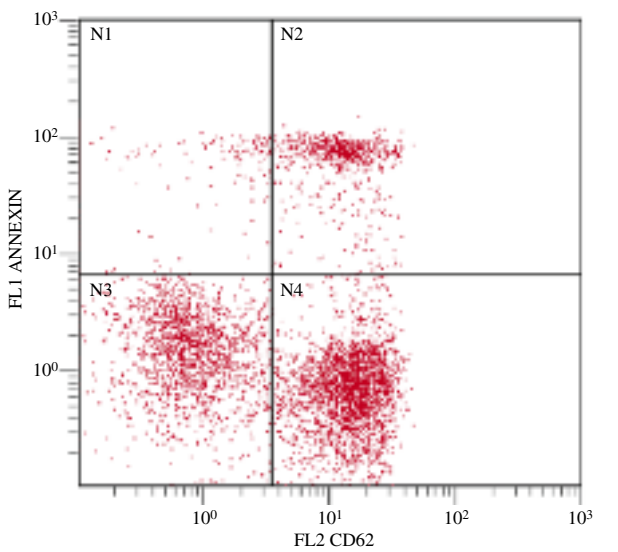

Fig. 3. Dual fluorescence analysis of PMVs (from gate S – Fig. 1) stained with monoclonal antibodies anti-CD62P and annexin V. In the area N2 are located PMVs with expression of CD62P and phosphatidyloserine (annexin positive)

Table 2. Comparison of number derived PMVs in nonstimulated and thrombin stimulated platelet rich plasma

Number of PMVs in group I				Number of PMVs in group II		
	Plasma medium	**Plasma 1 U thrombin**	**Plasma 2 U thrombin**	**Plasma**	**Plasma 1 U thrombin**	**Plasma 2 U thrombin**
Mediana	1738.0	1084.0	473.0	499.0	227.0	443.0
Q1	911.0	319.8	211.7	296.5	111.0	166.6
Q2	2639.0	1769.8	1207.5	1424.7	728.0	864.5
p		0.03		0.02	NS	NS

Group I – children without inflammation and clotting disturbances, group II – children with inflammatory reaction and increased concentration of D-dimer and other clotting disorders
NS. – differences non significant; results are presented as the median with quartile range
P-value calculated with Mann-Whitney U-test

blood concentration reversed correlation with $r = –0.96$ and $p < 0.001$ was found (Fig. 4). In this group D-dimer concentration had not significantly influence on number of PMVs (data not shown).

However, the comparison of the percentages of PMVs expressing CD41 and CD62P before and after thrombin treatment showed significant increase of positive PMVs in both groups of children (Figs. 5 and 6). As presented on Figures 5 and 6 increasing expression of phosphatidyloserine on the surface of PMVs after treatment of the platelets with thrombin was observed only in group I. Differences of the ability of platelets to release PMVs in the studied groups is illustrated on Figure 7. Untreated plasma of children in group II contained significantly higher percentages of CD41+ and Annexin positive PMVs in comparison to children in control group (Fig. 7A). Similarly, in the group of children with abnormal blood clotting the platelets after thrombin treatment produced higher per-

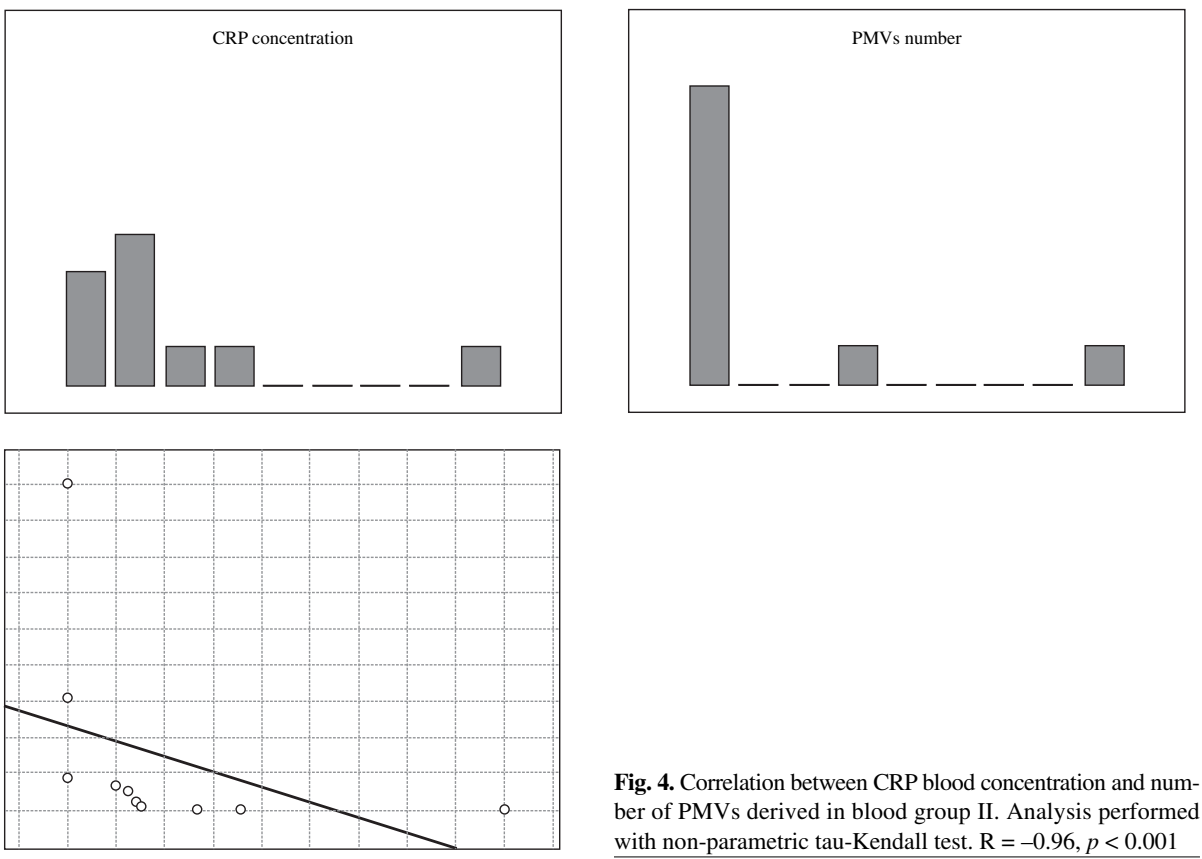

Fig. 4. Correlation between CRP blood concentration and number of PMVs derived in blood group II. Analysis performed with non-parametric tau-Kendall test. R = –0.96, $p < 0.001$

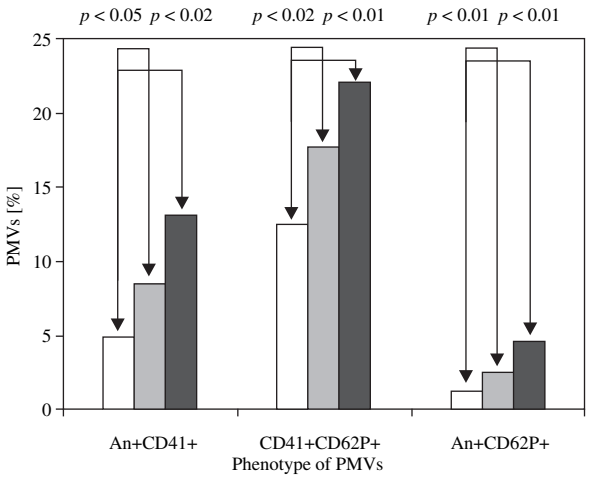

Fig. 5. Comparison of percentage of PMVs exposing CD62P and phosphatidyloserine (An+) together with CD41 released from peripheral blood platelets of 10 patients without signs of inflamation and disturbances of blood clotting (group I)

Plateletes incubated 1 hour in control medium (white bars) and medium suplemented with 1 U and 2 U of bovine thrombin (gray and black bars respectively). P-value calculated with Mann-Whitney U-test

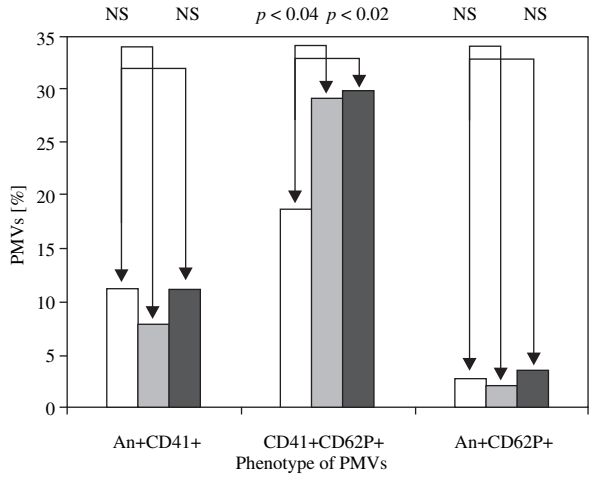

Fig. 6. Comparison of percentage of PMVs exposing CD62P and phosphatidyloserine (An+) together with CD41 released from peripheral blood platelets of 10 patients with signs of inflammation and abnormal results of blood clotting (group II)

Plateletes incubated 1 hour in control medium (white bars) and medium suplemented with 1 U and 2 U of bovine thrombin (gray and black bars respectively). P-value calculated with Mann-Whitney U-test

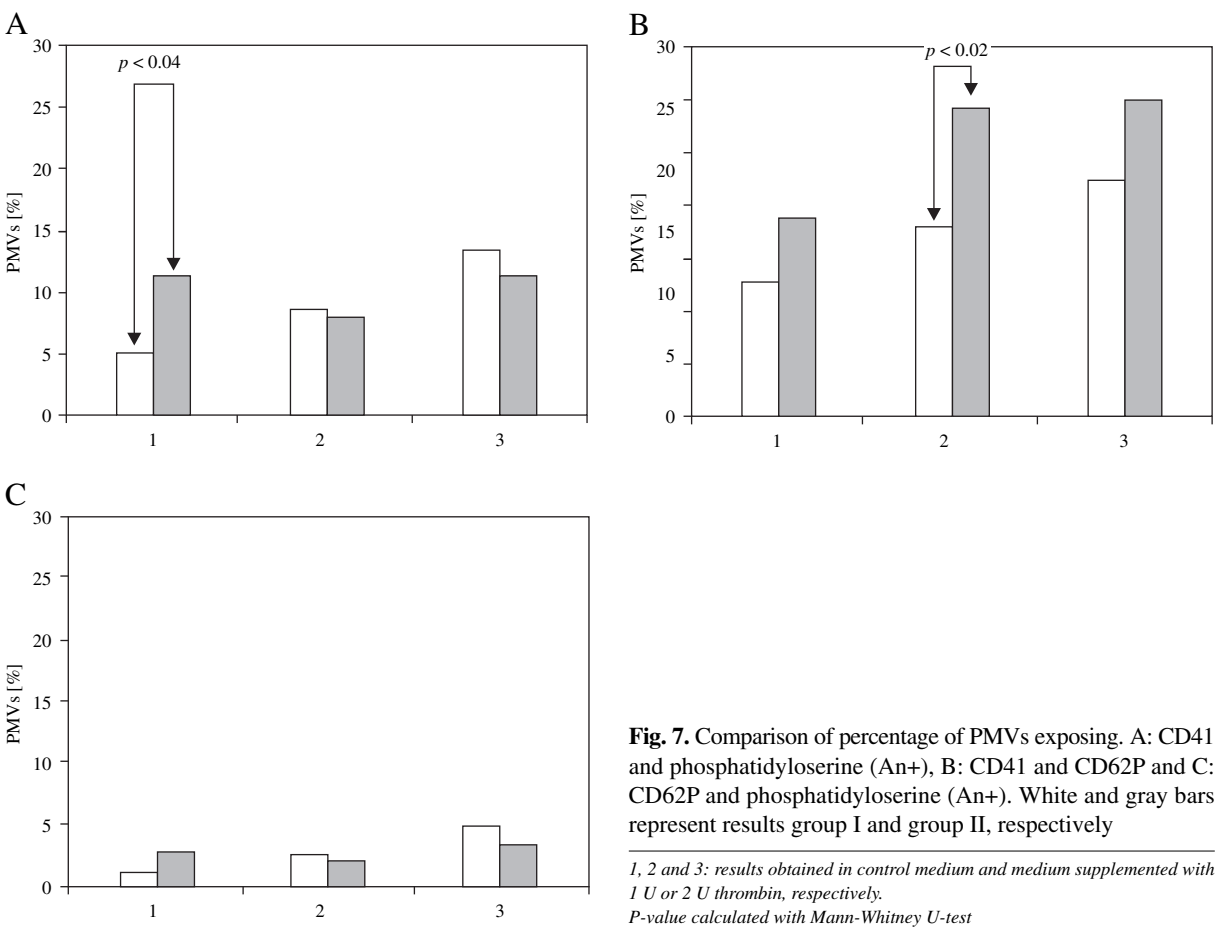

Fig. 7. Comparison of percentage of PMVs exposing. A: CD41 and phosphatidyloserine (An+), B: CD41 and CD62P and C: CD62P and phosphatidyloserine (An+). White and gray bars represent results group I and group II, respectively

1, 2 and 3: results obtained in control medium and medium supplemented with 1 U or 2 U thrombin, respectively.
P-value calculated with Mann-Whitney U-test

centage of CD62P positive PMVs than in control group (Fig. 7B). As presented on Figure 7C thrombin treatment did not influence expression of phosphatidyloserine on CD62P positive PMVs.

Discussion

Although many years passed since the first description of PMVs as a precipitable factor present in platelet free plasma or as "platelets dust" containing lipid rich particles there are different isolation protocols available in the literature [14-17]. Most investigators apply cell free plasma centrifugation with subsequent flow cytometric analysis. Flow cytometer enables measuring of count, size and membrane expression of characteristic proteins on the surface of PMVs. These proteins allow to distinguish cellular origin of MVs by interaction with specific antibodies. Using a blend of size calibrated fluorescent beads we obtained similar scatter distribution (Fig. 1) as was shown by Robert *et al.* [15]. The microparticles in gate S (Fig. 1) revealed positive reaction with annexin V (Fig. 3) and with monoclonal antibodies directed against CD41 and CD62P (Fig. 2).

It was noted that microparticles with membrane expression of CD41 are derived from megakariocytes rather than from platelets. In group II the number of PMVs CD41+ reversed correlated ($r = –0.96$, $p < 0.001$) with CRP concentration but not with level of D-dimer. But CD41 positive microparticles released from megakariocytes are CD62P negative [10]. It means, that all CD41+CD62P+ microparticles that were subject of our analysis were derived from platelets. Low number of annexin V positive PMVs observed in our study is in accordance with the observation made by Connor *et al.* [18]. They found that 80% of platelets-derived microparticles fail to bind annexin V. As is shown on Figures 4 and 6 the percentage of annexin V positive PMVs is very low in both studied groups. In non-stimulated platelets-rich plasma a significantly higher number of annexin V positive PMVs were released in samples taken from children with hallmark of inflammation than in control group (Fig. 6A); however, total number of PMVs was more than 3 times lower (Table 2). Inflammation process and the synthesis of pro-inflammatory cytokines activate platelets and PMVs delivery. The activation predisposes platelets and PMVs to adhesion and interaction with endothelial cells, matrix molecules as well as monocytes and neutrophils [6, 8, 10, 19, 20]. It may be a reason of lower number of free PMVs observed in plasma of children with increased concentration CRP. Presented results suggest that higher blood concentration of CRP with increased concentration of pro-inflammatory cytokines and fibrinogen decreased free PMVs deliveration as well as changed platelets reaction to thrombin stimulation.

In both studied groups the number of PMVs was decreased after stimulation with 1 U of thrombin. This phenomenon was probably dependent on procoagulative thrombin properties and was clearly seen in control group. In both studied group thrombin-stimulated platelets delivered more PMVs expressing activation marker CD62P but only in control group under influence of stimulation the number of CD41+ CD62P+ An+ PMVs increased (Figs. 5 and 6). This phenotype favors the appearance of dispersed haemostatic complications because phosphatidyloserine exposed on outer side of the cell membrane bind factor Xa-Va complex [4, 5, 7]. Moreover, PMVs may transfer specific platelets antigen CD41/CD61 into other cells [10]. In case of diseases with immunological disturbances these cells serve as targets for anti-platelet antibodies. Therefore the measurement of both number and phenotype, determined by flow cytometry, open a new space for diagnostics which may explain pathogenesis of hematological and immunological diseases.

References

1. Metcalfe P, Watkins NA, Ouwehand WH, et al. (2003): Nomonclature of human platelet antigens. Vox Sanq 85: 240-245.
2. Patel SR, Hartwig JH, Italiano JE Jr. (2005): The biogenesis of platelets from megakariocyte proplatelets. J Clin Invest 115: 3348-3354.
3. Lösche W, Heptinstall S (2007): Value of platelet activation markers as prothrombotic risk indicators. Transfusion Med Hemother 34: 34-42.
4. Oberle V, Fischer A, Setzer F, Lösche W (2007): Thrombous formation without platelets under inflammatory condition: an *in vitro* study. Platelets 18: 143-149.
5. Müller I, Klocke A, Alex M, et al. (2003): Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets. FASEB J 17: 476-478.
6. Perez-Pujol S, Marker PH, Key NS (2007): Platelet microparticles are heterogeneous and highly dependent on the activation mechanism: studies using a new digital flow cytometer. Cytometry A 71: 38-45.
7. van der Zee PM, Biró E, Ko Y, et al. (2006): P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infraction. Clin Chem 52: 657-664.
8. Kamath S, Blann AD, Lip GY (2001): Platelet activation: assessment and quantification. Eur Heart J 22: 1561-1571.
9. Benameur T, Andriantsitohaina R, Martínez MC (2009): Therapeutic potential of plasma membrane-derived microparticles. Pharmacol Rep 61: 47-57.
10. György B, Szabó TG, Pásztói M, et al. (2011): Membrane vesicles, current state-of-the art: emerging role of extracellular vesicles. Cell Mol Life Sci 68: 2667-2688.
11. Zwaal RF, Schroit AJ (1997): Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 89: 1121-1132.
12. Majka M, Kijowski J, Lesko E, et al. (2007): Evidence that platelet-derived microvesicles may transfer platelet-specific immunoreactive antigens to the surface of endothelial cells and CD34+ hematopoietic stem/progenitor cells implication for the pathogenesis of immune thrombocytopenias. Folia Histochem Cytobiol 45: 27-32.

13. Yuan A, Farber EL, Rapoport AL (2009): Transfer of microRNAs by embryonic stem cell microvesicles. PLoS One 4: e4722.
14. Shah MD, Bergeron AL, Dong JF, López JA (2008): Flow cytometric measurement of microparticles: pitfalls and protocol modifications. Platelets 19: 365-372.
15. Robert S, Poncelet P, Lacroix R, et al. (2009): Standarization of platetet-drived microparticle counting using calibrated beads and a cytomics FC500 routine flow cytometer: a first step towards multicenter studies? J Thromb Haemost 7: 190-197.
16. Nantakomol D, Chimma P, Day NP, et al. (2008): Quantitation of cell-derived microparticles in plasma using flow rate based calibration. Southeast Asian J Trop Med Public Health 39: 146-153.
17. Kim HK, Song KS, Lee ES, et al. (2002): Optimized flow cytometric assay for the measurement of platelet microparticles in plasma: pre-analytic considerations. Blood Coagul Fibrnolysis 13: 393-397.
18. Connor DE, Exner T, Ma DD, Joseph JE (2010): The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. Thromb Haemost 103: 1044-1052.
19. Ratajczak J, Wysoczynski M, Hayek F, et al. (2006): Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 20: 1487-1495.
20. Cocucci E, Racchetti G, Meldolesi J (2009): Shedding microvesicles: artefacts no more. Trends Cell Biol 19: 43-51.