Laboratory diagnostics of microparticles

PhD thesis

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Abbreviations

MB – microbead

MP – microparticle

MV – microvesicle

PS – phophatidylserine

PC - phosphatidylcholine

SM – sphyngomyelin

PhE – phosphatidyl-etanolamine

CD - cluster of differentiation

Cy5 – Cychrome (PE-Cy5 conjugate)

FITC - fluorescein isothyocianate

FS – forward scatter

LPS – lipopolysaccharide

PE – phycoerythrine

SC – side scatter

DIC – disseminated intravascular coagulation

EDTA - ethylene-diamin-tetra-acetic acid

FEV1 – forced expiratory volume measured in the first second

FVC – forced expiration vital capacity

Ig – immunoglobulin

ISTH SSC – International Society of Thrombosis and Haemostasis Standardisation sub-Committee

1. Introduction

Microparticles were first described by Chargaff in 1946 as precipitable factors that accelerate thrombine formation in platelet-free plasma. Twenty years later, Wolf gave them the name ’platelet dust’ and discovered that they have the ability to produce thrombine after the ultracentrifugation of plasma. As a result of the development of measuring methods from the 90s onwards, our knowledge about ’platelet dust’ has broadened and deepened considerably. These particles have since been called microparticles (MP) and microvesicles (MV). It has become clear later that besides *in vivo* platelets, they may originate from other cells and that they can be detected not only in the circulating blood but also in other body fluids e.g. urine, cerebrospinal fluid, synovial fluid and saliva.

1.1. Microparticles

Microparticles are defined as small 0.1-1.0 μm-size vesicular structures of cellular origin that contain a membrane skeleton but the nucleus is absent and the bodies lack synthetic capacity. Depending on their cellular origine and the sample preparation/shedding from the cell, they may express phophatidylserine (PS) in varying amounts on their outer membrane leaflet (International Society on Thrombosis and Haemostasis; Scientific and Standardization Commitee Meeting; Sydney, 2005). According to more recent data in the literature, there exist such MPs that do not express PS on their surface. MPs are released into the extracellular microenvironment during early stage of apoptosis, cell activation or exposure to high shear stress in physiological or pathological conditions. Consequently, their formation is the result of an active process. Their cellular origin could be distinguished based on their surface protein’s which are characteristic of the parent cells from which they detached. They may originate in the circulation from platelets, neutrophils, monocytes, lymphocytes, erythrocytes and endothelial cells. Inside, MPs may carry cytoplasm and nucleus (eg. mitochondrion, enzymes, transcription factors, mRNA and DNA) components.

1.2 The role of microparticles and methods used for their detection

MPs found in the circulation may fulfil several roles in the organism, both from a pathological and physiological aspect, according to their cellular origin, and process of formation. MPs of platelet origin (PMPs) possess a pro-coagulant activity that is 50-100

times higher, per surface unit, than that of activated platelets. Certain MPs may carry anticoagulant proteins on their surface, e.g. TFPI (tissue factor pathway inhibitor), Protein C, Protein S and thrombomoduline. MPs may also transport several immunologically active molecules that may be considered inflammatory mediators and possess pro-inflammatory characteristics. In vitro studies have shown that via the activation of the complement cascade, they can initiate an inflammatory process, the attachment and migration of leukocytes and the detachment of further MPs from various cells. MPs of platelet origin may increase the attachment of further neutrophils to the neutrophil cells that have already been attached. They are able to transport and transmit arachidonic acid which increases the attachment of monocytes to endothelial cells. MPs possess anti-inflammatory features as well. *In vivo* evidence that supports the pro-inflammatory characteristics of MPs primarily focus on the elevated number of MPs of various cellular origin measured in inflammatory processes.

At the beginning, ELISA, solid phase capture techniques, electron microscopy and flow cytometry were the methods available for the measurement of microparticles. As the interest in microparticles has increased, parallel arose the wish to characterise them and to define their physiological and pathophysiological roles that resulted in the advancement of novel measuring methods. These include: impedance-based flow cytometry, AFM (atomic force microscopy), TEM (transmission electron microscopy) and NTA (nanoparticle tracking analysis).

2. Aims

Laboratory aims:

- Based on available data from the literature, to develop a stable method for the isolation of microparticles and for their measurement on Beckman-Coulter FC500 flow cytometer.
- To establish a control group, to test the method on healthy volunteers with the aim of establishing the reference range.

Clinical aims:

- To study the clinical significance of MPs in septic patients.
- To study the clinical significance of MPs in COPD patients – with special focus on stable and exacerbated patients.

3. Materials and methods

3.1. Blood sampling

Taking into consideration the currently available literature data, anticoagulated, fasting venous blood samples were drawn with 21 G needles after short strangulation from the antecubital vein into 2.7 mL 3.8% (0.129 M) Na_3-citrate containing closed blood collection system tubes (Becton Dickinson). Upon blood collection, the first 3 mL blood was collected in a separate vial and discarded. The blood samples for MP measurements were transported immediately to the laboratory and processed within 1 hour.

3.2. Isolation of microparticles

There is no standard protocol for the isolation of microparticles and several methods have been used with different centrifugation time, cycles and forces. We developed our own method for the isolation of MPs. Blood was centrifuged at 800 ×*g* for 20 minutes at room temperature to obtain platelet-rich plasma. Then, 1.5 mL supernatant was transferred into a new test tube and centrifuged at 1,500 ×*g* for further 20 minutes to obtain platelet-poor plasma (PPP). Afterward, 1 mL PPP was further centrifuged at 1,500 ×*g* for 20 minutes in a new polystyrene tube to obtain cell-free plasma. The top 500 µL of cell-free plasma was transferred into an Eppendorf tube and pelleted at 18,000 ×*g* for 10 minutes. The supernatant was carefully removed leaving 25 µL of MP-rich plasma at the bottom of the Eppendorf tube. MPs 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) without $CaCl_2$. HEPES was obtained from Sigma-Aldrich Ltd. (Budapest, Hungary), while all other reagents of analytical purity were purchased from Reanal Ltd. (Budapest, Hungary).

3.3. Measurement of microparticles

Currently, there is no standardized method for MP analysis. Results acquired by different methods of measurement are difficult to compare. Nowadays, flow cytometry is the most frequently applied method in MP analysis. It provides the opportunity to parallel measure the number and the cellular origin of the MPs.

At our institute, the settings for MP measurements were performed on a Beckman Coulter FC500 flow cytometer. The cytometer has two laser sources: a standard argon-ion laser (488 nm) and an optional solid state (633 nm) HeNe laser. Fluorescent signals are detected

by 5 fluorescent channels: FL1=521nm/40, FL2=575nm/40, FL3=620nm/20, FL4=675nm/40, FL5=755nm/40. The collecting angle of the FS detector is 2-16°, which is not able to reliably distinguish particles below 0.4 μm from light and electrical background noise.

When our study began, there was no standardised flow cytometric method for MP measurement. Later the ISTH (International Society of Thrombosis and Haemostasis) accepted a recommendation which is very similar to the multi-step method developed by us.

SUMMARY OF OUR METHOD

3.3.a. Selection of the CD markers and fluorescent labelling

The selected CD markers, their cellular origin, the fluorescent dye used for labeling and the manufacturer's specification for our MP measurements are summarized in Table 1.

Table 1. Cellular origin of CD markers, fluorescent dye and the manufacturer's specification.

CD marker	Cellular origin	Fluorescent labelling	Manufacturer
CD61 (GPIIb/IIIa)	Platelet	FITC	Beckman-Coulter
CD41 (GPIIb/IIIa)		PE-Cy5	Becton-Dickinson
CD42a (GPIb/V/IX)		FITC	Becton-Dickinson
PAC1 (GPIIb/IIIa)		FITC	Becton-Dickinson
CD31 (PECAM-1)	Endothelial cell	PE	Becton-Dickinson
CD62E (ELAM-1/E-selectin)		PE	Becton-Dickinson
CD45	Pan leukocyte	FITC	Beckman-Coulter
CD13 (Aminopeptidase N)	Leukocyte (neutrophil, eosinophil, basophil, monocyte)	PE	Beckman-Coulter
CD14 (LPS-R)	Monocyte	PE	Beckman-Coulter
CD235 (Glycophorin A)	Red blood cell	PE	Becton-Dickinson
CD56	NK, LGL cell	PE-Cy5	Becton-Dickinson
Annexin V	Recognizes phosphatidylserine	FITC, Cy5	Becton-Dickinson
Mouse IgG1	Isotype control	FITC, PE, PE/Cy5	Becton-Dickinson

Abbreviations: Cy5: CyChrome (PE-Cy5 conjugate); ELAM-1: endothelial leukocyte adhesion molecule FITC: fluorescein isothiocyanate; Ig: immunoglobulin; LGL: large granular lymphocyte; LPS-R: lipopolysaccharide receptor; NCAM: neural cell adhesion molecule; NK: natural killer cell; PE: phycoerythrine; PECAM-1: platelet endothelial cell adhesion molecule.

3.3.b. Determining the microparticles size gate

For determining the MPs size gate, which is required to differentiate real events from electric background noise, 0.3-0.5-0.9 µm FITC labelled polystyrene microbeads were used (a gift of Soft Flow Ltd., Pécs, Hungary). Side scatter, forward scatter and fluorescence channels were set in logarithmic scale. For determination of the MPs number, known concentration ($1x10^5$/ml) of 3µm diameter counting microbeads (Becton-Dickinson, Hungary) were used.

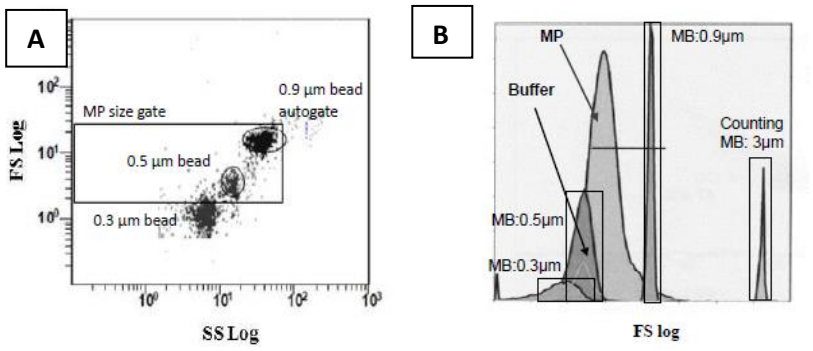

Figure 1. Determining the MP size gate with microbeads. Abbreviations: MB: microbead, MP: mikroparticle, FS: forward scatter, SS: side scatter.

The lower side of the gate was set below 0.5 µm bead as a threshold because the 0.3 and 0.5µm bead histogram displayed an overlap indicating that the cytometer would not be able to discriminate individual MPs between these size ranges. The upper side of the gate was set at the upper and right sides of the 0.9 µm bead cloud (Figure 1). In this MP gate, the buffer and the sample containing MPs can be clearly distinguished.

3.3.c. Determining AnnexinV-positive MPs within the microparticle size gate

Events in the MP gate were further discriminated by labelling with Annexin V. MPs were defined as Annexin V positive events in the MP gate with fluorescence intensity above the isotype control and sample-free buffer (Figure 2).

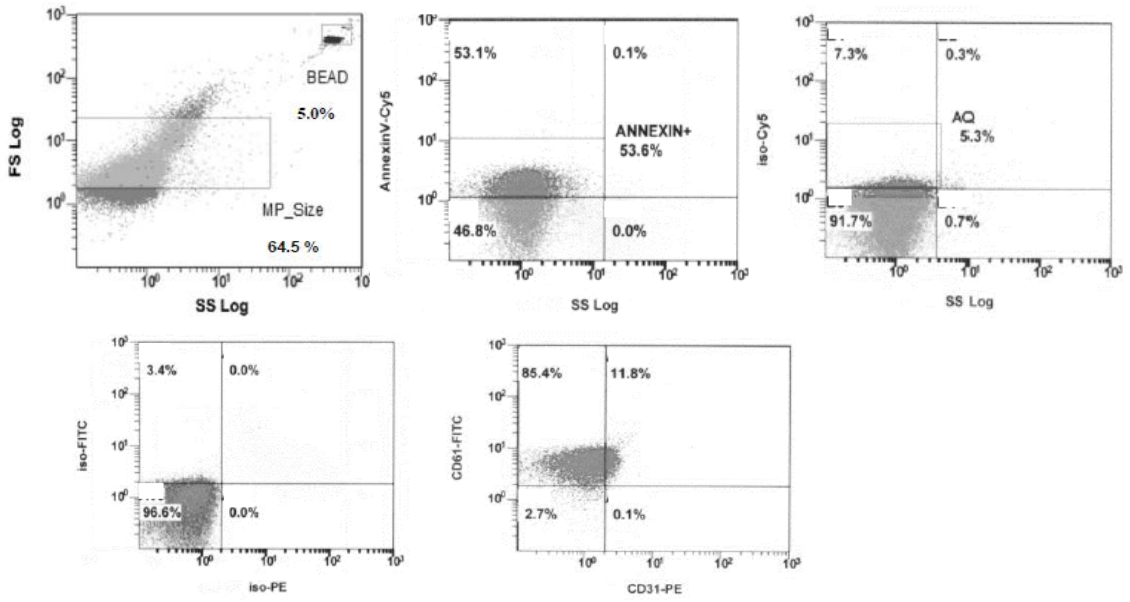

Figure 2. Determining AnnexinV$^+$ events within the MP size gate after removal of isotype control. Annexin V, platelet (CD61) and endothelial (CD31) marker$^+$ MPs.

3.3.d. Determination of the optimal labelling concentration of fluorescently-labelled CD markers and the labelling of MPs

In order to determine the optimal labelling concentration of fluorescently-labelled CD markers, a dilution sequence was prepared from antibodies with known concentrations, which were then used to label the sample-free Apo-buffer and the MP sample. The antibody-labelling concentration was considered optimal, if $CaCl_2$-labeled sample measurement events were clearly distinguishable from background and $CaCl_2$-free staining as well as from isotype controls. So far, MPs were defined as Annexin V-positive events in the MP gate with fluorescence intensity above the isotype control. To identify the cellular origin of MPs, we used adequately labelled CD markers of optimal labelling concentration. For sample labelling 10µl of MP suspension in $CaCl_2$-free Apo-binding puffer was incubated in 100µl Apo-binding buffer supplemented with 2.5mmol/l $CaCl_2$, with total 10µl antibody, previously diluted to optimal labelling concentration. Staining was incubated for 30 minutes at room temperature in dark chamber. All buffers were filtrated through 0.2µm membrane filters. Evaluation of results was performed with CXP software.

3.4. Positive and negative control measurements

As positive control, we used MPs isolated from pooled platelet-rich plasma collected from healthy volunteers after stimulation with calcium ionophore A23186 (Sigma Aldrich, 25 μmol/ml). As negative control, we used MPs stained with AnnexinV in the presence of 2.5 mmol/l EDTA (ethylene-diamin-tetra-acetic acid).

3.5. Reproducibility and the impact of the time passed from taking the samples upon MP quantities

In order to test the reproducibility of the method, three-three samples were taken from two healthy volunteers on two different occasions. The isolation of samples, their labelling with CD markers and the measurements were performed one after the other. In the case of the two samples, based on the calculated coefficient variants (<20%), the method was found to be reproducible in the case of each measured CD marker in terms of sampling, isolation, labelling and method of measurement. We also examined the extent to which the elapsed time after isolation influenced the total MP number and that of MPs labelled with different CD markers. We did not find any significant differences between the MP numbers in samples isolated immediately after blood sampling and in those isolated one hour later.

4. Application of the method on patients

4.1. Establishing the control group, reference interval calculation, testing the method

The control group consisted of 20 healthy volunteers, 10 males and 10 females. Median age was 57 years (49-63). Regarding age, as an inclusion criterion, we considered the ages of the patient groups we intended to study as a standard, compared to which we established a control group with a similar age range. Exclusion criteria included – as in the case of the patient group as well – major factors that could significantly alter MP amounts such as haematological diseases, cytostatic treatment in the past 3 months, high-dose, long-term steroid therapy, medications that alter platelet function, (e.g. acetyl-salicylic acid), blood transfusion or disseminated intravascular coagulation (with DIC score >5) in the previous few months and antiphospholipid syndrome (APS).

Table 2. shows the reference values for MP numbers measured in the control group as a whole and subgroups according to gender.

Table 2. Reference interval (median, 25th and 75th percentiles) in the control group (n=20), in females (n=10) and in males (n=10). Values are presented in MPx10^5/ml.

CD marker	**Control group**		**Females**		**Males**	
	Median MPx10^5/ml	25 - 75 percentiles	Median MPx10^5/ml	25 - 75 percentiles	Median MPx10^5/ml	25 - 75 percentiles
Total MP	**2.20**	(1.14 –5.98)	**2.03**	(1.24 –3.70)	**2.67**	(1.14 – 4.85)
CD61	**2.11**	(1.08 –5.87)	**1.94**	(1.19 –3.65)	**2.57**	(1.08 – 4.70)
CD41	**1.51**	(0.69 –5.39)	**1.44**	(0.83 –2.46)	**1.84**	(0.69 – 3.35)
CD42a	**0.21**	(0.05 –1.06)	**0.16**	(0.05 –0.32)	**0.29**	(0.07 – 0.79)
PAC1	**0.17**	(0.07 –0.94)	**0.13**	(0.07 –0.20)	**0.25**	(0.06 – 0.94)
CD31	**0.83**	(0.34 –3.42)	**0.76**	(0.34 –1.63)	**0.98**	(0.35 – 1.96)
CD62E	**1.77**	(0.90 - 5.62)	**1.68**	(0.93 - 3.17)	**2.12**	(0.90 - 4.11)
GlyA	**0.29**	(0.13 - 2.71)	**0.23**	(0.15 - 0.65)	**0.53**	(0.13 - 1.69)
CD45	**1.90**	(0.96 - 5.68)	**1.78**	(1.06 - 2.94)	**2.24**	(0.96 - 3.82)
CD13	**1.64**	(0.84 - 5.54)	**1.57**	(0.89 - 3.19)	**2.12**	(0.84 - 3.79)
CD14	**0.07**	(0.03 - 0.16)	**0.07**	(0.05 - 0.11)	**0.09**	(0.03 - 0.21)
CD56	**1.05**	(0.52 - 5.08)	**0.93**	(0.52 - 1.88)	**1.23**	(0.52 - 3.06)

CD markers showed no significant differences between males and females.

4.2. Testing the method on patients

4.2.1. The septic patients group

The study protocol was approved by the Regional Research Ethical Committee, University of Pécs (2406/2005). After being informed about the blood test and examinations in detail all patients signed a written consent form. 27 recently diagnosed (within 24 hours) severe septic patients with two- or multiple organ failure were enrolled into the study from our intensive care unit of the Department of Anaesthesiology and Intensive Therapy. Criteria for sepsis included: fever (≥38ºC) or hypothermia (≤36ºC), tachycardia (≥90/min), tachypnoe, altered state of consciousness, hyperglycaemia, leukocyte count (≥12,000 cell/μl or ≤4,000 cell/μl), elevated C-reactive protein (CRP)

(≥10mg/l) and serum procalcitonin levels (PCT) (≥5ng/ml). Exclusion criteria were as mentioned above for the control group. Primary endpoints included: withdrawal of consent and death during the period of the study. To follow-up the clinical status MODS (Multiple Organ Dysfunction Score) and SOFA (Sequential Organ-Failure Assessment) scores were calculated daily during the whole study period. Samples were drawn from all patients and MP measurements were performed on the day of admission to the ICU and on the third and fifh days.

4.2.1.a. Results

On all three measurement days (on days 1st, 3rd and 5th) significantly higher total MP count was detected in septic patients compared to the control group (all $p<0.01$). The number of $CD61^+$ and $CD41^+$ MPs showed a significant increase each day in the septic group compared to the control group (both $p<0.01$). By the 5th day, a mild decrease in MP number could be observed in both platelet markers and total MP number, but this was not significant. The platelet-derived $CD42a^+$ MP numbers also increased in the septic group on the 1st, 3rd and 5th days ($p<0.05$). However, the number of activated platelet-derived $PAC1^+$ MPs showed a significant decrease on the 1st and 5th days ($p<0.05$). Interestingly, $CD62E^+$ MPs of endothelial origin did not differ significantly comparing the septic and control groups (data not shown). Comparing the 20 survivors and 7 non-survior patients, we did not find any significant differences at any time point. Importantly, a significant difference was revealed between the number of organ failures and total MP numbers. The total MP number was significantly higher in patients with two-organ failure than in patients suffering from five-organ failure ($p<0.05$).

4.2.2. The chronic obstructive pulmonary disease (COPD) group

4.2.2.1. Prospective study on COPD patients

A total of 50 patients with COPD, who were treated at the 1st Department of Internal Medicine, Division of Pulmonolgy, University of Pécs, were recruited into this prospective clinical study between 2008 and 2010. Patients were classified into two groups: 1. patients with stable COPD (n=34) and 2. patients with acute exacerbation – AECOPD (n=16 patients). The diagnostic criteria and the GOLD stratification for COPD patients were made according to GOLD (FEV1 measured in the first second during forced

volume expiration after inhalation from a brochodilator - Recommendation of the Hungarian Pulmonology Society, GOLD stages, 2006):

I. mild: FEV1≥80 reference% (ref%)
II. moderate: 50 ref%≤ FEV1 <80 ref%
III. severe: 30 ref% ≤ FEV1 <50 ref%

} symptomatic or symptom-free

IV. very severe: FEV1 <30 ref% or FEV1 <50 ref% + chronic respiratory failure (PaO_2 <60 Hgmm +/- $PaCO_2$ > 50 Hgmm) or symptoms of cor pulmonale

Respiratory obstruction was verifiable in all four stages: FEV1/FVC (forced expiratory vital capacity) <70%. Definition of exacerbated patient was as follows: hospital admission and intravenous corticosteroid therapy were urgently initiated based on the worsening of the symptoms such as increased sputum, cough or signs of respiratory distress. COPD patients presenting for regular, annual check-up and without novel symptoms were considered as stable.

4.2.2.1.a. Results

Demographic and clinical data of COPD patients are shown in Table 3. A total of 19 healthy subjects served as controls (9 male and 10 female) with a median age of 57 (49-63) years. The number of smokers was 8 (42%).

Table 3. Demographic and clinical data in COPD patients (stable vs AECOPD).

	Stable COPD (n=34)	**AECOPD (n=16)**	***p*-value**
Age*	63 (54-70)	63 (56-67)	0.755
Males[#]	20 (59)	9 (56)	0.863
Chronic comorbidity			
DM[#]	2 (6)	5 (31)	0.03
CVD[#]	20 (58)	9 (56)	0.863
Smoking[#]	20 (59)	10 (62)	0.804
Medications			
Ca^{++}-channel-blocker [#]	8 (23)	10 (62)	0.009
β-blocker[#]	17 (50)	7 (44)	0.680
ACEI[#]	13 (38)	6 (37)	0.898
Statin[#]	2 (6)	3 (19)	0.320
ICS[#]	25 (74)	14 (87)	0.464
LABA[#]	26 (77)	14 (87)	0.698
LAMA[#]	32 (94)	14 (87)	0.245
Respiratory function			
FEV1/FVC*	0.56 (0.41-0.63)	0.49 (0.37-0.55)	0.179
FEV1 ref%*	0.52 (0.36-0.67)	0.56 (0.45-0.67)	0.925
GOLD stage			
GOLD I-II[#]	21 (62)	10 (62)	0.96
GOLD III-IV[#]	13 (32)	6 (37)	0.96
Routine laboratory parameters			
Cholesterol* (mmol/L)	5.50 (5.00-6.80)	4.95 (4.10-6.10)	0.151
RBC count* (T/L)	4.89 (4.43-5.22)	4.99 (4.49-5.29)	0.677
TCT count* (G/L)	232 (172-276)	267 (195-328)	0.112
WBC count* (G/L)	7.55 (5.76-9.76)	13.53 (8.99-15.85)	<0.001
hs-CRP* (mg/L)	5.65 (3.50-8.30)	9.67 (2.93-23.61)	0.114

Median (IQR) * and n (%) [#] are presented. Mann-Whitney U and Chi-square tests were used to compare variables. DM, diabetes mellitus; CVD, cardiovascular disease; ACEI, angiotensin converting enzyme inhibitor; ICS, inhaled corticosteroid; LABA, Long acting β-agonist; LAMA, Long acting muscarin antagonist; GOLD, Global Initiative for Chronic Obstructive Lung Disease; FEV1, Forced expiratory volume in 1 second; FVC, forced vital capacity; RBC, red blood cell; PLT, platelet; WBC, white blood cell; hs-CRP, high-sensitivity C-reactive protein.

All the measured MPs such as $CD31^+$, $CD62E^+$ with endothelial; $CD61^+$, $CD41^+$, $CD42a^+$, $PAC1^+$ with platelet; $GlyA^+$ with red blood cell, $CD45^+$, $CD13^+$ with leukocyte; $CD14^+$ with monocyte and $CD56^+$ with lymphocyte origin showed significant ($p<0.001$)

elevation in stable COPD patients compared to the controls (Table 4). Importantly, we detected statistically significant differences in patients with acute exacerbation (AECOPD) versus stable COPD patients: the number of $CD62E^+$, $CD41^+$, $CD42a^+$, $CD14^+$ MPs were significantly ($p<0.05$) higher in the AECOPD group (Table 4). These endothelial, platelet and monocyte derived MP numbers showed a remarkable elevation in acute exacerbation state of COPD patients.

Table 4. Number of microparticles in control subjects and patients with stable and AECOPD

CD marker ($X10^5$/mL)	**Control (n=19)**	**Stable COPD (n=34)**	**AECOPD (n=16)**	***p*-value; control vs. stable and control vs. AECOPD**	***p*-value; stable vs. AECOPD**
Annexin V+	2.18 (1.33-3.70)	53.74 (35.08-76.38)	61.15 (44.26-73.39)	<0.001	0.747
CD31+	0.79 (0.48-1.40)	10.35 (7.78-17.04)	15.59 (11.82-21.95)	<0.001	0.164
CD62E+	1.72 (0.97-3.17)	29.87 (26.85-40.58)	44.09 (33.43-59.21)	<0.001	***0.011***
CD61+	2.10 (1.22-3.65)	48.36 (32.02-73.68)	54.99 (38.18-65.73)	<0.001	0.992
CD41+	1.51 (0.90-2.46)	37.99 (32.69-52.59)	61.15 (44.25-73.39)	<0.001	***0.018***
CD42a+	0.17 (0.12-0.30)	1.32 (0.92-1.51)	3.19 (1.49-5.69)	<0.001	***0.002***
PAC1+	0.15 (0.09-0.23)	0.57 (0.28-3.05)	0.52 (0.15-0.76)	<0.001	0.182
GlyA+	0.28 (0.16-0.59)	8.21 (6.76-11.59)	11.93 (5.03-18.98)	<0.001	0.133
CD45+	1.88 (1.10-2.94)	37.67 (26.34-59.24)	43.21 (20.29-60.07)	<0.001	0.670
CD13+	1.62 (0.99-3.19)	30.37 (21.32-5,500)	41.44 (26.91-46.07)	<0.001	0.904
CD14+	0.07 (0.06-0.11)	0.29 (0.08-0.48)	0.624 (0.44-0.98)	<0.001	***0.008***
CD56+	1.02 (0.63-1.88)	43.99 (32.81-54.94)	61.15 (42.02-73.40)	<0.001	0.240

Median (IQR) data are presented. Kruskal-Wallis test was used to compare variables.

After dichotomizing patients based on GOLD stages, an increased number ($p=0.031$) of $PAC1^+$ MP was observed in severe cases indicated by GOLD III and IV. In addition, multivariate analyses showed that $CD62E^+$, $CD42a^+$ and $CD14^+$ MPs correlate inversely with FEV1/FVC (-0.406, -0.473, -0.440, $p<0.001$, respectively).

4.2.2.2. Retrospective study in the COPD patient group

In 2018 we performed the retrospective analysis of the COPD patient group we had examined in 2010 with the aim to detect correlations between MPs of different cellular origin and 7-year outcomes. Data on all the 50 COPD patients enrolled in 2010 (34 stable, 16 exacerbated), were available for follow-up that had been stored on the electronic database (eMedSol). We registered 7 years retrospectively all hospital admissions due to severe exacerbation, admissions to the A&E and date of death for each patient.

4.2.2.2.a Results

7-year mortality in total COPD population

We investigated the correlation between the number of MPs measured 7 years previously and survival in the entire COPD group. We found that the total MP numbers, the $CD61^+$ MPs of platelet and the $CD13^+$ MPs of leukocyte origine in the systemic circulation, measured 7 years ago were significantly ($p<0.05$) higher in non-survivors (n=20) than in survivors (n=30). Beside the above-described MP numbers, the $GlyA^+$ MPs with red blood cell origin and the platelet-derived $PAC1^+$ (activation marker) MP numbers were significantly (p=0.01;p=0.03) higher among non-survivors than in survivors in the whole COPD patient group. Interestingly in 2010, when we examined the amount of MPs based on GOLD stage, we found a difference only in case of one microparticle. The number of $PAC1^+$ MPs was significantly (p=0.031) higher in the more severe COPD patient group (GOLD III-IV), than in the GOLD I-II stages.

We performed multiple regression analysis in which we involved age, gender, FEV1/FVC, CRP, LABA, LAMA, red blood cell number, total MP numbers and $CD61^+$, $CD13^+$, $PAC1^+$ as well as $GlyA^+$ MP numbers. The analysis revealed that the red blood cell number (OR: 5.3, 95%CI: 1.009-28.324, $p<0.05$) and the $GlyA^+$ MP numbers of red blood cell origine (OR: 1.2, 95%CI:1.001-1.527, $p<0.05$) were independent predictors of 7-year mortality.

We examined with correlation analysis if the number of the peripheral blood elements affect the MP numbers. We found a positive correlation between $PAC1^+$ MP numbers and red blood cell number (0.317, p=0.03) and an inverse correlation between platelet number and $CD31^+$ MP amount (-0.485, $p<0.05$) in non-survivor patient group.

We retrospectively analysed whether there was a difference in terms of COPD severity registered 7 years ago and the number of exacerbations developed since then. Our results revealed that the number of exacerbations was significantly higher in non-survivors during the 7 year period, although, 7 years ago there was no difference between the two groups with respect to GOLD stages as established on the basis of FEV1 measurements.

The exacerbation markers of the stabile COPD population

Subsequently we were looking for MPs, which may indicate the frequent exacerbations (>3/year) in the stable COPD patients (n=34). According to our results in patients with frequent exacerbations the total MP numbers and the pan leukocyte $CD45^+$ MP numbers were significantly higher than in patients with rare exacerbations (<3/year) ($p<0.05$).
ROC analysis was performed to evaluate the predictive value of MP numbers and that of the circulating blood elements in stabile COPD population. Based on the ROC analysis, the pan leukocyte $CD45^+$ MP number (Area under the curve (AUC): 0.708, 95%CI: 0.532-0.888, p=0.047), the red blood cell number (AUC: 0.744, 95%CI: 0.566-0.922, p=0.020) and the platelet number (AUC: 0.759, 95%CI: 0.574-0.941, p=0.014) predicted mostly the frequent exacerbations. However, with multiple regression analysis we could not find any independent predictors.

Frequent exacerbations and survival

Finally, we were looking for MPs, which proved to be independent predictors of survival in patients with frequent exacerbations (<3/year) during the 7-year period follow-up. According to ROC analysis, the $CD45^+$ MP numbers at $\leq 8.5x10^5$/mL cut-off value predicted the survival with 73% sensitivity and 86% specificity (AUC: 0.81, 95%CI: 0.63-0.99, p=0.02). Multiple regression analysis, in which we involved age, gender, FEV1/FVC, CRP and $CD45^+$ MP numbers, revealed only the $CD45^+$ MPs as independent predictors of survival (OR: 0.75,95% CI:0.55-1.0, $p<0.05$).

5. Novel results

Laboratory results:

1. Starting out from available data from the literature, we designed a stable method for the isolation of microparticles and for their measurement by Beckman-Coulter FC500 flow cytometer.

2. We demonstrated that our method was reproducible based on CV% (<20%) in the case of all MP numbers labelled with a CD marker.

3. Our study proved that the time between sample-taking and the actual measurement (one hour after taking the sample) did not influence MP numbers for any of the CD markers.

4. We established a reference interval based on healthy individuals in the age group, with a mean age of 57 years (49-63) for the number of MPs of different cellular origin which may later provide a basis for comparison of patient groups falling within the same age-range.

Clinical conclusions:

The significance of MPs in septic patients:

1. Our study demonstrated that the total number of microparticles was significantly higher in septic patients on days 1., 3., and 5 following enrollments than in the control group.

2. In severely septic patients, platelet-derived $CD41^+$, $CD61^+$ and $CD42a^+$ MP numbers showed a significant increase on days 1., 3., and 5 following enrollments compared to the control group.

3. We found a significant decrease in the number of MPs carrying a platelet-derived $PAC1^+$ activation marker on days 1 and 5 of the study.

4. Regarding the numbers of MPs of different cellular origin, we did not find any significant differences between survivor and non-survivor septic patients during the follow-up period.

5. Our study revealed that the total MP number was higher in severely septic patients in the case of two-organ failure than in patients suffering from five-organ failure.

The significance of MPs in COPD patients:

1. Our study proved, that besides $CD31^+$ and $CD62E^+$ microparticles **of endothelial origin, total Annexin V^+ MP number**, **platelet-derived** $CD61^+$, $CD41^+$, $CD42a^+$, $PAC1^+$ MP numbers, **erythrocyte-derived** $GlyA^+$, **leukocyte-derived** $CD45^+$, $CD13^+$, **monocyte-derived** $CD14^+$ and **lymphocyte-derived** $CD56^+$ MP numbers were significantly higher in the circulation of patients with stable COPD or acute COPD exacerbation compared to healthy controls.

2. The present thesis is the first that describes that in the peripheral blood of patients with acute COPD exacerbation, besides $CD62E^+$ MP number of endothelial origin, **platelet-derived $CD41^+$, $CD42a^+$** and **monocyte-derived $CD14^+$ MP** numbers were significantly higher than those in patients with stable COPD.

3. Our study showed that the number of MPs carrying a platelet-derived $PAC1^+$ activation marker was significantly higher in patients with more severe COPD (GOLD III-IV) than in less severe cases (GOLD I-II).

4. In the entire COPD population, the number of **red blood cells** and the **$GlyA^+$ MPs** have been proved to be independent predictors of 7-year mortality.

5. In the stable COPD patient group, the pan leukocyte-derived **$CD45^+$ MP** number predicted the frequent exacerbations during the 7 years follow-up and in the patient group with frequent exacerbations it has been proven as an independent predictor of mortality.

6. List of publications

Publication the thesis is based on:

Tőkés-Füzesi M, Ruzsics I, Rideg O, Kustán P, Kovács GL, Molnár T. Role of microparticles derived from monocytes, endothelial cells and platelets in the exacerbation of COPD. Int J Chron Obstruct Pulmon Dis. 2018 Nov 15;13:3749-3757. doi: 10.2147/COPD.S175607. eCollection 2018. **IF: 2.917**

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IF: 2.498; **IF** based on equal (50%) contribution **1.249**

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IF: 1.922; **IF** based on (50%) contribution **0.961**

Cumulative IF**:** 4.113; **IF** on the basis of 50% contribution **2.056**

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