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Review/ Praca poglądowa

Methods for detection of microparticles derived from blood and endothelial cells

Metody oznaczania mikrocząstek pochodzących z komórek krwi i śródbłonka

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ABSTRACT

Microparticles (MP-microparticles) – cell membrane vesicles 0.1–1 μm diameter, released in response to activation or apoptosis, both in physiological and pathological conditions. They reveal a wide spectrum of biological activities, express cell surface antigens characteristic for cells of their origin. In this article reviewed quantitative and qualitative methods for detection of microparticles, presents pre-analytical conditions as an potential source of variability in the analysis of MP. In conclusion there are several methods for detection of microparticles but they are not standarized. Methods of microparticles detection need to be standarized to be clinically relevant.

Microvesicles are a heterogeneous group of membrane vesicles, measuring 0.1–1 μm in diameter that are released from the cell surface in response to activation or apoptosis [1–3]. In physiological conditions MVs are formed during cell maturation and aging, and increased number of circulating MVs has been associated with many pathological conditions [2, 4]. MVs are non-nucleated, cell-membrane coated vesicles containing the same surface antigens as their cell of origin [5].

Our knowledge on the morphology and function of MVs is still incomplete. This is due to the difficulties in separating MVs from other types of cells, the inability of most techniques to capture particles in a volume range of MVs, and low availability of costly and time-consuming methods for the detection of MVs [6]. The majority of studies were performed on MVs originating from platelets, endothelial cells, and monocytes [7].

Generation of microvesicles

The formation of MVs is a regulated process, leading to selective and concentrated release of cell contents to the surrounding environment. Both the parent cell and a trigger

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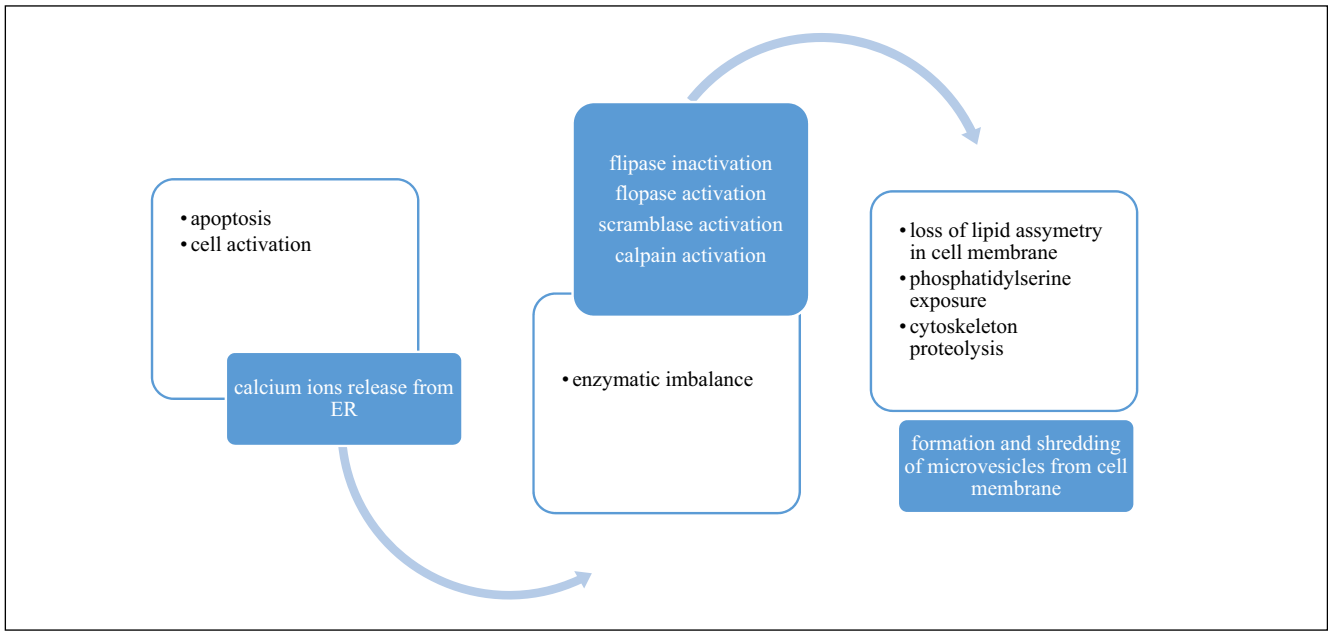

Fig. 1 – The formation of microvesicles

determine the number, size, and antigen composition of the released MVs [8]. Circulating MVs are released to the plasma from the surface of all types of blood cells: red blood cells, granulocytes, lymphocytes, monocytes, platelets, endothelial cells, and tumor cells. The number of MVs in the blood is the result of a dynamic balance between the release of apoptotic and activated cells, and their clearance from the circulation [8–10]. In healthy subjects, the majority of detected MVs come from platelets, whereas MVs originating from erythrocytes, leukocytes, and endothelial cells are much less abundant [7, 11]. Cell membrane is the first structure involved in the formation of the microvesicles. Under homeostatic conditions phospholipids, phosphatidylserine and phosphatidyloetanolamine, are arranged on the inner (cytoplasmic) layer of the membrane while phosphatidylcholine and sphingomyelin are localized in the outer layer. The asymmetry is a key factor for maintaining cell homeostasis and is regulated by an enzyme complex: flippase, floppase and scramblase. Flippase is responsible for keeping the phosphatidylserine and phosphatidyloetanolamine on the inner side of the membrane, while floppase keeps phosphatidylcholine and sphingomyelin on the outer side. Scramblase, responsible for the transport of phospholipids between the two monolayers of the cell membrane, is inactive in the steady state. Loss of phospholipid asymmetry and externalization of phosphatidylserine trigger the formation of MVs [7, 9, 12, 13]. Not all MVs exhibit phosphatidylserine on the surface, and their contents may vary depending on the cell of origin as well as the stimulus triggering the formation of MV [1]. The formation of MVs is depicted in Fig. 1: apoptosis and cell activation cause a sudden release of calcium ions by endoplasmic reticulum. The increased calcium ions flux results from enzymatic imbalance caused by the inactivation of the flippase, floppase and scramblase, leading to the loss of lipid asymmetry in the cell membrane and exposure of phosphatidylserine on the surface of the cell. The release of calcium activates protein proteases e.g. calpain leading to the proteolysis of cytoskeleton resulting in shedding of MVs from the surface of the cell membrane [7, 9, 12, 13].

Microvesicles are formed in response to the activation induced by various stimuli (thrombin, collagen, epinephrine, diphosphoadenosine) and ex vivo during preparation and storage of blood products for transfusion [14, 15]. Approx. 70–90% of circulating MVs originate from platelets [9, 16], 10–15% from endothelial cells [17], 4–8% from red blood cells [18], and their formation is an essential stage of red blood

Table I – The characteristics of microvesicles

1.	Formation triggered	By activation or apoptosis through blebbling of cell membrane
2.	Diameter	0.1–1 μm
3.	Nucleus	No
4.	Composition	Lipids and proteins, can contain mRNA, miRNA
5.	Structure	Outer layer contains phosphatidylserine, which in normal conditions is localized in the cytoplasmic layer
6.	Antigens	Specific surface antigens derived from the parent cell
7.	Origin	Platelets, endothelial cells, erythrocytes, monocytes, lymphocytes, and granulocytes
8.	Number	Depends on the balance between release and removal from the circulation
9.	Present in physiological conditions	Increased number in various pathological conditions

cell aging [19]. Basic characteristics of MVs are shown in Table I.

The role of microvesicles

Until recently the microvesicles were considered cell fragments without any significant biological functions. Currently, it is believed that they exhibit a wide spectrum of biological activity. The presence of the surface antigens and various contents such as mRNAs, miRNAs, and proteins suggests, that MVs are able to exert wide variety of biological functions [20, 21]. Biological properties of MVs largely depend on the cell of origin and the trigger that stimulated their production [2, 10]. The first described property of MVs was procoagulative activity, dependent mainly on the presence of phosphatidylserine and tissue factor on their surface. For this reason, MVs were initially related to coagulation activation, atherosclerosis, cardiovascular disease or venous thrombosis.

The MVs are involved in hemostasis due to their pro- and anticoagulative properties [4, 7, 8, 22]. As carriers of bioactive molecules such as mRNA, miRNA and proteins MVs play a key role in intercellular communication [20, 21], mediate cell-to-cell signaling, regulate inflammatory processes, vasospasm, fibrinolysis, and angiogenesis [23]. An increased number of microvesicles was observed in pathological conditions, such as diabetes, cancer, autoimmune disease, and sepsis [9, 14, 24, 25]. Increased number of endothelial-derived microvesicles is observed in patients with antiphospholipid syndrome, bleeding disorders and hematological diseases. MVs can be released from monocytes in response to various triggers, including proinflammatory cytokines. Neutrophil-derived microvesicles occur in healthy individuals as well as various pathological conditions [17]. MVs are involved in the response to hematopoietic stem cell transplant, cancer progression, and apoptosis induction [26]. Studies of microvesicles have contributed to the understanding of the mechanisms underlying the pathogenesis of many diseases i.e. sickle cell disease, thrombotic thrombocytopenic purpura or paroxysmal nocturnal hemoglobinuria [4]. Increased release of MVs was observed during blood components storage [27, 28], which suggests that they may be responsible for some of the negative side effects observed after blood transfusion [29, 30]. MVs derived from stored blood components are able to modulate the activity of macrophages, neutrophils and T cells [29, 31, 32], and MV-activated neutrophils might be directly involved in the pathogenesis of TRALI [33]. Storage of blood components may also contribute to the formation of MVs after transfusion [34].

Sample preparation

All preanalytical steps, regardless the method used, from blood sampling to sample freezing, should be considered a source of variation in MV analysis. To avoid the loss of MVs and artifacts, strictly defined rules of blood sampling and preparation of samples must be complied [35]. MVs can be analyzed directly in the platelet-poor plasma obtained by serial centrifugation of citrate-treated blood or isolated from the plasma by ultracentrifugation. Preferably, MVs analysis should be performed in freshly prepared plasma to avoid loss and damage of the MVs during freezing and thawing [10, 36].

There are three key preanalytical factors influencing MVs measurement:

(1) time to the first centrifugation of the sample;
(2) shaking the samples during transport;
(3) centrifugation parameters [20, 37].

In addition, the number and properties of MVs are affected by such factors as:

(1) blood sampling method;
(2) the diameter of the needle;
(3) application of the tourniquet;
(4) anticoagulant used;
(5) the time between blood sampling and plasma isolation, which is in part dependent on the anticoagulant used.

Preparation of the samples:

(1) Blood sampling:
- on an empty stomach, between 8 and 10 a.m.,
- from elbow flexure veins;
- use plastic tubes,
- use large diameter needle (19–21 G), to avoid hemolysis in vitro, platelet activation, and MVs formation;
- without the use of the tourniquet; light turniquet can be used only to locate the veins;
- use sodium citrate as an anticoagulant, because of its low ability to activate the cells and generate artifacts in comparison to EDTA and heparin (3.2%, 105–109 mM or 129 mM – 3.8%), ACD and CTAD may also be used (CTAD inhibits the activation of platelets without affecting their function). It is not recommended to use EDTA, heparin (as it induces activation and aggregation of platelets in the blood, does not chelate calcium ion chelates, does not prevent the generation of MVs after sampling), and hirudins.
- To avoid artifacts caused by vein damage during venipuncture, the first few ml after sampling (2–7.5 ml) should be discarded [7, 10, 36–39].

Sample preparation procedure:

(1) Blood after sampling should be centrifuged as soon as possible (preferably within 30 min to 1 h from sampling) to isolate the plasma. The maximum time from sampling to centrifugation is 2 h [7, 10, 36–39]. If necessary, before processing blood samples should be stored at room temperature, without mixing.
(2) Transport: the samples should be transported at room temperature (20–25 °C), in the transport container in vertical position. Rapid shaking and freezing during transport are not permitted [37].
(3) Storage: due to the fact that, in most cases, samples must be stored before analysis, fast freezing of the samples in liquid nitrogen at −80 °C and further storage at a temperature below −80 °C until analysis are recommended. Such conditions protect the structure and function of the MVs. Maximum time for storage of the frozen samples is 1 year. As far as possible, all of the samples intended for the analysis of MVs should be stored for the same period of time [10, 36].

(4) Thawing: thaw the samples in a water bath at 37 °C, which reduces the formation of ice crystals [5, 10, 38–40].
(5) Centrifugation: the cells can be activated very easily during the preparation of test samples, so the optimal measurement of circulating MVs requires thorough removal of platelets, red and white blood cells. Erythrocytes, platelets, and a large number of cell fragments are effectively removed during centrifugation at 1200–2000 × *g* for 15–20 min [37–39, 41]. Depending on the centrifugation speed, MVs might be lost in the pellet or supernatant. There is also a risk of contamination of the samples with the residual platelets, that forms during freezing and thawing cycle and can lead to an artificial increase in MVs number. To avoid generation of MVs ex vivo two-step centrifugation should be performed. The first centrifugation is intended to remove cells and platelets (1500–10 000 × *g* for 5–20 min). The second centrifugation is intended to obtain MVs pellet (13 000–100 000 × *g* for 30–60 min). It is recommended to perform two centrifugations for 15 min at 2500 × *g* [39], followed by another centrifugation at 13 000 × *g* for 2 min to obtain platelet-poor plasma. The largest number of MVs can be obtained during centrifugation at 20 000 × *g* for 20–30 min. Breaks should be avoided. After the centrifugation steps plasma should be collected gently to avoid the damage to the platelet layer [36–38, 41].

w?>In order to remove the residual platelets, filtration using 0.8 μm filters might be performed, however, the procedure may activate the platelets, induce MVs fragmentation, and in consequence lead to significant loss of MVs [17].

Detection methods

Analysis of microvesicles includes four essential steps: isolation, detection, typing and counting [10]. There are many methods of MVs detection described in the literature, aimed to determine their size, number, morphology, biochemical composition, origin, physical characteristics, and activity [42]. The individual methods differ in analytical and pre-analytical confounding factors influencing the differences in MVs measurement. The methods used for qualitative and quantitative MVs analysis are technically challenging. One of the main issues is lack of internal validation [10, 43]. Microvesicles can be analyzed using optical and non-optical methods [42] (Table II).

I. Optical methods

1. Optical microscopy

In this method the sample is illuminated by visible light, and the light scattered on the MVs is collected through the lens of the microscope and focused on the CCD camera (charge-coupled device). Optical microscopy allows to measure the size and morphology of the MVs larger than 200 nm. Quantification of microvesicles in the specified volume is possible, but very time consuming. This method does not provide any information on the biochemical composition or cell of origin. The time needed to analyze 10 000 MVs is a couple of hours. Therefore, it is a time consuming and low-throughput method [42]. Due to the small size of MVs and artifacts formed due to Brownian motions, optical microscopy is used in conjunction with image analysis software in NTA method [44, 45].

2. Fluorescent microscopy

It is a type of optical microscopy optimized for the detection of fluorescence. It detects surface proteins on MVs using fluorescent-labeled probes. If auto fluorescence is used, MVs size can be specified as a fluorescence signal proportional to the size of the particles; however, if fluorescent probes are used, the amplitude of the probe fluorescence is not proportional to the size of the particles. Therefore, this method does not allow to measure the size of the MVs. Fluorescent microscopy enables quantitative evaluation of the MVs with specific properties, on the assumption that all MVs of a given type are stained. The method is time consuming, low-throughput and the average measurement time is 1 h [42].

Highly Sensitive Fluorescent Microscopy (HSF) allows detection of submicro- and nano-MVs. This technique provides high detection sensitivity due to the high excitation and quantum efficiency of fluorescence. Detection of small MVs using HSF seems to be promising [46]. Fluorescence microscopy after FITC-labeled annexin V staining allows the observation of formation and shedding of microvesicles from the cell membrane [47]. Fluorescence microscopy, due to constant advances in optical imaging, is a promising technique for MVs characteristics; however, its sensitivity in nano-MVs detection is limited

Table II – Methods of microvesicles detection

Optical methods	Non-optical methods
Optical microscopy	Transmission electron microscopy (TEM)
Fluorescent microscopy	Atomic force microscopy (AFM)
Dynamic light scattering (DLS)	Impedance-based flow cytometry
Flow cytometry	Western blotting (WB)
Nanoparticle Tracking Analysis (NTA)	Enzyme-linked immunosorbent assay (ELISA)
Fluorescence NTA (F-NTA)	Functional assays
Raman spectroscopy	Surface plasmon resonance based imaging microscopy (SPRi)
Stimulated emission depletion microscopy (STED)	Surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS)
Laser scanning confocal microscopy (LSCM)	

by fast Brownian motions of the small particles in the fluid [48].

3. Dynamic light scattering (DLS)

DLS is based on the analysis of the intensity of light scattered by particles moving due to Brownian motions. Brownian motions are random, disordered, continuous movements of the particles in the fluid, caused by collisions with particles of the solvent. Speed distribution of the particles depends on the temperature and the viscosity of the liquid and the diameter of the particles. In general, the smaller the particles, the faster the Brownian motions. If the beam falls on the surface of the particle, the light is scattered in all directions, which is measured by a detector positioned in an appropriate distance. Brownian movements cause fluctuations in the intensity of the scattered light. The rate of the changes in the intensity of the scattered light depends on the size of the particles and is used to calculate the size of the MVs. The data is transformed using appropriate mathematical algorithm and in consequence the information registered by the detector regarding the changes in the intensity of the scattered light is converted into the particle size distribution graph (percentage of particles in relation to their size). The result depends on the mathematical algorithm applied. DLS allows to measure all particles at the same time, shows the average size and detects particles measuring from 1 nm to 6 μm. MVs size distribution measured using DLS favors the presence of a small number of large particles, which scatter the light more efficiently than the small ones [38, 49]. In a few minutes, this method is able to measure the relative and absolute size distribution of MVs in a small sample volume. The method is suitable for the determination of the particle size in monodisperse samples. Detection in polydisperse samples is less accurate and might be affected by the presence of a small number of larger particles, e.g. platelets or other pollutants. Two populations of particles can be analyzed at the same time only when the diameter of the particles varies at least twofold. DLS does not provide information on the biochemical composition and the origin of MVs, and is targeted to detect larger particles, therefore can ignore smaller microvesicles [42, 49–51].

4. Flow cytometry

Flow cytometry is the most common method of identification, quantification, and size assessment of the MVs. In flow cytometry, MVs are detected in two stages: based on the intensity of light scattering and using fluorescently-labeled antibodies against specific surface antigens. Flow cytometry allows simultaneous analysis of morphology and subtype determination of thousands of MVs per second. In this method, MVs cause scattering of the laser light, which is measured by the detectors: one detector positioned in line with the laser beam measures the dispersion of the light (forward scatter, FSC), the second detector, perpendicular to the laser beam measures the dispersion of the light (side scatter, SSC). FSC indicates the size of the particle, while SSC provides information about the internal complexity of the cell. The intensity of the light scattering depends on the size of the particles, their shape, refractive index, and absorption. The biochemical information is obtained by correlation between FSC and SSC. Proper determination of structure and size of MVs requires the use of fluorescently-labeled calibration beads of known size. Identification of the size of the MVs is done by comparing the intensity of light scattering measured for MVs with intensity emitted by the beads of known size. Total number of MVs can be specified using commercially-available beads of known concentration, that are added to a specific volume of the sample [38, 41, 42, 50, 52]. Prior to flow cytometry analysis, platelet-poor plasma or microvesicles suspension is labeled with fluorescent monoclonal antibodies against specific surface antigens of the cell of origin. These fluorescent probes are used to measure biochemical and biological properties of the MVs as well as to quantify and assess the subtype of the vesicles. In addition, to confirm phospholipid properties of MVs, annexin V or lactadhedrin can be used. Annexin V is usually labeled with phycoerythrin (PE), and antibodies to identify the origin of the MVs are labeled with fluorescein isothiocyanide (FITC) [36, 53]. The main advantage of flow cytometry is the double labeling to determine the origin of the MVs. Using antibodies against several cellular antigens, it is possible to characterize a large number of antigens on a single MVs. One of the advantages of flow cytometry is the possibility to analyze a large number of samples in a short time [35]. However, it has some limitations. Namely, it does not provide detailed information on the morphology of the cells [42], does not detect the full range of MVs, particles of smaller size are detected with less effectiveness [54], and the obtained results can vary even when analysis is performed using identical systems [50]. Microvesicles measuring 0.1–0.4 μm are too small to be detected by most of the available flow cytometers and clusters of small MVs might be counted as one event [42]. The results obtained using flow cytometry depend on the type of device, its settings, data analysis, as well as preparation and storage of the sample, which may directly affect the level of MVs. Although the use of antibodies against the surface antigens usually allows you to specify the origin of the MVs, under certain conditions, when the particles contain a small amount of the antigen typically present in the cell, the ability to bind the antibody is limited, specific fluorescence is low, and in consequence MVs might not be detected [35]. Flow cytometry does not require isolation of MVs, which prevents loss and retains the morphological properties of the MVs [41].

5. Nanoparticle Tracking Analysis (NTA)

NTA is a method of direct detection and visualization of particles in suspension, which allows you to specify the size and the number of particles in the studied sample. NTA enables you to specify the relative and absolute size distribution of the particles in the sample. In this method, the particles are illuminated by a laser beam and light dispersion caused by Brownian motions of these particles is analyzed by optical microscope. The particle motion is tracked using a microscope coupled with CCD camera. NTA relates the speed of movements

to the size of the particles. The average speed of the particles, the size and the total number of MVs is calculated by image analysis software [44, 45]. NTA measures absolute particle size distribution in the range from 50 nm to 1 μm and is accurate for particles larger than 50 nm. Detection of smaller particles is not possible due to microscope detection limit. There is a possibility to obtain information on absolute particle size distribution, after calibration of the system using beads of known number and size [42, 44]. NTA can detect particles smaller than those detected by conventional flow cytometer, and therefore it might be considered an alternative to flow cytometer for quantification of MVs. Because the particles are analyzed in suspension, there is no risk for morphological changes during fixation. NTA can be used to analyze all MVs [44]. This method, however, has some limitations. Although it can measure the particle in the sample in details, the presence of large particles reduces the number of small particles detected by the software. NTA has a limited use for clinical samples, is slow and time consuming. It does not provide any information on the biochemical composition and the origin of MVs. Moreover, it is not able to distinguish between cell-derived MVs and other particles i.e. high-density lipoproteins [38, 51, 55].

6. Fluorescent NTA (F-NTA)

   This method is similar to the NTA, but is based on the fluorescence of the particles in the liquid. It measures an absolute size distribution and the fluorescent signal of the molecules in the liquid. F-NTA is a very sensitive method, because fluorescence intensity is much higher than the intensity of white light scattering. Fluorescent particles are analyzed individually in real time. The labeled particles are quantified and measured. The number of labeled particles is compared to the total number of particles measured in light scatter mode [44]. F-NTA seems to be adequate to fast measurement of size, number, biochemical composition, and the origin of MVs. It is specifically useful to identify the characteristics of MVs directly in biological fluids [42]. F-NTA enables the characterization of fluorescently-labeled MVs through identification of their surface markers [49, 56, 57].

7. Raman spectroscopy

   It is a spectroscopic method, based on the inelastic scattering of monochrome, usually laser light, directly using fluorescent probes or indirectly using mono- and polyclonal antibodies [46, 56]. In this method the sample is illuminated by monochromatic laser light. The vibrations of the particles in the sample change the wavelength of the scattered light, which is detected by a specialized and sensitive spectrometer. The pattern of vibration is specific to the molecule, what allows to specify the biochemical composition of MVs without any staining. Raman spectroscopy is a quantitative technique, where signal strength is linearly proportional to the number of particles. In microvesicles that fit in the size of the probe, the intensity of the Raman signal is proportional to the volume of a single vesicle. The method allows to distinguish MVs from lipid particles of similar size, or small platelets. It can specify the size, number and composition of a single MV, without labeling. The method is expensive, has limited availability and is very time consuming (analysis of a single sample takes a few hours) [42, 56]. The main advantage of this method is fast and non-invasive analysis of the biochemical composition of MVs [46].

8. Stimulated emission depletion microscopy (STED)

   STED is a type of fluorescent microscopy characterized by high spatial resolution, allowing to specify the size of the MVs. High resolution STED and fluorescent labeling of microvesicles can potentially be used to obtain information on the morphology and distribution of the labeled receptors on the surface of larger microvesicles. This method is very time consuming and requires several hours to detect and characterize the MVs. The method is still being developed [42, 58].

9. Laser scanning confocal microscopy (LSCM)

   LSCM is a modern variation of fluorescent microscopy using laser as the light source to increase the resolution of the obtained images. In this method the individual subtypes of MVs are identified on the basis of the expression of specific antigens. MVs obtained by ultracentrifugation are labeled with antibodies conjugated with fluorescein, and to prevent Brownian motions, the microparticles are fixed using annexin V. Similarly to other optical methods, the results depend on the specificity and affinity of the antibodies against the target antigen as well as the density of the antigen on the surface of the MVs. LCSM enables to obtain the morphological information i.e. the size and the structure of the MVs. This method is time consuming, requires several hours of analysis per sample and is not suitable for direct determination of MVs in plasma. LSCM allows direct visualization and evaluation of the morphology of the MVs. One of the advantages is detection in a small volume of sample [38, 48, 59].

II. Non-optical methods

1. Transmission electron microscopy (TEM)

   TEM visualizes isolated MVs and allows you to specify their structure [49]. Transmission electron microscope uses electrons to obtain images. It is characterized by much higher resolution, allowing to specify the size and morphology of the MVs. TEM is not quantitative, but is mainly used to assess the composition and morphology of the MV membrane. It is not suitable for direct detection of MVs in plasma, as it requires fixation and dehydration of the sample, which has a direct impact on the size and morphology of MVs. In this method, it is necessary to increase the number of MVs in the sample by ultracentrifugation, and in consequence the quantification of MVs in the original sample is not possible. The use of colloidal gold-labeled antibodies allows to obtain the information on the antigen composition of the MVs. This method requires time-consuming sample preparation and the measurement requires several hours [38, 42, 54, 60]. It provides information on the morphological features of MVs, i.e. size, membrane structure, cytoskeleton [50, 61]. It is mainly used to visualize the MVs and validate other measurements [38].

Cryoelectromicroscopy is a variant of electron microscopy used to analyze MVs in temperatures below minus 100 °C. This method does not require staining and fixation of samples before analysis; however, the use of this method to study the MVs requires further investigation [46].

2. Atomic force microscopy (AFM)

AFM allows to detect a single MV and specify their size in 3D [49]. Atomic force microscope is equipped with a cantilever with a precise, ultra fine tip that is a few μm long and less than 100 Ð in diameter, and is used to scan the surface of the sample. In order to generate a three-dimensional, high-resolution image, the microscope scans the surface of the sample while the detector equipped with laser measures the deflection of the cantilever moving along the sample. The measurement of the deflection allows to generate a 3D image of the topography of the surface. The strength of the interaction between the tip and the sample depends on the distance between them. The advantage of this method is the possibility to detect the MVs directly in liquid, which allows to keep the sample in a physiological state. To distinguish MVs from the surface, this method requires strong binding of the particles to a flat surface what may influence morphology and hinder the determination of the correct diameter. Mica, an insulating mineral, coated with antibodies, is used to bind the MVs to the surface. This method requires the isolation of the MVs from the plasma and concentration of the sample prior to the measurement, which affects the morphology and the number of MVs. Fraction of the isolated MVs is incubated to bind MVs to the surface coated with antibodies [62]. The number of MVs on the surface is calculated using an image processing software. As the efficiency of binding to the surface of the MVs is not known, the number of particles cannot be accurately measured. AFM allows to detect small MVs and determine the phenotype but cannot obtain information on functional properties of MVs [38, 42, 49, 63, 64]. The measurement requires several hours (about 2 h per sample), the method is very labor-intensive [50, 64].

3. Impedance-based flow cytometry

It is a fast, non-optical method to identify, count, and determine the size of the MVs. It uses the Coulter principle to count and measure individual particles in a liquid within a few seconds, by detecting changes in electrical resistance produced by particles in suspension [65]. It consists of two chambers separated by an insulating membrane containing a single channel. The chambers are filled with electrolyte and an electrode is placed in each of them. The particles introduced to the channel induce reduction in electric current, which is measured as a voltage pulse. The size of the impulse is proportional to the size of the detected particles. MVs can be detected directly in the platelet-poor plasma using fluorescent antibodies. The lower detection limit is 300 nm, and in consequence MVs less than 300 nm are detected less efficiently. This method does not provide any information on the morphology, biochemical composition or origin of the particle, but can be combined with fluorescent flow cytometry [42]. MVs size distribution can be assessed using fluorescein-labeled antibodies, assuming that fluorescent signal amplitude is proportional to the volume or surface of the particles. To measure the MVs, the system is calibrated using fluorescent polystyrene beads of uniform size. The limit of detection depends on the diameter of the flow chamber [38, 56, 65].

4. Western blotting (WB)

WB is a method that allows to specify the origin of the MVs depending on the presence of various markers [6]. Western blot analysis consists of five stages: electrophoretic separation of proteins, transfer of the proteins to the nitrocellulose or polyvinylidene fluoride membrane, labeling using primary antibodies against the specific antigen, incubation with the secondary antibody against the primary antibody, and finally, visualization [57]. This method, however, requires the use of further complementary methods such as NTA or electron microscopy [46].

A large number of MVs to perform WB analysis, what limits the applicability of this method for serum samples [61, 66]. Moreover, WB allows to study specific subtypes of MVs, but do not give any information about the size and number of MVs [44].

5. Enzyme-linked immunosorbent assay (ELISA)

ELISA assay is a simple and reproducible method to measure MVs. This method is based on the binding of microvesicles by monoclonal antibodies conjugated with fluorescein in the multiwell plate. Annexin V or antibodies against surface antigens (so called catching antibodies) are used to specifically bind plasma MVs, and detection antibodies against surface antibodies are used for the detection of the "cought" antibodies [67]. ELISA allows quantitative analysis of MVs indirectly, using the measurement of total amount of phosphorus or anionic phospholipids. The advantages of MVs detection using ELISA are: the possibility to examine a large number of samples at the same time, no restrictions on size, wide availability of the method, and quantitative assessment of MVs. This method has also some disadvantages: it does not analyze all types of MVs, detects soluble antigens, it does not provide information about the size, and finally, non-specific binding of annexin V limits its usability [7, 43, 50]. In this method, it is necessary to use fresh plasma, because the freezing and thawing causes an increase in the number of annexin-binding MVs [68]. ELISA allows you to specify subtypes of MVs and is a high-throughput method [38, 52].

6. Functional assays

Functional assays characterize microvesicles based on their specific property. These methods are used for indirect determination of the number of MVs based on the measurement of the procoagulant or prothrombinase activity. The main disadvantage of these methods is the measurement of a single biological activity only. It is impossible to analyze the size of the studied MVs [49, 50, 69–71]. The method is based on the measurement of thrombin generation for indirect quantification of procoagulant MVs. The measurement of the total number of procoagulant MVs in plasma depends mainly on the exposure of procoagulant phosphatidylserine on the sur-

face of MVs, which binds annexin V. MVs-TF activity assay quantifies the level of circulating procoagulant MVs coated with TF by measuring the formation of MVs-TF-dependent factor Xa [72]. Functional assays have two major limitations: they do not assess the size and number of MVs in the sample. The advantage of the method is the measurement of all MVs, regardless the size and origin. In addition, the assays are easy to perform and allow to analyze large number of samples. As of today, there are limited and inconsistent data on the correlation between the flow cytometry and functional assays in terms of MVs analysis. The lack of a positive correlation is not surprising, considering the fact that highly sensitive method, AFM, is able to detect 1000-times more MVs than flow cytometry. Due to the fact that flow cytometry does not allow to detect small MVs, it is understandable why the results obtained in functional assays or using flow cytometry are not correlated [64].

7. Surface plasmon resonance based imaging microscopy (SPRi)

   SPRi is a quantitative method allowing to assess the size of the biological nanoparticles. It can be useful to solve the majority of problems in MVs analysis [6]. SPRi is a highly sensitive technique of biochemical analysis that does not require any staining. Plasmon resonance, the principle of the method, is associated with high resolution diffraction generated on the surface a thin metal surface [64]. SPRi equipment consists of an optical part containing radiation source, transformer that converts changes occurring on the surface of the metal to changes of the refractive index and a system processing and recording the data. SPRi apparatus is usually equipped with a CCD camera to capture the reflected light. High contrast signal between the cell edges and the surface makes it easier to identify the edges and the cells. This method do not allow to visualize the cells, however, makes it possible to count them [73, 74]. Quantitative interpretation of SPRi results can improve the analysis of MVs and overcome the limitations of flow cytometry in terms of low detectability of small MVs. Simultaneous use of SPRi and fluorescent microscopy improves the sensitivity and selectivity of the method, which may improve the identification of small MVs originating from different cells that have been considered so far to be cell fragments [46, 75, 76].

8. Surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS)

   SALDI-MS is a high-throughput technique capable to detect the analytes of low molecular weight, including MVs. The basic principle of MVs detection using SALDI-MS is similar to mass spectrometry; however, SALDI-MS uses organic matrix to prevent interference between the sample and matrix molecules after application of laser pulses [57]. The main advantage of this method is the possibility to analyze MVs from various biological fluids without special sample preparation. SALDI-MS measures the size of the MVs with lower limit of detection approx. 10–30 nm what allow to study small microvesicles. However, the role of SALDi-MS in the detection of MVs requires further investigation [6, 46].

Identification of the origin of MVs

Lipid and protein composition of MVs is similar to the cell of origin. Surface antigens allow to distinguish different subpopulations of MVs and identify the origin of the vesicle [3, 7, 77]. A list of antigens used to identify the subtype of MVs is shown in Table III. Identification of the origins of MVs isolated from plasma is possible using flow cytometry, microscopy or WB. These methods provide detailed information about the presence or absence of specific antigens in small samples [61, 78]. Identification of the origin of MVs is performed using fluorescently labeled monoclonal antibodies against specific surface antigens. These antibodies can distinguish MVs derived from platelets, white blood cells, red blood cells, and endothelial cells [38]. The fluorescence signal depends on the specificity and affinity of antibodies to the target antigen and the number of antigens present on the surface of the microvesicle. The correlation between the fluorescence and the scattered light is used to identify subtypes of MVs. Flow cytometry is the most commonly used method for the determination of the origin of the MVs. Fluorescence detectors in flow cytometry allow simultaneous detection of more than two antigens on a single MV. Platelet-derived MVs are detected by a single staining using antibodies against platelet antigens such as CD41 or CD42b, or using a combination of anti-CD41/CD42b. In addition to CD41 and CD42b, anti-CD31 antibody can be used for the detection of platelet-derived MVs. CD41 and CD42b are expressed on platelets only, while CD31 is present in large amount on the endothelial cells and other cells such as monocytes and granulocytes. The most common method of endothelium-derived MVs staining is a combination of CD31 and anti-CD41 or anti-CD42b antibodies. Because CD31 is a common marker of both platelets and endothelial cells, platelet-derived MVs are CD31+/CD41+, while endothelium-derived MVs are CD31+/CD41−. The advantage of this combination is high specificity. Other combinations can also be used, such as: CD61 for platelet-derived MVs and CD144 for the endothelium-derived MVs. Detection of leukocyte-derived MVs is difficult due to the variability of surface markers in different populations. Leukocyte-derived MVs can come from neutrophils, monocytes as well as B and T cells. Leukocyte markers include CD11b/CD18, CD11a/CD18, CD14, CD66, CD62L, or annexin [10, 35]. Anti-CD235a antibodies are used to detect erythrocyte-derived-MVs [79].

Quantification of MVs

The total number of MVs can be specified using flow cytometry, using commercial, fluorescently-labeled beads of known concentration, that are added to the specific volume of the sample. The number of MVs and beads together with the volume of the sample are used to determine the total number of MVs [10, 38, 41, 42, 50, 52]. Flow cytometry using fluorescein-conjugated antibodies against surface antigens and annexin V allow efficient counting of MVs in suspension [46]. Although not all MVs express phosphatidylserine on the surface, annexin V staining enables to distinguish

Table III – Antigens used to identify specific MVs subsets according to cell of origin

MV subtype	Antigen	Alternative name
CD41	GPIIb	
Platelet-derived MVs	CD42a	GPIX
	CD42b	GPIbα
	CD61[a]	GPIIIa
	CD62P[a]	P-selectin
Eythrocyte-derived MVs	CD235a	Glycophorin A
Leukocyte-derived MVs	CD45	Pan-lymphocyte antigen
Monocyte-derived MVs	CD14[a]	LPS receptor
Neutrophil-derived MVs	CD66b	
Lymphocyte-derived MVs	CD4[a]	
	CD8[a]	
	CD19	
	CD20	
Endothelium-derived MVs	CD31[a]	PECAM-1
	CD62E	E-selectin
	CD105[a]	Endoglin
	CD144	VE-cadherin
	CD146[a]	Muc18

[a] Shared antigen with other cell type.

MVs from large fragments of cell membrane and is widely used to measure the total number of MVs. Staining of the MVs with annexin V depends on the concentration of calcium ions and the amount of phosphatidylserine in cell membrane. Instead of annexin V it is possible to use lactadherin, which is more sensitive to changes in the expression of phosphatidylserine and detects the MVs in the absence of calcium ions [80, 81]. Determination of the number of MVs is also possible using AFM, NTA, and F-NTA. These methods, including flow cytometry, are based on the use of specific antibodies to the surface antigens of the MVs. Quantification of MVs using F-NTA and AFM requires high affinity antibodies. Quantification of MVs with these methods requires further development. Raman spectroscopy is a quantitative technique, where signal strength is linearly proportional to the number of particles. This method is very time consuming, expensive, and its availability is limited [42]. ELISA assay for the measurement of total phosphorus or anionic phospholipids can be considered an alternative to flow cytometry for quantification of MVs [43]. The quantification of MVs requires correlation of the results with standard curve.

Standardization of MVs research

The growing interest in the role of microparticles requires standardization of methods used for their isolation and analysis. Standardization is necessary to measure and compare the number, origin and biological activity of MVs in physiological and pathological conditions [82]. The methods used for isolation, qualitative and quantitative evaluation of the MVs has not been standardized so far [6, 83]. Standardization is difficult because of small size and heterogeneity of microvesicles. Moreover, new methods for the detection of even the smallest MVs in biological fluids are constantly developed. These methods, employing modern flow cytometry assays, provide information on the mechanisms involved in the generation and biological functions of MVs. In the past few years, a few attempts to standardize cytometric methods for MVs studies have been made. It has been shown, that to obtain consistent, reproducible results of MVs measurement stable analytical conditions should be maintained [79].

One of the attempts to standardize MVs measurement was made by the International Society of Thrombosis and Haemostasis (ISTH). In 2008, Robert, Poncelet and colleagues described a reliable and reproducible method for measuring platelet-derived MVs using Beckman Coulter Cytomics FC500 flow cytometer. They used a mixture of fluorescent beads of known size and showed that it is possible to count the MVs reproducibly on different machines of the same type. It was the first step toward the standardization of MVs measurement methods [41]. In 2010, ISTH Scientific and Standardization Subcommittees (SCC Vascular Biology, DIC and Hemostasis & Malignancy) attempted to standardize the flow cytometry method for the measurement of platelet-derived MVs bigger than 0.5 μm. In this project, various types of cytometers were calibrated using a set of synthetic, sub-micron beads measuring 0.5 μm, 0.9 μm, and 3 μm. The study involved 40 laboratories from 14 countries. Each of them received a plasma sample with three different concentrations of MVs and studied it using the same batch of antibodies for MVs staining. The results confirmed that calibration strategy using beads is useful and the quantification of MVs by flow cytometry is influenced not only by analytical factors (i.e. laser, antibodies, device type, calibration of instruments), but also factors related to the preparation and storage of the sample. These studies have shown that the standardization of platelet-derived MVs quantification using flow cytometry is possible, but depends on both the intrinsic characteristics of the flow cytometer as well as calibration strategy. The calibration beads are useful standards that allow for the qualification of the device, but do not constitute a universal standard in quantification of MVs. The attempts to standardize quantification of MVs using

Table IV – The advantages and disadvantages of the methods used to detect MVs

Method	Advantages	Disadvantages
Optical microscopy	- size assessment - morphology assessment - number of MVs	- time consuming - low throughput - it is not possible to measure the size and morphology of MVs of less than 200 nm
Fluorescent microscopy	- size assessment - number of MVs	- time consuming - low throughput
DLS	- size assessment - measurement of the relative and absolute MVs size distribution - fast	- it does not provide information on the biochemical composition and cell origin - less accurate in the polydispersion samples - may exclude small MVs
Flow cytometry	- allows analysis of a large number of MVs in a short period of time - number assessment - determination of origin - size assessment - availability - small sample volume - analysis of different subtypes of MVs at the same time - possibility to use multiple markers for the determination of the origin of the MVs	- inaccurate for 100–400 nm particles - small MVs may count as one - does not provide detailed information on the morphology of the cells - determination of the origin of the MVs depends on the specificity of the antibody
NTA and F-NTA	- precise measurements - assessment of the number of MVs - determination of the absolute and the relative distribution of MVs - detection of small particles - analysis of samples in suspension - fast - in combination with detection of fluorescence allows to assess the origin of the MVs	- limited availability - time consuming - requires clean-up of the sample from cell debris and plasma proteins
Raman spectroscopy	- size assessment - assessment of the number of MVs - determination of the biochemical composition without staining	- limited availability - time consuming - expensive
STED	- size assessment - morphology assessment	- limited availability - time consuming - requires improvement
LSCM	- determination of origin - size assessment	- time consuming - it is not suitable for the determination of MVs in plasma
TEM	- high resolution - size assessment - morphology assessment - phenotype assessment using colloidal gold labeling	- limited availability - inability to determine the number of MVs - requires dehydration and fixation of the sample, - not suitable for direct detection of MVs in plasma - time consuming
AFM	- assessment of the number of MVs - determination of origin - accurate measuring with a three-dimensional view of the MV structure, - high resolution - detection of single MVs - detection in liquid allowing to keep the sample in physiological condition	- limited availability - determination of the origin of the MVs requires using the surface coated with antibodies - requires isolation of the sample before the analysis - artifacts due to impurities and plasma proteins - low throughput - time consuming
Impedance-based flow cytometry	- fast - assessment of the number of MVs - size assessment - measurement directly in the plasma	- does not provide biochemical information - MVs less than 300 nm are detected with lower efficacy
WB	- determination of origin - semi-quantitative method	- inability to determine the number of MVs - requires a large sample size

Table IV (*Continued*)

Method	Advantages	Disadvantages
ELISA	- determination of origin - assessment of the number of MVs - availability - high throughput - there are no limits to the size of the measured particles - measurement directly in the plasma	- inability to determine the number of MVs - does not allow the analysis of all MVs - detects soluble antigens - require the use of fresh plasma - does not give information on the size and total number of MVs in plasma - quantitative assessment based on the presence of a single antigen
Functional assays	- availability - the assessment of biological activity - determination of the relative number of MVs - easy to perform - measurement of all MVs regardless of the size and origin - possible to study a large number of samples	- inability to determine the size of MVs - inability to determine the origin of MVs - indirect quantification - measures a signle biological activity
SPRI	- quantitative assay - size assessment - no need tp stain the sample - detects small MVs	- does not provide detailed information on the morphology of the cells - identification of small-sized MVs possible while simultaneously using fluorescent microscopy
SALDI-MS	- high throughput - allows to assess small size MVs - the possibility to analyze MVs in various biological fluids, - no sample preparation - determination of origin	- inability to determine the number of MVs - requires improvement to be used in the assessment of the MVs

flow cytometry failed [82]. In 2014, ISTH SSC Vascular Biology ISTH launched a new initiative to standardize the cytometric method, however, the results of this study have not been announced yet [55]. The next step made by ISTH is to standardize quantification of MVs smaller than 300 nm [84].

To analyze MVs in clinical diagnostics it is necessary to standardize not only device settings but also pre-analytical conditions [79]. Despite significant progress in the analytical phase, many pre-analytical variables may have a critical impact on the determination of MVs, i.e. blood collection, centrifugation conditions, the use of fresh/frozen samples [37, 38]. Therefore, the main priority is to standardize pre-analytical conditions. In 2015, in addition to attempts to standardize the analytical conditions, ISTH attempted to standardize pre-analytical conditions for the determination of platelet-derived MVs [85]. Two pre-analytical protocols have been used: any protocol used by the laboratory or a specific protocol common for all laboratories. In the common protocol, the blood was taken on an empty stomach in the morning from 08:00 to 11:00, by venipuncture at ulnar flexion with 21 gauge needle and light tourniquet. The blood was drawn to a plastic tubes containing citrate (3.2%) at a minimum volume of 3.5 ml. The first 2–3 ml of blood were discarded and the samples were gently mixed. The samples were transported in a transport container, in a stable vertical position and stored at room temperature (20–24 °C). Time to the first centrifugation was maximum 2 h. The samples were spun down at 2500 × *g*, at room temperature, for 15 min. The plasma was collected gently to a plastic tube leaving 1 cm, and then centrifuged for a second time at 2500 × *g*, at room temperature, for 15 min. The plasma was frozen in liquid nitrogen and stored at −80 °C until analysis. The use of this protocol allowed to reduce interlaboratory variability, however, it still remained too high to implement the method into routine clinical practice. Low reproducibility of the results is observed even in cases when a specific pre-analytical protocol is strictly implemented, what suggests that there are other, unidentified parameters influencing the number of MVs. Moreover, there is a need to standardize specific pre-analytical conditions for various subpopulations of MVs, including erythrocyte-, leukocyte- and endothelium-derived MVs [82, 85].

In the last 5 years, 2 methods of reproducible determination of platelet-derived MVs in plasma samples were presented. Both methods are based on flow cytometry with reference beads of various sizes. Both systems provide reproducible method for the analysis of platelet-derived MVs. However, these methods work only on certain cytometers, which is incompatible with the requirements of standardization [84].

Conclusions

Various methods are used to analyze MVs, and each of them has some pros and cons – see Table IV. These methods are often difficult to access, low-throughput or do not provide both qualitative and quantitative data. The exact measurement of the MVs is difficult due to the lack of standardized testing methods. The results of MVs measurements vary between different laboratories, not only because

of the discrepancies in the pre-analytical steps, but also in the analytical methods used to measure the MVs [10]. Review of the literature clearly indicates the need for further development of the MVs studies. Measuring the MVs in reproducible way is still a challenge. Characterization of MVs remains difficult due to the small size and diversity of the particles in terms of their phospholipid content and antigen profile. In addition, there is still no agreement regarding the best markers to define subpopulations of MVs derived from vascular endothelium, leukocytes or tumor, which has the highest predictive value [8]. Considering all the methods mentioned above, flow cytometry seems to be the best technique to study MVs because of the possibility of multiparametric analysis. It provides not only quantitative, but also qualitative information, thereby it allows to identify the origin of cell-derived MVs and despite many limitations it still remains the most commonly used technique for the detection and analysis of the MVs [50]. Microscopic methods, ELISA, and functional tests are routinely used, although they do not offer the same high-quality information as flow cytometry. Measurement of MVs can be crucial in the diagnosis and therapy of many diseases, and the ability to compare the obtained data is a necessary to explore the full potential of the MVs. However, standardization of methods used to analyze MVs is required to implement MVs assessment into clinical practice.

Authors' contributions/ Wkład autorów

According to order.

Conflict of interest/ Konflikt interesu

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Ethics/ Etyka

The work described in this article have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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