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Review

Cell-derived microvesicles in cardiovascular diseases and antiplatelet therapy monitoring — A lesson for future trials? Current evidence, recent progresses and perspectives of clinical application

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ABSTRACT

Circulating cell-derived microvesicles (MV) represent a subject of increasing interest in recent years as potential effectors in thrombosis, inflammation and vascular injury. Although several studies demonstrated an association between MV plasma concentrations and clinical manifestations of atherosclerosis as well as a clear effect of cardiovascular pharmacotherapy on MV formation pattern, the application of this promising biomarkers in clinical cardiology has been hindered so far due to heterogeneity of the hitherto studies employing non-standardized methodologies.

Recently great progresses have been done and international initiatives were started to unify the pre-analytical and analytical procedures, improve the comparison of measurements between the laboratories and increase detector sensitivity of flow cytometry — a golden standard for MV assessment. Likewise, the concept of a "therapeutic window" of P2Y12 inhibitor therapy was introduced, as the prognostic significance of bleeding consequences is equally important with that of ischemic events, particularly with the expanding use of more potent P2Y12 inhibitors. In this review we summarize currently available studies on circulating MV in terms of cardiovascular diagnosis, risk stratification and influence of antiplatelet agents on the MV release to postulate possible future role of MV as supplementary biomarker in monitoring of individual response to antiplatelet therapy. Methodological pitfalls faced in the previous studies and obstacles that need to be addressed before further trials and translation of MV-based assays into clinical practice were defined.

1. Introduction

In the quest for novel, reliable biomarkers in the diagnosis of cardiovascular diseases (CVD) circulating cell-derived microvesicles (MV) have become a subject of increasing interest in recent years as potential effectors in thrombosis, inflammation and vascular injury. Several studies have suggested an association between concentrations of cell derived MV and such conditions as stable coronary artery disease (SCAD), acute coronary syndromes (ACS) and other clinical manifestations of atherosclerosis [1], in which the reported increased levels of MV plasma concentrations have been considered as indicators of platelet activation and endothelial dysfunction [2]. Interestingly, the effects of a variety of drugs, including antiplatelet agents, on MV formation have been also described. Nonetheless, due to some methodological obstacles there are still no firm conclusions regarding the utility of MV measurements in clinical setting.

Recently significant advancement has been done and international initiatives were started to standardize the pre-analytical and analytical procedures and improve the detector sensitivity of flow cytometry to allow for comparison of the results from different instruments [3–7] — a condition that has to be fulfilled in an attempt to develop MV-based biomarker assays in cardiology. MV analyses have been already translated into diagnostic oncology, where a protein present on the tumor-derived MV, called glypican-1, proved to be useful for the early diagnosis of pancreatic cancer [8].

The aim of this review is to summarize currently available studies on circulating MV in terms of CVD diagnosis and cardiovascular risk assessment, define methodological pitfalls faced in the previous studies and obstacles that need to be addressed before wider application of MV

Abbreviations: MV, microvesicles; CVD, cardiovascular diseases; SCAD, stable coronary artery disease; ACS, acute coronary syndromes; EV, extracellular vesicles; CD, clusters of differentiation; PMV, platelet-derived MV; EMV, endothelial cell-derived MV; MMV, monocyte- derived MV; EryMV, erythrocyte-derived MV; NMV, neutrofile-derived MV; GP, glycoprotein; GPIIb/IIIa, glycoprotein IIb/IIIa receptor; GP IX, glycoprotein IX; GP Ib, glycoprotein Ib; GP IIIa, glikoprotein IIIa; HPR, high-on treatment platelet reactivity; VASP, vasodilator-stimulated phosphoprotein; RI, refractive index; NTA, nanoparticle tracking analysis; RPS, tunable resistive pulse sensing; PS, phosphatidylserine; TF, tissue factor; DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid.

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measurements to clinical practice as well as to review the great progresses that have been already done in the flow cytometry technique of MV assessments.

In addition, by the review of reports on the effect of antiplatelet agents on the MV formation *in vitro* and *in vivo* the authors will discuss a possible future role of MV measurements in the monitoring of individual response to antiplatelet therapy, once the currently pursued unification of methodology and a better comparison of assessments are achieved to reveal a full potential of MV as a novel biomarker. Since a number of publications on circulating MV raise exponentially, we sought to summarize only the most relevant papers, published in international, peer-reviewed journals. We focused on the studies that determined MV concentrations using flow cytometry, as – despite its still relatively low sensitivity – this technique remains the gold standard for MV measurements [3,5–7].

2. Cell-derived vesicles — classification, characteristics and subtypes

Cell-derived vesicles are fragments of outer cell membrane, which are released into the cell environment by all prokaryotic and eukaryotic cells [3,9]. Although there is currently no consensus on the classification of vesicles, the three most extensively investigated subtypes of extracellular vesicles (EV) are exosomes, MV and apoptotic bodies, which partially differ in size, morphology and biochemical composition [3,9] (Fig. 1). However, there is currently no consensus on the nomenclature and properties of different EV subtypes, because (1) the sizes of exosomes (50–100 nm), MV (20–1000 nm) and apoptotic bodies (1000–5000 nm) partially overlap, (2) they are released simultaneously from the same cell types, and (3) they have a similar morphology when assessed with transmittance electron microscope (TEM) [3,9]. Nevertheless, it is recognized that MV (1) are spherical particles surrounded by a phospholipid bilayer (membrane), (2) have a diameter < 1 μm, (3) retain cytoplasmic components of their parent cells, and (4) expose specific transmembrane receptors such as integrins derived from the parent cells [9] (Fig. 2).

MV are found in all body fluids, including plasma, whole blood, umbilical cord blood, urine, saliva and cerebrospinal fluid, as well as in conditioned culture media [10]. MV plasma concentrations are dependent on constitutional factors such as age and sex with elevated levels in pediatric and geriatric populations, as well as in men [9,10]. Increased MV levels were found after intense physical activity, during pregnancy, in obese and smoking patients [9,10]. Vesicles are referred to as important mediators of intercellular communication thanks to their capacity to transfer proteins, lipids and nucleic acids between the cells, and thus, to influence various physiological and pathological processes both in parental and in recipient cells [3,9,10]. Under steady-state conditions, vesicles seem to regulate hemostasis, inflammatory processes and endothelial function [11]. In stress conditions, upon cell activation or apoptosis an increased release of vesicles derived from circulating blood cells and vessel wall components (endothelial cells, smooth muscle cells) has been observed [11]. According to the widely accepted principle, MV expose phosphatidylserine (PS) and other negatively charged phospholipids on their surface, which are thought to bind activated clotting factors, including TF, and to create a source of circulating thrombin [10]. Nevertheless, it should be underlined that PS exposure is highly increased due to pre-analytical procedures, and that a substantial part of MV derived from healthy volunteers is PS negative [12].

About 70–90% of circulating MV derive from platelets (PMV) and megakaryocytes, however these vesicles are also abundantly released from activated or apoptotic endothelial cells (EMV), erythrocytes (EryMV), neutrophils (NMV), monocytes (MMV) or smooth muscle cells [3,9,10]. The various subtypes of MV seem to not only differ in biochemical composition and specific CD antigens exposed on their surface (Table 1), but also exert different functions. For instance, PMV and EryMV are thought to trigger thrombus formation by promoting thrombin generation in a factor XII-dependent manner, whereas MMV facilitate coagulation predominantly *via* TF [13,14]. MV released from leukocytes and endothelial cells can in turn stimulate the expression of proinflammatory genes in vascular endothelium, leading to the production of cytokines and leukocyte–endothelial adhesion. Thus, vesicles are considered to promote inflammation and coagulation along with modifying endothelial function, which all contribute to the development of atherosclerosis [15]. In the course of prothrombotic conditions, as well as some chronic and acute inflammatory conditions, such as SCAD, acute myocardial infarction, peripheral vascular disease and

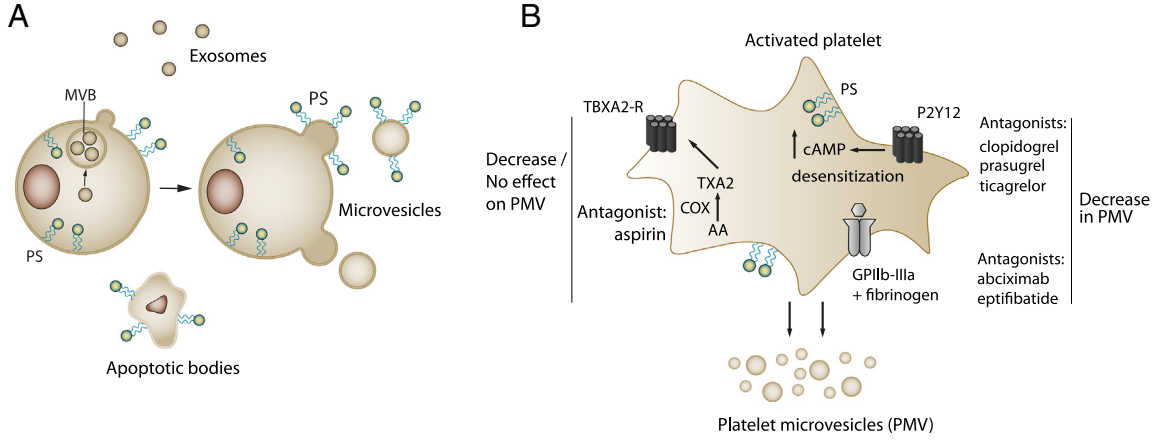

Fig. 1. (A) Formation of extracellular vesicles. Extracellular vesicles could be classified into three main classes: Exosomes which are formed inside the cell within the multivesicular bodies (MVB) and released upon MVB fusion with plasma membrane (PM). Exosomes carry the parent cells' specific markers, however, expose little or no phosphatidylserine (PS); microvesicles (MV) are released by outward budding of the PM. Upon cell activation or apoptosis, cytoskeleton is reorganized and PS, normally located at the inner side of the membrane, is exposed at the cell surface, facilitating MV generation. Nevertheless, MV formation is possible also without PS externalization. Apoptotic bodies are released as blebs of apoptotic cells, following such processes as increase in PM permeability, fragmentation of DNA and changes in the mitochondrial membrane potential. They expose PS on their surface, however, compared to MV, apoptotic bodies are usually observed to enclose more abundant organelles and nuclear fragments involving both nucleic acids and histones. (B) Potential effects of antiplatelet drugs on MV release from activated platelets Antagonists against P2Y12 receptor, such as clopidogrel, increase the intraplatelet concentration of cyclic adenosine monophosphate (cAMP), thereby decreasing platelet sensitivity to activation and the subsequent platelet vesiculation. Antagonists against GPIIb-IIIa receptor, such as abciximab, block binding of fibrinogen to the GP IIb-IIIa receptor, thereby preventing platelet aggregation and the second wave of platelet activation. Both P2Y12 antagonists and GPIIb-IIIa antagonists were shown to reduce the rate of MV generation [41,48,49,50,52,55]. The relationship between inhibition of the cyclooxygenase (COX) pathway with aspirin and reduction of MV release remains controversial and requires further investigation. Only receptors within the scope of this review have been presented. AA — arachidonic acid; TXA2 — thromboxane A2; TBXA2-R — thromboxane A2 receptor.

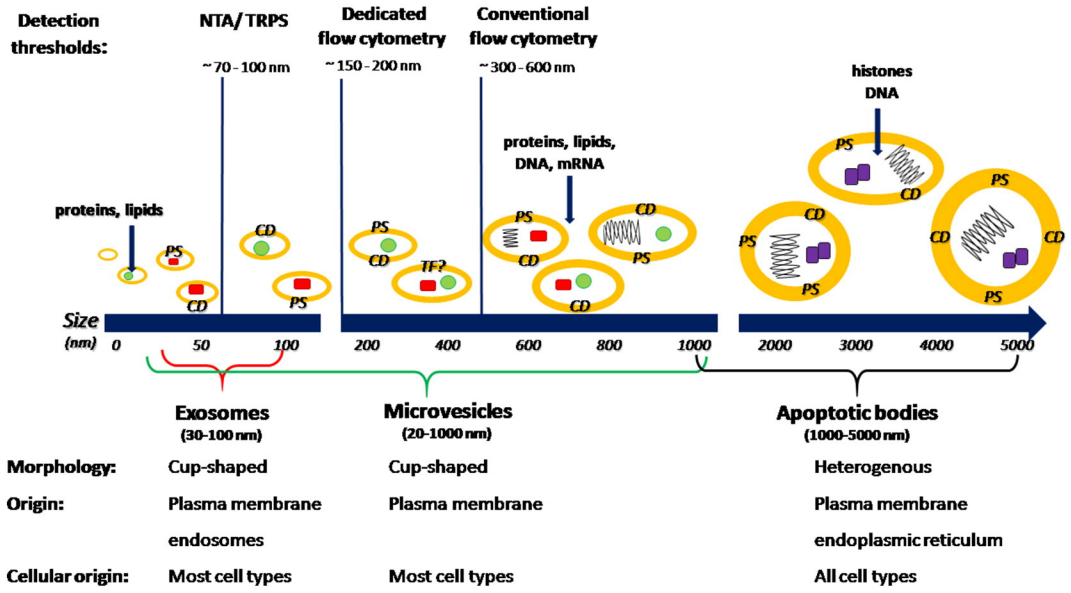

Fig. 2. A summary of extracellular vesicles' subtypes and the thresholds of currently available diagnostic modalities. NTA — nanoparticle tracking analysis, RPS — tunable resistive pulse sensing, PS — phosphatidylserine; CD — clusters of differentiation; TF — tissue factor; DNA — deoxyribonucleic acid; mRNA — messenger ribonucleic acid; [3,6,8,10,70].

cerebrovascular ischaemia, elevated plasma concentrations of MV were observed [16–25]. Recent studies suggested that MV levels not only are indicative of the severity of the disease, but also may contribute to the progression of both chronic and acute cardiovascular conditions, providing a novel link between inflammation and thrombosis [26].

3. Microvesicles in the diagnosis of cardiovascular diseases

MV made their way into the headlines of journal articles in many fields of medical research as potential sensitive, non-invasive biomarkers indicating the degree of activation or apoptosis of platelets, leukocytes, erythrocytes and endothelial cells. Thus, there have been attempts to measure their concentrations in cardiovascular disease not only to diagnose the clinical entity, but also to assess its severity.

A correlation between EMV levels and the severity of coronary artery disease (CAD) was first observed more than 10 years ago in a prospective, case-controlled study comparing patients with CAD and healthy volunteers [16]. Importantly, the study groups did not differ in terms of lipid-lowering therapy, which is now known to influence plasma MV concentrations [17]. EMV expressing CD31 were able to discriminate patients with CAD from control subjects, with 2.5-fold higher levels observed in the course of SCAD than in healthy volunteers and a further 12-fold increase in ACS [16]. Interestingly, in patients with first myocardial infarction (MI) higher plasma CD31+ EMV were observed than in patients with a history of ACS. In this subpopulation, EMV positive for CD31 were able to discriminate between MI and unstable angina (UA). As CD31 is present both on endothelial cells and on platelets, the authors used double labeling with a PMV marker anti-CD42 to exclude counting PMV as EMV. Both EMV enumerating CD51 and PMV positive for CD42 were significantly higher in CAD population than in control subjects, however they failed to differentiate subgroups of CAD (ACS, MI, UA, SCAD). The authors concluded that EMV exhibiting CD31 may serve as a useful marker of acute myocardial injury in the course of CAD, whereas EMV CD51+ and PMV CD31+42+ are indicative rather of a chronic ischemia.

In a more recent study, it was observed, that a total count of MV of different origin, including EMV (CD31+, CD51/61+ and CD34+), PMV (CD42+), MMV (CD14+) and MV derived from leukocytes (CD45+) was able to discriminate patients with MI from those with SCAD and healthy volunteers [18]. Additionally, levels of PMV exposing CD42 and TF (CD142) were significantly higher in SCAD than in a control group, thus corresponding to the severity of CAD. However, due to the small sample size and lack of information regarding cardiovascular medications in the analyzed population, it is relevant to remain cautious interpreting the results.

Another prospective, observational study performed in CAD population undergoing elective or urgent percutaneous coronary intervention (PCI) with stent implantation also found significantly higher levels of EMV (CD31+,CD42-), PMV (CD31+,CD42+) and MV released from apoptotic endothelial cells or platelets (CD31+, annexin+) in MI than in SCAD population [19]. However, no differences were seen between ST-elevation MI (STEMI) and non-ST-elevation MI (NSTEMI) or between SCAD alone or SCAD with concomitant peripheral artery disease (PAD), assessed by ultrasound study of carotids and lower limb arteries [19]. Of note, statins and antiplatelet therapy with aspirin and clopidogrel were equally distributed within the study groups, except for GPIIb/IIIa antagonists, administered only to unstable MI patients. These findings support the view that MV release correlates rather with the severity of atherosclerotic disease than with its anatomical extension or with MI pathophysiology, which is different in STEMI and NSTEMI.

Studies on MV role in patients presenting with cerebrovascular disease and PAD yielded similar results. In a group of 68 patients with acute ischemic stroke EMV of different phenotypes (CD144+/CD41a−, CD31+CD41a−, CD62E+ and annexin V+/CD62E+) were significantly higher than in age- and sex-matched healthy control subjects [20]. Moreover, levels of CD144+/CD41a− EMV correlated

Table 1
Surface antigens exposed on the surface of microvesicles of different cellular origins.

MV subtype	Surface markers
PMV	CD31, CD41 (GPIIb/IIIa, αIIb β3), CD42a (GPIX), CD42b (GPIb), CD61 (GPIIIa), CD62P (P-selectin), CD63
EMV	CD31, CD51 (vitronectin receptor, αv β3), CD54 (ICAM-1), CD62E (E-selectin), CD105, CD106, CD144 (VE-cadhedrin), CD146,
MMV	CD14, CD54
NMV	CD 66b (CEACAM-1)
EryMV	CD 235a (glycophorin A)

MV — microvesicles, PMV — platelet-derived MV, EMV — endothelial cell-derived MV, MMV — monocyte-derived MV, EryMV — erythrocyte-derived MV, NMV — neutrofile-derived MV; GP — glycoprotein; GPIIb/IIIa — glycoprotein IIb/IIIa receptor; GP IX — glycoprotein IX; GP Ib — glycoprotein Ib; GP IIIa — glicoprotein IIIa [16–25,27].

with stroke severity, assessed in NIH (National Institutes of Health) stroke scale, thereby providing evidence of a relationship between endothelial disorder and acute cerebral ischemia, whereas annexin V+/CD62E+ EMV level corresponded to the stroke subtype, evaluated in OCSP (Oxfordshire Community Stroke Project) score, thus pointing out a correlation between EMV with this phenotype and location of ischemia lesions. Correspondingly, PMV CD 61+ concentrations were significantly elevated in 112 patients with acute ischemic stroke, as compared to healthy controls [21]. Moreover, PMV were associated with the infarct volume in large-artery atherosclerosis, assessed by magnetic resonance imaging, which is consistent with the previous findings. Similarly, in subjects with PAD, levels of PMV positive for CD42b and CD61 were higher in patients with intermittent claudication compared to healthy controls [22], showing a further increase in critical limb ischaemia, which is indicative of an escalating prothrombotic state. Nevertheless, one should be aware that PMV positive for CD61 (platelet fibrinogen receptor subunit) are now known to derive not only from platelets, but also from megakaryocytes. Hence, this PMV CD61+ subtype may not be indicative solely of platelet activation and cerebral or peripheral ischaemia [23].

MV released from leukocytes (LMV), although less frequently found in plasma, comprise the major MV subpopulations in human carotid plaque extracts [24]. It was observed, that in patients with a significant (>70%) carotid artery stenosis, increased levels of LMV exposing CD11b/CD66b and CD15 were associated with carotid plaque instability, assessed after thromboendarterectomy by histological analysis [24]. Similar findings have been observed for CD31+/41− EMV, which predicted plaque instability in a group of 42 patients undergoing endarterectomy, and for MV exposing annexin V+, which distinguished between patients with carotid artery disease and healthy volunteers [25]. This observation suggests a clinically relevant possibility to use either of these biomarkers to identify subjects with carotid artery disease who are at the highest risk of neurologic events and thus require an urgent intervention.

4. Microparticles in cardiovascular risk assessment

Quantification of MV of different cellular origin was acclaimed as a promising tool to improve risk stratification for future cardiovascular events. In a cross-sectional study performed on a group of 190 healthy men increased levels of PMV exposing CD42a and CD42b were associated with an increased 10-year-risk of developing CAD assessed in Framingham Score [28]. However, in this study MV concentrations were assessed using an ELISA kit, which refrains from direct comparison of the results with MV flow cytometric measurements due to the different sensitivity of both methods. Also, a threshold of high 10-year cardiovascular risk set by the authors at 8% is arbitrary, as in clinical setting it is settled at 20%.

Similarly, EMV (CD144+) were found to be independent predictors of future cardiovascular events in a group of 387 patients at high risk for CAD (with ≥2 cardiovascular risk factors). Patients with higher EMV concentrations experienced significantly more cardiovascular events (cardiovascular death, nonfatal MI, UA, ischemic stroke, or coronary revascularization) during 36 months of observation, as compared to those with lower EMV concentrations at baseline [29]. Addition of EMV assessments' to the Framingham model improved risk stratification, with further amendment following addition of B-type natriuretic peptide (BNP) and high sensitivity C-reactive protein (hsCRP). Moreover, the authors measured EMV concentrations also in patients at low risk for CAD (<2 cardiovascular risk factors) and in patients with ACS, stating again that it is possible to differentiate between subsequent stages of CAD using EMV assessment. Importantly, EMV plasma concentrations were measured by flow cytometry immediately after blood withdrawal, so that additional EMV release during freeze–thaw procedure of plasma sample could have been avoided.

In a group of 200 subjects with SCAD, an increased concentration of EMV positive for annexin and CD31 was an independent predictor of cardiovascular events, comprising cardiovascular death, the need for revascularization and the occurrence of a first major adverse cardiovascular or cerebrovascular event (MACCE) during the observational period of 6 years [30]. However, due to the presence of CD31 both on endothelial cells and platelets, the authors admit to have additionally measured a proportion of CD31+ PMV.

Correspondingly, in 51 patients with STEMI undergoing primary PCI with stent implantation, concentrations of MV derived from erythrocytes (Annexin-V+, CD235a+) were approximately doubled in comparison with those measured in a reference population, and corresponded to the risk of the composite clinical endpoint, comprising death or the need for resuscitation, hemodynamically relevant sustained ventricular tachycardia, nonfatal MI, need for revascularization, acute HF or rehospitalisation for cardiac causes within 6 months after the index event [31]. Interestingly, none of these associations were found for PMV (annexin+, CD41+).

A significant correlation between increased levels of EMV positive for CD62E and a risk of major cardiovascular events and hospitalization was observed also in a group of patients with a history of stroke during 36-month observational period [32]. On the contrary to the previous studies, concentrations of other kinds of EMV(CD31+/annexin+ or CD31+/CD42−) were not predictive of cardiovascular outcomes [32].

Interestingly, the PMV and EMV negative predictive value cannot be extrapolated to all other MV subtypes. In a group of 172 patients with NSTEMI, concentrations of MV derived from monocytes (MMV) (CD11b+) were negatively associated with the extent of CAD assessed using the SYNTAX score [33]. Moreover, in 77 patients who were followed up for one month after coronary stent implantation, lower MMV concentrations were found in subjects who experienced a recurring cardiovascular event (cardiovascular death, acute or subacute stent thrombosis, recurrent ACS and stroke), compared to patients with no complications [33]. Regarding the fact, that MV derived from leukocytes are found in large amounts in atherosclerotic plaques, the authors have concluded that MMV are captured during plaques or thrombus formation in patients with more severe CAD or with CAD thrombotic complications. As a result, these patients present with lower MV concentrations.

In a recent study using virtual histology intravascular ultrasound (VH-IVUS) to evaluate the association between the concentrations of circulating MV and the composition of coronary plaque in SCAD patients increased levels of circulating PMV positive for CD31, CD42b and CD146 were associated with the amount of necrotic core in the target lesion, suggesting a potential role of MV as a biomarker for detecting unstable plaque in patients with stable angina. However, it should be noted that in this study the concentrations of MV were measured using a solid-phase capture assay combined with the prothrombinase assay to provide a functional assessment of the procoagulant potential of isolated circulating MV [34].

It is important to underline that MV are not specific biomarkers for atherosclerotic disorders, as their concentrations predicted cardiovascular outcomes in patients with heart failure (HF) as well. In a study that aimed to assess the risk of future cardiovascular events in 388 patients with chronic HF, concentrations of EMV positive for CD144, CD31 and annexin V, as well as EMV positive for CD31 and annexin V significantly correlated with fatal and non-fatal cardiovascular events, as well as the occurrence of death from any cause after median 2.3 years of follow-up [35]. However, whether EMV with apoptotic fenotype only present the level of apoptosis of endothelial cells in chronic HF patients, or contribute actively to endothelial dysfunction as well, remains still an open research question. Likewise, it should be noted that MV are non-specific biomarkers of cell activation and apoptosis, which may confound the association between elevated MV concentrations and presence of a particular disease. The main trials on

Table 2
A summary of main clinical trials on potential use of microvesicles in diagnosis of cardiovascular disease and in cardiovascular risk stratification.

Study population	MV fenotype	Effect	References
Microvesicles in the diagnosis of major complications of atherosclerosis			
Patients with coronary artery disease	EMV (CD31 +/CD42 −)	Discriminating between acute coronary syndrome, stable angina and healthy volunteers.	Bernal-Mizrachi et al., 2005 [17]
Patients with coronary artery disease	EMV (CD31 +, CD34 +, CD51 +/61 +), PMV (CD42 +), MMV (CD14 +), LMV (CD45 +)	Discriminating patients with acute myocardial infarction from subjects with stable angina and healthy volunteers.	Stępień et al., 2012 [18]
Patients with coronary artery disease undergoing elective or urgent percutaneous coronary intervention	EMV (CD31 +,CD42 −), PMV (CD31 +,CD42 +), EMV and PMV (CD31 +, annexin +)	Discriminating patients with acute myocardial infarction from subjects with stable angina; no differences between ST-elevation MI (STEMI) and non-ST-elevation MI (NSTEMI) or between SA alone or SA with concomitant peripheral artery disease	Biasucci et al., 2012 [19]
Patients with acute ischemic stroke	EMV (CD144 +/CD41a-)	Correlation with stroke severity	Li P, et al. 2015 [20]
	EMV (CD62E +, annexin +)	Correlation with stroke subtype	
Patients with peripheral artery disease	PMV (CD42b +, CD61 +)	Discriminating patients with critical limb ischaemia, intermittent claudication and healthy controls.	Tan et al., 2005 [22]
Patients with carotid artery stenosis > 70%	LMV (CD11b +/CD66b +, CD15 +) EMV (CD31 +/41-)	An indicator of carotid plaque instability	Sarlon-Bartoli at al., 2013; [24] Wekesa at al., 2014 [25]
Microvesicles in stratification of cardiovascular risk			
Healthy volunteers	PMV (CD42a +, CD42b +)	Association with a 10-year-risk of developing coronary artery disease assessed in Framingham Score	Ueba et al., 2010 [28]
Patients at high risk for CAD (with ≥2 cardiovascular risk factors)	EMV (CD144 +)	Independent predictors of future cardiovascular events during 36 months of observation	Nozaki et al., 2009 [29]
Patients with stable coronary artery disease	EMV and PMV (CD31 +, annexin +)	Independent predictor of adverse cardiovascular events during 6-year observational period	Sinning e al., 2011 [30]
Patients with non-ST-elevation acute coronary syndrome	MMV (CD11b +)	Inverse association with the early recurrence of cardiovascular events	Faille et al., 2011 [33]
Patients with STEMI undergoing primary percutaneous coronary intervention	EryMV (CD235a +, annexin +)	Association with the occurrence of the composite clinical endpoint during 6-month observational period	Giannopoulos et al., 2014 [31]
Patients with a history of stroke	EMV (CD62E +)	Predictor of adverse cardiovascular events during 36-month observational period	Lee et al., 2012 [32]

CD — clusters of differentiation; PMV — platelet derived microvesicles, EMV — microvesicles derived from endothelial cells, EryMV — microvesicles derived from erythrocytes; MMV — microvesicles derived from monocytes; LMV — microvesicles derived from leukocytes.

potential clinical utility of MV in early diagnosis of cardiovascular disease and in cardiovascular risk assessment are summarized in Table 2.

5. Influence of antiplatelet therapy on microvesicles' formation

There are numerous preclinical and clinical studies suggesting the influence of aspirin, P2Y12 and GPIIb/IIIa antagonists on MV release (Fig. 1). It is important to underline, however, that the hitherto conducted clinical trials differed in terms of assessed MV subtypes and applied methodology (Table 3). Nevertheless, we present recent findings in this area, as a starting point for consideration of MV assessments' as a potential novel diagnostic modality to monitor the efficacy of antiplatelet therapy. One may assume that such measurements could be possibly used to assess clinical response to clopidogrel in order to individualize periprocedural pharmacotherapy in patients undergoing PCI. In addition, the observed modifications of MV formation presented below might be also partially responsible for some of the described pleiotropic effects of some antiplatelet agents [36].

5.1. Aspirin

In the group of 15 male patients with SCAD it was demonstrated that aspirin (acetylsalicylic acid, ASA) in a standard dose of 100 mg once daily lowered the levels of PMV and EMV after 8-week therapy by 62.7% and 28.4% respectively, in contrast to selective cyclooxygenase-2 inhibitor etoricoxib at a dose of 90 mg administered once daily in the control group [37].

On the other hand, in another recently published study treatment with aspirin at a dose of 100 mg once daily for 15 days in a group of 81 patients with type 2 diabetes failed to translate into decrease of CD41 + Annexin + PMV levels [38]. Similarly, in a prospective study by Lubsczyk et al., the same dose of aspirin administered for one week did not reduce the concentrations of circulating PMV and EMV in healthy individuals, compared to placebo [39]. One may argue that the MV levels in healthy subjects are already known to be lower than in cardiovascular diseases, and as a consequence, the influence of antiplatelet administration might not be as substantial as in conditions associated with higher risk of thrombosis. Nevertheless, in patients with atrial fibrillation treated with aspirin (150 mg/daily) as well as in subjects with acute ischaemic stroke with documented much higher PMV concentrations than healthy controls, treatment with aspirin (100 mg/daily) or cilostazol (200 mg/daily) did not affect MV concentrations [40]. Such a heterogeneity of results might be related to the duration of the treatment, baseline characteristics of relatively small study groups and different methodologies of MV measurements. Indeed, in the study assessing population of stroke patients an ELISA technique was used, whereas the majority of studies assessing circulating MV concentrations applied flow cytometry.

5.2. Clopidogrel in stable coronary artery disease

The influence of clopidogrel on MV formation has been analyzed by several authors in patients with SCAD and ACS. Ryu and Kim reported that clopidogrel administration was associated with decreased EMV (CD31 +/CD42 −) formation induced by indoxyl sulfate in supernatants

Table 3
A summary of the main clinical trials on the influence of antiplatelet therapy on microvesicles formation.

Study population	MV fenotype	Effect	References
Aspirin			
15 male patients with SCAD treated with ASA or etoricoxib	PMV (CD42b+/CD31+/annexin+) EMV (CD42b−/CD31+/annexin+)	Reduction in the number of PMV and EMV in response to the ASA therapy	Bulut, et al., 2011 [37]
81 patients with DM2 before and during ASA treatment.	PMV (annexin+/CD41+)	No reduction in the PMV levels	Duarte et al., 2015 [38]
13 healthy male nonsmokers treated with ASA or placebo daily for 7 days	EMV (CD34+/ CD 142+/annexin+) PMV (CD41a+)	No reduction in EMV and PMV levels	Lubsczyk et al.; 2010 [39]
Clopidogrel			
26 SCAD patients treated with clopidogrel and not receiving aspirin or any antithrombotic agent	EMV (CD51+) PMV (CD42+/CD31+)	Inverse relationship between clopidogrel serum levels and the amount of PMP.	Franca et al. [42]
In vitro study with supernatants of human umbilical vein endothelial cells	EMV (CD31+/CD42−)	EMV generation induced by indoxyl sulfate effectively suppressed by clopidogrel	Ruy et al., 2011 [41]
200 patients with ACS after PCI	PMV (CD42a-+/CD62P+)	PMV concentrations higher in patients with HPR (assessed by impedance aggregometry)	Kaffian et al., Platelets 2015 [43]
40 ACS patients after 5 days of clopidogrel therapy	PMV (CD61+)	Higher production of PMV in patients with HPR (defined by VASP) despite clopidogrel administration	Kalantzi et al. 2012 [47]
51 patients with ACS and 61 sex- and age-matched healthy controls	PMV (CD61+/ CD62P+/ CD142+)	PMV concentrations decreased after initiation of clopidogreland at 6-month follow-up	Skepholm et al., 2012 [48]
12 patients with ACS}	PMV (CD42a+/annexin+) stimulation with TRAP	Decreased TRAP-induced PMV release	Behan et al., 2005 [49]
Prasugrel			
Healthy volunteers Samples preincubated with active metabolite of prasugrel or cangrelor	PMV (CD42a+, annexin+) stimulation with TRAP and collagen	Reduction of MV production in a concentration-dependent fashion	Judge et al., 2008 [50]
GPIIb/IIIa antagonists			
50 STEMI patients	PMV (CD42+), EMV (CD31+) LMV (CD11a+)	decrease in PMV, EMV concentrations(day 1) and LMV (day 6) after abciximab administration	Morel et al., 2004 [53]
Healthy volunteers	PMV (CD42a+, annexin +) stimulation with TRAP and collagen	prevention of PMV production in response to collagen stimulation observed for aspirin, cangrelor, abciximab TRAP-induced MV affected only by abciximab and/or cangrelor	Judge et al., 2005 [52]
38 patients undergoing elective PCI receiving abciximab *vs.* 75 PCI patients not receiving this drug	PMV (CD61+)	abciximab prevented periprocedural increase of PMV concentrations	Crafts et al., 2003 [55]

HPR — high-on treatment platelet reactivity; VASP — vasodilator-stimulated phosphoprotein; TRAP — thrombin receptor-activating peptide.

of human umbilical vein endothelial cells' *in vitro* [41]. Likewise, in a relatively small, prospective study including 26 patients with SCAD investigating the effects of clopidogrel therapy, alone or combined with atorvastatin, in patients who were not receiving aspirin or any antithrombotic agent, an inverse correlation between clopidogrel serum levels and the amount of PMV (CD31+/CD4+) was found [42]. Nevertheless, the total plasma levels of EMV and PMV remained unchanged, despite administration of either clopidogrel or a potent statin.

5.3. Clopidogel in acute coronary syndromes

Kafian et al. investigated the association between patients' response to clopidogrel, assessed by whole blood impedance aggregometry, and circulating PMV in 200 patients with ACS who had undergone PCI and received a loading dose of aspirin and clopidogrel before the procedure [43]. This study revealed for the first time that levels of both CD42a- and CD62P positive PMV were almost two-fold higher in patients with high-on treatment platelet reactivity (HPR) compared with patients without HPR, which might have indicated ongoing platelet activation despite clopidogrel treatment. Interestingly, patients with a documented lower platelet reactivity had significantly fewer PMV exposing CD62P than patients with HPR or those with adequate platelet reactivity to clopidogrel [43]. Cut-off values for HPR to clopidogrel in this study were set according to data from large prospective trials [44–46].

These observations are consistent with the previous study by Kalantzi et al. [47], who assessed PMV concentrations in 40 ACS patients, showing greater release of circulating CD61+ PMV in patients with HPR 5 days after initiation of clopidogrel treatment. The older method of platelet function testing – VASP (vasodilator-stimulated phosphoprotein) – has been employed to define HPR for this study. Similarly, previous studies with smaller sample size have proved that a reduction in circulating PMV levels is observed following the start of treatment with antiplatelet agents. In the study by Steppholm et al., in ACS patients plasma concentrations of PMV exposing CD61, CD62P and CD142 were respectively 2.5, 6.0-, and 5.0-fold higher than in controls receiving aspirin only [48]. Interestingly, PMV concentrations decreased significantly 24 h following initiation of treatment with clopidogrel and subcutaneous anticoagulation and decreased even further six months later. Likewise, in a previous pilot study by Behan et al. in platelet-rich plasma of 12 ACS patients treated with clopidogrel (300 mg loading dose plus 75 mg daily) significantly inhibited TRAP (thrombin receptor-activating peptide) — induced aggregation, procoagulant activity (evaluated with annexin V binding) and PMV (CD42a+) release production using aggregometry and flow cytometry [49]. However, one should be aware that common PS exposure at MV surface is currently questionable at best, as so are PMV procoagulant properties, assessed in the cited study. Nevertheless, the above observations suggest that PMV may potentially be evaluated also as possible biomarkers of clinical response to clopidogrel.

It should be also noted that in the study by Kafian et al. the levels of PMV exposing CD62P (P-selectin) were significantly higher in patients with HPR, in contrast to the levels of PMV exposing CD154 (CD40

ligand), which did not differ between the HPR and no-HPR groups [43]. CD62P is a platelet activation marker, which plays an important role in the recruitment of leukocytes by binding to its ligand, that is also expressed on endothelial cells. The observations regarding CD62P-positive PMV indicate increased platelet activation, which is in agreement with the data from studies with multiple electrode aggregometry. Moreover, this molecule is known to be a marker of MV derived specifically from platelets, but not from megakaryocytes [23].

5.4. Prasugrel, ticagrelor and cangrelor

Newer antiplatelets' influence on MV formation has been investigated solely in *in vitro* studies so far [50]. Preincubation of blood samples of healthy volunteers with an active metabolite of prasugrel (R-138727), a third generation thienopyridine acting as an irreversible inhibitor of the P2Y12 receptor or with the first intravenous antagonist of this receptor–cangrelor resulted in a reduction of collagen- and TRAP-induced procoagulant activity assessed by PMV (CD42a+, annexin +) formation [50].

To date, no data on the influence of prasugrel, ticagrelor or cangrelor are available. The ongoing clinical trial TIGER-M (*Evaluation of Ticagrelor Anti Platelet and Pleiotropic Effects in Patients Undergoing Percutaneous Coronary Intervention for an Acute Coronary Syndrome*) is aimed to assess the impact of ticagrelor, on the level of MV and microRNAs in patients with NSTEMI, compared with clopidogrel [51]. The authors of this study postulate that a higher clinical efficacy of ticagrelor results from more pronounced effect of this drug on the levels of MV and microRNA. Another study assessing the impact of switching from clopidogrel to prasugrel or ticagrelor on MV release in associations with standard platelet function testing analyses will be submitted by the authors of this review soon.

5.5. GPIIb/IIIa antagonists

In the *in-vitro* studies preincubation of healthy volunteers' blood samples with either aspirin, cangrelor or abciximab prevented the production of PMV in response to collagen stimulation and combinations of all agents resulted in even further inhibition. It should be noted that although TRAP-induced MV formation was not affected by aspirin administration, it was reduced in the presence of a P2Y12 or GPIIb/IIIa antagonist [52].

In 2004 Morel et al. presented the results of a study investigating whether an additional treatment with abciximab or eptifibatide infusion could reduce the amounts of procoagulant MV, as the measures of platelet activation and prothrombotic status, in 50 STEMI patients treated with primary PCI who obtained abciximab, as compared to patients who did not receive this drug [53]. At the end of abciximab infusion up to 73% decrease in PMV (CD42+) and EMV (CD31+) levels as well as lower concentrations of MV derived from leukocytes (CD11a+) were observed. Interestingly, this effect was not found for eptifibatide, which might be explained by its shorter half-life, lower affinity to the receptor and reported lack of influence of this drug on platelet shedding [54].

As a specific inhibition of platelet shedding by abciximab has been recently demonstrated in *in vitro* studies, with a comparable reduction of shear stress-induced platelet aggregation achieved with either eptifibatide or abciximab [53,54], the authors of the cited study concluded that the observed lower PMV concentrations revealed in patients treated with abciximab indicate to platelet shedding as a possible target for this antiplatelet agent. In addition, this drug might be also able to reduce endothelial cell activation and membrane shedding through binding to avb3 integrin [55]. The results of this study pointed also to some possible anti-inflammatory effects of abciximab that decreased the concentration of LMV in further observation (0.12 ± 0.04 *vs.* 0.56 ± 0.12 in STEMI patients treated with PCI without GPIIb-IIIa antagonist infusion) [53].

Noteworthy, in subjects undergoing elective PCI it has been also demonstrated that abciximab decreases periprocedural increase in PMV (CD61+) concentrations, which was not observed among PCI patients not receiving this drug [56]. Such observations were made despite the fact that abciximab-treated patients required more complex PCI procedures[56].

6. Circulating microvesicles for monitoring of antiplatelet therapy?

In the light of previous preliminary findings, demonstrating influence of antiplatelet drugs on MV plasma concentrations, one may assume that the next step would be to apply these biomarkers as a novel modality to monitor the efficacy of antiplatelet therapy. Although routine assessment of individual platelet reactivity is currently not recommended in the guidelines, it is important to underline that a substantial number of patients present an inadequate pharmacological response to standard regimes of clopidogrel. Dependent on the platelet function testing used (light transmission aggregometry [LTA], point-of-care testing modalities including multiple electrode aggregometry, VerifyNowTM assay, vasodilator stimulated phosphoprotein [VASP] phosphorylation assay, Platelet Function Analyzer [PFA-100 TM]) this number raises up to 25–50% of patients receiving clopidogrel [57–60], who are consequently not sufficiently protected from ischaemic major adverse cardiac events (MACE), including acute and subacute stent thrombosis after PCI [57–61]. Prasugrel and ticagrelor feature more potent and predictable P2Y12-inhibition, as compared to clopidogrel, which translates into improved ischaemic outcomes, however, this comes at the price of increased risk of major bleeding [62–65]. A routine measurement of platelet reactivity has not been widely implemented and recommended in the guidelines, as no significant improvement in clinical outcomes by personalized antiplatelet therapy has been demonstrated in the majority of prospective double-blind randomized trials [43–45]. Nevertheless, it has been argued that the exclusion of patients with high risk of thrombotic complications may have been responsible in part for the negative study results and intensification of antiplatelet therapy might improve clinical outcomes in this subpopulation [66]. Such an assumption has been supported by the results of a recent metaanalysis and some clinical trials assessing the net clinical benefit, indicating the need for individualized antiplatelet approach in order to prevent thrombotic events without an increase in bleeding rates [67–69], and thus, leaving the question of clinical utility of antiplatelet therapy monitoring still not completely answered. It should be also noted that different study populations, follow-up periods, treatment strategies, platelet function testing modalities, therapy modifications, study endpoints and blood sampling time points applied in the above studies as well as the fact that all such *ex vivo* tests do not reproduce the complexity of platelets' activation *in vivo*, make the concept of individualized antiplatelet therapy even more difficult for conclusive assessment.

In the view of increased understanding of the platelets' pathophysiology, attempts to evaluate the efficacy of antiplatelet therapy using specific markers of platelet activation, such as PMV, appear justified. It should be noted that the results of the last decades' studies on the influence of antiplatelet therapy are difficult to compare as they present considerable heterogeneity in methodology, and some of the studies lack a sufficient information on such parameters like assay variability, gating strategy, figures. Nevertheless, they clearly showed that administration of antiplatelet agents changes the MV formation pattern. As the goal of unification of the measurements' techniques and improving detection of MV has been recently internationally recognized, it is expected that the sensitivity of flow cytommetry will substantially increase next years and the comparison of analyses between laboratories and instruments will become feasible [3,70]. As a consequence, a qualitative as well as quantitative assessment of the MV could possibly

constitute another practical modality of assessment of individual response to antiplatelet therapy in the future.

7. Obstacles to overcome before wider clinical application as biomarker

A wider application of cell-derived MV in the diagnosis and prognosis of cardiovascular risk, as well as antiplatelet therapy monitoring has been limited due to the lack of standardized methods for their quantification. [4,71]. The results obtained from previous studies were influenced by multiple pre-analytical and analytical factors, such as (1) blood drawing conditions (size of the needle used to obtain a blood sample, anticoagulant, presence of hemolysis), (2) patient-dependent factors (lipid load in patient's blood, concomitant medications), (3) speed, time and temperature of centrifugation while preparing samples for analysis, (4) conditions of samples storage, (5) isolation protocols, (6) monoclonal antibodies used for determination of MV origin, and (7) detection technique used to analyze MV concentrations [71,72]. However, great progress has been done to improve the accuracy and reliability of MV measurements in the recent years, including (1) development of standard operating procedures for handling and storage of human body fluids for MV analysis, (2) increase in sensitivity of MV detection techniques, and (3) development of methods to measure MV morphology and biochemical composition [3].

It is recommended to collect blood from the antecubital vein using a relatively large needle (21-G) and a vacutainer system with minimal venous occlusion into tubes containing trisodium citrate. Importantly, the first 2.5 ml should be discarded to avoid the effects of the vascular injury caused by the venepuncture. The samples should be centrifuged twice within 15 min after blood collection at 1560 $\times g$ for 20 min in 20 °C) to obtain platelet-rich plasma [73]. After each centrifugation step the supernatant (approximately 55%) should be removed. After the second step aliquots of plasma should be snap-frozen in liquid nitrogen and stored at −80 °C up to one year [73]. Prior to the measurements, samples should be thawed on melting ice for 1 h. It is advised to separate MV fraction in a single-step using size-exclusion chromatography, instead of serial high-speed centrifugation, which is known to decrease the number of MV due to their clumping [74]. Although all the standardization of all the pre-analytical procedures is of paramount importance for inter-laboratory comparisons of MV measurements, the greatest progress has been done in MV detection techniques (Table 4.) [3,75].

8. Recent progresses in measurements' techniques

Among various detection techniques applied for MV measurements, fluorescent flow cytometry remains a preferred method for both quantitative and qualitative assessment of circulating MV [3,5,6,7,9,73–75]. This technique allows for identification of different types of MV based on their size, granularities, as well as the presence of specific antigens on the surface of MV, which are recognized by monoclonal antibodies labeled with fluorophores [3,7]. The fluorescence flow cytometry is more sensitive than scattering flow cytometry due to the signal-to-noise ratio, which in case of nanometer-sized particles is higher for fluorescence than for scattering [3,7]. One of the most significant drawback of conventional flow cytometers was the low detection threshold of 800–2400 nm, which allows to detect only 1–2% of the total MV load present in body fluids [76]. However, numerous amendments have been proposed over the past decade to improve the sensitivity of these methods. In 2010, the International Society of Thrombosis and Haemostasis (ISTH) made an attempt to increase the sensitivity of flow cytometry. In order to determine the cytometric size gate of MV, it was recommended to use a mixture of polystyrene beads of known size (Megamix beads; BioCytex, Marseille, France), similar to the size of MV [77]. The proposed gating strategy, however, did not bring the expected increase in MV detection threshold, as the refractive index (RI) of synthetic polystyrene beads is much higher that the RI of MV, and consequently the signal coming from MV remains approximately 10 fold weaker than the signal from polystyrene beads [7,78].

At present, in order to improve the detection of MV with the use of conventional flow cytometers, it is recommended to (1) calibrate the flow cytometer with use of silica beads, which have RI similar to MV, (2) select the flow cytometer detector with the largest collection angle (usually side-scatter detector) to measure MV, (2) dilute the sample in order to avoid swarm detection (counting of multiple small vesicles as one single event), and (4) use de-ionized water to set a gate in order to reduce background noise [7,76]. These adjustments allow to detect MV of 270–600 nm. Additionally, flow cytometers of newer generations and detection threshold of about 150–190 nm have become commercially available [70]. However, due to limited clinical accessibility, such

Table 4
Flow cytometry in the assessment of extracellular vesicles. A summary of limitations and strengths of the method together with the recent methodological advancements [3–6,9,10,70–77].

Limitations	Strengths	Advancements
Low detection threshold of conventional flow cytometers (800–2400 nm)	The most widely available tool in clinical laboratories to investigate single particles in body fluids	Increased sensitivity of newer instruments (detection of EV > 180 nm)
Not straightforward data interpretation and direct data comparison between laboratories:	Multiparameter measurements of EV	Development of metrology-certified reference materials, calibration with silica beads
a) flow cytometers differ in optical configuration and sensitivity		
b) results are expressed in arbitrary units		
No possibility of point-of-care testing	Provision of biochemical information on the cellular origin of EV	Dedicated software developed to correct for differences in optical configurations of the flow cytometers used, and to correct for differences in light scattering between reference materials and EVs
User-dependent method	Relatively high speed of measurements (flow rate of thousands per second)	Educational initiatives, standards and protocols for instrument characterization and fluorescence calibration released by ISAC[a]
Centrifugation and re-suspension or sucrose gradient centrifugation required to separate free dyes or dye aggregates from EV-associated dyes is prone to induce EV aggregation and measurements' artifacts	Low sample volume needed for testing	Use of lipid dyes followed by their removal by sucrose gradient ultracentrifugation before detection
		Fluorescence triggering to increase the sensitivity of flow cytometers
		Possibility of verification of quantitative measurements by novel techniques, such as NTA and TRPS

[a] International Society on Advancement of Cytometry; EV — extracellular vesicles.

instruments still remain for research use rather than for routine clinical application.

Owing to extensive research attempting to standardize MV measurements in the recent years, novel detection techniques have become validated. These methods comprise transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS), which allow for isolation of MV smaller than conventional or dedicated flow cytometry (Fig. 2.) [9,70]. However, none of these techniques provides insight into MV cellular origin and biochemical properties. Thus, their usefulness is limited in clinical practice, where not only a quantitative, but also a qualitative characterization of these biomarkers is necessary. On the other hand, methods based on electrophoretic separation of plasma proteins (Western blot, proteomic approach) require large volumes of analyzed material, which limits their use in clinical setting as well [79]. As a result, despite some limitations, flow cytometry remains a method of choice for both experimental and clinical studies on MV. However, the other available methods may serve as a useful tool to determine total sample EV concentration and to provide information on the accuracy of flow cytometric measurements.

9. Conclusions and future directions

Both the standardization of the pre-analytical steps and the sensitivity of MV flow cytometry analysis have greatly improved in the past decade, thus making it possible to compare the inter-laboratory results of experiments and trials on MV utility as novel biomarkers in cardiovascular diagnosis and cardiovascular risk stratification. One should also bear in mind, that the lack of unified methodologies used in the preceding studies have led to numerous publication artifacts, such as (1) an assumption of common phosphatidylserine (PS) exposure on MV surface, whereas a substantial part of MV presents PS negative, (2) an assumption that the majority of PMV (CD61+) derives from platelets, whereas these PMV are abundantly released also from megakaryocytes, or (3) a belief that MV are specifically prothrombotic, whereas nowadays it is considered that they serve as important mediators of intercellular communication, contributing not only to thrombus formation and inflammation, but also to physiological haemostatic and inflammatory responses As a result, the current knowledge on MV role in physiological and pathological processes is fragmentary, leaving this field still unexplored. Nonetheless, there are still no conclusions regarding the cut-off values for the levels of MV that can be considered pathological, and the reported concentrations range from 10^4 to 10^{12} per ml, due to the different minimal threshold of detection techniques [3,7,9,70,75–77]. A Flow Cytometry Working Group was initiated in 2015, which will collaborate in an attempt to improve the detection and comparison of MV cytometric analyses. Among the most pivotal optimizations that were postulated, there is a need for alongside use of other techniques like NTA and TRPS, and the development of standards and references for scatter and fluorescence in flow cytometry measurements [80]. Hence, in the coming years it is to be expected that rapidly expanding research on MV with the number of publications growing exponentially is likely to develop new point-of-care and cost-effective solutions to integrate MV measurements into laboratories and clinical routines.

Conflict of interests

The authors report no relationships that could be construed as a conflict of interest.

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