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Role of Platelet Microparticles in Blood Diseases: Future Clinical Perspectives

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Abstract. Platelet microparticles (PMPs) are released from the resting or activated platelet membrane, which includes a variety of proteins, mRNA, miRNA, lipids, and other substances. PMPs are involved in cell communication in vivo and are potential markers for multiple diseases. This narrative review focuses on the extraction, identification, and intercellular role of PMPs, with emphasis on the lesser known aspects of PMPs, namely, PMPs and complement systems, and their role in some diseases and disorders of women such as miscarriage and polycystic ovary syndrome, as well as the application of proteomics. For clinical purposes, we should first consider the importance of PMPs quantification and then what treatment should be given in response to increased quantities of PMPs as observed in many diseases. Currently, methods for quantification of PMPs are still in the experimental stages. Proteomics testing will expand biomarker discovery as well as future PMPs diagnostic and therapeutic applications.

Key words: platelet microparticles, therapeutic applications, disease, proteomics.

Introduction

In 1949, Chargaff et al. first observed that platelet-free plasma contains a precipitable factor, which can promote thrombin generation [1]. Peter Wolf then described the existence of elemental "cellular dust" that was capable of supporting coagulation [2]. Further studies have shown that platelet activation encompasses the exocytosis of platelet granule contents and the formation of microvesicles (MVs; also called microparticles (MPs) or ectosomes) [3]. Platelets may also release smaller microvesicles called exosomes (40 to 100 nm in diameter) that may not have sedimented with MPs upon centrifugation at 20,000 x g [4]. The two types of vesicles are different in their size, formation process, lipid composition, and content. The analysis showed that isolated exosomes were selectively enriched in the tetraspan protein CD63. Membrane proteins such as the integrin chains aIIb-b3 and b1, GPIba, and P-selectin were predominantly present on microvesicles [3]. As the most abundant microparticles in the blood of healthy individuals, platelet microparticles (PMPs) are shed from the plasma membranes of platelets and have been associated with various physiological and pathological processes in vivo, including angiogenesis, cell-cell communication, and biomarkers that are diagnosed in a variety of diseases [4].

The abilities of PMPs prompted scholars to investigate the possible intercellular transfer of PMPs and assess their role in several diseases. In this review, the term microparticle will appear when appropriate because it is the most commonly used, although, in the relevant research areas, scholars also considered the term microvesicles. PMPs have been implicated in the complement system, which may cause a decrease in inflammatory response and thrombosis. Proteomics for PMPs, which act as a biomarker in the process of disease, should be considered. The purpose of this review is to discuss the significance of PMPs extraction and detection, PMPs in pathophysiology, the relationship between complement and PMPs in some diseases, and the role of PMPs in the diagnosis and treatment of some diseases in women, as well as the advantages and challenges of studying PMPs with proteomics in diseases **(Figure 1)**.

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Table 1. *Main methodologies for assessing platelet microparticles.*

Methodologies	Reference
Flow cytometry	
ELISA	[20]
Immunofluorometric assay	[21]
Microscopy (TEM, SEM, AFM)	
Dynamic light scattering	[22]
Nanoparticle tracking analysis	[23]
Tunable resistive pulse sensing	[24]
Raman spectroscopy	[25,26]
Proteomics	

Extraction and Detection of PMPs. To ensure the overall potential of PMPs usage in clinical diagnosis/disease prognosis, PMPs isolation and identification must first be addressed, though most studies were based on mixed EVs populations [5]. Accurate counts of PMPs in circulation depend on different handling methods that trigger PMPs production. The most widely used method for PMPs isolation is differential centrifugation. It is important to acknowledge that centrifugation, storage conditions, and anticoagulants affect PMPs measurements and unavoidably cause pre-analytical variations in the counts and sizes of PMPs determined by flow cytometry (FCM) [6]. Centrifugation is a critical step in the measurement of microparticles. Livshts et al. attempted to use the velocity of a particle sedimenting under centrifugation to form a general equation. An "individual" differential centrifugation protocol can be selected by relatively simple theoretical estimates [7]. Relatively low-speed centrifugation (13,000 x g for 2 minutes) reduces exosomes contamination. Any method that requires ultracentrifugation is quite challenging in the hospital setting [8]. Using conventional isolation steps, platelet-rich plasma (PRP) was obtained after 20 minutes (min) of centrifugation at 200 x g. Platelet-poor plasma (PPP) was obtained after 15 min centrifugation at 3000 rpm [9]. In order to prevent loss and phenotypic changes, many studies directly used blood plasma for MPs measurements during the isolation procedure [10]. Nevertheless, in order to prevent clotting, using PMPs that have been subject to high-speed centrifugation is advised.

Of concern is cryopreservation, as some scholars have found that it alters the membrane and cytoskeletal protein profile of platelet microparticles [11]. It has also been reported that Ca^{2+} concentrations could affect annexin V binding to MPs and its enumeration. Montoro Garcia et al. do not recommend using CaCl2 concentrations above 10 mM in citrated conditions to avoid nonspecific adsorption of membrane-bound proteins [9]. The use of citrate and EDTA during centrifugation not only inhibits the coagulation cascade by chelating Ca^{2+}, but they also inhibit complement [12]. There are also many emerging isolation methods that are constantly being developed, such as PRotein Organic Solvent PRecipitation (PROSPR), which can be used to rapidly isolate extracellular vesicles (EVs) from small blood volumes in human plasma. PROSPR includes the precipitation of soluble proteins in plasma and the retention of lipid-encapsulated EVs in cold acetone [13]. By adapting methods for isolating viruses using polyethylene glycol, exosomes and other EVs also were purified [14]. From cell culture media and human plasma, EVs can be purified using ultrafiltration (UF) followed by the use of heparin-affinity beads [15]. For the isolation of EVs, the use of a polyethylene glycol/dextran aqueous two-phase system is also a novel and effective method [16].

Several previously reported methods for analyzing PMPs and exosomes have been used for research [17,18]. Flow cytometry is the most widely applied technique for MPs analysis [9] and has been considered as the 'gold standard'. Although vesicles can be detected and classified by fluorescent labeling, this technique is limited by a lack of adequate standardization. Of interest is a rapid analysis detection method using an Apogee A50 microflow cytometer (MFC), which can be used to determine differential CD47 expression in blood-derived individual circulating exosomes [19]. ELISA [20], immunofluorometric assay[21], microscopy, dynamic light scattering (DLS) [22], nanoparticle tracking analysis (NTA) [23], tunable resistive pulse sensing(TRPS) [24], Raman spectroscopy [25] and tip-enhanced Raman spectroscopy (TERS) [26], and proteomics also can be used for microparticle analysis **(Table 1)**.

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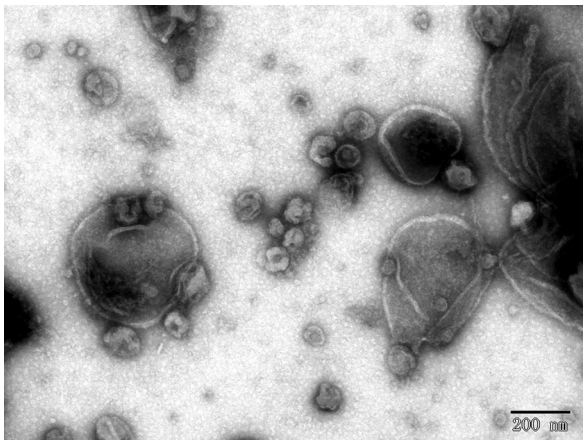

Figure 1. *Electron microscopic image of PMPs.*

Shinji Miyamoto et al. used a captured enzyme-linked immunosorbent assay (ELISA) to measure PMPs generated during open heart surgery [27]. PMPs were detected using antibodies related to platelet antigens. The results showed that activated PMPs were adsorbed on a 0.8-μm filter, but circulating PMPs were not adsorbed, which indicated that a new ELISA needed to be developed [20]. Fluorescence-activated cell sorting (FACS) and atomic force microscopy (AFM) were used by Leong et al. to obtain the first quantitative nanoscale images of PMPs ultrastructure [28]. Yuana et al. found that compared to FCM detection, the number of MPs that can be detected using AFM is 1000 times higher [29]. A microfluidic system combined with AFM detection was developed by Ashcroft et al., who demonstrated that the size distribution of CD41-positive MPs is strong for high-speed centrifugation and dilution [30]. Nevertheless, AFM surface scanning is also time-consuming. To successfully use this method as a diagnostic tool, it is necessary to increase the throughput by developing high-speed AFM [31]. A separation method based on acoustofluidics was reported, which enables to isolate exosomes directly from whole blood with its advantages to label-free and contact-free [32].

Raman spectroscopy can be used to analyze the biomolecular composition of biological vesicles. It is capable of detecting molecular vibrations that provide information regarding the structure and environment of a molecular species [33]. The potential of Raman spectroscopy in clinical applications is increasingly well known, as the high sensitivity of Raman scattering can greatly assist with clinical research. The most common clinical target studied by Raman spectroscopy is cancer [25]. NTA and DLS utilize the Brownian velocity of nanoparticles and then obtain the hydrodynamic information regarding the particles through the Stokes-Einstein equation. NTA can directly obtain the particle velocity and then subsequently measure the particle size, while DLS indirectly detects the particle velocity by the scattered light frequency. Based on the differences in particle size, DLS can distinguish between single nanoparticles and nanoparticle dimers, oligomers, or aggregates, which enables DLS to be a potential analytical tool for quantitative immunoassays [22]. The particle-particle analysis of TRPS technology has the advantages of great sensitivity and high resolution in the characterization of nanoparticle dispersions, which facilitates further exploration of toxicology and nanomedicine [34]. Proteomics in PMPs research will be described later in a separate section.

Cellular Interactions of PMPs. Faille et al. observed that internalized PMPs were distributed in endosomes and lysosomes after cell entry, which indicates that endocytosis plays a major role in PMPs entry into endothelial cells [35]. PMPs initially bind to an exposed subendothelial matrix, providing a substrate for further platelet adhesion via GP IIb/IIIa–fibrinogen bridging [36].

PMPs are accepted as an important means of intercellular communication; however, the mechanisms underlying PMPs internalization in recipient cells are poorly understood. Anne-Claire Duchez et al. observed that MPs and their cargo are internalized by activated neutrophils in the endomembrane system via 12(S)-hydroxyeicosatetranoic acid [12(S)-HETE] [37]. Caspase-3 was determined to be present in PMPs and platelets during storage in vitro. Whether PMPs-induced apoptosis of human macrophages is due to the transfer of caspase-3 is still not confirmed [38]. PMPs promote the proliferation, survival, migration, and tube formation in human umbilical vein endothelial cells (HUVECs). The main contributor may be the lipid component of PMPs, and the protein component may be secondary [39]. PMPs increased the adhesion of

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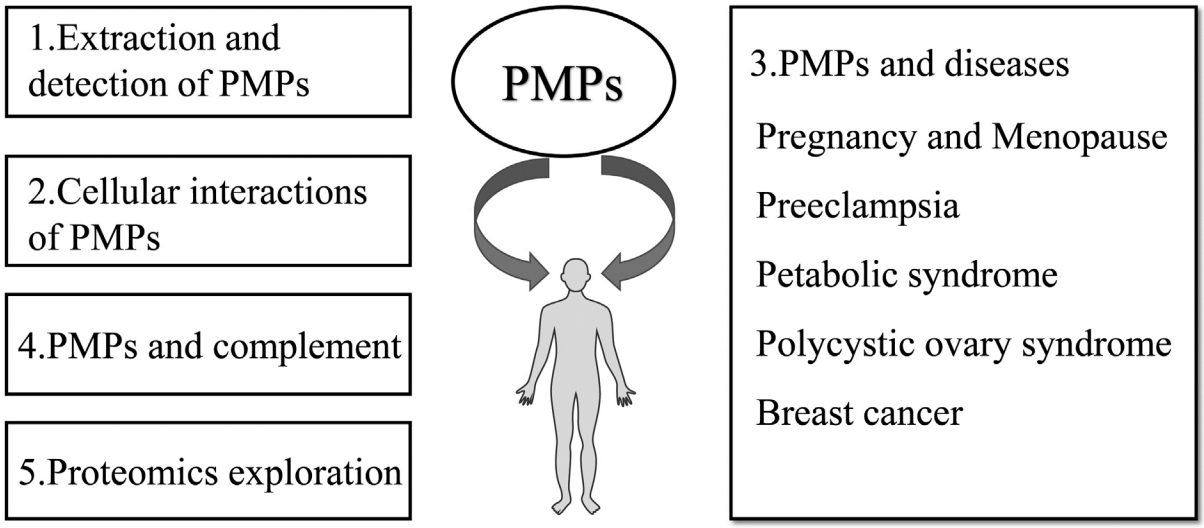

Figure 2. *PMPs contributions to disease diagnosis and therapy as biomarkers.*

monocytes to HUVECs in a time- and dose-dependent manner. The maximum adhesion of resting monocytes to MPs-stimulated HUVECs and the maximum adhesion of monocytes to resting HUVECs were observed after 5 hours and 24 hours of stimulation with MPs [40].

PMPs use regulatory T cells (Tregs) to inhibit IL-17 and IFN-γ production and stimulate Treg stability in an inflammatory environment. PMPs inhibit Treg plasticity in a P-selectin- and partially CXCR3-dependent manner [41]. In diseases with elevated PMPs concentrations, PMPs may enhance leukocyte aggregation and accumulation on selectin-expressing substrates [42]. Platelet vimentin may regulate fibrinolysis in plasma and thrombi by binding platelet-derived vitronectin·type 1 plasminogen activator inhibitor complexes [43]. Staphylococcal superantigen-like protein 5 (SSL5) induced PMPs (SSL5-PMPs) to bind to monocytes, enhancing MCP-1-induced monocyte migration [44].

Developmental endothelial locus-1 (Del-1) can act as a bridging molecule between integrin on endothelial cells and phosphatidylserine-containing microparticles. The present experiments show that Del-1 secreted by the endothelium plays a role in the clearance of phosphatidylserine-expressing microparticles. Del-1–deficient lung and liver endothelial cells displayed significantly impaired uptake of PMPs. However, there were no significant differences in uptake by wild-type and Del-1/splenic endothelial cells. A Del-1 deficiency may contribute to the procoagulant state only during times of pathological increases in MPs generation [45].

Volume transmission (VT) is a major mode of communication in the central nervous system (CNS) that takes places in the extracellular fluid and the cerebral spinal fluid through diffusion and the flow of molecules such as neurotransmitters and EVs [46]. Currently, there is no research being performed on the role of PMPs in volume propagation. However, in the basal work of Simons and Raposo [47], it was proposed that EVs (exosomes and exfoliated vesicles) mediate the specific form of VT in the CNS. Exosomes appear to be the main vesicle vectors of VT, but larger microparticles are also involved.

PMPs and Diseases. Initially, due to procoagulant activity, PMPs were studied with wide concern. Recent studies have also investigated their participation in the pathophysiological status of many diseases as opposed to health, in particular, in vascular disorders [17]. Circulating PMPs have been detected in a variety of clinical syndromes of platelet activation [48]. PMPs affect many disease processes, including tumor formation, transcellular interactions within the nervous system [49], cardiovascular disease [50], hemolytic disease, chronic hepatitis C [51], HIV [52], rheumatoid arthritis [53], psoriasis [54], ankylosing spondylitis [55], pregnancy [56,57], miscarriage, and

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preeclampsia [58]. In other reviews, these diseases and disorders have been described in detail. Therefore, herein, we will focus on PMPs involved in pregnancy and female disease research.

In the human placenta, extravillous trophoblasts (EVTs) induce the remodeling of the maternal arteries because this process contributes to the successful establishment of pregnancy. Immunohistochemistry showed that platelets were only detected in the spiral arteries of pregnant women, and these platelets are likely to have been activated and to have released various bioactive substances [59]. Studies have shown that EVs specifically induce thrombus inflammatory responses in the placenta. After EVs injection, activated platelets are particularly accumulated in the placental vessel bed [60]. Other EVs and syncytiotrophoblast microparticles (STBMs) were measured in the state of pregnancy (in pregnant women with preeclampsia (PE)). The analysis showed that most EVs are derived from platelets [61]. Flow cytometry showed that the interaction between platelets and syncytiotrophoblast extracellular vesicles (STBEVs) increased when thrombin activates platelets, indicating that low levels of STBEVs may bind to platelets in a normal pregnancy, whereas in activated follicles in PE, activated platelets may have a greater affinity for STBEVs. Differences in the microparticle-exosome ratio and STBEVs composition can also explain the heterogeneity of maternal symptoms [62].

Platelets are also involved in human corpus luteum formation [63]. EVs change their levels and makeup during antral follicle development [64]. As a hypertensive pregnancy disorder, preeclampsia is still the leading cause of maternal and neonatal morbidity and mortality [65]. Lok et al. found that MPs initially decrease and subsequently normalize during pregnancy, and reduced numbers of PMPs result from decreased platelet counts [66]. However, some studies showed higher concentrations of PMPs [67]; the differences between these studies are likely due to the different preeclampsia subtypes and their underlying mechanisms. Pre-pregnancy maternal platelet activation can augment endothelial dysfunction and inflammation via platelet-derived EVs (PEVs), facilitating the progression to preeclampsia [65].

There are two unique events that women undergo, pregnancy and menopause, where there is a certain association that exists between the various hormonal changes and the risk of cardiovascular disease. Changes also occur in platelets and PMPs, which indicates that women should consider hormone supplements [68]. Oral menopausal hormone treatments may confer a greater risk of thrombosis than transdermal products [69]. The concentration of circulating microparticles for those with metabolic syndrome (MS) was found to vary with the individual components of MS [70]. PMPs were found to be elevated in patients with polycystic ovary syndrome (PCOS), and they are associated with increased annexin V binding and an altered miR expression profile [71]. Microvesicles are released in abundance from the placenta during pregnancy. By transferring miRNA, proteins, and lipids between cells, endometrial-embryo communication can be performed during the peri-implantation period [72]. In addition to placental-derived microvesicles, PMPs also play an important role in the communication between maternal vascular cells and the placenta. Compared with patients with benign breast tumors, PMPs levels in breast cancer patients are associated with tumor infiltration [73]. Exosomes derived from amniotic fluid stem cells prevented ovarian follicular atresia in chemotherapy-treated mice via the delivery of microRNAs containing highly enriched miR-146a and miR-10a [74]. The proangiogenic effects of exosomes have been demonstrated in endometriosis by exerting autocrine/paracrine effects [75].

PMPs and Complement. Complement activation can be mediated by platelet activation, and complement CP and AP are involved in causing this activation [76]. It has been demonstrated that when complement is added to platelet-rich plasma that contains the C-terminus of platelet surface H, platelet activation and thrombotic response occur [77]. Thrombin, human coagulation factor (F) XIa, Xa, and IXa, and plasmin were effective in cleaving C3 and C5 [78]. The complement system is activated in the vasculature during thrombotic and inflammatory conditions [79], via secretion of their contents onto endothelial surfaces. By supporting the classical pathway of complement activation, PMPs transport the complement component to the site of vascular injury and support local inflammation [80]. C5 is associated

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with platelet aggregation and coagulation system activation and is a key molecule for lethal thrombosis induced by extracellular histones. Histones induce platelet aggregation and coagulation disorder, and the coagulation disorder, in turn, promotes C5 cleavage [81].

The genetic variants in complement factor H (FH), complement component 2, complement component 3, and complement factor B are known to be associated with age-related macular degeneration (AMD). Visual deterioration is more prominent in neovascular AMD with choroidal neovascularization (CNV). Jo et al. found that the anti-C5 antibody inhibited CNV by binding to the kMG4 domain of C5 and subsequently preventing C5 to be cleaved to effector proteins [82]. The high plasma concentration of complement factor C3 is a risk factor for human cardiovascular disease. C3 may act as a biomarker for insulin resistance after bariatric surgery [83]. Cancer cells within the cerebrospinal fluid (CSF) produced C3 in correlation with clinical course, and Boire et al. found that cancer-cell-derived C3 activates the C3a receptor in the choroid plexus epithelium to disrupt the blood-CSF barrier, thus proving therapeutically beneficial in suppressing leptomeningeal metastasis [84].

In the model of venous thrombosis induced by tissue factor (TF)-dependent flow restriction, the complement factor was found to significantly contribute to platelet activation and fibrin deposition. C3 deficiency leads to prolonged bleeding in the inferior vena cava as well as reduced thrombosis, thrombus size, fibrin and platelet deposition, and platelet activation in vitro [85]. Systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and antiphospholipid antibody syndrome (APS) are associated with complement component dysfunction, which has common pathogenic characteristics in tissue inflammation, thrombosis, and peripheral tolerance to autoantigens. FH can adjust these events with the assistance of C3 and C5 invertase decay accelerators [86]. The complement system also involves the clearance of PMPs. Immune adhesion is a complement-mediated host defense that removes particles from the circulation quickly, and several complement proteins in the plasma can be conditioned for immune adhesion [87]. It was observed that PMPs immediately increased after transfusion, and annexin V-bound MPs or PMPs ($CD61^+$) disappeared faster than the occurrence of the platelet cycle [88]. With regard to complement activation and regulation, there are several important differences between mice and humans [12].

Proteomics Exploration. In the production, treatment, and recovery of disease, the protein domain may be most susceptible to interference; however, compared with 2-dimensional gel electrophoresis (2-DE) analysis, the proteomics approach provides a more equitable platform for a comprehensive analysis of the entire proteome within a certain physiological timeframe [89]. Proteomics holds special promise for biomarker discovery [90].

A high-resolution 2-DE-based proteomic analyzed PMPs with different stimulated platelet activation and the results showed that 30 protein features were differentially regulated [91]. A 2D-difference gel electrophoresis (DIGE)-based proteomic analysis was used to compare the platelets released following platelet activation with thrombin or collagen, and it was observed that 122 protein spots were differentially regulated between both conditions. The study demonstrated that glycoprotein IV (GPVI) has a major effect on collagen-induced platelet activation/aggregation [92]. Gel filtration chromatography was used to separate PMPs to facilitate identification of active protein and lipid components. PMPs can be divided into several size classes according to different needs [93]. The proteomic approach was used to identify membrane proteins present on MPs in endothelial progenitor cell cultures. The results showed that platelet proteins were also present in endothelial progenitor cell (EPC) cultures [94].

Clinical and pathological characteristics of acute coronary syndromes (ACSs) may involve a number of diverse mechanisms. A number of studies have described proteomics methods to identify new biomarkers associated with ACSs, including biomarkers associated with platelet metabolism, intravascular thrombus proteomics studies, and proteomics of membrane particles released from activated cells, mainly by platelets [89]. The principal enabling technology for proteomic discovery is mass spectrometry (MS). Technology platforms for proteomic biomarker discovery include gel electrophoresis, time-of-flight mass spectrometry, liquid chromatography, and activity-based functional proteomics. Each method has its strengths and limitations.

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Compared to LC-MS/MS-based approaches, 2D-PAGE has limited sensitivity, reproducibility, and throughput. As a throughput- and time-saving procedure, a nano-liquid chromatography (nano-LC) technique coupled off-line with spectrometric measurement, MALDI-TOF-MS/MS, has been proposed [95]. The detection of iTRAQ proteomic analysis identified STBMs' key proteins can as potential biomarkers [96].

An enormous challenge resides in the obvious fact that it is necessary to clearly recognize that the proteome is a dynamic rather than a static entity [97]. First, the separation process or the process of ultracentrifugation may lead to different results, and it is important to consider whether the last step of an isolation protocol yields proteins that reflect the true composition of EVs. Obtaining two or more proteome datasets, including quantitative proteomics analysis, may result in identification of the final protein [98]. For biokinetic studies, highly purified vesicle populations are important. Possible side effects may be affected if EVs, which are a source of diagnostic and prognostic markers, exhibit an energy difference between subtypes and if impure samples are used in clinical settings **(Figure 2)** [99].

Conclusion. Platelet microparticles are released by different stimuli, and the biological information they carry is also different. The extraction methods for PMPs and detection technologies still have no recognized standard specification, which can be attributed to the different combinations of the abilities of biomolecules. Lipoprotein particles can be detected by various EVs evaluation methods, but cannot be separated from EVs by any currently available purification technique [100]. For clinical purposes, the quantification of PMPs is still a challenge today, although some other emerging methods have been tried. Through cell communication, PMPs play a variety of roles in the biological pathophysiological environment through biologically active molecules, surface receptors, and genetic information. This interaction between cells can be used for the development of drug delivery systems and clinically applied to additional diseases. Some scholars have developed PMPs-nanofoam (PMN) that can protect encapsulated thrombolytic drugs from *in vitro* digestion and effects resulting from the drug being uptaken elsewhere in the body [101]. PMPs have been shown to have specific expression in a variety of diseases, whether it affects the cellular content or the cellular surface substances. There is great potential for biomarkers to be developed using proteomics, and proteomics is more practical in view of the biologically active molecules that associate with PMPs and the surface receptors of PMPs. However, it is necessary to take into account several shortcomings in current research, such as the analysis of sample design and instrumentational analysis of the lack of standardization, and the technical gap between the clinical and the current body of knowledge with respect to PMPs.

Proteomics is currently used for the realization of precision medicine and the detection of rare genetic defects in diseases, and in the future, a variety of groups (including proteomics, genomics, metabolomics, etc.) will likely form an interactive network that will be used for more dynamic and comprehensive analyses of organisms. An improved method for understanding EVs biology as well as EVs quantification, storage, and molecular characterization will greatly enhance future prospects for EVs-based diagnostic and therapeutic applications. At present, there is considerable controversy regarding whether the EVs dose should be defined by the number of vesicular granules, the amount of vesicular protein, or the expressed dose as a proportion of vesicles to protein. This problem must be addressed if EVs are to be clinically administered.

Much cellular communication involves regulating the immune system. Cell communication can be mediated by direct cell-to-cell contact, and often through soluble factors such as hormones, cytokines, and inflammatory mediators, which can communicate with a large number of responsive cells at local or scattered sites. PMPs contain a variety of proteins, unique lipids, and nucleic acids, but the role of PMPs as they act upon recipient cells is combined and diverse, and it is difficult to separate the role of each component in the vesicle. Recent studies have shown that PMPs stimulate hematopoietic cells and transfer platelet-specific receptors to the surface of other cells. Increasing numbers of studies have reported that monocyte hematopoietic cells, monocytes, neutrophils, plasma cell-like dendritic cells, and endothelial cells can uptake MPs. Cerebral endothelial cells that uptake MPs may be involved in circulating MPs

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clearance. During stroke recovery, brain endothelial cell-derived exosomes actively perform brain remodeling by communicating with brain cells, including neurons and glial cells, as well as distant cells in other organs. PMPs interact with target cells in blood or blood vessels, and the characterization of biologically active molecules (such as mRNA, miRNA, proteins, lipids, and metabolites) that characterize PMPs may assist with the identification of pathophysiological mechanisms of potential disease.

Clinically, PMPs have been used as a biomarker for a variety of diseases. Future studies should focus on the role of cell vesicles in the human body, as well as their extensive role in cell communication. Multiple studies help to explain the specific PMPs associated with clinical disease, as well as the choice of specific markers. We should actively search for methods that can be used to extract pure PMPs suitable for clinics and develop instruments that can detect diagnostic PMPs. The particle tracking technique and dynamic light scattering technique used to characterize nanoparticle size should be further improved for clinical application. In the course of disease, the changes in PMPs are very important and could be used to determine their role in the body's physiological and pathological state.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (no.81473387 and 81503291). We thank School of Pharmacy, Anhui University of Chinese Medicine for their excellent technical assistance. The authors have no conflict of interest to declare.

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