# Extraction and identification of platelet-derived microparticles

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**Abstract.** Microparticles are carriers of signals for intracellular signal transduction. These carriers include proteins, mRNAs, microRNAs and other bioactive substances. Platelets are a major source of circulating microparticles, and microparticles are closely associated with the development of certain cardiovascular diseases. In the present study, a method for separating, extracting and identifying platelet-derived microparticles was developed and differences in the expression of surface proteins on microparticles harvested from platelets stimulated by vortexing or treatment with thrombin was investigated. The counts, composition, sizes and inner structures of microparticles were determined using flow cytometry and transmission electron microscopy. Additionally, it was demonstrated that platelets could be readily activated, and a large quantity of microparticles with varying complex compositions, structures and sizes were derived from activated platelets. High purity platelet-derived microparticles were obtained by gradient centrifugation. However, the microparticles derived from platelets stimulated by thrombin treatment or vortexing differed significantly in the levels of CD63. The present study aimed to provide improved options for the extraction and identification of microparticles.

## Introduction

Platelets are derived from mature megakaryocytes, and are biconvex in shape, when they have not been activated, have no nuclei and have a diameter of 2-4 $\mu$m. Platelets have a short lifespan, typically 1-2 weeks. In addition to hemostasis, platelets serve an important role in pathophysiological processes, including angiogenesis, atherosclerosis, tissue regeneration and immune regulation (1,2).

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Platelet-derived microparticles (PMPs) are submicron particles with a diameter of 0.1-1 $\mu$m that are secreted by activated or apoptotic platelets (3). Particles of <0.1 $\mu$m in diameter are called exosomes, while particles >1 $\mu$m in diameter are termed apoptotic bodies (4,5). Microparticles have been demonstrated to act as carriers of signals for communication between cells (1). However, because of their small size and the diverse structures and phenotypes of PMPs, there are considerable challenges in handling and characterizing these particles (6).

In the present study, the traditional method of extracting PMPs was improved upon, and differences in the expression of molecular markers on the surface of PMPs were investigated. Transmission electron microscopy (TEM) was used to identify platelets and their microparticles. Platelets were readily activated using a simple protocol, and a large quantity of microparticles of varying compositions, sizes and structures were derived from the activated platelets. A high degree of purity of PMPs was attained through gradient centrifugation, and additionally it was demonstrated that the standard practice of using flow cytometry to identify PMPs may underestimate the actual number of microparticles.

## Materials and methods

*Sample acquisition*. Platelet-rich plasma (PRP) was obtained from healthy volunteers from the blood station of Changhai Hospital affiliated to The Second Military Medical University between May and December 2015. Volunteers provided informed consent for the collection of blood samples and the protocol used in the present was approved by The Ethics Committee of Changhai Hospital. Blood (200 ml) was collected in the morning at the blood station prior to breakfast from each volunteer. There were 32 volunteers, 23 males and 9 females. The age of volunteers was 20-40 years old. The free-flowing technique using a 16G needle was employed for blood collection to prevent platelet activation. The initial centrifugation of the PRP, as described below, was performed <2 h after collection. Aspirin (0.5 g; Sigma-Aldrich; Merck KGaA) was dissolved in 1 ml DMSO, sterilized by filtration through a bacterial filter (0.22 $\mu$m), and the filtrate was added to each unit of PRP.

*Separation and extraction of PMPs*. PRP was dispensed into a 15 ml centrifuge tube and washed using 5 ml of 4.2 mM ethylene

diamine tetra-acetic acid per tube. The tubes were centrifuged at 150 x g for 15 min at 22˚C and the pellet containing the red blood cells was discarded. The supernatant was further centrifuged at 1,000 x g for 10 min at 22˚C and the supernatant was discarded. The pellet containing the platelets was resuspended in 1 ml of Hepes-$NaCl_2$ buffer (10 mM HEPES, 0.85% $NaCl_2$, pH 7.4) and transferred to a 1.5 ml tube. Each tube contained 10-12 million platelets. Thrombin (Sigma-Aldrich; Merck KGaA) was added at a final concentration of 0.1 IU/ml to tubes to activate the platelets, each tube was mixed gently and evenly, and subsequently placed in a 37˚C cell incubator for 90 min. Other tubes were vortexed at room temperature for 1 min (oscillation frequency 2,800 beats/min) to activate platelets. The suspension was centrifuged at 3,200 x g for 15 min at 4˚C and the supernatant was transferred to a new 1.5 ml tube. The supernatant was further centrifuged at 20,000 x g for 90 min at 4˚C and the supernatant was discarded, leaving the pellet which contained the PMPs.

*Flow cytometry*. Data was analyzed by CellQuest v5.1 software (BD Bioscience). Each tube of PMPs was resuspended in 200 $\mu$l binding buffer (BD Bioscience) and mixed evenly. Polystyrene microspheres with a diameter of 1 $\mu$m (Sigma-Aldrich; Merck KGaA) were diluted 1:1,000 with PBS and 5 $\mu$l was added to each tube. As a control, one tube of 200 $\mu$l binding buffer with 5 $\mu$l microspheres was left blank. The solution was then incubated at 4˚C for 15 min in the dark with 20 $\mu$l CD63-phycoerythrin (PE; cat. no. 556020; BD Bioscience), CD61-PE (cat. no. 555754; BD Bioscience), CD62P-allophycocyanin (APC; cat. no. 550888; BD Bioscience), CD40L-PE (cat. no. 555702; BD Bioscience), CD41-APC (cat. no. 303710; BioLegend, Inc.) or 5 $\mu$l Annexin V-FITC antibodies (BD Bioscience).

*TEM*. Platelets with PMPs were fixed in 1 ml 2% paraformaldehyde and 2% glutaraldehyde at 4˚C for 6 h. After fixing, the samples were centrifuged at 1,000 x g for 10 min at 4˚C and supernatant containing the fixative was discarded. The precipitate was washed five times with PBS and centrifuged again at 1,000 x g at 4˚C for 10 min. The precipitate was resuspended in plasma (supernatant obtained from the second centrifugation) and centrifuged at 1,000 x g at 4˚C for 30 min. The majority of the supernatant (plasma) was discarded, leaving a small volume. The samples were fixed using 1% osmium tetroxide at 4˚C for 2 h, rinsed once with PBS and centrifuged at 1,000 x g for 10 min at 4˚C. Gradient dehydration was performed as follows: 70% acetone for 15 min, 80% acetone for 15 min, 90% acetone for 15 min, and 100% acetone for 10 min (x2). The pellet was embedded in a transparent capsule no. 3 (Electron Microscopy room of Second Military Medical University) with epoxy resin and oven-dried at 45˚C for 12 h and 60˚C for 36 h. The embedded block was sliced into 70 nm thick sections with an ultramicrotome and placed on a copper mesh covered with polyvinyl formal film. Melted wax was dripped on to a sterile Petri dish to form a wax plate, a few drops of lead dye solution were dropped on to the wax and the copper mesh with the sample was placed on top of the lead dye droplet and incubated for 15 min at room temperature. The copper mesh was taken from the lead dye solution, washed three times with distilled water, dried with filter paper and observed by TEM. The specimens were examined using an electron microscope (HT7700; Hitachi, Ltd.) at an operating voltage of 100 kV (magnification, x1,000-5,000). Images were analyzed using ImageJ v1.8.0 software (National Institutes of Health).

*Statistical analysis*. SPSS v19.0 statistical software (IBM Corp.) was used for data analysis. All data are expressed as the mean ± standard error of the mean. A two-tailed Student's t-test was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*PMPs can be separated by gradient centrifugation*. There is no standard protocol to extract PMPs as far as the authors are aware. However, a protocol for the isolation of PMPs from blood samples was recommended (Fig. 1A). In the present study, gradient centrifugation was used to extract PMPs, with some adjustments. During this process, red blood cells and the majority of platelet fragments were removed from the sample and the resulting precipitate containing the PMPs could be observed at the base of the tube (Fig. 1B). However, it should be noted that a portion of microparticles were lost during the gradient centrifugation.

*Flow cytometry results of PMPs*. The distribution ranges of the particles with different diameters were divided (Fig. 2A): R1 means 1.0 $\mu$m polystyrene microspheres; R2 means particles smaller than 1.0 $\mu$m; R3 means particles larger than 1.0 $\mu$m. Flow cytometry analysis of PMPs demonstrated that platelets were markedly sensitive to both physical and chemical stimuli. Pretreatment with aspirin effectively reduced the activation of platelets; however, the attenuation of activation was not complete (Fig. 2B and C). PRP without aspirin pretreatment derived a portion of PMPs, which were lost during the gradient centrifugation, then after totally being activated, the PMPs finally obtained were obviously fewer than PRP with aspirin pretreatment (Fig. 2D). Treatment with thrombin or vortexing stimulated the release of a large number of microparticles from the PMPs. The majority of the precipitates obtained by gradient centrifugation contained particles <1 $\mu$m in diameter, although it was possible that platelet fragments or large vesicles were also present (Fig. 2E and F).

Labeling with Annexin V or the five platelet surface markers (CD61, CD62P, CD63, CD40L and CD41) demonstrated that the targets were present on the surface of the microparticles at high levels in the PMPs obtained from platelets stimulated by thrombin or vortexing. There was no significant difference in the expression of surface markers found between PMPs from the thrombin-stimulated platelets compared with the vortex-stimulated platelets, except in the case of CD63, which was significantly higher in the PMPs from vortex-activated platelets (55.38±5.27% vs. 43.50±3.86%; $P<0.05$; Fig. 3).

*TEM of platelets and PMPs*. Platelets which had not been pretreated with aspirin displayed an increased level of activation after centrifugation compared with aspirin-treated platelets and the untreated platelets possessed extended pseudopodia compared with the aspirin-treated platelets (Fig. 4A and B). The distal portion of the pseudopodia gradually separated from the platelet body to form a membrane chain (Fig. 4C).

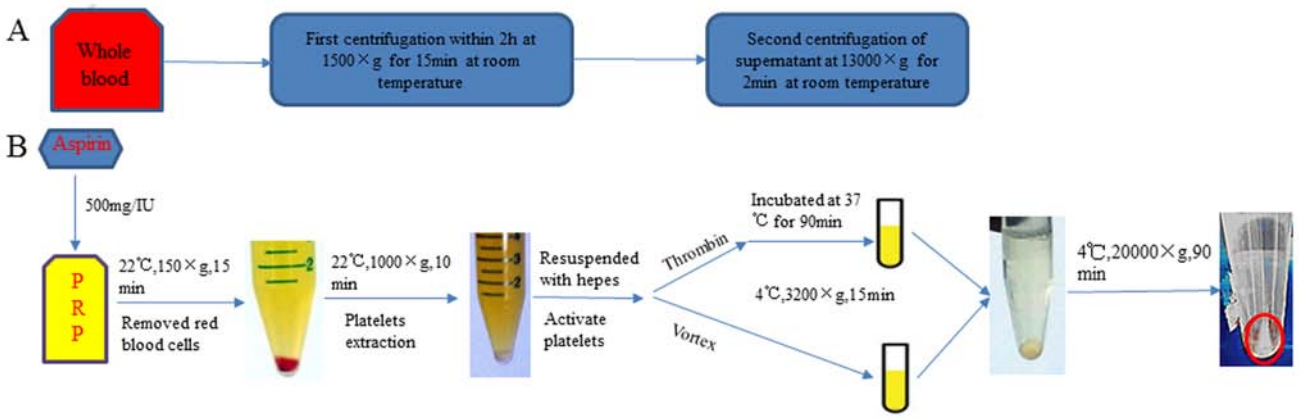


Figure 1. Protocol for extracting and purifying PMPs. (A) The recommend protocol for PMP extraction. (B) Protocol used in the present study for PMPs extraction. The precipitate in the red circle were finally obtained PMPs. PMPs, platelet-derived microparticles; PRP, platelet-rich plasma.

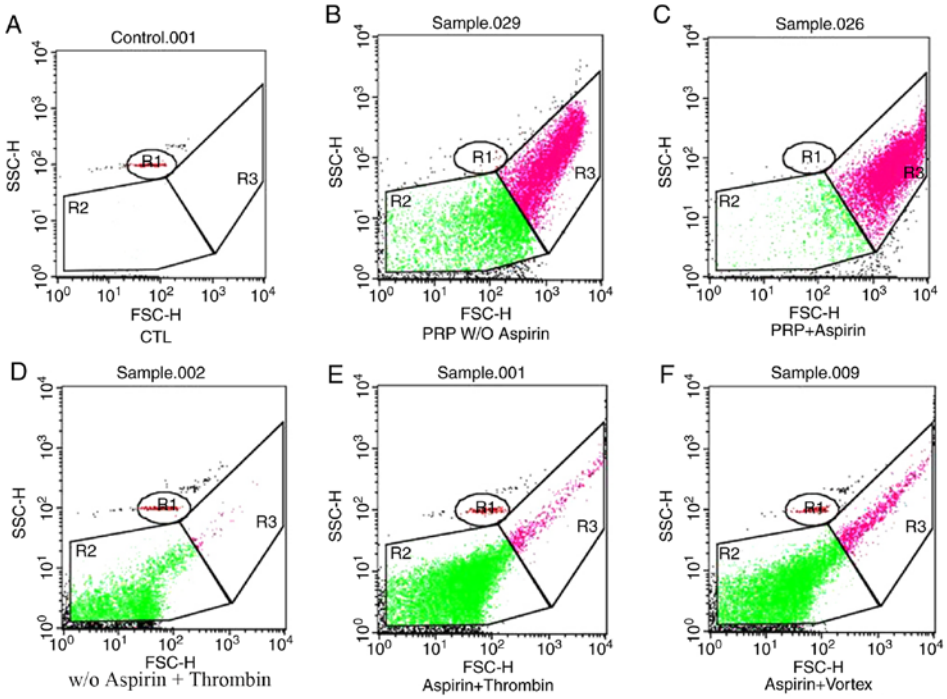


Figure 2. Flow cytometry analysis of purified PMPs. FSC/SSC plots of PMPs. The gate was set such that the upper right limit on the FSC was 1.0 $\mu$m. (A) Control sample. (B) PRP not pre-treated with aspirin after the first centrifugation. (C) PRP pre-treated with aspirin after the first centrifugation. (D) PMPs harvested from PRP not pre-treated with aspirin and stimulated with thrombin. (E) PMPs harvested from PRP pre-treated with aspirin and stimulated with thrombin. (F) PMPs harvested from PRP pre-treated with aspirin and stimulated by vortexing. R1, 1.0 $\mu$m polystyrene microspheres; R2, <1.0 $\mu$m particles; R3, >1.0 $\mu$m particles; PMPs, platelet-derived microparticles; PRP, platelet-rich-plasma; FSC, forward scatter; SSC, side scatter; w/o, without.

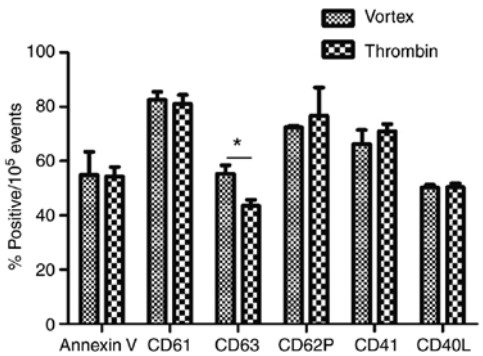


Figure 3. Flow cytometry analysis for marker staining of platelet-derived microparticles stimulated by thrombin or by vortexing. *P<0.05.

Eventually, the membrane chain broke and the microparticles were secreted. Treatment with thrombin and vortexing activated platelets resulting in the release of PMPs (Fig. 4D and E). PMPs have different shapes and complex ultrastructures, and their contents, membrane structure and electron density vary. In a similar way to medicine capsules, PMPs contain biologically active substances such as α-granules, glycogen granules and mitochondria (Fig. 4F).

## Discussion

Microparticles secreted by platelets are involved in the regulation of many physiological and pathophysiological processes

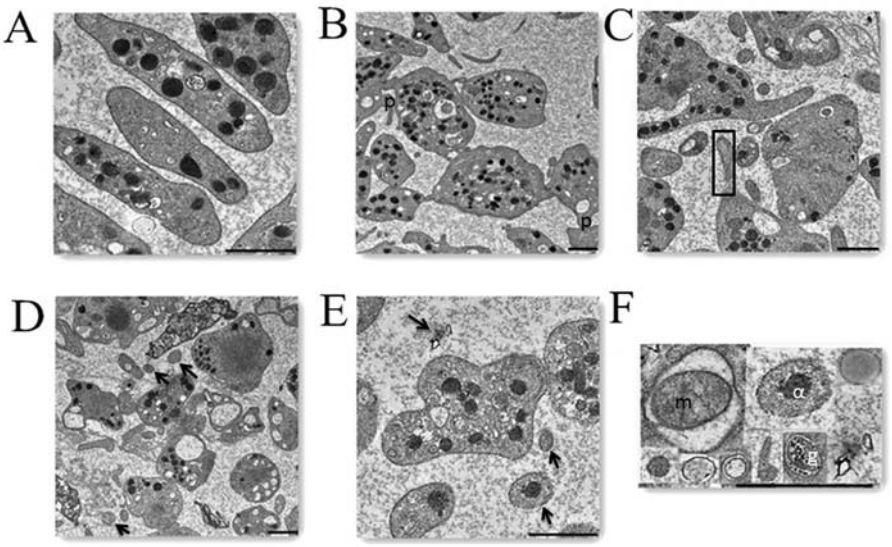


Figure 4. Transmission electron microscopy of platelets and PMPs. (A) Platelets pre-treated with aspirin had relatively stable structures. (B) Platelets not pre-treated with aspirin had extended pseudopodia. (C) A microparticle being released from a platelet (black box). (D) Released PMPs from platelets activated using thrombin. (E) Released PMPs from platelets activated by vortexing. (F) PMPs with different shapes, contents, and membrane structure. The arrows indicate PMPs. Scale bar, 1.0 $\mu$m. PMPs, platelet-derived microparticles. α, α-granules; g, glycogen granules; m, mitochondria.

in the body, including coagulation, vasomotor regulation, cell proliferation, differentiation, apoptosis, inflammation, the immune response and the transmission of signals between cells (1,6). Studies have demonstrated that different kinds of cells can secrete microparticles, including red blood cells, lymphocytes, platelets, endothelial cells and tumor cells; however, the microparticles derived by platelets account for 70-90% of microparticles present in the circulatory system (7-9). Extracting PMPs is a difficult and labor-intensive process. A two-step gradient centrifugation method is recommended to extract PMPs from blood samples, although a standardized approach is not available. In theory, the longer and faster the final centrifugation step, the more particles of smaller sizes can be obtained, although there may be an upper limit. In addition, increasing the number of gradient layers will concurrently increase the purity of the sample, with the caveat that there will also be an increase in the loss of the desired product. Therefore, determining the optimal combination of centrifugal speed, duration and number of gradient layers, to maximize the purity and minimize the loss of microparticles requires further investigation.

In the present study, adjustments were made to the most frequently used two-step gradient centrifugation method. PRP was obtained from the blood station at Changhai Hospital affiliated to The Second Military Medical University, and met China's national quality standards, reducing the proportion of other cells obtained. Residual red blood cells were removed from the PRP in the first centrifugation step, further improving the purity of the platelets. The precipitation of the platelets was achieved by centrifugation, after which the platelets were activated using thrombin or by vortexing. To increase the purity of PMPs further, two additional centrifugation steps were used. However, there was still a small proportion of unwanted particles >1 $\mu$m in diameter in the final precipitate, possibly platelet fragments or large vesicles. PMPs are particles with a 0.1-1 $\mu$m diameter that are secreted by activated or apoptotic platelets (3). Therefore, a 1 $\mu$m diameter was set as the upper limit for microparticles in the flow cytometry experiments performed in the present study. Platelets can be activated with ease, with activation resulting in the release of a large number of microparticles (10). Platelets continue to release microparticles when they are inactive, although at a lower level (10). Previous studies have demonstrated that collagen, thrombin, lipopolysaccharide, viruses, immune complexes, temperature changes and shear stress could all activate platelets, resulting in the release of microparticles (7,11-15). To minimize the loss of microparticles during transportation and centrifugation, antiplatelet activation or anticoagulant drugs can be added before further activation of platelets, of which aspirin is probably the most economical (16). A previous clinical study reported that aspirin significantly reduced the number of PMPs in circulation in patients with coronary heart disease (17). The present study additionally demonstrated that although aspirin did not completely prevent platelets from being activated, it markedly inhibited the secretion of PMPs. In the PRP samples not pre-treated with aspirin, there was a notable increase in the release of microparticles following centrifugation. This fraction of microparticles was discarded with the supernatant during the gradient centrifugation, and the final number of microparticles extracted was markedly decreased compared with the aspirin pre-treated samples.

Murphy and Gardner (18) studied the effects of temperature on platelets and demonstrated that platelets maintained *in vitro* at 22˚C had an improved structure and function. Bode and Knupp (19) demonstrated that platelets lost more glycoproteins and formed more microparticles at 4˚C. Therefore, platelets in the present study were extracted at 22˚C and the final ultracentrifugation step was performed at 4˚C to obtain the PMPs.

The mechanism of microparticle production is not fully understood and may be related to the asymmetric loss of proteasomes and membrane phospholipids (20,21). A previous

study demonstrated that during normal blood flow, activated platelets form a membrane chain downstream of the blood flow, and eventually the membrane chain breaks, releasing microparticles (22). In the present study, platelets were observed to form a membrane chain following activation. Eventually the membrane chain broke and microparticles were released. There are a number of methods used to identify PMPs and their associated markers. After activation, the intracellular calcium concentration of platelets is elevated, and non-selective ion channels on the cell surface and on the mitochondria, in addition to the stimulation of some enzymes, promote the flipping of phosphatidylserine; when present on the extracellular facing side of the cell membrane, phosphatidylserine subsequently acts as a signal for phagocytosis on apoptotic cells (23-25). Therefore, Annexin V is commonly used as a marker for detecting microparticles. However, studies have shown that ~50% of the microparticles in circulation do not present phosphatidylserine on their surface (26). The use of Annexin V alone as a marker of microparticles may, therefore, underestimate the number of microparticles present (26). In the present study, Annexin V positive particles accounted for ~50% of the particles obtained. Therefore, the use of Annexin V alone as a marker for microparticles may be inadequate and may result in a large underestimation of the number of microparticles.

A limitation of using flow cytometry to detect microparticles is the accuracy of detection for sub-200 nm particles (27). TEM is the most accurate and reliable method for identifying microparticles; however, the preparation of samples is a time-consuming process and requires high quality specimens. In order to determine whether the extracted microparticles are derived from platelets, it is also necessary to identify platelet surface-specific molecular markers. In the present study, CD41, CD61, CD62P, CD63 and CD40L were used in combination with Annexin V to perform a single step detection of microparticles from platelets activated by vortexing or thrombin treatment. Differences in handling and storage methods may result in changes to the surface markers present on PMPs (28,29). The present results demonstrated that all six markers were expressed on the surface of PMPs, with CD63 expression found to be significantly higher in microparticles derived from vortex-stimulated platelets compared with microparticles from thrombin-stimulated platelets. In a previous study, CD63 was used as a biomarker for the detection of exosomes (30). However, in the present study, exosomes were not able to be detected by flow cytometry. Brisson *et al* (31) demonstrated that the majority of larger extracellular vesicles, up to 1 $\mu$m in diameter, also expressed CD63. Whether the difference in CD63 levels on PMPs obtained from the two different stimulation methods was a result of differences in the presence of large vesicles is unknown and requires further study. Yuana *et al* (32) studied microparticles in fresh plasma using electron microscopy. The results demonstrated that microparticles existed in various shapes, including round, drop-shaped, tubular and cup-shaped. In the present study, based on the results of TEM, PMPs also displayed a variety of shapes, sizes, contents, ultrastructure and electron densities. After activation, platelets secrete particles and tend to disintegrate (33). In the present study, the diameter of microparticles was 200-600 nm. Other differences observed in the TEM images include the presence of either a single or double layered membrane, α-granule content, the presence of glycogen granules and the presence of mitochondria. The majority of the particles observed were circular, oval or almost round. The size of microparticles is associated with their contents. Microparticles containing organelles typically have a larger diameter and irregularly shaped particles may be a result of the handling process (32,33). To the best of our knowledge, a certain shape or size of PMP has not been attributed to a particular function. Difficulties in isolating specific types of PMPs has hampered progress in understanding differences in function.

In conclusion, high purity PMPs may be obtained by gradient centrifugation, although a small fraction of platelet fragments or large vesicles may remain. A higher purity of PMPs can be achieved if a 1 $\mu$m filter is used. At present, the use of flow cytometry to detect PMPs based on Annexin V may lead to inaccurate results. TEM is more accurate in identifying microparticles; however, the technical limitations, labor-intensive preparation process and considerably lower throughput make TEM less convenient. Determining the best method to use towards identifying PMPs may be best decided on a per case basis; it may be possible to use TEM on a small sample of purified PMPs to confirm the results of flow cytometry. Difficulties in identifying PMPs may be a result of the diversity of PMPs, and this diversity may additionally underlie the range of functions attributed to PMPs. Therefore, further studies are required to elucidate the function of PMPs and to improve the methods for their identification.

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**Availability of data and materials**

All data generated and/or analyzed in the present study is included in the published article.

**Authors' contributions**

JZ, XXZ, BZ and JG conceived and designed the experiments. JG, CF and XS performed the experiments. JG and SZ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript

**Ethics approval and consent to participate**

Patients provided informed consent for the collection of blood samples and the protocol used in the present was approved by the Ethics Committee of Changhai Hospital.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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