Platelet extracellular vesicles as biomarkers for arterial thrombosis

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SPECIAL REVIEW: PLATELET MICROVESICLES

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Abstract

Arterial thrombosis is a major and global cause of human death and disability. Considering the socioeconomic costs of arterial thrombosis, identification of biomarkers to predict and detect arterial thrombosis at an early stage is an important public health goal. Platelet extracellular vesicles (PEV) are a new candidate biomarker of arterial thrombosis. PEV can be measured in biorepositories, thereby offering the possibility to validate PEV in multicenter clinical trials. PEV analysis has been hitherto hampered by lack of standardized methodology, but substantial technological improvements of PEV detection techniques have been achieved recently. However, before PEV emerge from research tools to clinical applications, a number of issues should be clarified. To facilitate validation of PEV as biomarkers of thrombosis, we discuss (i) *whether* PEV are useful as biomarkers of thrombosis, (ii) *why* previous conclusions on PEV concentrations, composition and functions require re-evaluation, and (iii) *which* questions have to be answered before PEV become clinically useful.

1. Introduction

Arterial thrombosis is responsible for two major and global causes of human death and disability: acute myocardial infarction (AMI) and acute ischemic stroke [1]. Currently, there are no tools available to predict or diagnose arterial thrombosis at an early stage, and hence, there is a quest for novel biomarkers to facilitate early recognition of thrombotic events [2,3].

Although the term "biomarker" is commonly used as an objective indication of a medical state which can be measured accurately and reproducibly [2], in cardiovascular literature, this term specifically refers to soluble biomolecules present in blood [3]. Biomarkers in blood may provide insight into patient's hypercoagulable state, which is a hallmark of arterial thrombosis. Because insight into developing arterial thrombosis cannot be provided by either clinical examination or imaging, analysis of biomarkers in blood is indispensable to predict, diagnose, and monitor thrombosis [3].

A reliable biomarker (i) has a sensitivity and specificity above 90%, (ii) has an established kinetics of release and clearance, (iii) can be assayed with an established technique, and (iv) has been validated in multicenter prospective clinical trials [3,4]. Biomarkers that are currently used to diagnose AMI, such as cardiac troponin or creatine kinase MB isoenzyme (CK-MB), suffer from several limitations. For example, an elevated concentration of cardiac troponin may result from conditions other than AMI, and cardiac troponin is detectable in blood of healthy individuals when high-sensitivity assays are being used [4]. Moreover, cardiac troponin and CK-MB are released *after* cardiomyocyte necrosis and hence do not reflect the *preceding* or *early* phase of atherothrombosis [4]. At present, the diagnosis of acute ischemic stroke is based exclusively on neurologic deficits and brain imaging, but no clinically applicable and relevant biomarkers exist [5,6].

Because activation of platelets plays a key role in thrombosis, measuring platelet function may provide novel biomarkers of arterial thrombosis [7]. Out of numerous platelet function methods, aggregometry-based tests such as light transmission aggregometry are dominant in clinical practice [8]. However, aggregometry provides information on platelet functionality in response to exogenous and soluble agonists, which do not mimic the process of platelet activation *in vivo*. Thus, so far results from platelet function tests *in vitro* have limited value as biomarkers of arterial thrombosis.

Alternatively, biomolecules or metabolites released from activated platelets in blood may provide clinically relevant information for early detection of arterial thrombosis [9]. One of these biomarkers is platelet-derived extracellular vesicles (PEV), encompassing platelet microparticles/microvesicles and exosomes [10]. Table I compares established biomarkers to diagnose arterial thrombosis and PEV. In contrast to cardiac troponin or CK-MB, PEV may fulfil the requirements for a biomarker of *developing* arterial thrombosis because (i) PEV are released from activated platelets and platelet-rich thrombi and therefore may reflect developing arterial thrombosis [11], (ii) PEV expose specific platelet antigens and thus can be identified as being platelet specific [12], and (iii) disease-dependent changes in the biochemical composition of PEV may be useful for differential diagnosis of AMI and acute ischemic stroke [13]. Because identification and characterization of PEV directly in human plasma have recently become feasible [14], and because PEV can be measured in biorepositories, thereby facilitating validation in multicenter clinical trials [15], it is likely that the requirements for PEV to become a reliable biomarker can now be fulfilled.

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Table I. Comparison of established biomarkers to diagnose arterial thrombosis and platelet extracellular vesicles (PEV) [4–6,10]. AMI: acute myocardial infarction; AIS: acute ischemic stroke; CK-MB: Creatine Kinase MB isoenzyme; NA: not applicable.

Biomarker	Thrombotic condition		Pathology		Release and clearance	Pathological cut-off values	Validation in clinical trials
	AMI	AIS	Myocardial necrosis	Developing thrombus			
Cardiac troponin	Yes	No	Yes	No	Known	Known	Yes
CK-MB	Yes	No	Yes	No	Known	Known	Yes
PEV	Yes	Yes	NA	Yes	To be established	To be established	To be validated

The role of PEV in physiological and pathological processes has been summarized previously [16,17]. In this review, we discuss the significance of PEV as biomarkers for prediction and early diagnosis of arterial thrombosis, in comparison with other used biomarkers, outline methodological pitfalls in hitherto performed studies, and highlight challenges to be overcome before PEV can be clinically applied. Overviews of recent technological advancements to measure concentrations and functional properties of PEV in body fluids are to be expected in subsequent reviews from the current series.

2. Platelet extracellular vesicles as biomarkers of arterial thrombosis

Table II provides an overview of compounds exposed on—or released from—activated platelets, including (i) biomarkers of single and overall platelet activation and (ii) biomarkers that can be measured in fresh blood samples and in biorepositories.

2.1. Biomarkers of single platelet activation

Biomarkers of single platelet activation include activation-induced changes in surface expression and/or conformational changes of transmembrane receptors, and platelet-leukocyte aggregates (PLA) which are formed when activated platelets bind to leukocytes [18,19]. At present, platelet surface receptor profile and PLA, analyzed by flow cytometry, are considered the most reliable markers of platelet activation *in vivo*, because these biomarkers (i) are platelet specific, (ii) are independent of the platelet count and thereby applicable to thrombocytopenic patients, and (ii) reveal the interaction between platelets and leukocytes, thereby promoting vascular inflammation and atherosclerosis [18,19]. However, there are three major drawbacks of this approach. Firstly, flow cytometric analysis of platelet activation state and PLA requires freshly collected blood, which hampers validation of these biomarkers in multicenter clinical trials where storage and transport of samples for analysis are required. Secondly, exposure of platelet activation markers is a dynamic process and sensitive to proteolysis [18], so that the measured activation status after labeling *in vitro* does not reflect the original platelet activation status immediately after blood collection. Finally, PEV exposing platelet activation markers such as P-selectin may bind to P-selectin glycoprotein ligand-1 (PSGL-1)-exposing leukocytes, similar to activated platelets, and PLA are unable to distinguish platelet-leukocyte complexes from PEV-leukocyte complexes [19]. Due to these drawbacks, neither platelet surface receptor profile nor PLA are clinically applicable as biomarkers of arterial thrombosis. PEV retain the advantages of platelet surface receptors and PLA, because PEV (i) are platelet specific [10], (ii) allow to determine single platelet activation status independently from the platelet count when visualizing the parental PEV-releasing platelet [20], and (iii) are involved in the interaction between platelets and leukocytes due to exposure

of P-selectin [10]. To which extent activation markers exposed on PEV are sensitive to proteolysis is unknown. However, if PEV are measured directly in (diluted) plasma, the risk of proteolysis of activation markers exposed on PEV seems small, because plasma contains (i) abundant protein substrate, (ii) protease inhibitors, and (iii) calcium-chelating agents, which all hamper plasma protease activity.

2.2. Biomarkers of overall platelet activation

Overall platelet activation is reflected by molecules secreted from platelet granules, synthesized *de novo*, or shed from the platelet surface upon activation [9]. These molecules can be measured in biorepositories using commercially available immunoassays [21,22]. However, the reliability of such measurements is questionable at best, because (i) platelet-specific chemokines are secreted already at room temperature unless a special anticoagulant is used [21], (ii) soluble platelet receptors and thromboxane A_2 are released from extra-platelet sources [22], and (iii) methods applied to analyze soluble biomarkers lack standardization [23]. Finally, some "soluble" markers are in fact not a single marker but a mixture of different forms that are measured in a single assay. For example, "soluble" P-selectin in platelet-free plasma is present not only as a truly soluble (non-membrane bound) form, but is also exposed on PEV [19]. To which extent these different forms of "soluble P-selectin" behold different clinical information, however, is unknown.

Likewise biomarkers of overall platelet activation, PEV are also released at room temperature in case of artifactual platelet activation, and from this reason, it is recommended to (i) centrifuge the sample twice to prepare platelet-free plasma and thus to reduce the risk of small platelet contamination and/or the release of PEV from such platelets and (ii) standardize the interval between blood collection and handling throughout the study so that obtained results can be compared between samples. However, PEV offer an advantages over the available biomarkers of overall platelet activation, because PEV are truly platelet specific, whereas biomarkers of overall platelet activation are not [18,19].

3. Formation and clearance of platelet extracellular vesicles

One of the most important issues before validating PEV as biomarkers is to firmly establish the (i) PEV cellular origin, (ii) mechanisms of PEV formation, and (iii) PEV clearance.

3.1 Cellular origin and composition of platelet vesicles

PEV were identified in human plasma and serum as being derived from platelets in 1967 [24]. Consequently, the cellular origin of PEV was established by measuring the exposure of specific platelet receptors, such as glycoprotein (GP) IIb-IIIa (CD41/CD61; integrin αIIbβ3) or GPIb (CD42b) [10]. Because GPIIb-

Table II. Biomarkers of platelet activation *in vivo* [9–10].

	Single platelet activation	Overall platelet activation
Fresh samples	Surface receptors/molecules: • CD63 • CD40L • GPVI • GPIIb-IIIa conformation • PS exposure • P-selectin (CD62p) Platelet-leukocyte aggregates **Platelet extracellular vesicles**	Secreted biomarkers: • platelet factor 4 • serotonin • trombospondin-1 • β-thromboglobulin Synthesized biomarkers: • thromboxane A2 • 11-dehydro-thromboxane B2 Shed soluble biomarkers:
Biorepositories	 **Platelet extracellular vesicles**	• sCD40L • sGPVI • sGPV • sP-selectin (CD62p) **Platelet extracellular vesicles**

Platelet extracellular vesicles (PEV) are the only platelet biomarkers (i) that can be analyzed both in fresh blood samples and in biorepositories and (ii) may reflect both activation of single platelets and the overall platelet activation, depending on the applied technique. CD: Cluster of Differentiation; GP: Glycoprotein; PS: Phosphatidylserine; s: soluble.

IIIa and GPIb are also exposed on megakaryocytes, these receptors are *identification* markers of EV from either platelets or megakaryocytes, but they are not markers of platelet *activation* [25]. In contrast, typical platelet activation receptors such as P-selectin (CD62p), which are exposed only on activated platelets, are thought to be truly unique for PEV from activated platelets because such receptors are not detectable on EV from megakaryocytes [25,26].

Over decades, detection of PEV and other blood-borne EV was based on the exposure of phosphatidylserine (PS). The exposure of PS depends on pre-analytical conditions as blood collection, handling and storage of plasma [15]. In many studies, the exposure of PS has been overestimated due to incomplete removal of platelets upon preparation of platelet-free plasma and the following freeze–thaw cycle [27]. Furthermore, the almost invariably reported elevated levels of PS-exposure and concentrations of PEV in patients' plasma samples compared to controls may—at least in part—be due to systematic differences in collection and handling of blood samples. For example, in daily clinical practice, the time between patient blood collection and handling is often longer than controls. Finally, in studies where PEV were isolated from plasma by centrifugation, we now know that PEV aggregate [27,28]. Because PEV aggregates are impossible to resuspend, the detection of PEV aggregates was favored in older studies, where instruments used to detect PEV were insensitive, with single vesicles often below the instrument detection limit. Consequently, hitherto data on PEV concentrations and PS exposure have been questioned in recent studies showing that PEV constitute up to approximately 25% of all vesicles in bloodstream, instead of 90% as concluded before [29], and that in fresh and non-centrifuged blood samples, most PEV above 1 μm in diameter do not expose PS [30]. To which extent PEV below 1 μm in diameter expose PS remains unknown.

PEV which are truly derived from activated platelets and do expose PS may provide a surface for assembly of tenase and prothrombinase complexes, hence being directly involved in coagulation [11]. If true, this implies a causal relationship between increased PEV concentrations and thrombosis, thus strengthening the utility of PEV as biomarkers. However, the formation and degradation of an arterial thrombus are a dynamic process, and to

which extent PEV reflect either one or both phases of this process remain to be elucidated. It is likely that there are several distinct subpopulations of PEV in the bloodstream, which may play different roles in coagulation *in vivo*, as well as may reflect different stages of thrombosis.

3.2. Formation of platelet vesicles

Out of numerous molecular events reported to be involved in PEV formation, binding of fibrinogen to activated GPIIb-IIIa seems to be indispensable, at least *in vitro* [31]. If so, PEV are released *after* reversible platelet aggregates have been formed, which is a first step towards arterial thrombus formation. In atherosclerosis, formation of reversible platelet aggregates and thrombi may be induced by adhesion of platelet GPIIb-IIIa and GPIb to P-selectin and von Willebrand factor (vWF) exposed on dysfunctional endothelial cells [32]. Correspondingly, in 40% of patients with AMI coronary thrombus is formed more than 24 hours *before* clinical manifestations of acute coronary occlusion, and the presence of such old thrombi increases the risk of death two times during median follow-up time of 2.5 years [33]. Because PEV may directly reflect the dynamic process of *in vivo* thrombus formation/degradation at a presymptomatic stage, PEV may potentially allow to diagnose coronary thrombosis *before* the myocardial damage occurs, which is not detectable with any of the currently available diagnostic tools.

3.3. Clearance of platelet vesicles

Because predictable kinetics is among the attributes of a valuable biomarker, it is important to know how long a biomarker is present in bloodstream. Although the half-life times of PEV have been studied in a few experiments [26,34], in none of these experiments, sensitive analytical methods have been used, and hence, no firm conclusions can be drawn on the rate of PEV clearance *in vivo*. PS is thought to be a key trigger for clearance of (P)EV [35]. However, PS exposure is often an artifact due to the presence of residual small platelets and/or centrifugation, and therefore, it is likely that artificially induced exposure of PS may have affected the clearance of PEV in preceding studies. Moreover, because composition of (P)EV

vary with disease state, it is likely that (P)EV half-life times and mechanisms of (P)EV clearance are dependent on pathophysiological conditions, such as the presence of inflammation. Finally, it remains to be established whether increased concentrations of PEV in thrombosis are due to increased formation of PEV, less efficient clearance, or a combination of both.

4. Methodological pitfalls in hitherto studies on platelets extracellular vesicles

Increasing fascination with PEV as potential biomarkers results from numerous reports on elevated concentration of PEV in a variety of diseases, including thrombotic disorders [36–40], diabetes mellitus [41–43], autoimmune disease [44–46], and multiple types of cancer [47–49]. Elevated concentration of PEV has been observed by flow cytometry [36–49], enzyme-linked immunosorbent assay (ELISA) [50], and functional assays such as the factor X-activated clotting time assay (XACT) [51]. Moreover, correlations between the disease activity and PEV concentrations and/or their function have been demonstrated. For example, PEV concentrations increased stepwise with the severity of coronary artery disease [37], stroke volume [40], activity of rheumatic arthritis [46], and progression of malignancy [48,49]. Increase in concentrations of PEV irrespective of the clinical condition implies two hypotheses: either PEV are an entirely non-specific biomarker, and as such provide no clinically relevant information about the underlying pathology, or—as mentioned before—there have been systemic methodological errors in the pre-analytical and or analytical phase of PEV detection. In the light of recent findings, we are prone to think that many of the previously reported findings were subject to substantial errors and artifacts that have affected downstream PEV detection.

4.1. Inter-individual variability in concentrations of platelet vesicles

Concentrations of PEV have been reported to depend on age, gender, body mass index (BMI), physical exercise, sleep pattern, diet, nicotine abuse, hormonal status in women, and concomitant pharmacotherapy [52]. Moreover, multiple drugs have been reported to influence concentrations of PEV, including antiplatelet drugs [53], statins [54], antihypertensive drugs [54], and oral antidiabetic drugs [55]. Because the effects of these factors on PEV release were established only during the last decade and were not taken into account in the earlier studies on PEV in many diseases, this may have also affected previously reported results.

4.2. Inter-study variability in concentrations of platelet vesicles

Flow cytometry represents the mainstay to analyze (P)EV in clinical setting, because in contrast to other methods of EV analysis, such as nanoparticle tracking analysis, resistive pulse sensing or electron microscopy, flow cytometry (i) enables analysis of thousands of individual (P)EV per second, (ii) provides information on the biochemical composition of (P)EV, (iii) and is widely accessible [56,57]. Figure 1 shows the concentrations of PEV detected with flow cytometry in acute coronary syndrome [35–38], diabetes mellitus [40–42], autoimmune disease [43–45] and healthy controls [35–38, 40–45] over the last two decades. The three types of disorders have been chosen due to their distinct pathophysiology. Figure 1 shows that the reported concentrations of PEV increase both in patients and in control groups at least 10-fold every 10 years, *irrespective* of a disease pathophysiology. Additional details on PEV concentrations shown in Figure 1 are available in Table 3 in the online data supplement. This
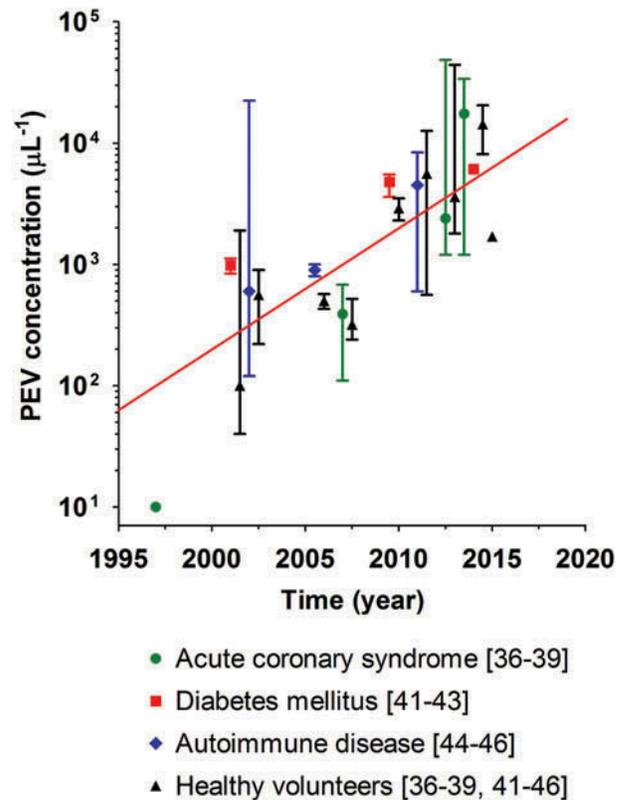

Figure 1. Reported concentrations of platelet extracellular vesicles (PEV) detected with flow cytometry in acute coronary syndrome, diabetes mellitus, autoimmune disease, and healthy volunteers on a log scale versus time. Data are presented as mean ± standard deviation or median (range). The red line shows a 10-fold increase in PEV concentrations every 10 years. However, PEV concentrations seem to increase even more.

remarkable increase in PEV concentrations is likely to result from improved sensitivity of the flow cytometers over time. For example, flow cytometers applied in older studies (approximately 1995–2005) were able to detect far less than 1% of all PEV present, which was due to the discrepancy between their detection limit (800–2400 nm) [57] and the size of PEV (>90% below 500 nm in diameter, with a major part between 100 and 250 nm) [58]. In fact, "PEV" detected by older flow cytometers may have been either aggregates of PEV caused by (ultra)centrifugation, or remnants of platelets due to centrifugation and freeze-thawing, or multiple small PEV that were detected as a single event by swarm detection [59]. Flow cytometers dedicated to EV analysis, which allow to measure EV down to 150–190 nm in diameter, detect another one order of magnitude higher concentrations of (P)EV than conventional flow cytometers [36,37]. Table 3 (online data supplement) also shows that the conclusions on significant differences between PEV concentrations in patients and control groups are not consistent throughout the studies. Hence, both conclusions on PEV absolute numbers and significant differences in PEV concentrations between patients and controls require re-evaluation in future studies.

5. Challenges before clinical application of platelet extracellular vesicles as biomarkers

The main challenge before clinical application of PEV as biomarkers is the standardization of PEV isolation and detection methods. Recent efforts to standardize the methodology of (P)EV analysis have been brought together in International Society of

Extracellular Vesicles in 2011. Since then, (i) standard operating procedures for handling and storage of human body fluids for EV analysis have been established [15,52], (ii) a method to isolate EV from body fluids has been developed [60], and (iii) technological improvements of PEV analytical techniques have been introduced, largely in the field of flow cytometry [14]. One should bear in mind, however, that improved detection of (P)EV will affect previously developed protocols for collection, handling, and storage of blood or fractions thereof. Increased sensitivity of currently available detection techniques allows to differentiate between *in vitro* induced changes triggered by sample collection and handling, and true *in vivo* platelet activation status. Hence, it is likely that standardization of study protocols will start anew before (P)EV may finally reach clinical applications.

It is currently recommended to measure PEV in (diluted) platelet-free plasma. However, isolation of (P)EV from plasma is a challenge due to the presence of proteins and lipoproteins of overlapping density and/or size [60]. This challenge may be overcome by isolating (P)EV from plasma of fasting donors with size-exclusion chromatography (SEC), which enables separation of (P)EV > 75 nm in diameter from >99% of the soluble plasma proteins and >95% of plasma high-density lipoproteins. Because PEV < 75 nm in diameter cannot be yet detected by flow-based techniques, the fraction containing PEV < 75 nm in diameter is usually discarded. SEC has a recovery of up to 90% and does not result in clumping of PEV and therefore offers advantages when compared to centrifugation [60]. Although SEC facilitates proteomics and electron microscopy, this technique is relatively laborious, and for clinical studies measuring PEV directly in (diluted) plasma, for example with flow cytometry, may be preferred.

Limitations of the first generation of flow cytometers have been largely overcome by (i) diluting the sample to reduce swarm detection (large number of PEV entering a laser beam simultaneously) [59], (ii) using de-ionized water prior to PEV analysis to rinse the flow cell and reduce background noise from the instrument [14], (iii) calibrating the flow cytometer with beads of similar refractive index (similar amount of scattering light) to PEV [61], and (iv) triggering on fluorescence, instead of light scattering [28]. However, the flow cytometry field is expanding further. Next generation of flow cytometers, which currently are being developed, are likely to enable analysis of most (P)EV present in body fluids, hence assuring for the reliable PEV analysis in clinical studies [62].

6. Summary

Figure 2 shows the number of publications and landmark discoveries on PEV versus time. There are two major implications in Figure 2. First, Figure 2 shows a shift in the nomenclature, with the terms "platelet microparticles/microvesicles" being gradually replaced by the term "platelet extracellular vesicles." The term "platelet extracellular vesicles" cover all types of vesicles released by platelets, including exosomes and microparticles/microvesicles. Because differentiation between these vesicle subtypes in plasma is not yet feasible, the term "extracellular vesicles" have been recommended to facilitate exchange of information within the vesicle research community [10,12].

Second, Figure 2 shows that research interest on PEV has been grown vastly in the last five years, and that discoveries of the last five years re-evaluated many of the previous assumptions on PEV. This growing interest is driven by the perspective to apply PEV analysis to biorepositories, which is not applicable to any other biomarkers of platelet activation, and which creates a long-awaited chance to validate PEV in multicenter clinical trials. However, before this step will be made, a number of issues should
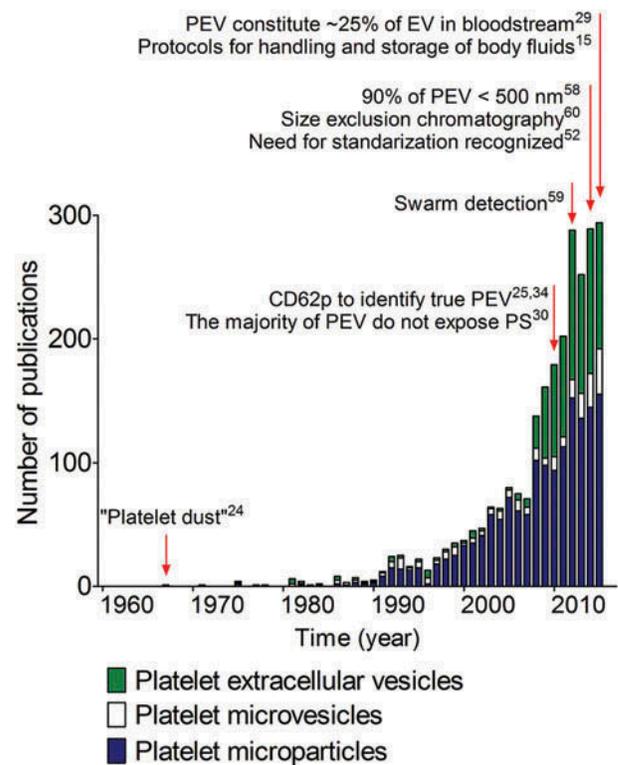

Figure 2. The number of publications on PEV versus time. Please note, that there is (i) a shift in the nomenclature, with the terms "platelet microparticles/microvesicles" being gradually replaced by the term "platelet extracellular vesicles" and (ii) an extensive increase in the research interest on PEV in the last decade, with landmark discoveries on PEV in the last five years.

be clarified. First of all, as for all platelet measurements, standardized blood collection and handling conditions are a prerequisite to obtain reliable PEV concentrations, and novel protocols to standardize pre-analytical and analytical variables are urgently required, based on the detection of (P)EV by novel and sensitive analytical techniques. Next, physiological concentrations and composition of PEV need to be established, before any threshold for PEV pathological concentrations can be defined. Further, more insight into the kinetics of PEV release and clearance is needed to correctly interpret data on PEV concentrations in clinical setting. Finally, it is crucial to investigate the extent of PEV involvement in thrombosis. Once these questions are answered, PEV are likely to make their way from research tools to clinical applications.

Declaration of interests

The authors report no declarations of interest.

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