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Blood platelets and cancer: the involvement of platelet-derived extracellular vesicles in tumour progression

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Introduction

A Brief History of Blood Platelets

Platelets are small anucleate cells that circulate in the blood stream. In haemostasis, they rapidly become activated, spread and coat the damaged blood vessel. The activated platelets become cohesive and release their granules' content, aiding the recruitment and stimulation of additional platelets. This process culminates with the formation of a platelet aggregate, or thrombus, that is stabilised by the activation of coagulation and fibrin deposition [1].

Max Schultze published the first accurate description of platelets as part of a study on to the white blood cells in 1865. He recognised platelets as a normal constituent of the blood and 'enthusiastically recommended' them as an object for further 'in-depth study of the blood of humans' (Fig. 1 a). In 1882, the Italian medical researcher Giulio Bizzozero observed platelets microscopically in the circulating blood of living animals and in the blood removed from the vessels. He showed that they were the first blood cell type to adhere to damaged vessel walls *in vivo* and, *in vitro* (Fig. 1 b) [2].

(a) **(b)**

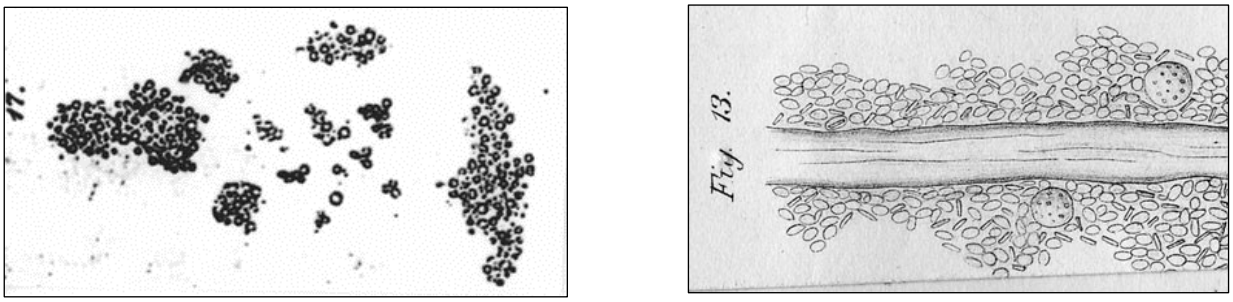

FIGURE 1. ***(a)*** *Schultze's figure 17. The granular formations which often occur in larger collections in normal human blood. (Schultze 1865).* ***(b)*** *Bizzozero's figure 13. A little fibre from a thread with which the blood of a dog was beaten for 45 s, examined in methyl saline solution. The fibre is covered with a deposit of blood platelets in which two white cells are noticeable. Zeiss 1/12 [2].*

From the discoveries of Schultze and Bizozzero, our understanding of platelet structure and function has strongly advanced thanks to several technological innovations. In 1962, the invention of the light transmission platelet aggregometer by Gustave Born [3] allowed the identification of the primary platelet agonists. The next historical advance in the understanding of platelet biology was the analysis of platelet–vessel wall interactions, employing new model systems developed by Baumgartner and colleagues. Importantly, these studies considered the effects of blood flow on platelet behaviour, revealing the critical role of platelets in thrombus formation at elevated shear rates [4].

The important functions of specific platelet membrane glycoproteins, as receptors for adhesive ligands in platelet adhesion, were elucidated using improved *in vitro* perfusion systems [5, 6]. These functions were evaluated by the quantitative analysis of receptor–ligand interaction and by the analysis of deficient platelets in particular receptors. Moreover, the generation of novel blocking antibodies and the specific inhibitors, and the increased analysis of genetic models in mice contributed to characterise the pathophysiological roles of platelets in haemostasis and thrombosis.

Blood platelets are implicated also in non-haemostatic functions including inflammatory responses, blood/lymphatic vessel separation in the embryo, and regulation of microbial infection [7, 8]. Furthermore, they play critical roles in cancer evolution, metastasis diffusion and tumour angiogenesis [9, 10].

The complex relationship between platelet and cancer has been established since the late 19th century, when the French physician Armand Trousseau linked venous thrombus formation (Venous thromboembolism, VTE) with an occult malignancy [11]. However, thrombosis is not merely a marker for the presence of a malignancy, rather it represents a relevant cause of death for cancer patients. The mechanisms that support thrombosis in cancer are only partially known, but many authors now point to the “platelet–cancer loop” as a pivotal mechanism in tumorigenesis [12].

The role of platelets in cancer growth and spread may represent an important therapeutic prospect to reduce cancer growth or spread.

The Biology of Blood Platelets

Platelet Morphology

After the erythrocytes, blood platelets are the most abundant cells in the circulation with a concentration of 150.000-400.000/µl. They are produced by polynucleated progenitor megakaryocytes (MKs), which release platelets from pseudopodia-like structures extended into the bone marrow sinusoids. Platelets survive for approximately 10 days in the circulation, before being destroyed in spleen and liver [13].

Platelets have a typical discoid shape and, in humans, have a diameter of ~4 µm, and a thickness of ~1 µm. The discoid shape of the platelets, together with rheological parameters of the flowing blood, ensures that the platelets circulate in a well-defined area in close proximity to the endothelial cell surface [14]. These factors ensure platelets to continuously monitor the endothelium for damages. Upon endothelial injury, platelets rapidly interact with the components of the subendothelium, driving the restore of vessel wall integrity [15]. Such interactions generate a series of activation signals which result in a change of platelet shape, secretion of granular contents and activation of the integrins responsible for the building of platelet plug. Platelet adhesion, aggregation, and secretory responses are the result of a series of complex processes.

Platelets are surrounded by an asymmetric plasma membrane that contains negatively charged phospholipids, as phosphatidylinositol and phosphatidylserine, in the inner leaflet. The platelet plasma membrane also contains several glycoproteins, including receptors for soluble agonists and adhesion molecules which are fundamental for platelet adhesion and activation [16]. The open canalicular system, an elaborate membrane system that is contiguous with the plasma membrane, facilitates membrane remodelling and shape changes that occur during platelet adhesion, filopodia formation, membrane tethering, and spreading. The dense tubular system, a reticular membrane network, that derives from the endoplasmic reticulum of megakaryocytes and serves primarily as a store for calcium ions (Ca2+).

In addition to mitochondria, peroxisomes and lysosomes, platelets cytoplasm contains a population of organelles that includes secretory alpha granules and dense granules.

Alpha granules are the most abundant secretory organelles in platelets and contain a large variety of adhesive proteins that are important for primary haemostasis [17]. These proteins are crucial for platelet adhesive properties and the building of a stable thrombus. Platelet alpha granules also contain a large number of mediators that function in coagulation, wound repair, inflammation,

and angiogenesis [18]. Alpha granules acquire cargo from biosynthesis as well as from endocytosis [19].

Dense granules are the second major secretory compartment of platelets. They contain small molecules such as ADP, ATP, serotonin, calcium, pyrophosphate, and polyphosphate [20]. Secreted ADP plays a crucial role in primary haemostasis, by potentiating platelet aggregation through the stimulation of the P2Y12 receptor.

Platelets are also characterized by three types of cytoskeletal elements: microtubules, the membrane skeleton, and the actin cytoskeleton. That complex cytoskeletal system provides a contractile machinery that, during the different phases of platelet activation, governs platelet shape change and spreading, emission of filopodia and lamellipodia, granule secretion, and clot retraction [21].

The role of platelets in haemostasis and thrombosis

Under physiological condition, the principal function of vascular endothelial cells is to interface with platelets to coordinate and inhibit haemostasis and thrombosis.

Traditionally, haemostasis is considered as a two-stage processes of platelet plug formation (primary haemostasis) followed by fibrin clot generation (secondary haemostasis).

Upon a vessel injury, platelets arrest at the site of endothelial damage thanks to the exposition of the subendothelial matrix components, and through platelet aggregation, create platelet plug that acts as the first barrier that prevent excessive bleeding. This initial process, named primary haemostasis, is followed by secondary haemostasis. In the secondary haemostasis, the activation of the coagulation cascade culminates with the production of thrombin, a serine protease that converts soluble fibrinogen into insoluble fibrin and also acts as a potent platelet soluble agonist. The consequence of these events is the growth in size of the blood clot and its stabilization by the coagulated fibrin. The later phases of haemostasis are characterized by the retraction of the clot, which largely depends on the contractility of platelet cytoskeleton, and vessel repair.

However, platelet activation and blood coagulation are strongly correlated processes, each contributing to haemostasis and thrombosis.

Platelet function is therefore essential for preventing blood loss during haemostasis, but on the other hand, platelet hyper-activation cause pathologic events.

The primary trigger for intravascular thrombosis is the rupture of an atherosclerotic plaque. Under physiological conditions, platelets circulate in the bloodstream without significant binding to

the vascular endothelium. Injury of the arterial wall exposes the thrombogenic subendothelial matrix and initiates rapid platelet and monocyte recruitment. Following plaque rupture or erosion, platelets are activated in response to subendothelial components and play a central role in developing an occlusive thrombus within the artery. The reduction of blood flow prevent the correct supply of oxygen and nutrients to downstream tissues. For these reasons, thrombosis occurring in heart and brain can cause dramatically severe conditions as myocardial infarction or stroke [22].

Platelets and platelet activation are best known for their key role in the pathophysiology of arterial thrombosis, but their function is less clear in venous thromboembolism (VTE). It was proposed that venous thrombi are formed over an intact vessel wall in a low shear system through multifactorial processes and are rich in fibrin and trapped red blood cells. Today, experimental studies show that platelets and endothelial activation are essential in initiation of thrombus formation and augmentation of venous thrombosis [23].

Platelets have an important role in the development of VTE in cancer patients. Cancer cells have the ability to induce platelet activation and aggregation, and thus platelets are recognized to be involved in VTE formation and also in the progression of the malignant disease [24]. In cancer patients, an elevated platelet count was found to be associated with risk of VTE [25].

Molecular mechanism of platelet activation

Surveillance by platelets of the vasculature for injury or infection triggers rapid functional responses that control haemostasis, wound healing, innate immune, and inflammatory responses. Central to these coordinated responses is the ability, within seconds to minutes, to adhere and activate, secrete from storage granules, and interact with other vascular factors and cell types.

Platelet adhesion to endothelial cells and exposed subendothelial matrix

Upon a vessel wall damage due to trauma, infection, inflammatory response, or other pathological condition, platelets are arrested at the site of injury, in a process known as platelet adhesion.

The reversible binding of endothelial molecules and platelet receptors keeps platelets into a close contact with the subendothelial surface, reducing their speed while they continuously roll in the direction of the blood flow. Slowly rolling platelets are eventually firmly arrested at the site of injury by the establishment of additional interactions between subendothelial adhesion molecules, such as collagen, von-Willebrand factor (VWF), laminin, fibronectin, and specific platelet receptors [26].

Endothelial cells can rapidly secrete the multimeric adhesive glycoprotein VWF, with high capacity to mediate platelet adhesion via platelet GPIbα [27]. Activated endothelial cells also express P-selectin which is another counter-receptor for GPIbα (Fig. 2 b) [28].

The precise role of endothelial matrix-platelet receptor interactions in platelet adhesion in health and disease is not entirely certain. It is apparent that platelet GPIbα-VWF and GPVI-collagen engagements are indispensable for initial adhesion of platelets and subsequent activation and aggregation. For example, GPIb- IX-V/GPVI bind VWF/collagen at elevated shear rates (such as in small arteries) and integrins or other collagen-binding proteins exert their activity at lower shear, typical of the slow flows in large vessels (Fig. 2 a).

Human platelet GPVI preferentially interacts with collagen types I and III. However, a series of publications highlighted a multiplicity of additional GPVI natural adhesive proteins. GPVI was reported to directly bind to laminin, thereby promoting platelet activation and spreading [29]. Other studies showing that fibrillary fibronectin can support platelet adhesion under flow conditions demonstrate the involvement of multiple receptor-ligand interactions leading to thrombus formation. Platelet adhesion to fibronectin initially involves platelet α5β1, with subsequent thrombus growth mediated by GPIb-IX-V, GPVI, and Toll like receptor-4 (TLR-4), the latter potentially via a direct interaction with fibronectin (Fig. 2 a) [30].

However, the most important platelet receptor is integrin αIIbβ3 which is critical for platelet aggregation, but also mediates platelet adhesion to VWF, fibronectin and immobilized fibrinogen.

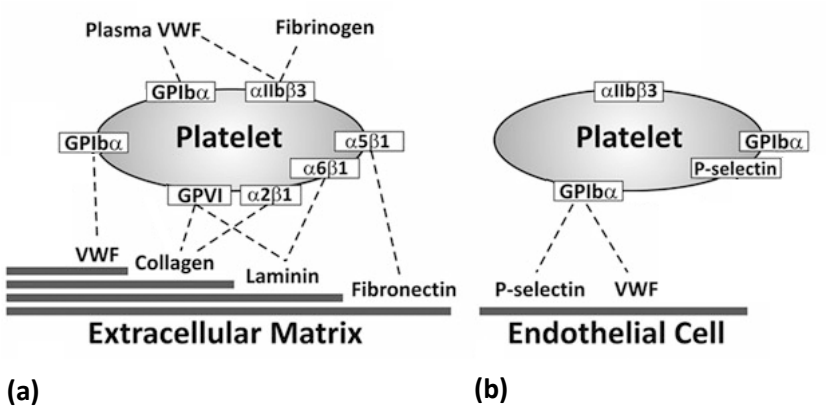

FIGURE 2. *Platelet adhesion networks. Platelet adhesion in the vasculature involves **(a)** platelet–matrix adhesion mediated by receptors on resting or activated platelets VWF, collagen, laminin, and/or fibronectin **(b)** platelet–endothelial cell adhesion mediated by GPIbα binding P-selectin/VWF on activated endothelium. Modified from [31].*

Platelet activation and signalling cascade

Adhesion receptors initiate signal transduction pathways mostly based on tyrosine phosphorylation cascades, supported by non-receptor tyrosine kinases.

In this context, the collagen receptor GPVI is the more potent mediator of platelet activation and the GPVI-dependent signalling pathway is well characterized [32].

The first event in GPVI signalling is the phosphorylation of two tyrosine residues located within a conserved motif in the FcRγ chain known as immunoreceptor tyrosine-based activation motif (ITAM). Upon ligand binding, the ITAM tyrosine residues are phosphorylated by the Src family kinases (SFK).

Binding of Syk to the phosphorylated ITAM leads to its activation through a combination of conformational change, phosphorylation by SFKs and autophosphorylation [33]. The binding and activation of Syk is a critical event in the formation of a signalling consisting of various adapter and effector proteins. Central to the formation of the signalosome is linker for activation of T cells (LAT). Phosphorylated LAT recruits the adapter protein SLP-76. LAT also binds class I phosphatidylinositol 3-kinase (PIK3) that catalyse the formation of the phosphotinositide 3,4,5-trisphosphate (PIP3) [34].

PIP3 recruits at the level of plasma membrane several proteins containing a PIP3-binding domain, including phospholipase Cγ2 (PLCγ2), phosphoinositide-dependent kinase 1 (PDK1) and the Ser/Thr protein kinase Akt. Akt is then activated by phosphorylation on threonine 308 and serine 473 mediated by PDK1 and by the cytosolic mammalian target of rapamycin complex2 (mTORC2), respectively. Active Akt regulates different cellular functions, largely through phosphorylation and inhibition of glycogen synthase kinase-3 (GSK3) [35].

Altogether LAT, SLP76 and PIP3 lead to the recruitment of the PLCγ2. Mice deficient in PLCγ2 are protected from experimental thrombosis but also exhibit a marked defect in haemostasis, which may reflect the well-documented role of PLCγ2 signalling downstream of GPIbα and αIIbβ3 [36].

Activation of PLCγ2 leads to hydrolysis of its substrate, phosphatidylinositol-4,5-bisphosphate (PIP2), to form the two second messengers, inositol-1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), that respectively trigger the release of Ca2+ from intracellular stores and activation of protein kinase C (PKC). At this level, the signalling pathways downstream ITAM receptors, including GPVI, and G protein-coupled receptors converge and act synergistically. The guanine nucleotide exchange factor, CalDAG-GEFI, senses increased levels of cytosolic calcium and facilitates the rapid but reversible activation of the small GTPase Rap1 [37]. Sustained activation of Rap1 results from PKC activation downstream of GPVI and feedback activation through the Gi-coupled receptor for ADP, P2Y12 [38]. Rap1 activation promotes integrin activation, thromboxane A2 generation and granule secretion, and the importance of this pathway is demonstrated by the fact that a deficiency in CalDAG-GEFI protects mice from collagen-induced thrombosis [39].

The classical excitatory soluble agonists (thrombin, thromboxane A2 and ADP) engage G protein-coupled receptors (GPCRs), which are seven-span transmembrane receptors that signal through heterotrimeric G proteins, consisting of α-, β-, and γ-subunits. Ligand binding to GPCRs induces exchange of GTP for GDP on Gα and its separation from the Gβγ subunits, each then promoting downstream activation of distinct pathways [40]. ADP is central to full platelet activation initiated by other agonists. It binds the purinergic P2Y1 and P2Y12 receptors on platelets [41], which are needed for a full aggregation response. P2Y1 associates with Gαq to regulate platelet shape change and granule secretion. P2Y12 associates with Gαi2 to repress ADP-mediated generation of cAMP, a negative regulator of platelet activation [42]. P2Y12 also promotes signalling through phosphatidyl inositide 3-OH kinase (PI3-K) independently of PKC [43], and supports the slow, irreversible phase of platelet aggregation [44].

Activated platelets synthesize and release thromboxane A2, which is generated de novo following the release of arachidonic acid from membrane phospholipids through the sequential enzymatic activities of phospholipase A2 (PLA2), cyclooxygenase- 1 (COX1), and thromboxane synthase that liberate and convert arachidonic acid to thromboxane A2 [45].

Thromboxane A2 is a clinically important platelet agonist, as suggested by the widespread use of aspirin in the primary prevention of cardiovascular events. Aspirin inhibits COX1 and thromboxane production in platelets. Thromboxane A2 binds the TP receptor and signals through

Gq and G12/13 leading to shape change and intracellular calcium mobilization [46]. Full platelet aggregation in response to thromboxane A2 is dependent on released ADP [47].

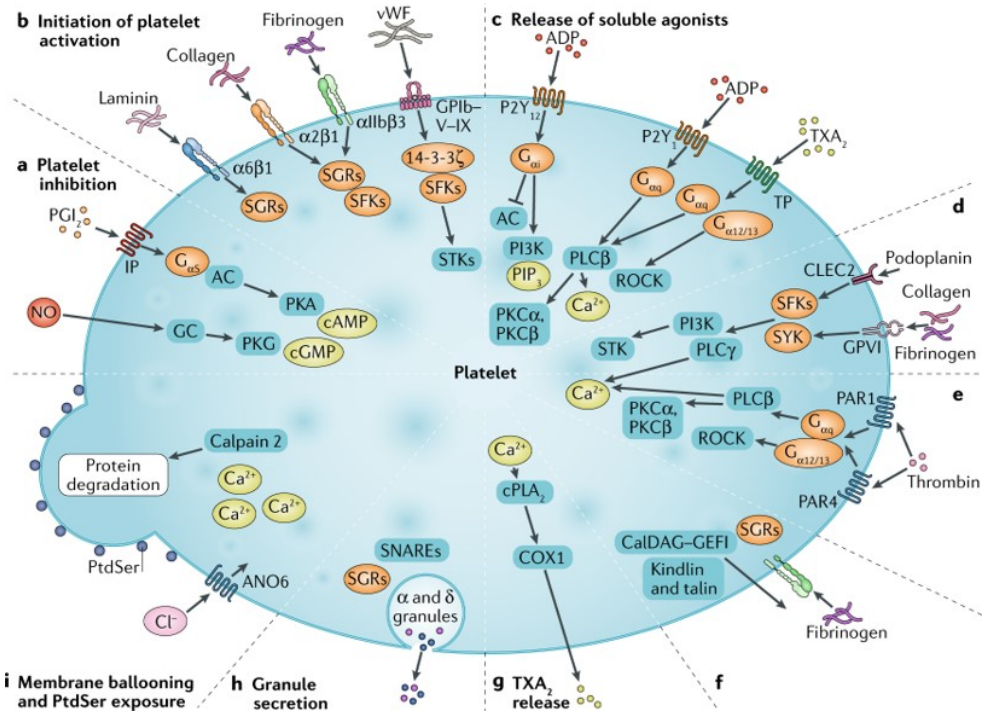

FIGURE 3. *Major signalling events and responses during platelet activation* [48]

Platelet activation is accompanied to the exposure of negatively charged phospholipids on the outer leaflet of plasma membrane which offers a support for the catalytic conversion of the zimogen prothrombin into the active serine protease thrombin by the prothrombinase complex.

Thrombin is a “strong” agonist that binds with high affinity to the non-GPCR, GPIb-V-IX, as well as to the protease-activated GPCR receptors, PAR1 and PAR4 in humans. Platelet activation through PAR receptors is mediated by Gαq and Gα13 [49]. Simultaneously, thrombin supports the process of blood clotting by converting soluble fibrinogen into insoluble fibrin [50].

Under normal circumstances, none of these agonists is available to circulating platelets at sufficient concentrations to surmount normal restraints to platelet activation, arguing that a minimal threshold trigger must exist to initiate the release or generation of these soluble agonists at effective microenvironmental concentrations.

Platelets also express prostaglandin (PG)-specific GPCR which are responsible of maintaining platelet quiescence. The production of PGI2 and nitric oxide (NO) by endothelial cells, leukocytes, and platelets leading to production of cyclic AMP [51] and cyclic GMP in platelets [52]. cAMP and cGMP activate protein kinase A (PKA) and protein kinase G (PKG), respectively, which through their

serine kinase activity serve to inhibit many critical enzymes involved in platelet activation. Two important substrates of PKA are PLCβ and CALDAG-GEFI, the principal GTP exchange factor for Rap1b in platelets [53].

Platelet aggregation

In the later phases of platelet activation, all the signal transduction pathways triggered by membrane receptors cooperate to promote platelet aggregation, the first key step for thrombus formation. Platelet–platelet interactions following stimulation with soluble agonists such as thrombin or ADP are mediated by activation of a platelet-specific β3 integrin, αIIbβ3, which is constitutively and highly expressed on the resting platelet surface, but in an inactive conformation unable to bind fibrinogen [54]. Platelet activation leading to conversion of integrin αIIbβ3 into the active state with a high affinity for soluble fibrinogen, which acts as a bridge between platelets. This adhesive ligand enables platelet–platelet aggregate formation.

Regulation of integrin αIIbβ3 is often divided into two phases: inside-out signalling, which controls integrin activation state and ligand binding, and outside-in signalling, which is triggered by ligand binding leading to the generation of inward signals that promote further platelet responses, including activation of additional integrins, cytoskeletal rearrangements, platelet secretion, and the development of platelet procoagulant activity [55].

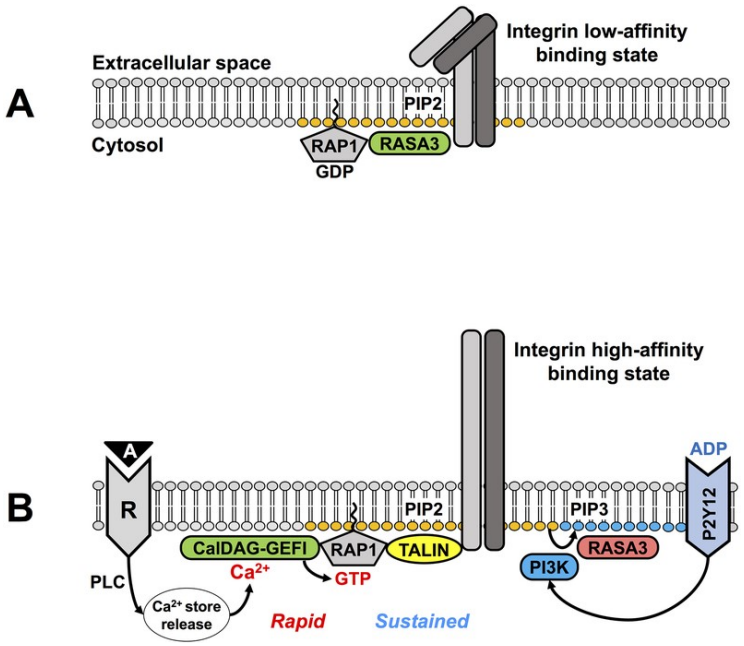

FIGURE 4. *Working model of integrin regulation [56].*

The central signalling event for platelet aggregation is the stimulation of PLC isoforms by adhesion receptors and GPCRs which leads to IP3-mediated cytoplasmic Ca2+ increase. Ca2+ directly stimulates the guanine nucleotide exchange factor CalDAG-GEFI, which in turn induces the small GTPase Rap1b to exchange of GDP with GTP leading to its full activation. The Rap1-GDP state is promoted by Rap GTPase activating protein RASA3. Upon platelet stimulation through the P2Y12 ADP receptor and activation of PI3K, the inhibitory effect of RASA3 on Rap1b is blocked [57].

GTP-bound Rap1b, through the contribution of the cytoskeletal protein talin, triggers the conversion integrin αIIbβ3 into the active state and the binding of soluble fibrinogen that mediates aggregation. The occupation of integrin αIIbβ3 by fibrinogen stimulates “outside-in” signalling pathways that further potentiate platelet activation and strengthen the platelet-platelet interaction, therefore avoiding thrombus embolization. Integrin αIIbβ3-dependent pathways also regulate the later phases of haemostasis, such as clot-retraction and vessel healing [13].

Extracellular vesicles (EVs)

The intercellular communication is a fundamental aspect of multicellular organisms. It can be mediated by direct cell to cell contact or by secreted molecules, but in the last twenty years a third mechanism has emerged. It involves the intercellular transfer of extracellular vesicles (EVs) released from the plasma membrane of cells. The release of apoptotic bodies during apoptosis has been long known [58], while the release of EVs from perfectly healthy cells gave new evidence on the studies of intercellular communication.

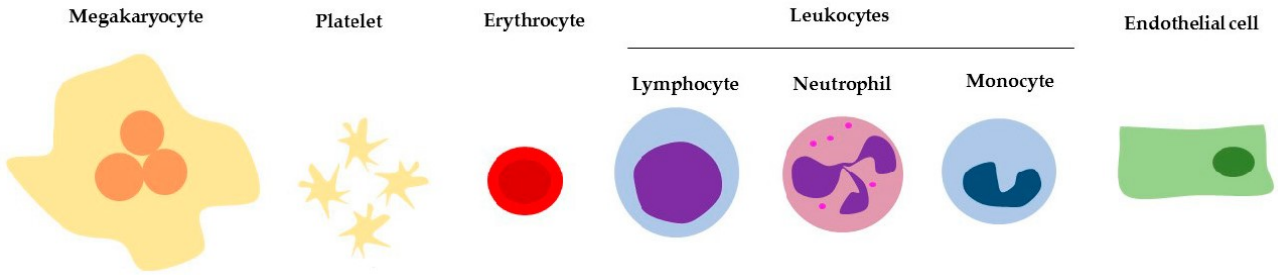

FIGURE 5. *EVs are produced by different cell types. Modified from [59].*

EVs are released from different type of cells, including leucocyte, platelets, erythrocytes, mast cells, neurons, intestinal epithelial cells, endothelial cells, and breast cancer cells detected in human and animal body fluids (Fig.5) [60, 61]. They have been classified in three groups: exosomes, microvesicles (MVs) and apoptotic body. Exosomes are the smallest EVs (50-150 nm in diameter) and are released upon multivesicular bodies exocytosis, MVs are produced by budding of the plasma membrane with heterogeneous size (100 nm-1 µm in diameter). Apoptotic bodies (1-5 µm in diameter) are generated by cells during apoptosis. More recently, the scientific community improves a list of "minimal information for studies of extracellular vesicles 2018" (MISEV2018). MISEV2018 indicates to use the operational terms for EV subtypes that referrer to a) physical characteristics of EVs, such as size ("small EVs" (sEVs) and "medium/large EVs" (m/lEVs), with ranges defined, for instance, respectively, < 100nm or < 200nm [small], or > 200nm [large and/or medium]) or density (low, middle, high, with each range defined); b) biochemical composition (CD63+/CD81+-EVs, Annexin A5-stained EVs, etc.); or c) descriptions of conditions or cell of origin (podocyte EVs, hypoxic EVs, large oncosomes, apoptotic bodies) in the place of terms such as exosome and microvesicle that are historically burdened by both manifold, contradictory definitions and inaccurate expectations of unique biogenesis [62]. However, differences between properties such

as size, morphology, density and protein composition seem insufficient for a clear distinction [63]. In this case, it is recommended to use terms such as extracellular particles (EP) [62].

Extracellular vesicles, acting as a reservoir of lipids, proteins, nucleic acids, have been shown to contribute to a large variety of pathophysiological processes including senescence [64], coagulation [65], inflammation [66], migration [67], tumorigenesis [68] and infection [69]. As they are found in body fluids, they are easily accessible and might represent useful diagnostic biomarkers and/or target for therapeutic applications [70].

Extracellular vesicles isolation and characterization

The purification of EVs represents a critical aspect. This is due to the small size of vesicles and to the difficulty in depleting sample from non-vesicle contaminants. Over the years, several strategies have been developed to optimize extracellular vesicle isolation. Differential ultracentrifugation is the most used. Increasing centrifugal force allow to separate EVs from debris and intact cells thanks to their difference in size and density. In combination with ultracentrifugation or alone, size-exclusion chromatography allows EV separation from high density lipoproteins (HDL) [71]. Finally, the affinity-based methods are based on the interaction of EV surface molecules with antibodies, lipid-binding protein, either biotinylated or coupled to magnetic beads [72].

The structural characterization of EVs after purification is another criterion to consider in EV classification. Electron microscopy, and recently cryo-transmission electron microscopy, allow to assess EV size and morphology and to identify cellular origin. Also, atomic force microscopy (AFM) make possible to investigate structural properties generating a 3D-image of the EV surface [71].

Dynamic light scattering and nanoparticle tracking, both based on the same principles, determine the size and concentration of particles in suspension by tracking their Brownian motion with a dark field microscope. For larger EVs, flow cytometry can be employed with fluorescent antibodies or probes for the recognition of EV surface marker [73]. Because conventional flow cytometry cannot discriminate vesicles smaller than 300 nm, a novel high-resolution flow cytometry-based method has been developed for quantitative high throughput analysis of individual nanosized vesicles [74].

Molecular composition of extracellular vesicles

Protein content

The protein content of EVs is generally associated with cell type origin. For instance, EVs produced by several tumour cells are positive for small GTPases suspected to play an essential role in EVs release by the cells [75]. Actin has been detected in neutrophil and red blood cell derived vesicles [76, 77]. In particular, extracellular vesicles originated by endothelial cells, neutrophils and tumour cells (i.e. prostate cancer and breast cancer cells) are enriched with proteolytic enzymes, such as matrix metalloproteinases, allowing tissues microenvironment remodelling which is essential for angiogenesis, tissue repair and cancer cell invasion [76, 78]. Adhesion molecules are commonly found in EVs and they can direct stimulate the recipient cells or promote the vesicle internalization. For example, P-selectin is found in platelet-derived microvesicles (or platelet-derived extracellular vesicle, PEVs) and PSGL-1 is detected in macrophage-derived vesicles able to fusion with platelets [79]. Finally, EVs could help the distribution of oncoproteins between tumoral cells. This event leads to the transfer of oncogenic activity, including activation of transforming signalling pathways, morphological transformation and increase in adhesion-independent growth capacity [80].

Nucleic acid content

The study of RNA in EVs represent a growing field of research. It was demonstrated that the cargo of EVs include both mRNA and miRNA and that vesicles-RNA could be translated into proteins by target cells. Many RNAs that were isolated with EVs were found to be enriched relative to RNA profiles of originating cells, indicating that RNA molecules are selectively incorporated into EVs [81]. For instance, EVs from endothelial progenitor cells are loaded with mRNAs associated to PI3K/Akt signalling pathway, which drives the angiogenesis in endothelial cells and promote organization in capillary like-structures [82]. Analysis of RNA from EVs, demonstrated that, in addition to mRNA and miRNA, vesicles also contain a large variety of other small noncoding RNA species [83].

Several investigations have pointed out that, when comparing MVs with exosome, the latter is the richest reservoir for almost all RNAs [84], indicating that the intercellular transfer of nucleic acids is most important in exosome than in MVs. There is no indication on the presence of DNA in MVs, on the contrary it was found in apoptotic bodies and exosomes [85].

Lipid content

Lipid are the basic structural constituent of EVs but a few reliable data on lipid content of vesicles are available. Sphingolipids (SLs) are enriched in MVs released by some tumour cells and human bone marrow-derived mesenchymal stem cells[86], whereas are less enriched in PEVs and not at all in MVs from red blood cells. Regarding phospholipid (PLP) content, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) appear to be depleted from MVs, phosphatidylcholine (PC) seems to present a similar content as plasma membrane. No certain information can be found on cholesterol content [87].

Some studies reveal that the lipid composition of MVs is similar to plasma membrane, while others display enrichment or depletion in lipid content, leading the idea that MVs can originate from specific plasma membrane sites.

Biogenesis, cargo selection and release of EVs

In addition to size, structure, content and biological source, EVs are classified also by their mechanism of production and release. Apoptotic bodies represent the type of EVs released during programmed cell death (apoptosis) by membrane blebbing [88]. Exosomes are formed in multivesicular endosomes (MVEs), or multivesicular bodies (MVBs), and released after fusion with the cell membrane. Instead, MVs originate by direct budding from the plasma membrane through a mechanism supported by cytoskeletal remodelling. Despite exosomes and MVs biogenesis required different cellular machineries, some mechanistic elements may be shared.

Exosomes are released after fusion of multivesicular endosomes (MVEs) with the plasma membrane and early endosomes (EEs) are the starting point for MVE maturation. Association of proteins with the EE membrane defines the identity of the organelle. The cytosolic surface of the EE membrane contains clathrin and components of the ESCRT responsible for sorting of proteins into MVEs [89]. Cargo molecules include, activated growth factor, adhesion molecules, ion channels and other proteins [90]. The depletion of ESCRT machinery components results in abnormal endosome. Although, an ESCRT-independent mechanism also exists.

The last step of exosomes biogenesis, the exocytosis, is poorly understood, but a few molecular players involved in this process have been identified, including the small GTPases of the Rab family (Rab11, Rab27, and Rab35) and the SNARE complex [91].

The biogenesis of microvesicles will be discuss in deep in the next paragraph.

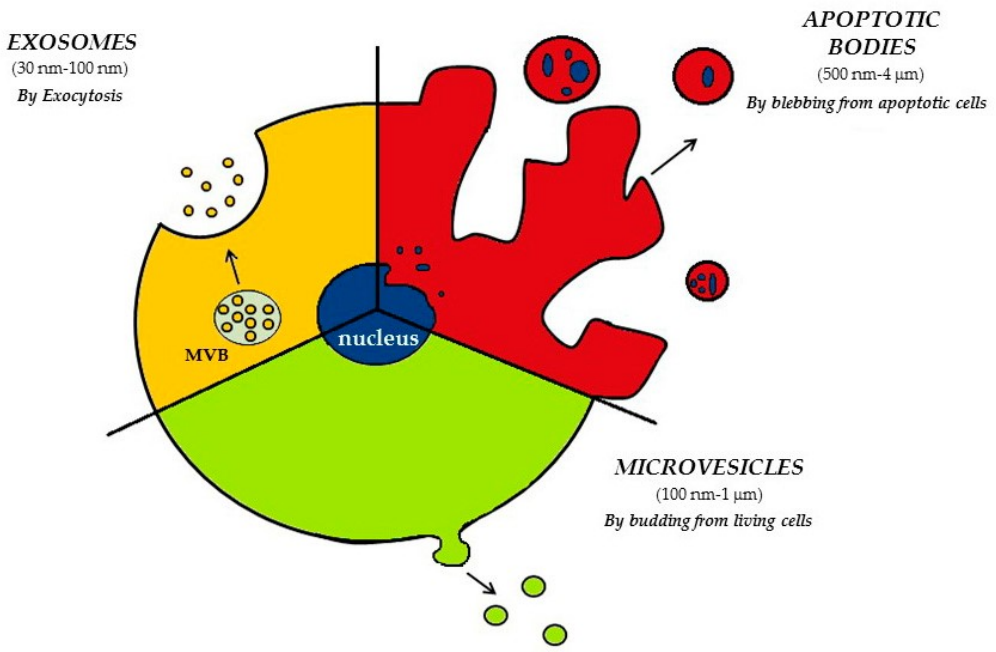

FIGURE 6. *Schematic mechanism of EVs and apoptotic bodies biogenesis. Modified from [59].*

Microvesicles

Most of the knowledge on mechanism of MVs biogenesis comes from observations on cancer cells [92]. The vesiculation from plasma membrane, seems to initiate with the influx of Ca^{2+}, activating calpains which disrupt the membrane cytoskeleton with protrusion formation [93]. Changes in lipid composition of the plasma membrane contribute to the membrane curvature, thus facilitating the outward-budding vesiculation [94].

The mechanisms of MVs production and release involve multiple partners, which depend on cell type and stimulus. Ras superfamily GTPases are recognized to be major mediators in MV biogenesis. Activated RhoA promote actin-myosin contraction, required for MV formation through the signalling pathway of ROCK and ERK [95]. Indeed, activated ARF6 promotes the recruitment of ERK to the plasma membrane, which phosphorylates myosin light-chain kinase (MLC kinase) and subsequent phosphorylation of MLC. Actomyosin is able to contract at the neck of MV allowing MV release [75]. Another pathway, initially thought to only play a pivotal role in exosome formation, implies the endosomal sorting complex required for transport (ESCRT) which participate to the release of MVs. Some protein of ESCRT can be relocated from the endosomal membrane to the plasma membrane where they mediate the MVs release. Tumour susceptibility gene 101(TSG101) supports vesicle budding through the interaction with arresting domain-containing protein-1 (ARRDC1). Moreover, ESCRT complex III is involved in the late phases of MV biogenesis and mediates the detachment of the vesicle from the plasma membrane [96]. More, the purinergic receptors of

ATP, a ligand release by many types of cells, have an important role to induce intense release of vesicles [97].

In the past, oligomerization of a cytoplasmic protein in addition to plasma membrane anchors (myristoylation and palmitoylation), appear sufficient to drive proteins into budding MVs. Interestingly, recent studies provide the evidence that different proteins, including Adenosine diphosphate-ribosylation factor 6 (ARF6), Ras-related protein 22a, vesicle-soluble NSF attachment protein receptor, Vesicle-associated membrane protein 3, T-cell internal antigen 1, and Argonaute2, have been involved to drive proteins and nucleic acids into MVs [92, 96].

Function of EVs

In the past very diverse biological function have been attributed to EVs, and it is now accepted that exosomes and MVs represent important vehicles of intercellular communication between close or distant cells. Upon shedding, vesicles seem to be important for transport and release of signalling molecules which induce functional response and promoting physio-pathological changes.

After release, EVs seems to interact only with cells that they recognize specifically. The nature of interaction with target cells is highly important and, in many cases, is restricted to the surface mediated to receptor binding [98]. In this case, the consequences of interaction are limited to activation of signalling pathway. In other cases, binding is followed by direct fusion of the shedding vesicles with the plasma membrane of target cell or the internalization of the vesicles, which is regulated based on type of cells [99]. The endocytosed EVs can be destinated to lysosomes or, alternatively, they can fuse with endosomes ensuing discharge of their content in the cytosol. As third possibility, they can be regurgitated to the extracellular space after endosome fusion with plasma membrane.

EVs are initially recognized as important mediator factors in the studies of blood coagulation and tumour growth processes [100]. Furthermore, recent studies revealed new processes, such as inflammation and angiogenesis, in which EVs play an important role.

The role of PEVs in coagulation processes is thought to be mediated by tissue factor (TF), a key activator of the coagulation cascade [101]. The formation of PEVs leads the exposition of anionic phospholipids, such as PS, which contribute actively in the procoagulant activity of microvesicles [102]. In addition, EVs released from activated platelets may, in turn, induce activation of other platelets, amplifying the coagulative and thrombus formation processes.

It is now largely recognize the role of shedding vesicles in inflammation [103]. At the later time of the inflammatory response, EVs exert their pro-inflammatory activity mediating the transfer of chemokines receptors and stimulating the release of other mediators, such as interleukin-6 and chemotactic proteins, which induce and strengthen the inflammatory response [103]. Recently, it has been proposed that shedding vesicles have an important role in the pathogenesis of various inflammatory diseases. For example, rheumatoid arthritis is characterized by abundant EVs release from fibroblast in the synovia [103].

EVs are involved in tumour progression

Tumour cells but also other cells in tumour microenvironments, such as macrophages and neutrophils, secrete EVs which contribute to tumour progression, promoting angiogenesis and tumour cell migration in metastases [104]. Tumour-derived vesicles also bring immune-suppressive molecules, which inactivate T lymphocytes or natural killer cells [105]. The metalloproteinases coat the surface of vesicles and with their protease activity digest the extracellular matrix promoting migration and metastasis of tumour cells [106].

The digestion of extracellular matrix favours angiogenesis, which have a pivotal role in the support of tumour growth and malignant cell survival. The angiogenic effects are also induced by the transfer of mRNAs and miRNAs via EVs released by tumour cells and taken up by endothelial cells [107]. Finally, transfer of drug molecules in the membrane of shedding vesicles decrease their cell cytoplasm levels and thus can contribute to drug resistance process [108].

Clinical application

The main role of extracellular vesicles is the intercellular communication mediated by their specific interaction with target cells. In addition, they have major roles in the pathogenesis of diseases. Shedding vesicles contribute to the thrombotic manifestation of several pathological conditions, such as in cancer patients, and to sustain the pathogenesis of various inflammatory diseases.

The recognition of their role in a growing number of diseases is stimulating both at the diagnostic and therapeutic level. The knowledge of the molecular specificity of the vesicles will be instrumental in the identification of their pathogenetic roles and to their potential use as diagnostic tools. In this context, there is a great need to improve the standardization of isolation and analysis of EVs, including identification of specific markers. Once isolated, it is essential to determine vesicle diversity in a specific physiological process or disease, while providing a comprehensive proteomic and lipidomic analysis in comparison with the plasma membrane from which they originate.

Peripheral blood is a rich source of circulating EVs, which are easily accessible through a blood sample. An analysis of EVs in peripheral blood could provide access to of biomarkers of great diagnostic and prognostic value.

Because EVs are released into the bloodstream and this release increases on cellular activation, as well as in many pathologic conditions [109-111], a mere enumeration of EVs may indicate the presence of an aberrant process. The use of blood samples is largely used in the clinic because blood harbours a large number of biomarkers and other biologically relevant molecules. In addition, most tissues will contribute to this molecular reservoir due to dense vascularization of the body. In line with this, the analysis of EVs in peripheral blood is likely to provide an indicator of the systemic health status, which can be used in clinical settings.

The use of EV analysis encompasses several advantages, such as the protection of the EV cargo of proteins and RNA from degradation, thus rendering them intact and functional [107]. This has proven to be particularly significant for the use of miRNA as valuable biomarkers because most RNA in blood exists as cargo of EVs. Moreover, EVs appear to have a relatively long half-life in blood [112] and they can likely be transported from any location of the body to the bloodstream, thus making them easily accessible for analysis, compared with biopsies.

In further relation to cancer, protein and RNA biomarkers have been identified in EVs from the peripheral circulation. Accordingly, several cases reveal that differentially expressed miRNAs can significantly identify patients with cancer compared with control individuals (Table 1) [109, 111, 113].

However, EVs can be identified and the phenotype and cargo can be analysed and interpreted into clinically relevant information renders a sophisticated panel of diagnostic and prognostic value of a disease. Furthermore, EV size distribution, phenotype, or cargo content can seemingly change according to the progression of a disease [109-111, 113-115].

miRNA	Cancer Type
miR-1	NSCLC
miR-15b	Melanoma
miR-17-3p	Colorectal cancer
miR-21	DLBCL
	Glioblastoma
	Ovarian cancer
miR-25	NSCLC
miR-30d	NSCLC
miR-92	Colorectal cancer
	Ovarian cancer
miR-93	Ovarian cancer
miR-141	Prostate cancer
miR-155	DLBCL
	Breast cancer
miR-182	Melanoma
miR-210	DLBCL
miR-223	NSCLC
miR-486	NSCLC
miR-499	NSCLC

DLBCL = diffuse large B-cell lymphoma
NSCLC = non–small cell lung cancer

TABLE 1. *miRNA are differentially expressed in different cancer conditions [116].*

Platelet-derived extracellular vesicles

Production and composition of PEVs

Platelets can be activated by several physiological (thrombin, collagen, and lipopolysaccharides) and non-physiological stimuli (Ca^{2+} ionophore), and their activation is accompanied also by the release of platelet-derived extracellular vesicles (PEVs), which include exosomes and membrane-derived microparticles [117]. PEVs represent in the blood the 70-90% of all circulating extracellular vesicles [118] and their size range from 100 nm up to 1 μm in diameter [119]. More recently the term medium/large PEVs has been adopted to indicate the heterogeneous population of microparticles released from stimulated platelets [120].

The size of vesicles strongly depends on the stimulus that induced their release [121]. For the same reasons, the cargo composition of PEVs is strictly related to the trigger which induce the activation of platelets [122, 123]. PEVs contain different families of proteins (membrane receptors, signalling proteins, enzymes, proteases), nucleic acids (including mRNA and miRNA), cytokines, lipids, growth factors, and other components.

On PEVs surface are generally expressed platelet membrane receptors such as P-selectin, GPIb-IX-V and integrin αIIbβ3. The latter could be present in the inactive or active conformation, depending on the stimulus that induced platelet activation and the consequence PEVs release. The active conformation of integrin αIIbβ3 on PEVs enhance the interaction with platelets or endothelium, suggesting an important role in haemostasis in the development of thrombotic events [102]. Moreover, PEVs express negatively charged phospholipids on their surface, contributing to haemostasis process but also to thrombotic disorders. Negatively charged phospholipids, such as phosphatidylserine, have strong procoagulant activity. They participate in the assembly and activation of coagulation cascade components, supporting and enhancing thrombin production and fibrin formation [102].

PEVs also transport bioactive lipids which act as second messengers [122]. Phospholipase A2 cleaves the phospholipids on PEVs membrane and promotes the release of arachidonic acid, which is transformed in TxA2, a potent platelets and endothelial cell activator [124].

PEVs in intercellular communication

The first vesicles identified as mediator in physiological processes were those released by platelets. In 1967, Peter Wolf described the platelet-derived extracellular vesicles (PEVs) as procoagulant derivates of platelet, giving them the name "platelet dust" [125].

PEVs are an important mechanism of intercellular communication by delivering biological signals and information. The events that lead the interaction of PEVs and target cells are not completely understood, but several crucial aspects have been investigated. PEVs can directly induce functional responses of target cells thanks to lipids and membrane proteins which are expressed on their surface. Additionally, PEVs can transfer receptors, proteins, miRNAs and other molecules to recipient cell by their internalization or membrane fusion [126]. The transfer of platelets receptor to target cells profoundly affect the behaviour of target cells. PEVs provide molecules to respond to extracellular stimuli which normally have no effect on these cells [127, 128]. For example, the transfer of integrin αIIbβ3 to hematopoietic cells and neuthrophils facilitate cell adhesion to fibrinogen [129] and enhances inflammatory response to GM-CSF [130] respectively. The fusion between PEVs and cell membranes allow to transfer PEVs cargo to cells. Noteworthy, the miRNA exchange plays a crucial role in intercellular communication, leading the way for new therapies [126].

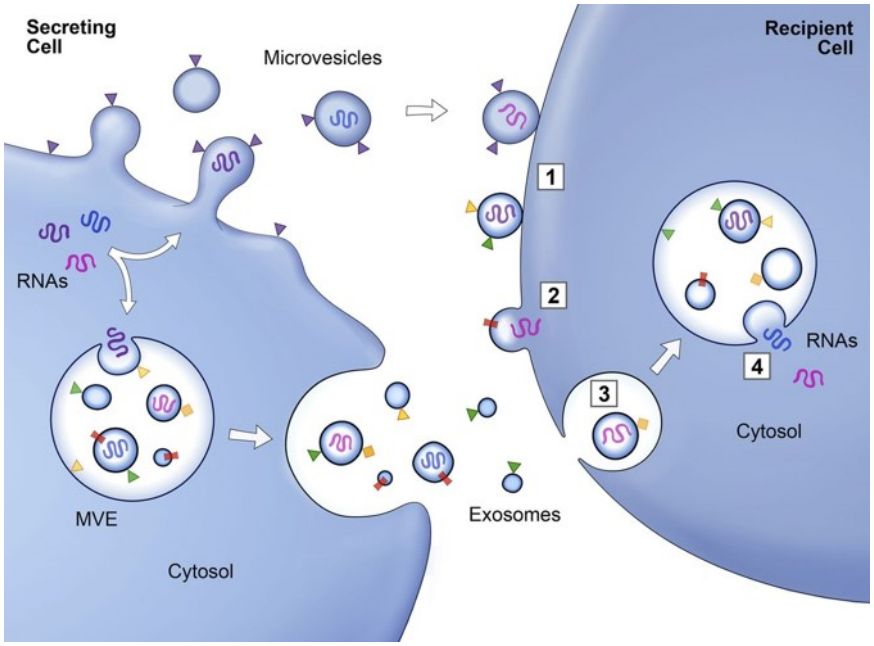

FIGURE 7. ***Schematic of molecules transfer by EVs.*** *MVs and exosomes may dock at the plasma membrane of a target cell (1). Bound vesicles may either fuse directly with the plasma membrane (2) or be endocytosed (3). Endocytosed vesicles may then fuse with the delimiting membrane of an endocytic compartment (4) [131].*

Laffont and colleagues demonstrated that PEVs delivered regulatory miRNA (miR-233) to endothelial cells, regulating their gene expression [132]. PEVs transport also proangiogenic factors which regulate the angiogenesis stimulating endothelial cell proliferation, survival, migration, and novel tube formation [133, 134]. It was observed that PEVs can stimulate antigen-specific B cells triggering antibody synthesis. This indicates that activated platelets, by releasing PEVs, can regulate the immune response [135].

The clinical impact of PEVs

Considering their role during intercellular communication by the transfer of functional molecules, PEVs are consider important actors in the development of several pathological conditions and clinical disorders [136]. A part their classical involvement in thrombosis and haemostasis, PEVs are key factors in the development of atherosclerosis, infectious diseases, immune thrombocytopenic purpura (ITP), rheumatoid arthritis, systemic Lupus Erythematosus (SLE), and cancer [121, 136].

PEVs have a pivotal role in haemostasis, in fact defects in PEVs generation were associated to defects in coagulation and bleeding [121, 137]. Conversely, high level of circulating PEVs were found in patients with coronary diseases and myocardial infarction experiences. In fact, PEVs were observed to improve thrombus formation and potentiate the coagulation [138].

Atherosclerosis is a major cause of arterial thrombosis and a leading cause of death. PEVs play a crucial role in atherosclerosis: they interact with endothelial cells and the subendothelium during plaque formation, recruit activated platelets and leukocytes in the injured area of the vasal wall, modulate monocyte-endothelial interactions, and induce smooth muscle proliferation promoting the atherosclerotic plaque formation [139, 140].

The role of PEVs in cancer will be discuss in the next chapter.

Platelets and Cancer

For a long time, platelets were described as major effectors of haemostasis and thrombosis. After correlation between platelets and cancer by Armand Trousseau in 1865, in the last 30 years there has been increasing evidence that platelets deal a pivotal role in the diffusion of cancer and in cancer-associated thrombosis [141]. Venous thromboembolism (VTE) is common complication in cancer patients and pancreatic, bladder or lung cancer are associated with the highest risk of VTE [142]. The unexpected appearance of VTE may be an indicator light of an occult malignancy and is associated with a poor prognosis [143, 144]. Thus, cancer-associated VTE constitutes the second cause of mortality in patients with cancer after the cancer itself [145, 146]. The risk of VTE in cancer patients is highly increase in the first few months after diagnosis and in the presence of distant metastases [147]. The process of metastasis may be facilitated by embolization of the local microvasculature with platelet-tumour cell aggregates [148, 149], as patients with cancer are at a higher risk for thrombosis.

Today, almost the totality steps of metastatic process have linked with platelet involvement. The first experimental evidence of the link between platelets and cancer was provide in the 1960s, with the demonstration that a low platelet count was correlated to antimetastatic effect [149]. Since them, thrombocytopenic agents, platelets inhibitors, and anticoagulants have been used to slow down tumour spread.

This link is a real cross-talk between platelets and cancer, which can influence platelet count and turnover. Most cancer patients have activated circulating platelets, without necessarily having detectable coagulopathy, which is a critical state for cancer progression [150]. Platelets are a huge reservoir of biomolecules that, upon activation, release the biomolecule content on their granules that participates to malignancy progression.

The abilities of tumour to activate and aggregate platelets give them numerous advantages into bloodstream. Activated platelets create a physical cloak which protects circulating tumour cells (CTCs) against immune system and mechanical insult of blood flow. By releasing soluble factors, such as transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF), platelets suppress the cytolytic action of natural killer cells and favour pro-survival tumour signals. Moreover, platelets induce invasive properties and transfer adhesive molecules which will interact with the endothelium participating in the formation of early metastatic niches [122, 151].

Recent studies have demonstrated that cancer cells can “educate” platelets by transfer of tumour-associated biomolecules and re-modulating platelet RNA profiles and phenotypes,

suggesting the concept of tumour educated platelets (TEPs) [152]. External stimuli, such as activation of platelet surface receptors and lipopolysaccharide-mediated platelet activation [153] induce specific splice variants of pre-mRNAs in circulating platelets, providing a specific mRNA signature into platelets [154, 155].

Effects of Cancer on Platelets

Cancer-induced thrombocytosis

Several clinical studies have demonstrated the presence of an elevated platelet count or thrombocytosis (higher than $400x10^9$ plts/L) in gastrointestinal [155], renal, breast, lung, colorectal and urogenital cancer patients [156-159]. The mechanism of cancer-induced thrombocytosis has not been thoroughly elucidated. The actual evidence suggests that primary tumours influence platelet production through a direct paracrine activity on megakaryocytes and over their abilities to educate platelets (Fig 8).

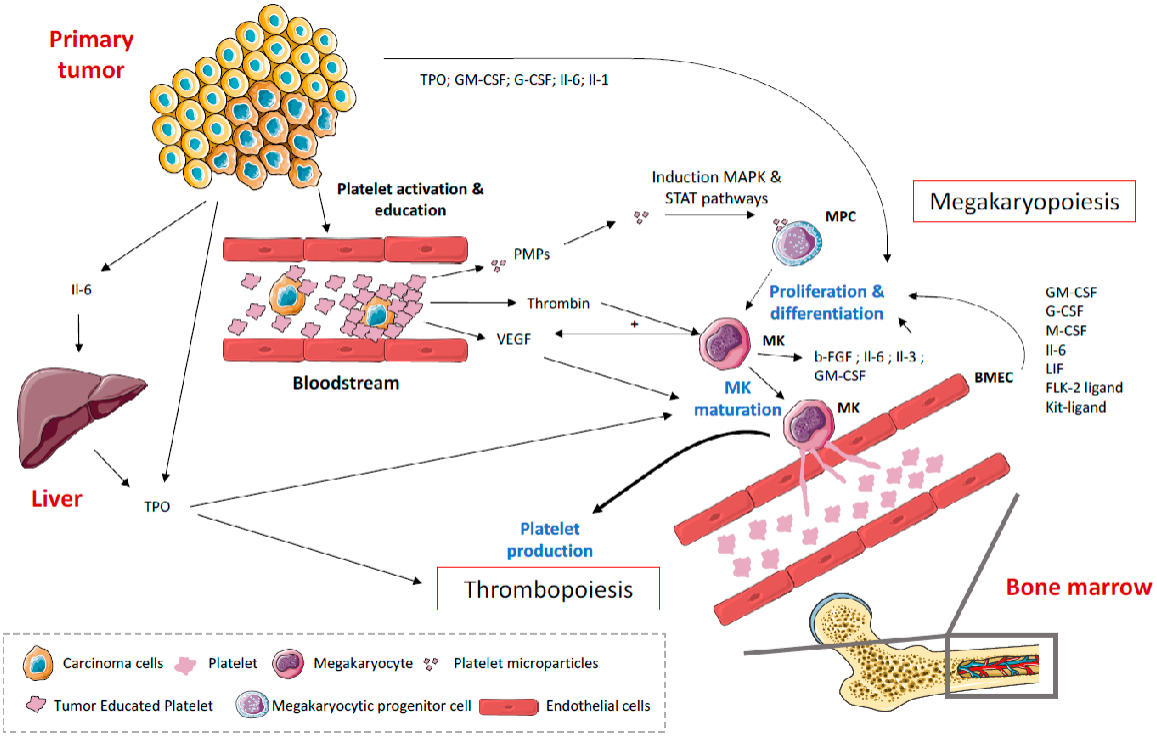

FIGURE 8. ***Mechanisms of cancer-associated thrombocytosis.*** *Summary of the mechanisms involved in the production of platelets mediated by the primary tumour. BMEC: Bone Marrow Endothelial Cells. GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; G-CSF: Granulocyte Colony-Stimulating Factor; M-CSF: Macrophage Colony-Stimulating Factor; Il-6: Interleukin-6; LIF: Leukaemia Inhibitory Factor; FLK-2: Fetal Liver Kinase-2; Kit-ligand (Steel factor); TPO: thrombopoietin [160].*

It has been shown that primary tumour can produce and secrete granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factors (GM-CSF), resulting in the stimulation of megakaryopoiesis and thrombopoiesis in cancer patients [161]. Moreover, carcinoma cells can directly produce thrombopoietin (TPO) promoting bone marrow megakaryocyte growth, differentiation and platelet production [162, 163]. In fact, elevated plasma levels of TPO, correlated with high level of interlukin-6 (Il-6), were related to thrombocytosis in ovarian and hepatocellular cancer patients [163, 164].

Beyond the abilities of primary tumour to secrete megakaryopoietic and thrombopoietic factors, they can influence platelet production directly through the activation of platelets. For instance, PEVs can promote proliferation, survival, adhesion and chemotaxis of hematopoietic cells through various intracellular signalling cascade [129]. Once again, the activated state of platelets and excessive thrombin generation participate in cancer-associated thrombocytosis. Megakaryocytes, stimulated by thrombin, increase the secretion of Vascular Endothelial Growth factor (VEGF). Through an autocrine-paracrine loop, VEGF potentiates megakaryocyte maturation and differentiation [165].

Tumour Cell Induced Platelet Aggregation or TCIPA

As mentioned above, tumour cells are able to activate platelets and the haemostatic system by direct interaction in the bloodstream or indirectly by EVs and secreted factors. This ability is responsible for the pro-thrombotic state of cancer patients and correlated with metastatic potential of cancer cells [166]. All the mechanisms involved in platelets activation and aggregation via tumour cells are called Tumour Cell Induced Platelet Aggregation (TCIPA). It is induced by released factors (ADP, TXA_2, thrombin, sCD40L, and MMPs), procoagulant proteins (TF and fibrinolytic proteins), extracellular vesicles (PEVs) and adhesive proteins.

Various cancer cell lines, including melanoma, neuroblastoma, ovarian and breast carcinoma cells can release ADP, supporting the TCIPA. The ADP binding to ADP-specific G-coupled receptors (P2Y1 and P2Y12) induce platelets shape change, TXA_2 release and aggregation [167]. The secretion of thrombin by several cancer cell lines is responsible for platelet aggregation [168]. Thrombin, a serine protease, represents the most potent activator of platelets. By their proteolytic activity, it cleaves platelet receptor PAR1 and PAR4, which lead to platelet activation and aggregation. Thrombin also converts soluble fibrinogen in insoluble fibrin during clot formation and activates other coagulation factors (factor V, VII, XI and XII) that amplify the coagulation response [169]. Different tumour cells, i.e. osteosarcoma cells, can secrete TXA_2. These support the evidence that

TCIPA hold a pivotal role in metastatic outbreak [170]. Numerous cancer types, such as melanoma and some carcinoma, co-express CD40 and CD40L. The bind of soluble CD40L (sCD40L), after CD40L cleavage and release, with platelet receptor CD40 induces platelet activation and aggregation [171]. The matrix metalloproteinases (MMPs) are enzymes involved in all the steps of cancer progression, from primary tumour to metastasis. Fibrosarcoma, colorectal and breast carcinomas express MMP-2 on the cell membrane surface, contributing to TCIPA [168, 172, 173].

TF is normally involved in the extrinsic pathway of blood coagulation and in endothelial cells and monocytes is expressed only after adequate stimulation. In numerous malignant cancers, TF is overexpressed and is correlated with progression to invasive cancer [174]. For these reasons, it represents the major effector of TCIPA and is responsible for the pro-thrombotic state of cancer patients [168]. Another mechanism involved in the TCIPA is the production of fibrinolytic system molecules by various tumour types. For instance, urokinase type plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1) are expressed in prostate, colorectal, breast and ovarian cancers [175-177]. The increased plasma levels of PAI-1 were related to cancer-associated thrombosis and negative prognosis [177].

In 1946 Chargaff and collaborators described for the first time the ability of PEVs to accelerate thrombin generation in platelet-free plasma [178]. Today, it is well known that TF positive tumour-derived EVs are associated to pro-thrombotic state in cancer patients [179]. Several studies report an increased level of circulating EVs, including PEVs, in numerous types of cancer such as gastric, pancreatic, colorectal, lung, ovarian and breast. These observations were related to an increased procoagulant activity in cancer patients [180]. Tumour cell-derived EVs express all factors and molecules involved in platelet aggregation and in activation of the haemostatic system. By the exposition of phosphatidylserine and the TF expression on their surface, EVs provide a pro-coagulant surface for thrombin generation. Thus, these observations indicate that EVs actively participate in cancer-associated thrombosis.

Tumour cells express a wide variety of adhesive molecules that allow their interaction with the blood cells, including platelets, immune cells and endothelial cells. It was well established that the inhibition of platelet integrin $\alpha_{IIb}\beta_3$ with blocking antibody reduce colorectal and melanoma cancer cell-platelet interactions in vitro and reduce metastasis in vivo. The interaction between platelets and cancer cells was decreased by RGDS (Arg-Gly-Asp-Ser) peptide suggesting an interaction via fibronectin and von Willebrand factor in $\alpha_{IIb}\beta_3$-dependent manner [181]. Moreover, it has been shown that TCIPA is promoted by the cancer cell integrin $\alpha v\beta_3$ binding with platelet

integrin $\alpha_{IIb}\beta_3$ [182]. More recently, it has been demonstrated that platelet integrin $\alpha6\beta_1$ directly bind colorectal and breast cancer ADAM9. This interaction leads to platelet activation, granule secretion and extravasation of cancer cells [183]. Following platelet activation and degranulation, P-selectin is rapidly expressed on the membrane and interacts with ligand PSGL-1 expressed mostly by leukocytes and cancer cells. In fact, Kim et al. showed that P-selectin deficiency in knock-out mice reduce tumour growth and metastasis in vivo [184]. The glycoproteins GPIbα, a component of platelet receptor GPIb-V-IX, was reported to contribute to TCIPA and tumour progression, but its role remains contradictive and should be verified.

Tumour Educated Platelets or TEPs

Platelets possess a large repertoire of RNA: mRNA (premRNA and mRNA), ribosomal RNA (rRNA), microRNA (miRNA), small nuclear RNA (snRNA), and transfer RNA (tRNA) [185-187] and their ability to translate mRNA into proteins has long been establish [188, 189]. In fact, platelet RNA may undergo a specific splice events in response to external signals in circulating platelets.

The concept of Tumour Educated Platelets is a novelty which can provides interesting tools in oncology and cancer diagnostics. Indeed, cancer cells and tumour microenvironment, such as stromal and immune cells, are able to release stimuli which activate platelets inducing specific splice variants of premRNA in platelets, resulting in platelets with a specific mRNA signature. The splice events in response to external signals and the capacity of platelets to ingest circulating mRNA can provide TEPs with a wide and specific mRNA repertoire [152, 190] (Fig. 9).

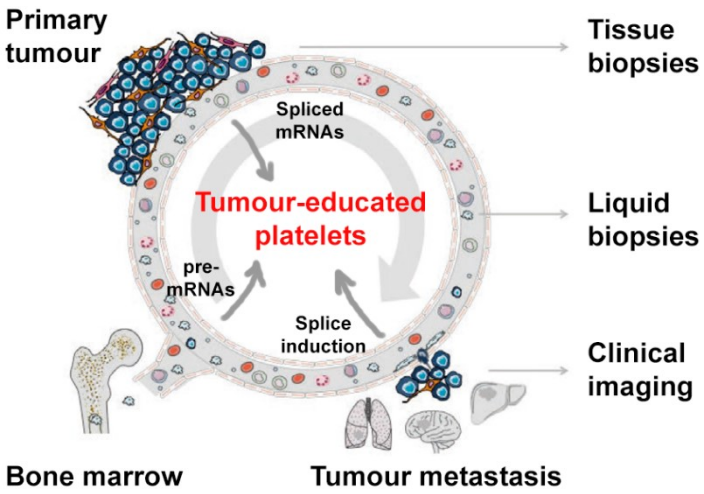

FIGURE 9. *Schematic overview of tumour-educated platelets (TEPs) as biosource for liquid biopsies [191].*

Changes in platelet RNA profile were reported in several cancer, such as lung, prostate, glioma, and breast carcinomas [190]. Molecular interrogation of blood platelet mRNA can offer valuable diagnostics information for cancer patients affected by different tumour types. By development of RNA-sequencing analysis, is now possible to distinguish cancer patients from healthy individuals with 96% of accuracy, suggesting that the emerging concept of TEPs may provide a potential tool for cancer diagnostic. Furthermore, by the analysis of TEP mRNA profile, it is possible to precisely distinguish mutant oncogenic drivers demonstrating that platelets can assist in determining tumour type and in selecting patients for targeted therapies [191]. But also, investigating partial or complete normalization of the platelet profiles following treatment of the tumour would enable to monitor the efficacy of treatment and disease recurrence. In addition, increasing evidence demonstrated the roles of miRNA in cancer biology. In 2012, Plé et al. described 532 different miRNA in platelets [187], which play important roles in their function. Moreover, platelets from myocardial infarction patients differentially expressed miRNAs compared to healthy subjects [192]. Together these observations suggest that miRNA fingerprint in platelets could be exploited as diagnostic tools in cardiovascular diseases and cancer.

Platelet contribution to cancer metastasis

Metastasis is the major cause of mortality in cancer patients. During metastatic propagation, tumour cells enter blood circulation to spread to distant sites [193]. The metastatic event can be divided into four major steps (Fig 10): Tumour cells leave the primary tumour and intravasate into the blood stream; they disseminate to distant sites transported by the blood stream; arrest in a distant organ and extravasate where they colonize the tissues forming secondary tumour.

Patients with cancer frequently present signs of thrombosis, which are most severe if tumour is progressed to a metastatic stage [146, 194]. However, thrombosis events are not detected in all cancer patients, but the increase of coagulation parameters and the enhanced platelet turnover are generally displayed.

These observations indicate a potential crosstalk between platelets, coagulation system and cancer spreading. Several animal models are used to investigate whether a functional relationship might exist between platelets and tumour progression. These studies yielded the evidence that platelets can guard tumour cells from the elimination action of immune system, promote cells arrest to endothelial wall and promote tumour angiogenesis. Thereby, the activation of platelet and the coagulation system have a pivotal role in the metastatic progression of cancer.

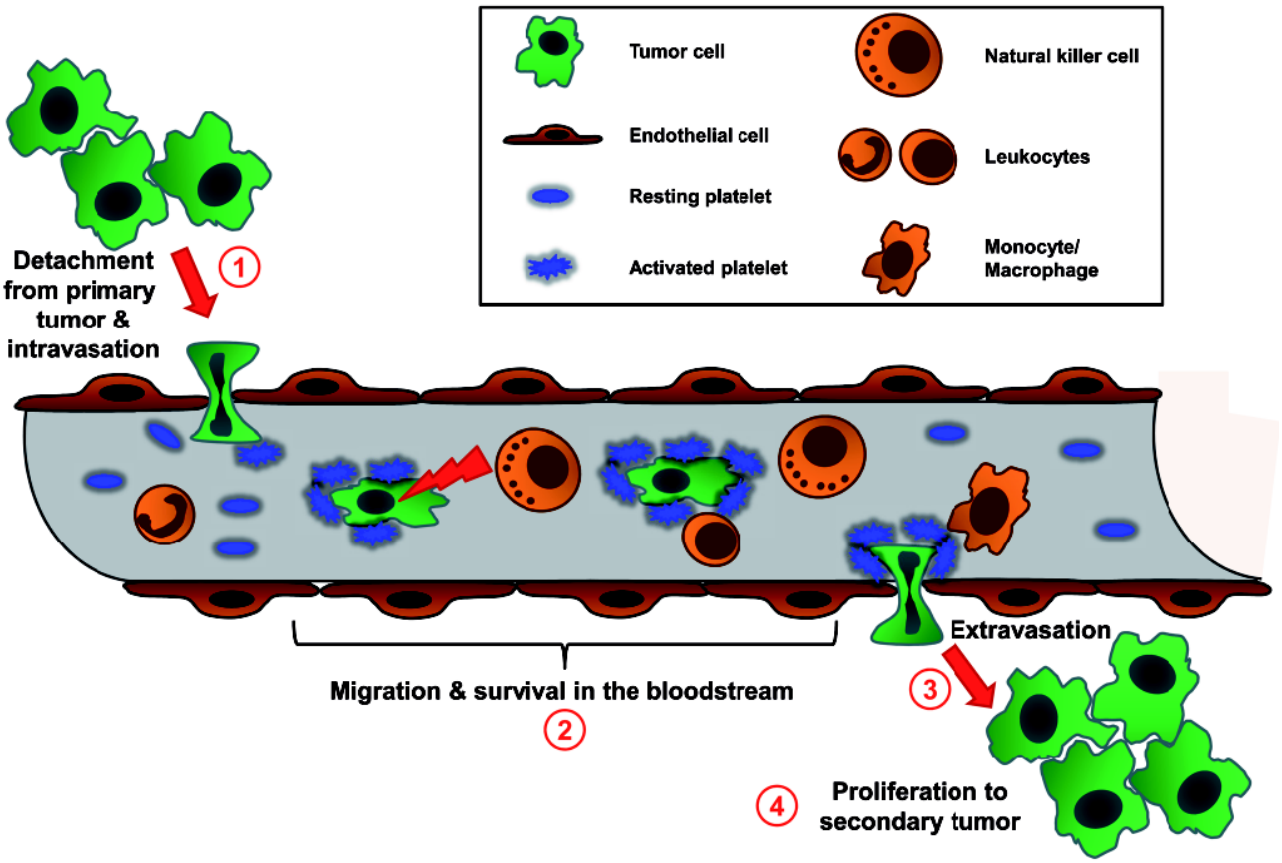

FIGURE 10. ***Overview of platelet contribution to tumour metastasis.*** *(1) Once tumour cells detach from the primary tumour and enter the circulation (intravasation), they are exposed to shear forces and immunological attack. (2) Most tumour cells are destroyed by natural killer cells, but some trigger platelet activation and the formation of a protective 'platelet coat'. (3) Platelets contribute to the attachment of metastatic cells in distant organs and to their extravasation. (4) Platelet-derived growth factors promote tumour cell proliferation to secondary tumours [195].*

Platelet-enhanced tumour angiogenesis

In 1971 Folkman and colleagues hypothesized that the possibility of tumour expansion is correlated to angiogenesis to prevent hypoxia-induced growth arrest and necrosis. Likewise, the growth of metastasis is highly dependent to the formation of newly vessels [196]. Now, it is well known that angiogenesis controls tumour growth and that platelets are crucial for angiogenesis and for the stabilization of new formed blood vessels [197].

Platelets store and release a number of angiogenic regulators, both pro-angiogenic (i.e. VEGF, PDGF, TGF and MMPs) [198] and anti-angiogenic factors (PF4, thrombospondin I, PAI and angiostatin) [199]. However, the pro-angiogenic effects prevail and these make platelets active players in tumour angiogenesis. Platelets released can promote tube formation of endothelial cells in vitro [200] and promote new vessel sprouting in an aortic ring model. In the tumour vessels, endothelial cells have an elevated expression of TF, which induce thrombin generation and

subsequent platelet activation. In turn, the activated platelets release angiogenic mediators that promote endothelial activation and proliferation and the subsequent formation of new vessels [199, 201]. The continuous release of angiogenic factors from platelets, contribute to stabilize tumour vessels preventing intratumorally haemorrhage and ensuring tumour growth [202].

It was shown that the concentration of VEGF in platelets of cancer patients is nearly double compared to healthy controls (216,000 pg/ml *vs* 413,00 pg/ml) [203] and that plasma fraction of stored platelets concentrates enhance the invasiveness of pancreatic cancer cell lines (MIA PaCa-2 and Pan02) and a breast cancer cell line MDA-MB-231 [204]. In addition to VEGF, platelets contain PDGF, which has been associated to tumour growth and angiogenesis [205, 206].

Despite many studies on the influence of platelets on tumour-angiogenesis, data are inconclusive. It remains to understand which cytokine exactly are released by activated platelets within intratumorally vasculature and which have an overall effect on local tumour growth and angiogenesis.

Platelets protect circulating tumour cells

The circulatory system is a hostile environment for circulating cancer cells (CCCs or circulating tumour cells – CTCs) and their survival before extravasation is fundamental for metastasis. Platelets and their activation have a crucial role in haematogenous tumour spreading. In fact, a knockout nuclear factor erythroid-derived 2 (NEF2) mouse model, which interrupts platelets production because of lack in megakaryocyte maturation, display a nearly complete inhibition of metastasis [207].

Platelets contribution to haematogenous metastasis depend on the ability of platelets to cooperate with coagulation factors to protect CTCs. When coagulation cascade activates thrombin, this protein cleaves fibrinogen in fibrin and induce the activation of platelets which, through adhesion molecules, bind soluble fibrinogen and support thrombus formation [208]. This entanglement composed by platelets and fibrin surround tumour cells, providing a physical barrier which protects them against the action of immune system [209, 210]. Leukocytes and natural killer (NK) cells are free in circulation and they can find in proximity to the metastasizing cells [211]. NK cells, with their cytolytic action, can disable CTCs by direct contact and lysis, but platelet adhesion to tumour cells, may protect them from the activity of NK cells. The fibrin formation, induced by tumour cell-associated TF, avoids CTCs recognition by NK cells and promote metastasis spreading through shielding tumour cells within the circulation [212]. Several mice model, lacking important protein for platelet activation or protein involved in haemostasis (i.e. fibrinogen), were mostly

unable to support metastasis. Other studies, where mice are treated with inhibitors of platelet aggregation, shown similar results [213, 214]. Notably, in presence of NK cells platelet activation is required for the survival of tumour cells in circulation [208], but the exact mechanism how platelets protect CTCs from NK cells still need to be defined. New hypotheses include that platelets exert a paracrine suppression of NK-mediated cytolytic activity [215]. Transforming growth factor-β (TGF-β) released from platelets caused a down-regulation of the immunoreceptor NKG2D on NK cells, diminishing NK granule mobilization, cytotoxicity and interferon-γ (IFNγ) secretion [216].

Platelets enhance tumour cell-vascular interaction

The adhesive and arrest processes of tumour cells in the vasculature of target organ are complicated to study *in vivo* because these events are rare in living animal models. Furthermore, the *in vitro* models reflect only certain aspects of the process and cannot mimic the complexity of the intravascular situation. Nevertheless, different studies focalized on tumour cell-vascular cell interaction into circulation of drug-treated or genetically modified animals have highlighted tumour cell arrest in target organ vessels [217]. These works have also produced information on the adhesion molecules involved in tumour cell anchorage at the endothelium and the following steps of tissue colonization.

Several observations suggest that selectins mediate the rolling and tethering of CTCs to vessel wall and the formation of crosslink between tumour cells, platelets and leukocytes. Selectins, a family of transmembrane cell adhesion molecules, are expressed by platelets, endothelial cells and leukocytes. Studies on metastasis indicate that tumour cells can take advantage of platelet selectin (P-selectin) to interact with the endothelium in a manner similar observed during the recruitment of leukocytes in inflammation [218]. Results on P-selectin-knockout mice indicate that tumour growth, at the early steps of interaction between tumour cells and pulmonary microvasculature, involves P-selectin function and platelets [219]. These observations are strengthened by the fact that metastasis can be attenuated by the anticoagulant heparin. Heparin blocks tumour cell binding to P-selectin on platelets and the following P-selectin-dependent tumour cell tethering [184, 219]. CTCs are exposed to the shear stress generated by the blood flow and CD44 is the main ligand on colon cancer cells that mediate the binding to platelets P-selectin. Some evidences also support that the binding between CD44 and fibrin stabilizes the platelet-tumour cells adhesion. Such model throw light on the hypothesis that tumour cells recruit platelets and leukocytes to form heteroaggregates, which may travel into blood stream and tether and roll along

endothelium in a P-selectin-dependent manner. The tethering process, which precedes tumour cell arrest, is a prerequisite for extravasation from the blood stream [220].

After the selectin-dependent rolling along the endothelium the firm arrest of tumour cells is mediated by the integrins. In this process, the main platelet integrin involved is the integrin αIIβ3. The platelet-tumour cell adhesion is mediated by integrin αIIβ3 and tumour cell receptors, such as integrin αvβ3. The integrin binding helps to overcome shear forces that are generated by blood flow and enhance tumour cell retention in microvessels [217]. The inhibition of αIIβ3 reduce tumour cell colonization, indicating that the integrin coordinates the platelet-tumour cells cohesion and adhesion which are fundamental steps for the growing of metastatic lesions [181, 221]. Therefore, during metastasis the activation of platelets and leukocytes can support tumour cells cohesion and their firm arrest at the vascular endothelium.

Another platelet receptor that potentially contributes to tumour progression is the GPIb-IX-V complex. It was shown that experimental metastasis of melanoma cells is reduced in mice lacking GPIbα [222], but no underlying mechanism was identified [223].

The metastatic process could produce microlesions in the endothelium with consequent collagen exposition. Collagen is avidly recognized by platelets integrin (i.e. α2β1) and by the platelet glycoprotein receptor for collagen VI (GPVI) [224], causing platelet activation, aggregation and amplification of platelet binding to collagen. How this process contribute to tumour metastasis is unclear, but metastatic colonization was found to be reduced in GPVI-deficient mice [225].

Extravasation

During inflammation, platelets are able to modulate the permeability of the endothelial cells to help immune cells to colonize the site of inflammation. Similarly, there is the possibility that platelets might assist tumour cells to leave the circulation end enter target tissues for metastatic colonization.

The vascular endothelium integrity may be directly or indirectly affected by growth factors released by platelets, including PDGF, TGFβ, EGF, insulin-like growth factor 1 (IGF1) and VEGF [226]. Other molecules released by platelets are the endothelial agonist sphingosine 1 phosphate (S1P), a potent inhibitor of vascular leakage [227] and lysophosphatidic acid (LPA). Both affect cytoskeletal rearrangement, actin changes, cell-cell communication, and influence endothelial permeability [228, 229].

In addition, platelets release potent bioactive factors such as serotonin and histamine which modulate vascular tone and vascular permeability, respectively. Serotonin induces either vasoconstriction or vasodilatation and histamine increases vascular permeability and augments the

extravasation of leukocyte. The shielding of tumour cell into platelet-leukocyte heteroaggregates and the arrest at the vascular endothelium generate a positive situation for tumour cells that my support their extravasation and invasion of target tissues. Platelets also release matrix metalloproteinases (MMPs) which contribute to tumour cell invasion by degrading the ECM and remodelling the surrounding tissue [230].

Thus, tumour cell extravasation could be enhanced by platelets, which may affect blood vessel integrity and ECM remodelling.

The role of PEVs in cancer dissemination

In the last years, the study of the host environment in cancer progression and diffusion has acquired great relevance. When cancer cells enter the bloodstream, before extravasate and produce new metastatic niches, receive several stimuli from the surround environment which may affect their aggressiveness.

Platelet contribution to cancer spreading has been known from several years, but only in the last period the possible role of PEVs in supporting this event has captured the interest of scientific community. Considering their important role in intercellular communication by delivering several regulatory molecules, PEVs have been suggest playing a pivotal role in cancer spreading [231, 232].

PEVs, similarly to platelets, may recover a role in angiogenesis, regulating endothelial cell proliferation, migration and chemotaxis *in vitro* [133]. The involvement of PEVs in the regulation of angiogenesis was also demonstrated *in vivo* by injection of vesicles in the heart of rats. The result was the formation of new tubular structures [233]. Moreover, lung cancer cells co-incubated with PEVs display an upregulation of angiogenic factors, such as MMP-9, VEGF, IL-8 and hepatocyte growth factor (HGF), implicated in tumour angiogenesis and vascularization [234].

PEVs also support the metastatic event by mediating the arrest and adhesion of circulating tumour cells to the endothelium wall. Thanks to the molecules expressed on their surface, PEVs may interact whit tumour cells. P-selectin express on the PEVs membrane mediates the interaction with glycoconjugates expressed by cancer cells. In addition, this binding helps tumour cells to escape from the immune system surveillance improving circulating tumour cells survival [231].

The interaction between PEVs and lung cancer cells resulted in the activation of pathways which are involved in cell proliferation and upregulation of mRNA associated to an increase of aggressiveness of various tumours *in vivo*. It was observed that PEVs stimulate MMPs production and lung cancer cell invasion. Janowska-Wieczorek and colleague demonstrated that the injection of LLC cells preincubated with PEVs in mice may increase the formation of metastatic foci compared to control mice, confirming the ability of PEVs to enhance metastasis of lung cancer cells [234, 235]. The same researchers also proved that PEVs are able to deliver the integrin αIIbβ3 to breast cancer cells, increasing cell ability to interact with endothelial cells. Moreover, the vesicles stimulated MAPK and Akt signalling pathways and enhanced the expression of MMPs, improving the invasive potential of breast cancer cells [236, 237].

It was demonstrated by Varon and collaborators that PEVs physically interact with prostate cancer cells and that vesicles potentiate cell ability to bind endothelial cells and matrix components.

Another publication showed that PEVs promote the production of MMP-2 from prostate cancer cells, contributing to increase their invasiveness [134, 238].

Only few investigations explored the prometastatic effects of PEVs on cancer behaviour and for this reason other investigation are required.

Platelet as pharmacologic target in anti-cancer therapy

The link between coagulation and cancer has been observed for centuries and considering the important implications of platelets in angiogenesis, tumour growth and procoagulant activity, they may represent a potential therapeutic target to inhibit tumour progression. The use of antiplatelet therapy for cancer treatment was proposed in 1968 by Gasic and collaborators. They showed that intravenous injection of neuraminidase resulting in thrombocytopenia was associated with decrease of metastasis in mouse model [149]. Therefore, anti-platelet agents could prevent cancer, decrease tumour growth and the metastatic potential, and improve the survival of patients. On the other side, there are studies that antiplatelet treatment may assist cancer development during prolonged anti-platelet/coagulant treatment in a phenomenon described as “cancer follow bleeding”. However, studies among populations with different cancer type did not confirm an increased risk of mortality in patients using antiplatelet therapy [239].

The debate around antiplatelet agents justify the rising of several studies to elucidate the role of platelet therapy in the anticancer management.

The most relevant process contributing to cancer spreading regulated by platelets is the tumour cell-induced platelet aggregation (TCIPA) [168]. The activation of platelet pathways are dependent of membrane receptor activation or inhibition during TCIPA. Several platelet receptors are involved in cancer progression [240]. In addition, attractive targets for antiplatelet treatment include arachidonic acid (AA) metabolism, COX enzymes, ADP receptor P2Y12 [241] and phosphodiesterases enzymes (Fig. 11).

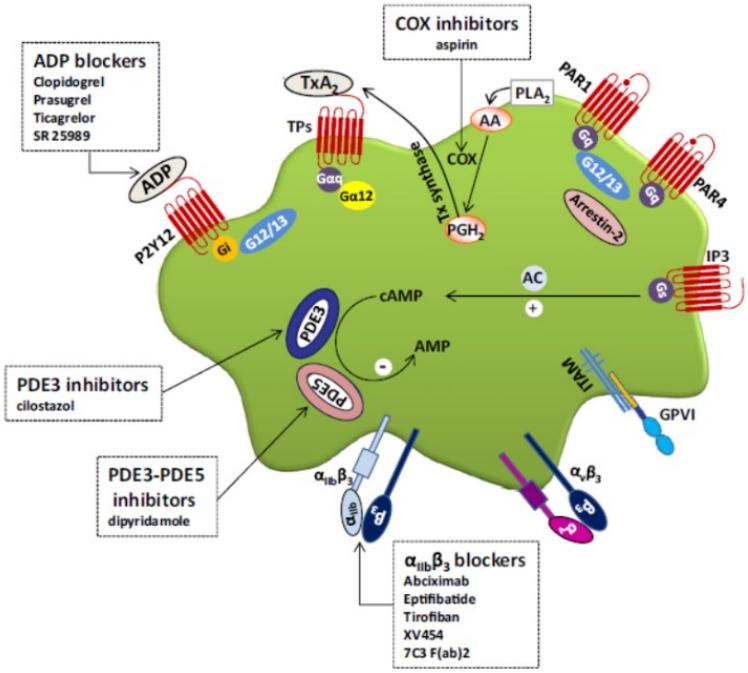

FIGURE 11. *Platelet expresses several structures such as transmembrane receptors and eicosanoid machinery which are involved in the crosstalk with other platelets and cancer cells. These are platelet target for pharmacologic treatments explored as anti-cancer therapy. Modified from [242].*

Cyclooxygenase inhibitor: Aspirin

The discovery of acetylsalicylic acid (aspirin) was at the end of the eighteenth century, but the understanding of their mechanism of action, which derives from the inhibition of arachidonic acid metabolism, came many years later.

Phospholipase A2, by cleaving phospholipid from cell membrane, produces arachidonic acid which is then transformed by cyclooxygenases (COXs) into PGH2 and rapidly converted into prostanoids [243]. The TXA2 synthase enzyme, which is constitutively expressed in platelets, synthetizes TXA2 from PGH2. TXA2 is a powerful-platelet activating agent and a vasoconstrictor [244, 245] and is also involved in cell migration and angiogenesis during tumour growth and chronic inflammation.

Aspirin induce an irreversible inactivation of COX-1 (through acetylation of Ser529) and COX-2 (through acetylation of Ser 516). The consequence of aspirin action is the suppression of prostaglandins and thromboxane production [246].

Pre-clinical studies

The concept that aspirin could be an effective weapon against cancer rises from the observation that tumour metastases are impaired in rats with thrombocytopenia [149] and that prostaglandin concentrations are higher in rat colorectal tumour tissue [247]. This was demonstrated when researchers shown that a daily administration of aspirin significantly reduce the incidence (% animals with tumours) and multiplicity (tumours/animal) of invasive adenocarcinomas of the colon as well as the size of adenocarcinomas in rats [248]. The effect of aspirin in human, administered at a dose equivalent to 80–110 mg/day, is the apoptosis of CRC cells in vivo by a mechanism which involves the downregulation of IL- 6/STAT3 signalling pathway [249].

The inhibition of COX-2, which is overexpressed in cancer cells [250, 251], is the main mechanism of the anti-tumour activity of aspirin, but also the inhibition of platelet adhesion to tumour cells is an essential step in the activity of immune surveillance [252]. In B16-F0 melanoma tumour bearing C57BL/6 mice, aspirin reduce the rate of tumour proliferation and growth. The hypothesis is that aspirin, beyond its effects on COX and platelets, reduced the overexpression of mir-4670-5p in tumour cells, which is involved in tumour progression [253]. The overexpression of mir-4670-5p in tumour cells was ascribed to their interaction with platelets, and aspirin suppressed this effect [254].

The combination of aspirin with antitumoral drugs acting on different tumour target is now widely explored.

Clinical studies

The evidence that regular aspirin use may prevent colorectal cancer (CRC) came from a several studies. Data analysis cumulating from at least 17 case-control studies in CRC, that involved over 30,000 subjects, concluded that regular use of aspirin was associated with a reduced risk of CRC [255]. Aspirin also prevents the development of cancer in hereditary CRC [256] and more recently was shown to prolong survival when aspirin treatment started after diagnosis in patients with mutated PIK3CA CRC [257].

At least 5 years of treatment was required to reduce colon cancer risk, and benefit was evident with aspirin doses as low as 75 mg/day, with no further increase of benefit with greater doses [258]. Subsequent, analyses showed that daily aspirin reduced death not only due to gastrointestinal cancers but also to several common cancers such as pancreatic, brain, lung, or prostate cancers [259]. Based on these reports, it was assumed that aspirin would cause an at least

10% reduction in overall cancer incidence during the first 10 years of treatment, balancing bleeding risk, cardiovascular and cancer benefits [260].

PDE inhibitors

Phosphodiesterases (PDEs) is a class of enzymes that catalyses the hydrolysis of cyclic AMP (cAMP) and cyclic GMP (cGMP) to inactive 5'AMP and 5'GMP forms, regulating the amplitude, duration, and compartmentation of cyclic nucleotide signalling [261]. cAMP and cGMP are potent inhibitor in platelets. More than 60 different isoforms of PDE have been described in mammalian tissues, and three of them, PDE2, PDE3, and PDE5 are expressed in platelets. Selective PDE inhibitors have been developed as anti-platelet agents and some of them are investigated in the treatment of cancer.

Cilostazol

Cilostazol, a 2-oxo-quinoline derivative, is a specific and strong inhibitor of PDE3 in platelets.

In the migration assay, cilostazol suppressed cancer cell invasion of human colon cancer cells (DLD-1), induced by fetal bovine serum indicating that cilostazol inhibits colon cancer cell motility [262].

Moreover, it was shown that the administration of cilostazol two hours before tumour cells HAL8 and HAL33 injection in nude mice, strongly impaired lung metastases while the treatment was ineffective when administered 72 h after tumour cell injection. This suggest that platelets play and essential role in the metastasis development [263].

Dipyridamole

Dipyridamole was initially used as a coronary vasodilator and now it is currently used in association with aspirin as anti-thrombotic drug in the prevention of stroke. Dipyridamole inhibits the reuptake of adenosine by red blood cells, enhancing plasma levels of this platelet inhibitory nucleoside. It acts as an inhibitor of PDE5 and PDE3, increasing intraplatelet cAMP and/or cGMP; and as an antioxidant by scavenging free radicals that inactivate cyclooxygenase, thus enhancing PGI2 biosynthesis [261, 264, 265].

Pre-clinical studies

In a breast cancer cell tumour mice model, administration of dipyridamole reduces metastases formation and macrophages infiltration in primary tumours by inhibiting Wnt, ERK1/2 -MAPK, and NFkB signalling pathways. These suggest that dipyridamole could be a promising agent for breast cancer treatment [266].

Clinical studies

Trials on potential use of dipyridamole to enhance chemotherapeutic efficacy in several cancers such as sarcoma, colorectal, breast, renal cell, and prostate cancers failed to show an improvement in response to chemotherapy.

P2Y12 inhibitors

ADP is a fundamental molecule for platelet function and purinergic receptors (P2 receptors) on platelets membrane act as receptor for ADP and trigger the aggregation of platelets [267]. In particular, P2Y12 Gαi-coupled ADP platelet receptor is essential for platelet activity. P2Y12 plays a pivotal role in thrombus growth and stability in vivo [268], amplifies and stabilizes platelet activation and aggregation triggered by several platelet agonist, plays a crucial role in granule secretion, integrin activation [269], and thrombus formation [270]. Mice model deficient for with P2Y12 gene (P2Y12−/−) have prolonged bleeding times and a reduction in platelet function [268].

The link between P2Y12 and tumour progression is not fully understood. Seems that, TCIPA favours ATP and ADP release by platelet dense granules that are accumulated in the tumour niche and contribute to interaction between tumour cells and platelets [167]. Furthermore, the ADP release from platelets activates the P2Y2 receptor on endothelial cells, favouring tumour cell extravasation.

Lung metastasis induced by Lewis Lung Carcinoma (LLC) cells in P2Y12-deficient mice are less evident, showing a role of platelet P2Y12 in tumour spreading [271]. P2Y12 ablation decreases the ability of LLC cells to induce TGFβ1-platelet release resulting in a diminished platelet-induced EMT-like transformation of LLC cells, crucial for LLC cell metastasis.

For this reason, P2Y12 is the main target of all anti-platelet ADP antagonists used.

Clopidogrel

Clopidogrel, a second-generation thienopyridine, is an oral P2Y12 inhibitor acting as a pro-drug that requires metabolic conversion to its active form by the liver, thus its onset of action is relatively slow. About 85% of the absorbed clopidogrel is metabolized into an inactive metabolite (SR26334) by plasmatic carboxylases. The remaining 15% is metabolized by a two-step biotransformation process dependent on cytochrome P450 isoenzymes in the liver [272]. During the first step, CYP2C19, CYP1A2, and CYP2B6 transform clopidogrel into 2-oxoclopidogrel, which is then hydrolysed by CYP2C19, CYP2C9, and CYP3A to R130964, the active metabolite that irreversibly blocks P2Y12 [273]. There is significant heterogeneity in the general population with regard to the

activity of the CYP2C19 allele, and this modifies the efficiency of conversion of clopidogrel to its active form thus affecting the anti-platelet effect [272].

Pre-clinical studies

P2Y12 signalling pathway is involved in the release pro-angiogenic factors (i.e. VEGF), suggesting an additional effect of P2Y12 antagonists on angiogenic protein release [274]. SR 25989, an enantiomer of clopidogrel, through an antiangiogenic process inhibits metastases of melanoma cells B16- F10 in a pulmonary metastatic C57BL/6 mice model [275]. In a model predisposing to hepatocellular carcinoma, the treatment with clopidogrel (~1 mg day/kg) prevents and delays hepatocarcinogenesis and significantly improves overall mice survival [276].

Clinical studies

Despite several studies analysed the anti-neoplastic effect of the anti-thrombotic treatment with clopidogrel, it was impossible to clarify their beneficial role.

Ticagrelor

Ticagrelor belongs to the cyclo-pentyl-triazolo-pyrimidine family and it is the first reversible oral P2Y12 receptor antagonists used in clinical.

Pre-clinical studies

Ticagrelor strongly reduces lung and liver metastases in intravenous and intrasplenic B16-F10 melanoma metastasis mouse models and improved survival of treated animals. A similar effect was observed in mice receiving intravenous 4T1 breast cancer cells, with significant reductions in lung and bone marrow metastases following intraperitoneal ticagrelor treatment by the blockade of ADP-mediated regulatory signalling between endothelium and platelets [277].

Clinical studies

There are no clinical studies directly assessing the effects of treatment with ticagrelor on cancer and metastasis.

GPIIb/IIIa inhibitors

The platelet GPIIb/IIIa (αIIbβ3) receptor belongs to the family of integrins and its ligands are fibrinogen and von Willebrand factor (VWF)[278]. The role of αIIbβ3 as fibrinogen receptor and the important role of fibrinogen in the final step of platelet aggregation, make this integrin a key target of anti-thrombotic therapy. Moreover, αIIbβ3 is one of the major platelet receptors involved in

TCIPA [279]. The importance of αIIbβ3 in cancer metastasis comes from studies in β3 (subunit of αIIbβ3) knockout mice. Knockout mice are refractory to bone metastasis after intra-arterial injection of B16-F10 mouse melanoma cells, suggesting that β3 integrins play a central role in tumour cell trafficking to the bone [280].

αIIbβ3 plays an important role in the formation of platelet-tumour cell aggregates. Tumour cells induce platelet aggregation by upregulating the expression of platelet αIIbβ3 and P-selectin, which bind to mucin-type glycoproteins on the surface of tumour cells, mediating platelet-tumour cell interaction. On the other side, platelets protect circulating tumour cells from immune system and release TGF-β1 that induces phenotypic changes in tumour cells with epithelial to mesenchymal-like transition, facilitating their extravasation and dissemination to distant sites during metastasis [281].

Successful transendothelial migration of tumour cells depends on cooperation between the mediation of tumour cell αvβ3 and platelet integrin αIIbβ3.

Pre-clinical studies

In a mouse model the decrease of metastasis is marked when mice were depleted of platelets, indicating the importance of platelet αIIbβ3 for the cross-talk with tumour cells [282].

In the experimental lung metastasis model induced by injection of Lewis lung carcinoma cells (LL2), a non-peptide oral αIIbβ3 antagonist (XV454) significantly inhibited tumour cell-induced thrombocytopenia and the number of lung tumour nodules [221]. Furthermore, snake venom RGD-containing peptides inhibits TCIPA in experimental colon adenocarcinoma models with a mechanism mediated by the blockade of platelet αIIbβ3 [283].

Clinical studies

Vitaxin, a monoclonal antibody that binds a conformational epitope formed by the αv and β3 subunits, was evaluated for the treatment of stage IV metastatic melanoma and of androgen-independent prostate cancer [284]. Vitaxin treatment shown an increase of median survival of metastatic melanoma patients with minimal side effects, but no inhibition of tumour growth or metastases was observed in the treatment of refractory solid tumour [285, 286].

Other αIIbβ3 inhibitors entered in clinical use as anti-platelet drugs have a promising anti-tumour activity in preclinical studies, and they are Abciximab and Eptifibatide [287].

Innovative therapeutic approaches

Platelets as drug carriers

The strong ability of platelets to interact with circulating tumour cells, leads to consider them as a potential anticancer drug carrier. The delivery of drugs using platelets may prolong drug circulation time, reduce immunogenicity and improve selective targeting[288]. The anti-cancer drug is encapsulated in platelets or nanoparticles covered with platelet membranes. In the first case the chemotherapy was loaded into platelets by natural uptake through the open canalicular system [289]. Loaded platelets were then infused in mice with cancer and an enhanced therapeutic efficacy and reduced adverse effects, compared to drug injected as such, was observed [290]. An improvement was the use of drug-loaded platelets conjugated with a monoclonal antibody directed against a specific cancer cell target. Platelets loaded with doxorubicin were employed for drug delivery to non-Hodgkin's lymphoma, lung adenocarcinoma (A563) and to Ehrlich ascites carcinoma in vivo in mice, showing that drug dose requirement is lower compared to the drug given as such [289].

Another interesting study was executed using hematopoietic stem/progenitor cells (HSPCs) genetically modified to express TRAIL, a cytokine known to induce apoptosis in tumour cells. HSPCs genetically engineered were transplanted in mice, generating platelets which express TRAIL. Mice were injected with human prostate tumour cells after 30 days from TRAIL-HSPCs transplant and it was shown that TRAIL-expressing platelets kill cancer cells *in vitro* and significantly reduce liver metastases *in vivo* [291].

In the second approach the nanoparticles, that contain anti-cancer doxorubicin, was coated with platelet membranes which were functionalized with TRAIL conjugation. The intra venous infusion of platelet coated nanoparticles in MDA-MB-231 tumour-bearing nude mice effectively eliminated circulating tumour cells and inhibited the development of metastases [292].

Platelet-derived extracellular vesicles

PEVs have a role in cancer progression. In fact, they promote primary tumour growth, stimulate cancer angiogenesis and contribute to the formation of metastases [293]. Considering their crucial role in cancer, PEVs could be exploited to inhibit tumour spreading.

In a recent study, platelets were conjugated with antibodies which recognize programmed-cell-death protein 1 ligand (PDL- 1) and were observed to release anti-PDL-1 antibody-microparticles that can interact with tumour cells which express PDL-1. Tumour cell-PDL-1, upon binding with its receptor PD1 expressed on T cells, induces lymphocytes inactivation allowing cancer cell evasion from immune surveillance. The antibody conjugated on platelets is an immune checkpoint blocker used in cancer immunotherapy that impedes the binding of PD1 with PDL1 thus preventing the inactivation of T cells. After the surgical resection of the tumoral mass to mice bearing B16-F10 tumour, the administration of modified platelet with anti-PDL1 significantly prolong overall mouse survival by reducing relapsed tumour and metastatic spread. The mechanism is mediated by the antibody express on microparticles which are released by platelets after activation in the surgical bed due to the cancer cells remained after resection of tumour [294].

Aim of the work

Metastasis remains the major cause of mortality in cancer patients and the limited understanding of mechanism that support metastasis causes the failure of antimetastatic therapies. Cancer spreading is strictly related to metastatic potential of cancer cells, but it is now recognized that the host environment is crucial to the development of metastasis [295]. Blood platelets are among the principal components of the host environment, and it is largely known that they play a fundamental role in tumour progression and spreading [274]. Activated platelets prolong tumour cells survival in circulation, support cancer cell extravasation and regulate angiogenesis in new metastatic site [208, 274]. Our research group previously demonstrated that platelet activation is accompanied by the release of a large amount of PEVs [296]. More evidences suggest that PEVs have a fundamental role in the platelet-dependent regulation of cancer progression and metastasis [59, 231]. Cancer patients, in particular at advanced stages of the disease, display an increase in circulating PEVs [110, 232, 297]. Moreover, hypercoagulative conditions of cancer patients are characterized by elevated level of PEVs, but they are also implicated in the interactions between tumour and host and are considered a marker for negative prognosis [298]. However, the effects of PEVs on cancer cell regulation are not well understood, and a series of conflicting results have been presented in several works. In general, it is recognized that PEVs support cancer cell progression by regulating angiogenesis, cancer cell aggressiveness and the escape from immune system surveillance [135, 234, 236, 238]. Anyway, some investigations suggested that PEVs may act as cancer suppressor [299, 300].

In this study, we compared for the first time the ability of PEVs to bind and to regulate four different types of breast cancer cells *in vitro*. We selected for this investigation the three metastatic cell lines MDA-MB-231, SKBR3 and MCF-7, and a cell line from ductal carcinoma BT474. PEVs were obtained after platelet stimulation with the physiological agonist thrombin.

Using multiple approaches, we have investigated the ability of cancer cells to interact and internalize thrombin-induced PEVs and we analysed the functional consequences of the interaction between PEVs and cancer cells. We focused our attention on key aspects of cancer biology involved in cancer dissemination, such as cell survival, cell cycle progression, cell proliferation, cell migration and invasiveness. We also investigated the possible signalling pathway involved in these events.

Materials and Methods

Materials

Apyrase, Thrombin, Trypan Blue solution 0.4%, 5(6)- Carboxyfluorescein diacetate N-succinimidyl ester (CFSE), Methylthiazolyldiphenyl-tetrazolium bromide (MTT), Paraformaldehyde (PFA), Phalloidin Conjugates TRITC (Phalloidin-TRITC), Triton X-100, Dimethyl sulfoxide (DMSO), Ethanol, Nonidet P-40 Substitute, Propidium Iodide (PI), Aspirin (ASA), U73122, 2-APB, BTP-2, and Y27632 were from Sigma-Aldrich. Prostaglandin E1 (PGE1) was from Santa Cruz Biotechnology. Dulbecco Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), 200 mM L-Glutamine 100×, Penicillin-Streptomycin solution 100×, Trypsin/EDTA 1× in PBS and BCA protein assay kit were from EuroClone. Hoechst-33342 was from Cell Signaling Technology. Ribonuclease I A Bovine Pancreas (RNase A) was from USB Corporation. Fura-2 acetoxymethyl ester (Fura- 2AM) was from Molecular Probes. The antibodies for GAPDH (V-18) and integrin αIIb (B-10) were from Santa Cruz Biotechnology. The antibody against phosphotyrosine (4G10) was from Millipore. The phosphospecific antibodies for phospho-PKC substrates (Ser), phospho-p38MAPkinase (Thr180/Tyr182), and phospho-MLC2 (Ser19), phospho-Akt (Ser473), phospho-ERK (Tyr202/204), and phospho-mTOR (Ser2481) were from Cell Signaling Technology.

Cancer cell culture

Human breast adenocarcinoma cell lines MDA-MB-231 and SKBR3 were provided by Dr. Livia Visai (Department of Molecular Medicine, University of Pavia), MCF-7 by Dr. Maria Grazia Bottone (Department of Biology and Biotechnology, University of Pavia), and BT474 by Dr. Riccardo Vago (San Raffaele Scientific Institute, Milan). Cancer cells were periodically cultured without antibiotics to verify the absence of bacterial contamination. All cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, split every 2 days, and used for the experiments within 10 passages. The count of vital cells was determined by Trypan Blue staining and phase contrast microscopy analysis.

PEVs isolation and quantification

Human platelets were purified after blood withdrawal from informed healthy volunteers recruited by Fondazione IRCCS Policlinico San Matteo. Buffy-coat was diluted by adding one-third volume of a 1:9 mixture of ACD (152 mM sodium citrate, 130 mM citric acid, and 112 mM glucose) and HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, and 12 mM NaHCO3, pH 7.4) and then was centrifuged at 120 × g for 15 min at room temperature in 5 ml aliquots. A volume of 1.5 ml of Platelet Rich Plasma (PRP) was recovered by each aliquot, and apyrase (grade VII, 0.2 units/ml) and PGE1 (1 μM) were then added to prevent platelet activation and ADP receptor desensitization. Platelets were recovered by centrifugation at 720 × g for 15 min and washed with 5 ml of PIPES buffer (20 mM PIPES, 136 mM NaCl, pH 6.5). Upon an additional centrifugation at 720 × g for 15 min, they were resuspended at the concentration of 3×10^8/ml in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, and 12 mM NaHCO3, pH 7.4) supplemented with 1 mM CaCl2, 0.5 mM MgCl2 and 5.5 mM glucose. Platelets were allowed to rest for 30 minutes before use.

To induce the release of PEVs, platelets were stimulated with the physiological agonist thrombin (0.2 U/ ml) for 30 min at 37 °C under constant stirring. Platelets were pelleted by low-speed centrifugation (750g, 20 min) and the supernatant was then centrifuged at 18,500g for 90 min at 10 °C to collect PEVs, that were finally resuspended in HEPES buffer. In some experiments, in order to obtain fluorescently-labelled PEVs, platelets were labelled with 3 μg/ml of CFSE for 10 min, before stimulation with thrombin. The protein content of the different preparations of PEVs was determined by BCA assay and used to set the amount of PEVs added to cells in all the experiments.

Concentration and dimension of purified PEVs were assessed by Nanoparticle Tracking Analysis (NTA) using NanoSight NS300 (Malvern Panalytical). Samples were diluted to the appropriate concentration and five videos of 60 s were recorded for each sample and analysed using NTA software (version 3.4; NanoSight Ltd.).

Analysis of PEVs interaction with cancer cells

MDA-MB-231 cells (5×10^4 cells/well), SKBR3, MCF-7 and BT474 cells (10^5 cells/well) were grown for 24 h on glass coverslips placed in 12-wells plate. Cells were left untreated or incubated for 24 h with 30 µg/ml of PEVs obtained from thrombin-stimulated CFSE-labelled platelets. Cells were subsequently washed with PBS (8 mM Na2HPO4, 2 mM NaH2PO4, 140 mM NaCl, pH 7.4) and fixed with 3% PFA in PBS for 10 min. Nuclei were counterstained with 1 µg/ml Hoechst-33342 and samples were examined using a Leica DM6 microscope. The interaction between PEVs and cancer cells was quantified by counting the percentage of cells associated with green-fluorescent PEVs. The internalization of PEVs by cancer cells was analysed by confocal microscopy using a Leica TCS SP8 Microscope and the collected images were analysed with LAS X software (Leica Microsystems Gmbh, DEU). For the analysis of PEVs/cancer cells interaction by flow cytometry, cells were incubated with fluorescent PEVs and then were washed twice with PBS, collected, fixed with PFA 3% for 10 min, and examined using BD FACSLyric flow cytometry (BD Biosciences).

MTT assay

Cancer cells were seeded in a 96-well plate (5×10^3 cells/well) and incubated for 24 h with increasing concentration of PEVs. MTT solution (5 mg/ml MTT in PBS) was added at the final concentration of 0.45 mg/ ml and incubated at 37 °C for 3 h. The MTT enters the cells and the mitochondria where it is reduced to insoluble, violet-coloured formazan. The formazan crystals are then solubilized by addition of DMSO for 10 min at room temperature and the released, solubilized formazan product is measured spectrophotometrically. The plate was read in a Bio-Rad microplate reader model 680 (Bio-Rad Laboratories, Inc., USA) using a test wavelength of 570 nm and reference wavelength of 650 nm. The degree of light absorption is dependent on the degree of formazan concentration accumulated inside the cell, assessing the mitochondrial dehydrogenase activity.

Analysis of cell viability

Cancer cells were grown for 24 h in 35 mm petri dishes and then incubated for 24 and 48 h with 30 µg/ml of PEVs obtained from thrombin-stimulated platelets. Cells were subsequently washed with PBS, harvested after incubation with trypsin, keep in the dark on ice for 15 min in PI staining solution (2 µg/ml PI in PBS + 2% FBS) and eventually examined using a BD FACSLyric flow cytometry (BD Biosciences). Cells negative for PI fluorescence were considered intact.

Cell cycle analysis

MDA-MB-231, SKBR3 and BT474 cells were grown for 24 h in 12- well plate and then incubated with increasing concentrations of thrombin-induced PEVs for additional 24 or 48 h. Cells were subsequently washed with PBS, harvested, fixed in cold 70% ethanol and kept 30 min at 4 °C. Cells were suspended and stained with the PI staining solution (0.5 mg/ml RNase A, 0.05% NP-40, 10 µg/ml PI in PBS) for 30 min at room temperature, and finally stored at 4 °C. Cell cycle progression was analysed using BD FACSLyric flow cytometry (BD Biosciences). Incorporated PI and associated fluorescence are proportional to the amount of cellular DNA. Cells that are in S phase contain an intermediate amount the DNA between cells in G1 and G2/M phases. The cells in G2 are approximately twice as bright as cells in G1. The percentage of cells in the different phases was determined using Watson pragmatic curve fitting algorithm.

Cell proliferation analysis

Cell proliferation was measured as previously described [301]. MDA-MB- 231, SKBR3 and BT474 cells were labelled with 2 µg/ml CFSE, grown for 24 h in 35 mm petri dishes and then incubated or not with 30 µg/ml of thrombin-induced PEVs for 24 and 48 h. Cells were subsequently washed with PBS, collected, and fixed with PFA 3% for 10 min. The extent of proliferation was evaluated using a BD FACSLyric flow cytometry (BD Biosciences).

Cell migration assay

The effect of PEVs on migration of cancer cells was evaluated using Falcon cell culture inserts (8 µm pore size) positioned in a 24-well plate. Cancer cells were serum starved for 6 h and then resuspended in DMEM added of and 0.5% FBS. Then cells were either left untreated or treated with increasing amount of PEVs and then transferred inside the inserts. DMEM containing 10% FBS was added to lower chamber. After 24 h, cells that moved through the porous membrane were stained with 0.5% crystal violet and counted at 20× microscope magnification, with an Olympus BX51 microscope (Olympus Corporation, JPN).

$[Ca^{2+}]_i$ measurements

$Ca2^+$ imaging was used to measure intracellular Ca^{2+} signals in PEVs-treated cancer cells as described elsewhere [302, 303]. MDA-MB- 231, SKBR3, and BT474 cells were loaded with 4 µM Fura-2/AM (1 mM stock in DMSO) in physiological salt solution (PSS) (150 mM NaCl, 6 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 10 mM Glucose, 10 mM HEPES, pH 7.4) for 1 h at 37 °C and 5% CO_2. After washing in PSS, the coverslip was fixed to the bottom of a Petri dish and were either left untreated or treated with 30 µg/ml thrombin-induced PEVs. The cells were observed by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, DEU), equipped with a Zeiss ×40 Achroplan objective (water immersion, 2.0 mm working distance, 0.9 numerical aperture). The cells were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot on-line the fluorescence from rectangular "regions of interest" (ROI) enclosing 20–30 single cells. $[Ca^{2+}]_i$ was monitored by measuring, for each ROI, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm [ratio (F340/ F380)]. An increase in $[Ca^{2+}]_i$ causes an increase in the ratio. Ratio measurements were performed and plotted on-line every 3 s. The experiments were performed at room temperature (22 °C).

SDS-PAGE and immunoblotting

MDA-MB-231, SKBR3, and BT474 cells were grown in 35 mm Petri dish, incubated with PEVs for 24 h and lysed with lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.25% deoxycholic acid, 0.1% SDS, pH 7.4, 1 mM PMSF, 5 µg/ml leupeptin, and 5 µg/ml aprotinin). Equal amount of total platelet proteins from the different samples were separated by SDS-PAGE and transferred to PVDF membranes. Membrane staining was performed using the different antibodies diluted 1:1000 in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 5% BSA and 0.1% Tween-20 in combination with the appropriate HRP-conjugated secondary antibodies (1:2000 in PBS plus 0.1% Tween-20). Quantification of band intensity was performed by computer-assisted densitometric scanning using Quantity One – 4.6.8 software.

Statistical analysis

All the reported figures are representative of at least three different experiments and the quantitative data are reported as mean ± SD or ± SEM. Comparisons between two groups were done using Student t-test, whereas multiple comparisons were performed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. p-Value less than 0.05 was considered statistically significant. Data were analysed using GraphPad Prism Version 5.01 software.

Results

PEVs characterization by NanoSight analysis

We calculated that, in our experimental conditions, about 100 PEVs were released by a single thrombin-stimulated platelet. A representative size distribution of PEVs, obtained by NanoSight analysis, is reported in Fig. 1. The size of purified PEVs obtained in every single preparation was in agreement with the results reported by Menck and colleagues [304]. In particular, 82 ± 2% of PEVs was in the 100–500 nm range. The average PEV diameter calculated by comparing different preparations was 171.7 ± 15.0 nm (n = 3). Based on previous determination of the amount of PEVs in the plasma of healthy subject and cancer patients [110, 304, 305], concentrations of PEVs in the range of 10–30 µg/ml, corresponding to about 0.7–2.1 × 10^9 PEVs/ml, were used.

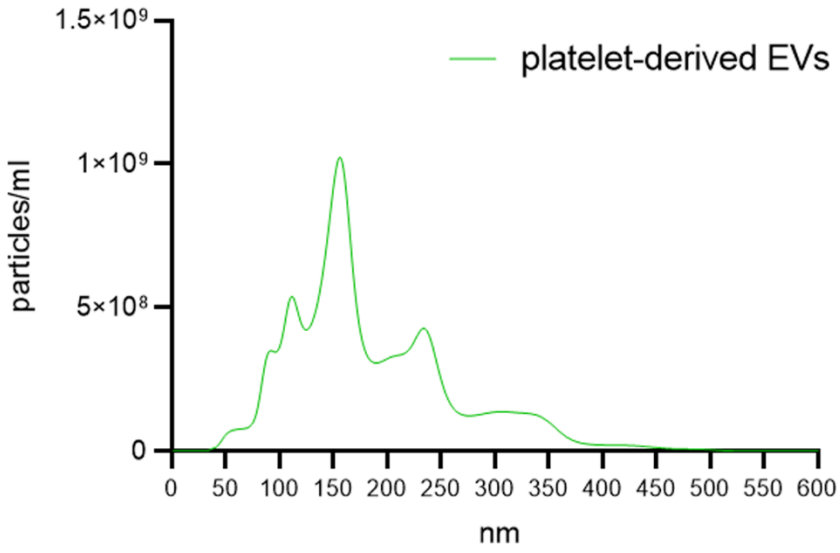

FIGURE 1. ***PEVs size distribution.***
Representative size distribution of platelet-derived extracellular vesicles (PEVs) obtained by NanoSight analysis is reported.

PEVs interact with breast cancer cell lines

PEVs, labelled with the CFSE fluorescent probe, were isolated after human platelet stimulation with the physiological agonist thrombin and cocultured with four breast cancer cell lines. The cell lines used were MDA-MB-231, SKBR3, MCF-7, and BT474. In these experiments we evaluated the ability of PEVs to physically interact with breast cancer lines. Analysis, performed in a fluorescence microscopy, revealed that MDA-MB-231, SKBR3, and BT474 cells efficiently interact with PEVs. Instead, MCF-7 cells are unable to bind PEVs (Fig. 2A). The quantitative analysis shown that about 60% of MDA-MB-231 and SKBR3 cells, and 35% of BT474 cells, were circled by green-fluorescently

vesicles. About MCF-7 cells, only the 10% of cells was found surrounded by PEVs, displaying their limited ability to bind vesicles (Fig. 2Aii).

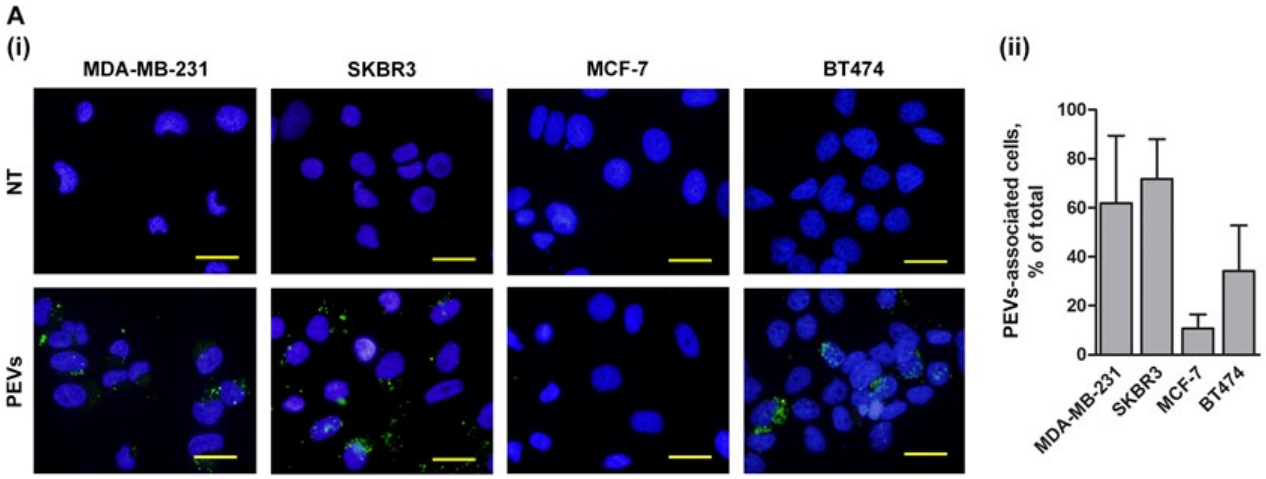

FIGURE 2: ***Interaction of breast cancer cells with PEVs***
(A) *MDA-MB-231 cells (5 × 10^4 cells/well), SKBR3, MCF-7 and BT474 cells (10^5 cells/well) were grown on glass coverslips and then incubated for 24 h with 30 µg/ ml of PEVs obtained from thrombin-stimulated CFSE-labelled platelets (green).* ***(i)*** *Representative fluorescence microscopy images upon 24 h of incubation. Cell nuclei were stained with Hoechst-33342 (blue); Scale bars: 50 µm.* ***(ii)*** *Quantification of the percentage of cells associated with fluorescent PEVs. Data are the mean ± SD of three independent experiments.*

The interaction between PEVs and cancer cells was also evaluated by flow cytometry approach. Upon co-culture with CFSE-labelled extracellular vesicles, a significant increase in the fluorescent signal was observed in MDA-MB-231, SKBR3, and to a lesser extent, in BT474 cells. Again, the fluorescent relative to MCF-7 cells was found unaltered if compare to untreated samples (Fig. 3A).
The analysis in confocal microscopy revealed that microvesicles were internalized by the cell lines which interact with them. Fig 3B shown that PEVs were localized on the same focal plan of nucleus and actin cytoskeleton in MDA-MB-231, SKBR3, and BT474 cells and they are preferentially accumulated in the cell cytoplasm in the perinuclear position. The analysis also confirmed the lack of internalization of PEVs in MCF-7 (Fig. 3B).

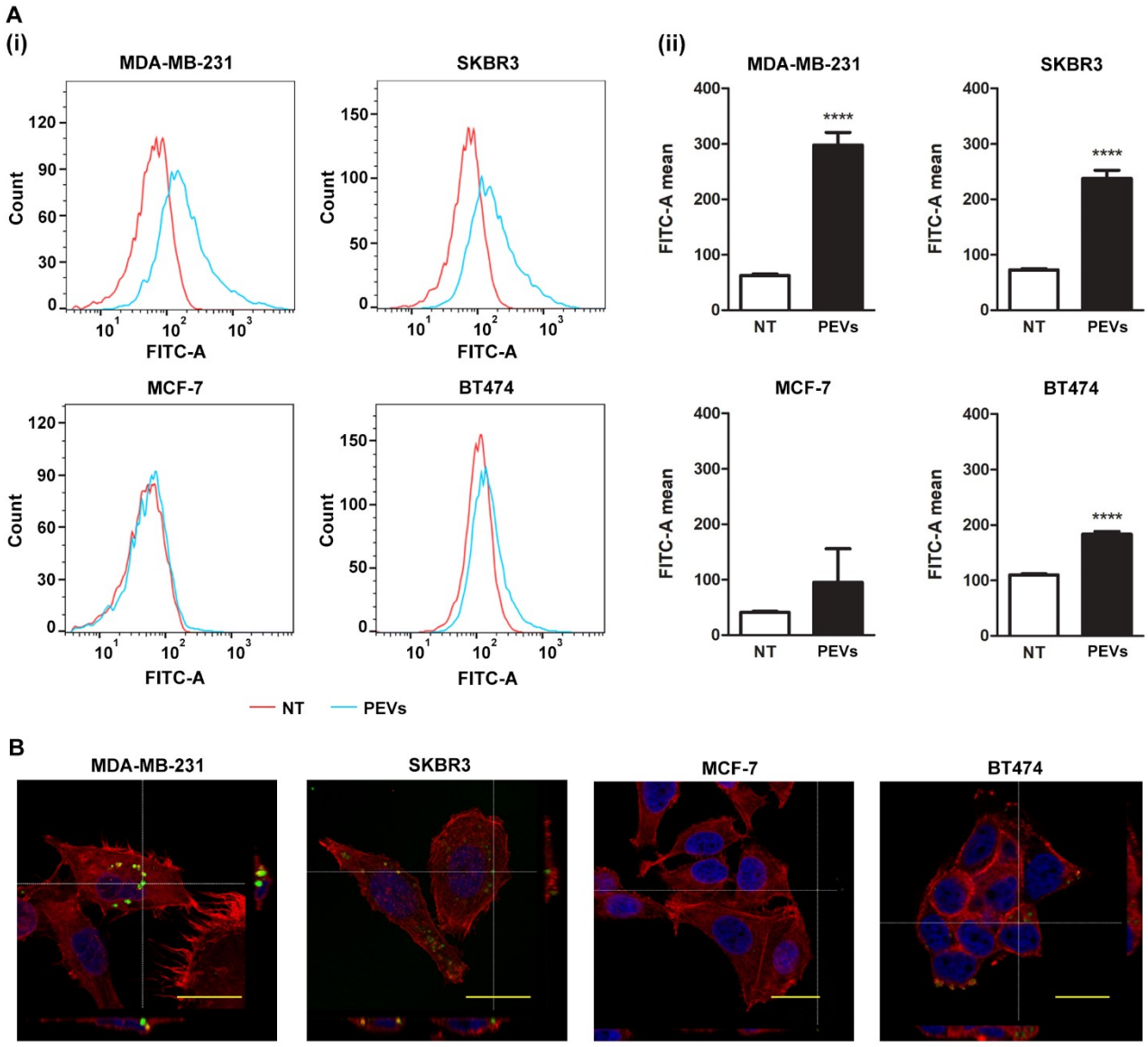

FIGURE 3: ***Interaction of breast cancer cells with PEVs***

(A) *Analysis of PEVs-cancer cells interaction by flow cytometry.* ***(i)*** *MDA-MB-231 cells (2 × 10^5 cells), SKBR3, MCF-7 and BT474 cells (4 × 10^5 cells) were grown for 24 h in 35 mm petri dishes and then incubated for 24 h with 30 µg/ml of PEVs obtained from CFSE-labelled platelets. NT = not treated. The representative overlapped histograms are shown.* ***(ii)*** *Quantification of the green fluorescent signal. The data are representative of the mean ± SD of three independent experiments with **** $p < 0.0001$.*

(B) *Confocal microscopy analysis of MDA-MB-231, SKBR3, MCF-7 and BT474 cells interacting with green fluorescent PEVs. Cell nuclei were stained with Hoechst- 33342 (blue) and actin filaments were stained with Phalloidin-TRITC (red). Representative confocal middle z-sections and orthogonal views are reported. Scale bar: 20 µm.*

To get far insight on the PEVs uptake, time course and dose-response analysis were performed with MDA-MB-231 cells through confocal microscopy and flow cytometry, respectively. The results revealed that the interaction of microvesicles with MDA-MB-231 cells was dose-related and already detectable with 10 µg/ml of PEVs upon incubation (Fig. 4A and B). In time-course analysis with 30

µg/ml of PEVs as experimental dose, we found that the binding with cancer cells was detectable after 1 hour of incubation and increase up to 24 hours (Fig. 4C). The confocal microscopy analysis also revealed a time-dependent internalization of PEVs, which is concomitant to decrease of cell surface-associate PEVs. This indicate that also internalization was time-dependent (Fig. 4D).

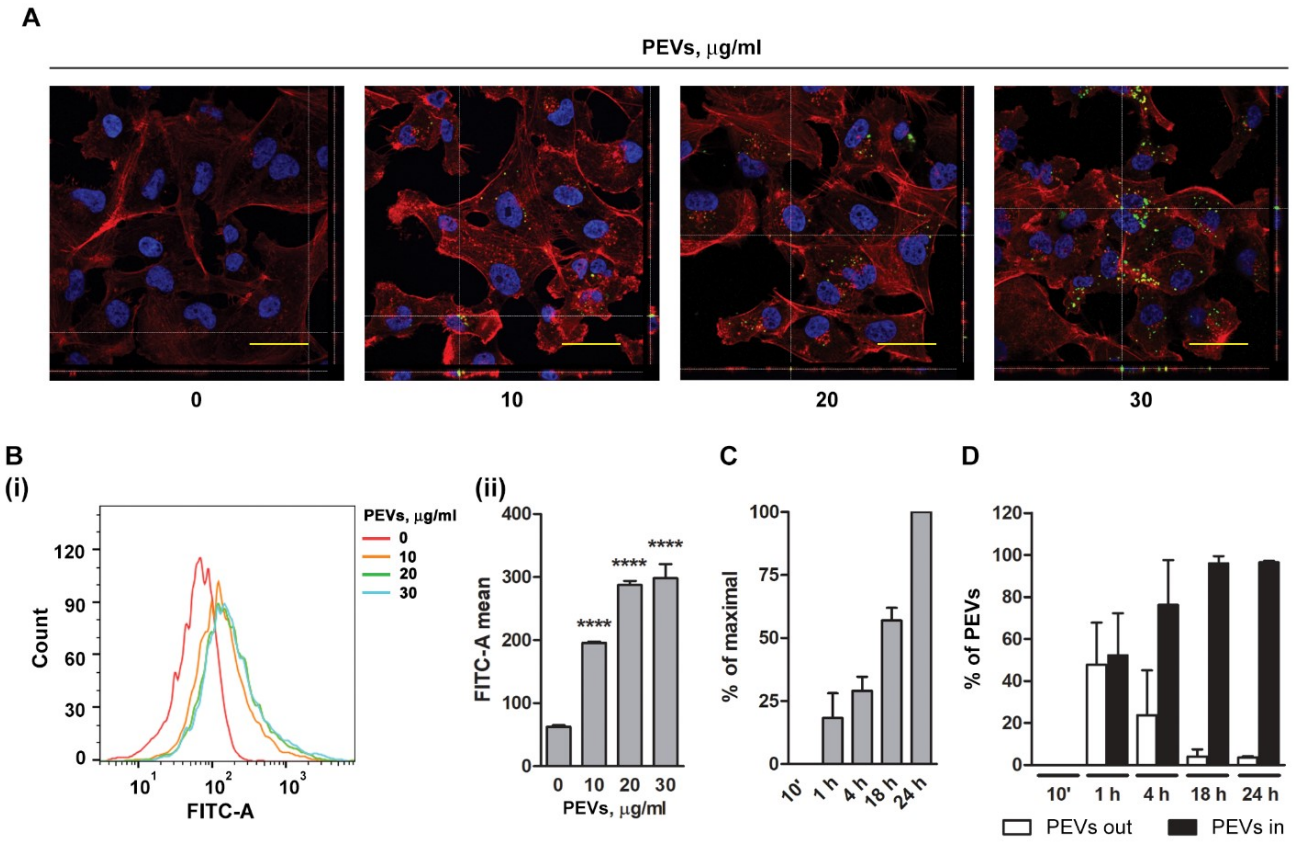

FIGURE 4: ***The internalization of PEVs by MDA-MB-231 cells is time and dose dependent.***

(A) *Confocal microscopy analysis of MDA-MB-231 cells interacting with the reported amounts of CFSE-labeled PEVs. Cell nuclei were stained with Hoechst-33342 (blue) and actin filaments were stained with Phalloidin-TRITC (red). Representative confocal middle z-sections and orthogonal views are reported. Scale bar: 20 µm.*

(B) *Flow cytometry analysis of MDA-MB-231 cells (2×10^5 cells) upon incubation with increasing amounts of CFSE-labeled PEVs (from 0 to 30 µg/ml) for 24 h. The representative overlapped histograms are shown in* ***(i)*** *and the quantification of the green fluorescent signal is reported in* ***(ii)****. The data are representative of the mean ± SD of three independent experiments with **** $p < 0.0001$.*

(C) *MDA-MB-231 cells were incubated with 30 µg/ml of CFSE-labeled PEVs for increasing times ranging from 10 min to 24 h and then analyzed by confocal microscopy. The number of vesicles associated to the cells at the different time points was counted in 10 separated fields. Data are expressed as percentage of the maximal value that is observed after 24 h of incubation. Data are the mean ± SD of three independent experiments.*

(D) *Quantification of the percentage of PEVs associated to the cell surface or internalized at the different time points. Values have been determined by counting the green fluorescent PEVs localized inside or around the single cells on 10 separated fields on confocal microscopy images. Data are the mean ± SD of three independent experiments.*

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By western botting and immunofluorescence analyses we observed that the cross-link between cancer cells and microvesicles contributed to transfer platelet components to recipient cells. The experiments highlighted that the specific platelet membrane marker integrin αIIb subunit, absent in untreated MDA-MB- 231, SKBR3 and BT474 cells, was detectable in cells treated with PEVs (Fig. 5A–B).

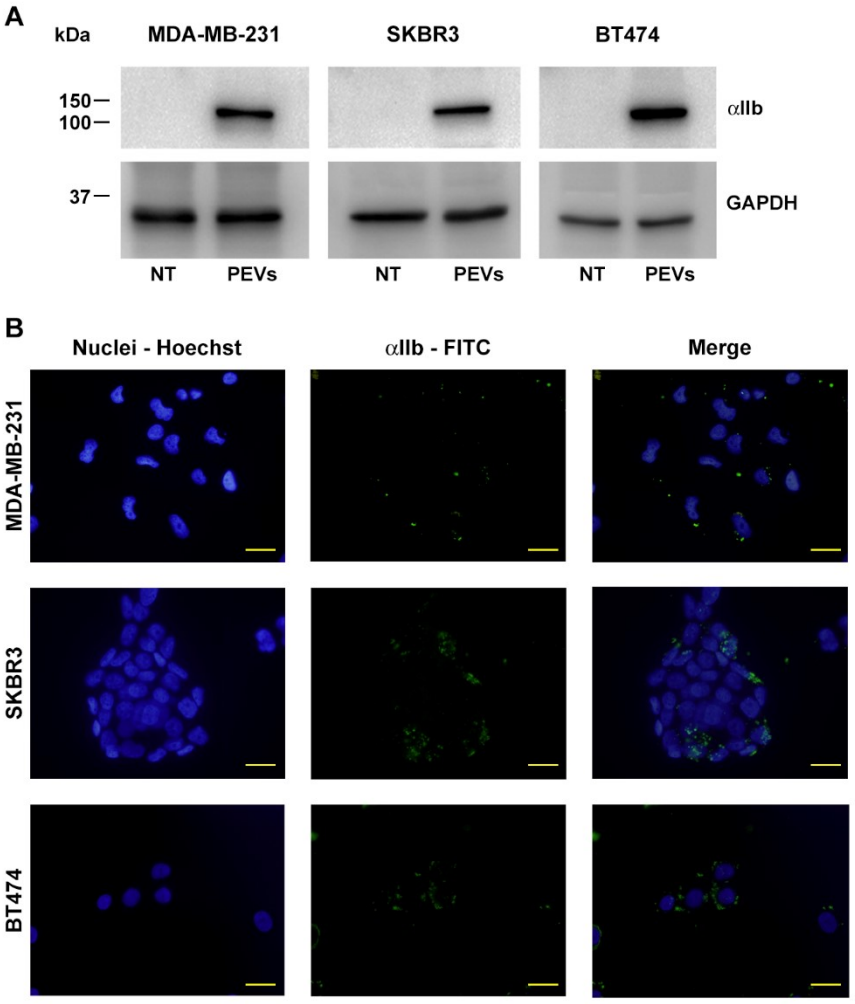

FIGURE 5. ***Transfer of platelets components from PEVs to recipient cells.***

(A) *Immunoblotting analysis of integrin αIIb in whole lysates of cancer cells, untreated (NT) or incubated with PEVs. GAPDH staining was performed as control for equal loading.*

(B) *Immunofluorescence staining of MDA-MB-231, SKBR3 and BT474 cells for platelet integrin αIIb treated with PEVs for 24 hours. Panel of representative images merged in the last column. Nuclei in blue and integrin αIIb in green. Scale bar: 50 µm.*

Exposure to PEVs triggers functional responses in breast cancer cells

Considering the important role of PEVs in intercellular communication and the microvesicles binding with cancer cells, the consequence on regulation of specific cellular processes was investigated.

The mitochondrial function, assessed by MTT assay, was not altered after the exposure of MDA-MB-231 or BT474 cells to increasing amounts of PEVs ranging from 10 to 30 µg/ml (Fig. 6Ai). By contrast, a significant decrease of mitochondrial dehydrogenases activity was found in SKBR3 cells treated with all the amounts of PEVs used. Considering that PEVs transport mitochondria [306], we tested their activity to exclude that the colorimetric signal was due to PEVs mitochondria. In our experimental condition, PEVs alone did not generate a significant colorimetric signal in the MTT assay (Fig. 6Aii).

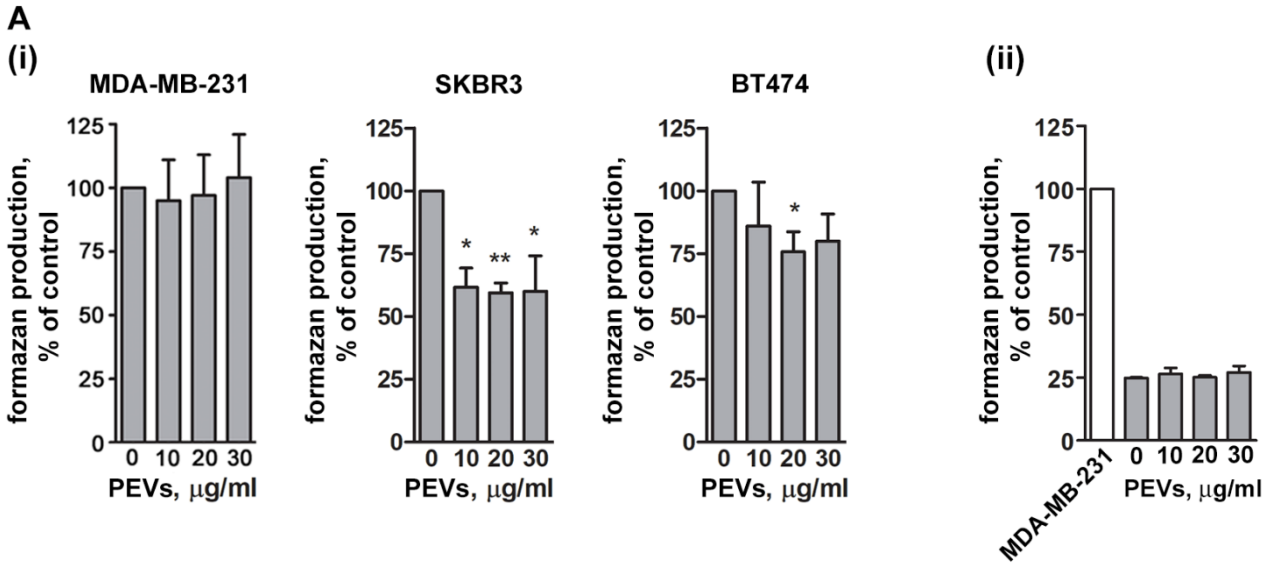

FIGURE 6. ***Effects of PEVs on mitochondrial activity.***
(A) (i) *Mitochondrial activity of MDA-MB-231, SKBR3 and BT474 cells incubated with the indicated amounts of PEVs for 24 h and was assessed by a colorimetric MTT assay.* ***(ii)*** *Mitochondrial activity of indicated amounts of PEVs alone. MDA-MB-231 cells were used as control. Results are reported as the mean ± SD of three different experiments with * $p < 0.05$ and ** $p < 0.01$.*

To assess that the reduction of mitochondrial dehydrogenases activity in SKBR3 was associated to cell death, the analysis of nonviable cells by flow cytometry with propidium iodide staining was performed. The analysis revealed that the exposure of SKBR3 cells to 30 µg/ml of PEVs, the highest amount of extracellular vesicles in our experiments, did not affect their viability after 24 or 48 h of treatment. As expected, also MDA-MB-231 and BT474 cells did not display an altered viability due to PEVs (Fig. 7).

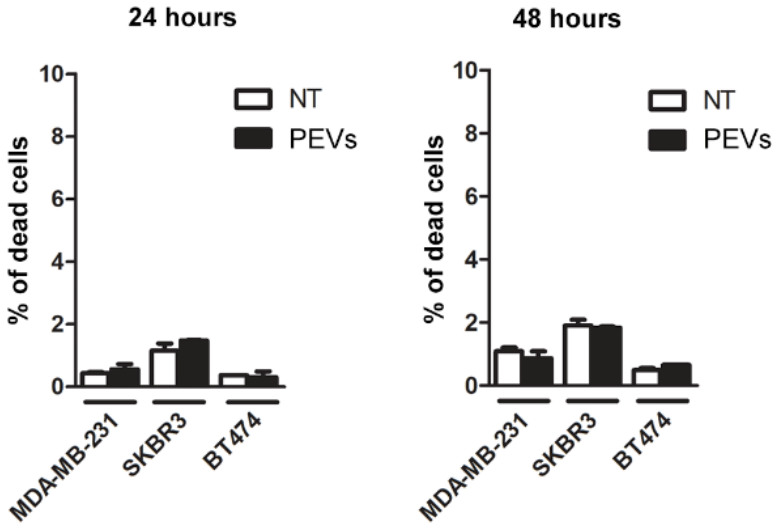

FIGURE 7. ***Effects of PEVs on cell viability.***
MDA-MB-231 cells (105 cells/dish), SKBR3 and BT474 cells (2 × 10^5 cells/dish) were incubated with 30 µg/ml of PEVs for 24 and 48 h, as indicated and cell viability was analysed by flow cytometry after PI staining. Quantification of the red fluorescence signal as percentage of dead cells. NT = control samples not treated with PEVs. Data are the mean ± SD of three independent experiments.

Afterward, the effect of PEVs was evaluated on cell cycle progression. Fig. 8Ai shows that the treatment with 10, 20 and 30 µg/ml of PEVs for 24 h did not induced any significant alteration in the distribution of the cell population in the different phases of cell cycle of MDA-MB-231, SKBR3 and BT474 cells. Interestingly, upon 48 h significant alteration was observed in the cell cycle of SKBR3 and BT474 cells. SKBR3 cells, with PEVs treatment higher than 20 µg/ml, shown minor but significant reduction of the percentage of cells in the G1 phase and a consequent accumulation of those in the S phase. Conversely, treatment of BT474 cells with 30 µg/ml PEVs significantly reduced the percentage of cells population in the S phase of about 10%, in association with a moderate accumulation of cells in the G1 and G2/M phases (Fig 8Aii).

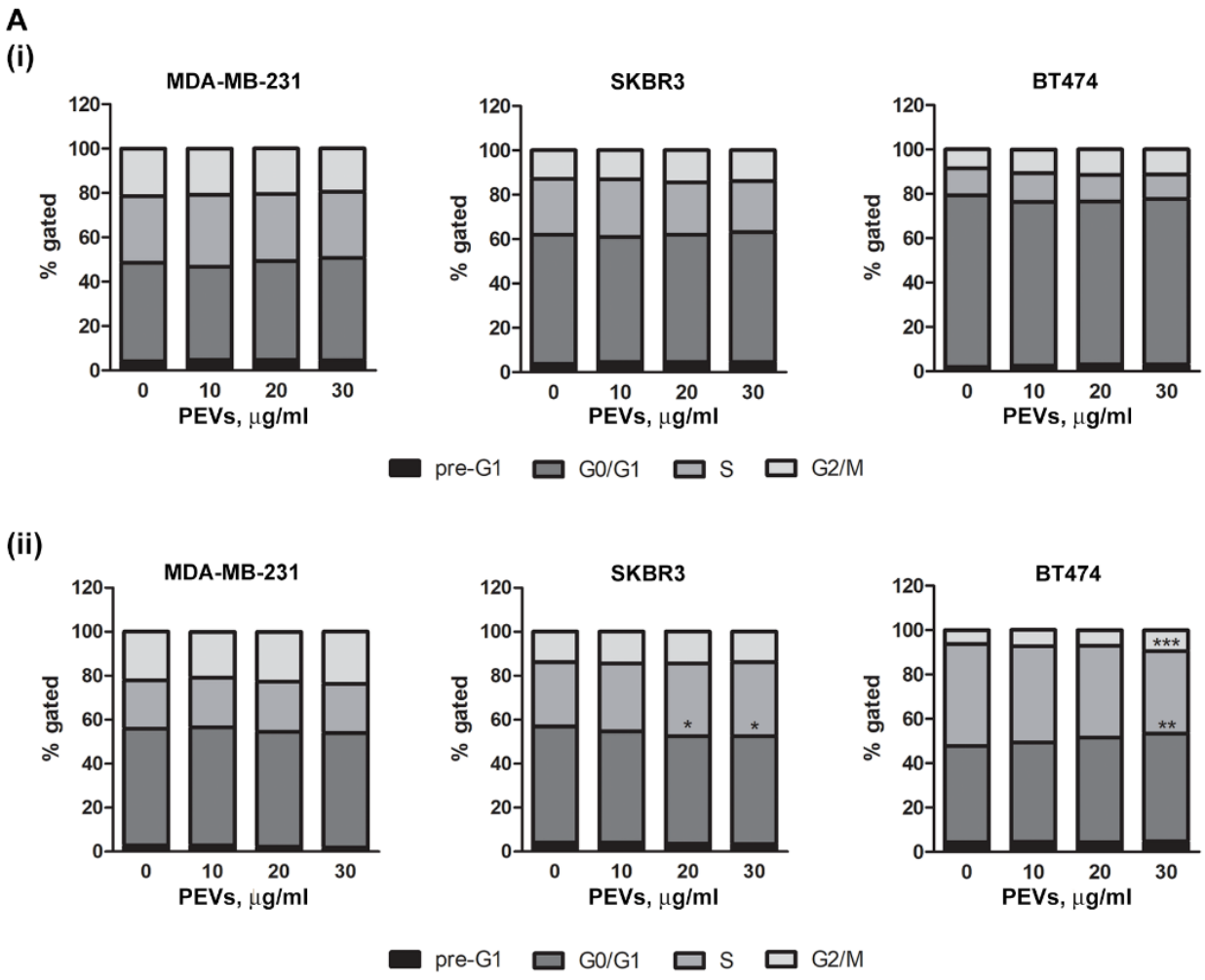

FIGURE 8. ***Effect of PEVs on cell cycle progression.***
(A) *MDA-MB-231, SKBR3 and BT474 cells were incubated with the indicated amounts of PEVs for 24* ***(i)*** *or 48 h* ***(ii)****. Cells were stained with PI and analysed by flow cytometry. Quantitative analysis of cell distribution in each cell cycle phase was performed from at least 2000 cells per sample. Each bar represents the mean of data obtained from three independent experiments with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.*

These slight changes in cell cycle progression were not associated in any evident alteration of cell proliferation. As analysed with a CFSE-based assay, coincubation of MDA-MB-231, SKBR3 and BT474 cells with 30 µg/ml of PEVs for 24 or 48 h did not stimulate or reduce the rate of cell proliferation (Fig. 9A).

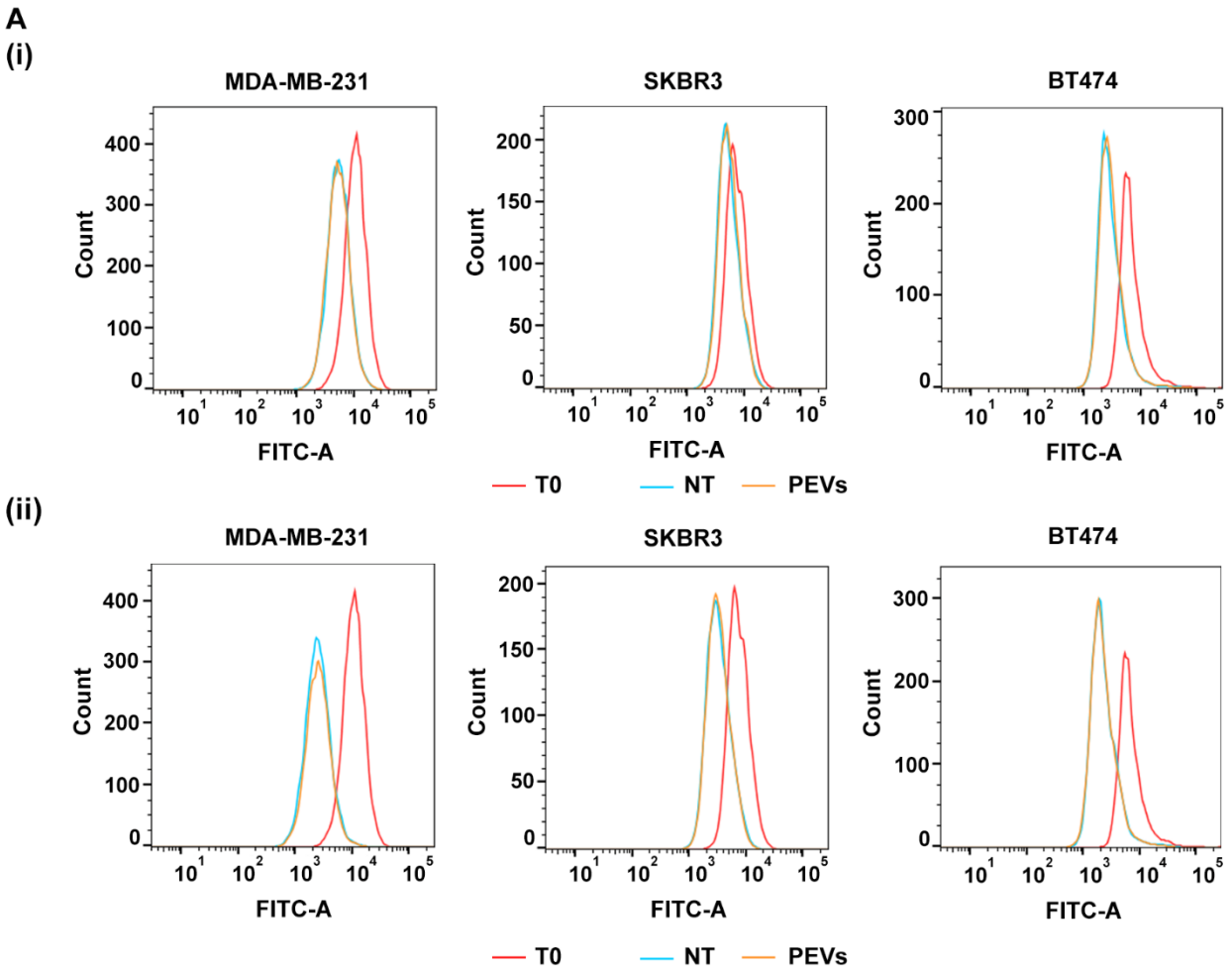

FIGURE 9. ***Effect of PEVs on cell proliferation.***
(A) *CFSE-stained MDA-MB-231 cells (10^5 cells/dish), SKBR3 and BT474 cells (2×10^5 cells/dish) were incubated with 30 µg/ml of PEVs for 24* ***(i)*** *and 48 h* ***(ii)****. The proliferation rate was measured by flow cytometry. NT = not treated with PEVs. Representative overlapped histograms are shown.*

Another critical process for cancer dissemination is cell migration. Using a transwell-based assay, we investigated the effects of PEVs on cancer cell migration of MDA-MB-231, SKBR3 and BT474. Interestingly, we found that PEVs affected cell migration depending on the nature of the recipient cells. Extracellular vesicles strongly potentiated the migration of MDA-MB-231 cells and in particular, 30 µg/ml of PEVs induced a three-fold increase in the number of migrated cells. Following the same experimental condition, PEVs did not stimulate the migration of BT474 cells and induced only a moderate, not significant increase of the percentage of migrating SKBR3 cells (Fig. 10A). PEVs also strongly affected MDA-MB-231 cell invasion potentiating cell invasiveness through ECM-coated membranes (Fig. 10B).

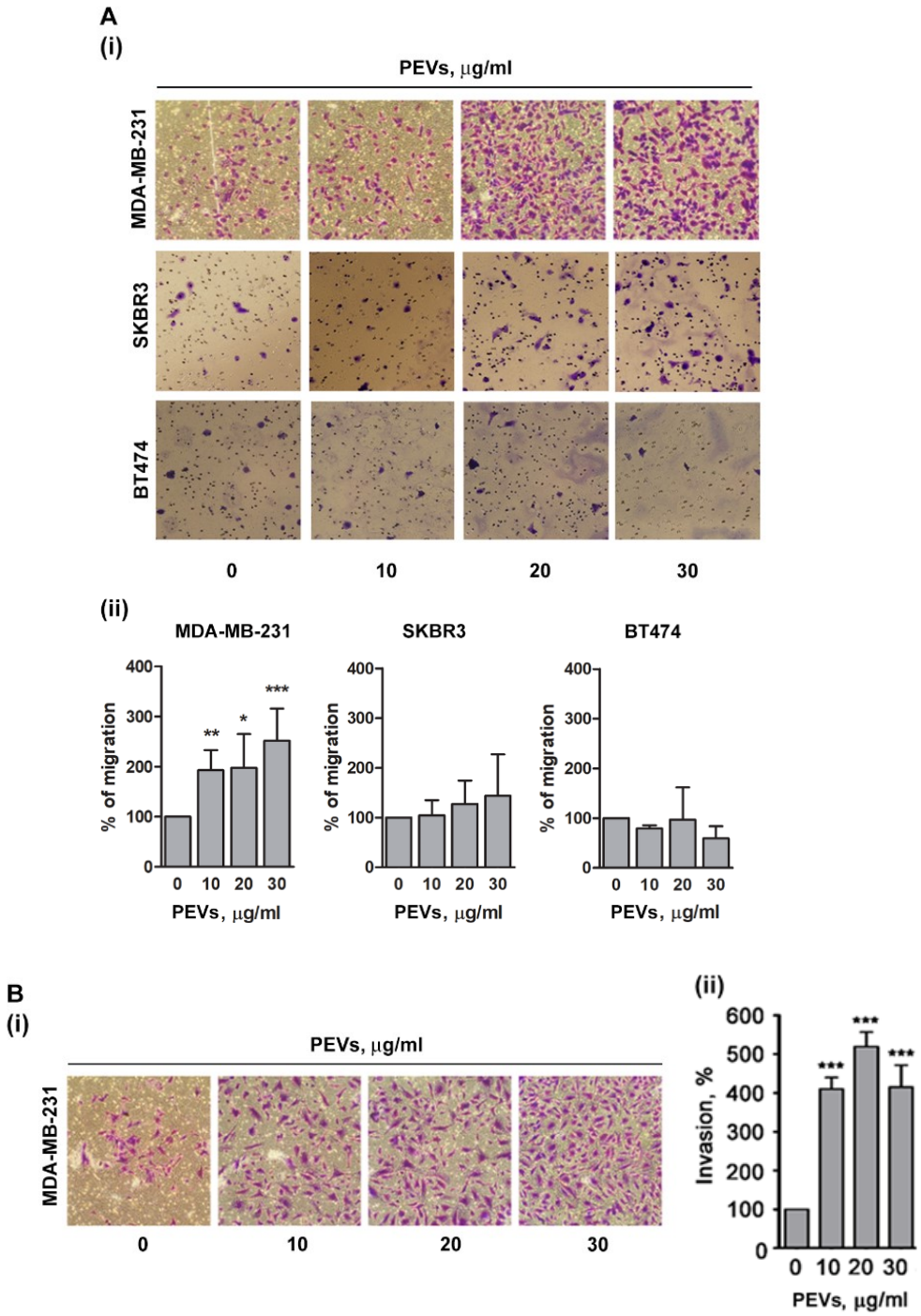

FIGURE 10. ***Effect of PEVs on migration of MDA-MB-231, SKBR3 and BT474 cells.***

(A) *MDA-MB-231 cells (5 × 10^4 cells/insert), SKBR3 and BT474 cells (2 × 10^5 cells/insert) were treated with increasing amounts of PEVs (from 0 to 30 μg/ml) and then transferred into cell culture inserts. Incubation was prolonged for 24 h and the cells migrated through the porous membrane were stained and counted. Representative images of migrated stained cells are reported in* ***(i)****, while quantification of the results is shown in* ***(ii)*** *as the mean ± SD of three experiments. Statistical significance of the differences was calculated between treated and untreated cells (sample 0). * p < 0.05, ** p < 0.01 and *** p < 0.005.*

(B) *PEVs potentiated MDA-MB-231 cells invasiveness. MDA-MB-231 cells were treated with increasing amounts of PEVs (0–30 μg/ml) and then transferred into cell culture inserts. The upper side of the insert was coated with 0.1 ml of Matrigel (50 μg/ml). Incubation was prolonged for 24 h and the cells that moved through the porous membrane were stained and counted. Representative images are reported in the panel* ***(i)****, while quantification of the results is shown in panel* ***(ii)*** *as mean ± SD of three experiments. Statistical significance of the differences was calculated between treated and untreated cells (sample 0). *** p < 0.005.*

When MDA-MB-231 cells were incubated with CFSE-labeled PEVs, migrated cells were found associated to fluorescent vesicles, indicating that cells had actually interacted with PEVs during the migration (Fig. 11).

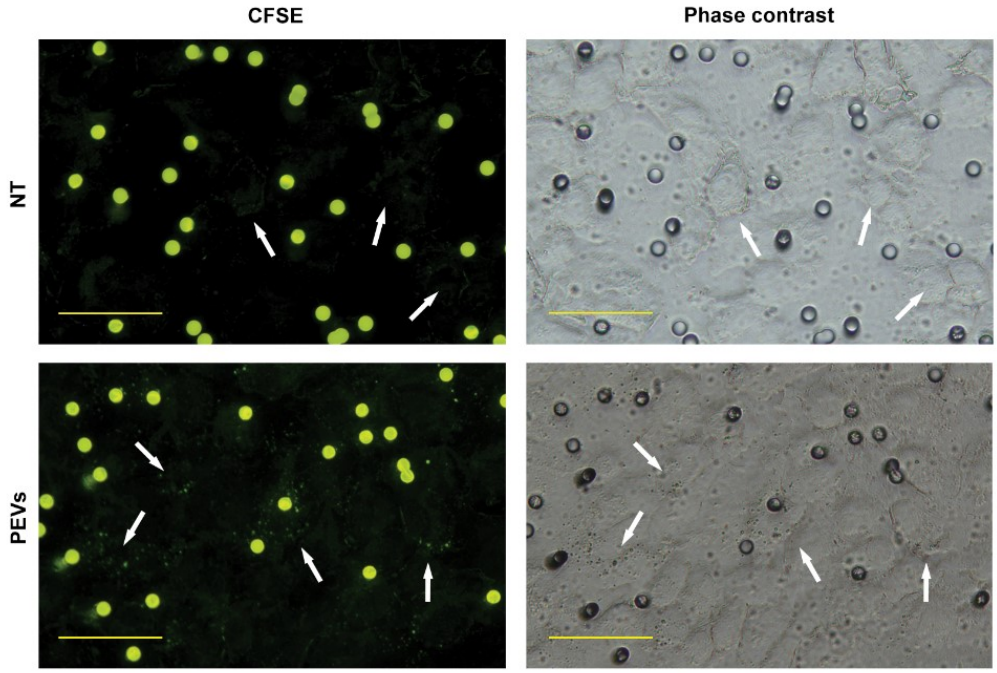

FIGURE 11. ***CFSE-labelled PEVs are associated to migrated MDA-MB-231 cells.***
MDA-MB-231 cells (5 x 10^4 cells/insert) were treated with 30 µg/ml of CFSE-labelled PEVs and then transferred into cell culture inserts. Incubation was prolonged up to 24 hours and the cells migrated through the porous membrane were visualized by phase contrast and fluorescence microscopy. Representative images of migrated cells are reported. The white arrows indicate the position of selected migrated cells. In the bottom panels, it is possible to appreciate that migrated cells are associated to fluorescent PEVs.

A shown in Fig. 12, the potentiation of migration of MDA-MB- 231 cells was not observed when cells were incubated with resting platelets, meaning that the enhancement of cell migration is specifically caused by the released PEVs. Moreover, aspirin strongly reduced cell migration induced by PEVs, suggesting the involvement of secondary messengers (Fig. 12).

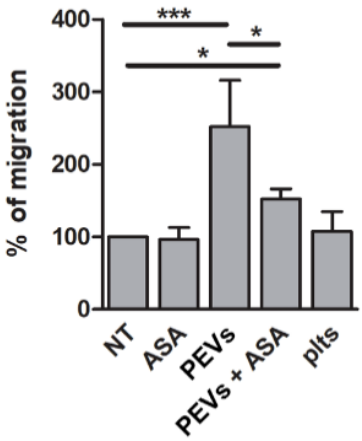

FIGURE 12. ***Effect of PEVs on migration of MDA-MB-231, SKBR3 and BT474 cells.***
*MDA-MB-231 cells (5 × 10^4 cells/insert) were treated with 30 µg/ml of PEVs in presence or absence of 200 µM aspirin (ASA), or incubated with 30 µg/ ml of intact platelets (plts), and then transferred into cell culture inserts. Incubation was prolonged for 24 h and the cells migrated through the porous membrane were stained and counted. Quantification of the results expressed as percentage of cell migration are reported. Data are the mean ± SD of three experiments. * $p < 0.05$ and *** $p < 0.005$.*

PEVs stimulate specific signalling pathways in cancer cells

Calcium (Ca^{2+}) represents one of the main modulators of cell activity. One of the earliest responses to extracellular stimulation is represented by a rapid increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [307]. Therefore, we assessed whether PEVs were able to elicit intracellular Ca^{2+} signals in breast cancer cell lines.

It was observed that 30 µg/ml PEVs triggered a biphasic increase in $[Ca^{2+}]_i$ in MDA-MB-231, SKBR3, and BT474 cells (Fig. 13A). In all cell types tested, PEVs evoked a rapid Ca^{2+} elevation which then reached a sustained plateau level. The plateau was maintained as long as PEVs were present in the perfusate, in fact PEVs washout caused a rapid decline of $[Ca^{2+}]_i$ to resting levels (Fig. 13Ai). However, the rising of peak was significantly higher in MDA-MB-231 cells compared to SKBR3 and BT474 cells (Fig. 13Aii).

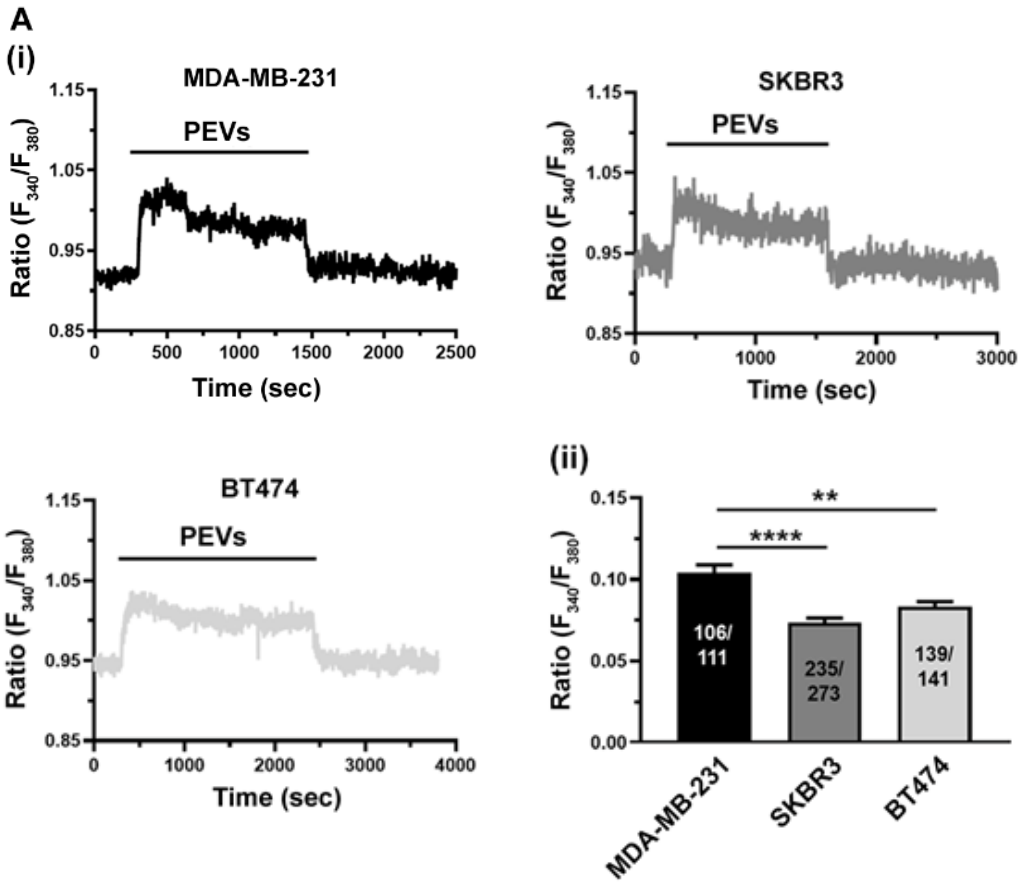

FIGURE 13. ***PEVs induce intracellular signalling responses.***

(A) (i) *Representative traces of PEVs-triggered a biphasic increases in [Ca2+]i in MDA-MB-231, SKBR3 and BT474 cells loaded with Fura-2/AM. The black bars above the traces indicate the time of exposure to PEVs (30 µg/ml).* ***(ii)*** *Mean ± SEM of the peak amplitude of the Ca2+ response to PEVs. ** p < 0.01, **** p < 0.0001.*

In MDA-MB-231 cells, the increase of $[Ca^{2+}]_i$ was significantly impaired after 2-aminoethoxydiphenyl borate (2-APB) or U73122 treatment, which block inositol-1,4,5-trisphosphate (IP3) receptors (IP3Rs) and inhibit phospholipase C (PLC) respectively (Fig. 14A). PKC, which is a major downstream effector of PLC and Ca^{2+} mobilization, was found rapidly activated after incubation with PEVs by measuring the phosphorylation of protein substrates in whole cell lysate (Fig. 14B). Noteworthy, the selective blocker BTP-2 did not reduce completely the increase of $[Ca^{2+}]_i$, indicating that the Ca^{2+} signal induced by PEVs partially depends on store-operated Ca^{2+} entry (SOCE) (Fig. 14A).

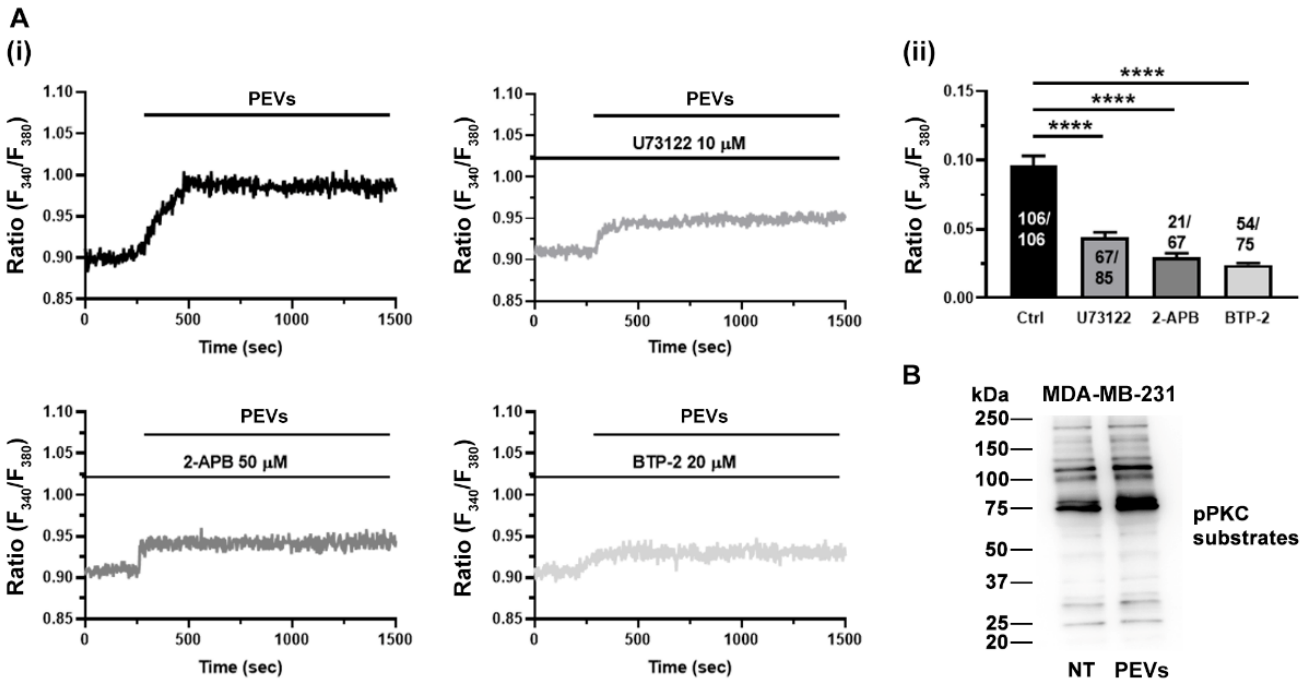

FIGURE 14. Inositol-*1,4,5-trisphosphate receptors (IP3Rs)-mediated Ca2+ release and store operated calcium entry (SOCE) are responsible for Ca2+ signals induced by PEVs in MDA-MB-231 cells.*

(A) *The [Ca2+]i increase induced by PEVs (30 µg/ml) was inhibited by the selective PLC inhibitor U73122 (10 µM, 30 minutes), by the IP3Rs antagonist 2-APB (50 µM, 30 minutes) and by selective SOCE blocker BTP-2 (20 µM, 20 minutes).* ***(i)*** *Representative traces of [Ca2+]i and* ***(ii)*** *quantification of the results as mean ± SEM of the peak amplitude of Ca2+ signals to PEVs. **** $p < 0.0001$.*

(B) *PEVs stimulate the rapid PKC-dependent protein phosphorylation. Phosphorylation of PKC substrates were evaluated by immunoblotting with a specific antibody on whole cell lysate of MDA-MB-231 cells left untreated (NT) or incubated with PEVs for 5 minutes. A representative immunoblot is reported.*

These results demonstrate that PEVs trigger intracellular signalling in breast cancer cell lines.

Next, we analysed the effect of PEVs on phosphorylation of several intracellular proteins potentially involved in the regulation of cell motility. No significant differences were detected in the tyrosine phosphorylation and PKC substrates phosphorylation profiles between controls and PEVs-treated samples (Fig. 15A–B). Likewise, the phosphorylation of the MAPkinase ERK, the kinase mTOR, and the protein Akt were unaffected to PEVs treatment (Fig. 15C).

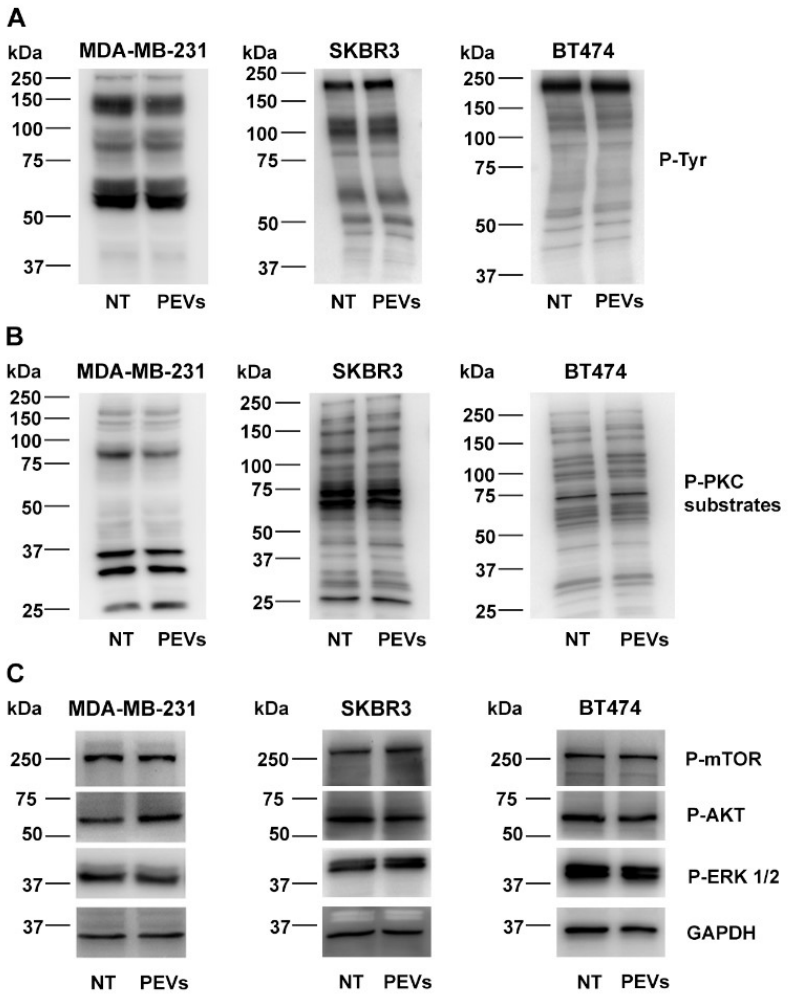

FIGURE 15. ***PEVs did not alter total protein tyrosine phosphorylation, PKC-dependent protein phosphorylation, and phosphorylation of mTOR, ERK and Akt.***

Protein tyrosine phosphorylation ***(A)*** *and phosphorylation of PKC substrates* ***(B)*** *were evaluated by immunoblotting with specific antibodies on whole cell lysate from MDA-MB-231, SKBR3 and BT474 cells left untreated (NT) or incubated with PEVs for 24 hours. Representative immunoblots are reported.*

(C) *Phosphorylation of the indicated selected signalling proteins in MDA-MB-231, SKBR3 and BT474 cells incubated with PEVs for 24 hours. Representative immunoblots with specific anti-phosphoprotein antibodies directed against the different substrates are reported, where GAPDH staining is for equal loading control.*

Interestingly, PEVs induced a strong phosphorylation of p38MAPK and myosin light chain 2 (MLC2) in MDA-MB-231 cells (Fig. 16A).

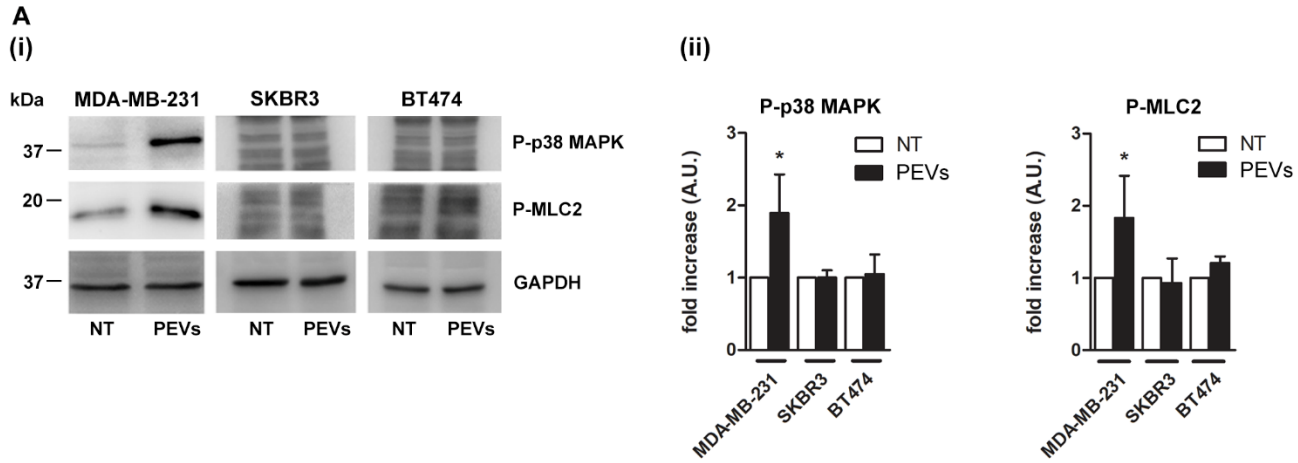

FIGURE 16.

(A) PEVs induce intracellular signalling responses.

*Phosphorylation of p38MAPK and MLC2 in MDA-MB-231, SKBR3 and BT474 cells incubated with 30 µg/ml of PEVs for 24 h. Representative immunoblots are reported in **(i)**, where GAPDH staining is for equal loading control. Quantification of the results performed by densitometric scanning is reported in **(ii)**, as fold increase (A.U.) of phosphorylation over basal (NT). Results are the mean ± SD of three different experiments. * $p < 0.05$.*

Searching the p38MAPK and MLC2 phosphorylation in intact PEVs, neither were found to be phosphorylated, indicating that the phosphorylated proteins were not simply delivered to recipient cells by PEVs (Fig. 17A). The kinetic of phosphorylation of the two proteins was analysed and it was different (Fig. 17B). The phosphorylation of p38MAPK raised after 30 min of treatment with PEVs and it was sustained for the entire time of incubation. Instead, phosphorylation of MLC2 was biphasic. It was detected more quickly, after 5 min of exposure to PEVs, decreased within 60 min and again peaked after 24 h of coculture (Fig. 17B). In agreement with the lack of effect on migration, no changes in the phosphorylation of either p38MAPK or MLC2 were detected in SKBR3 and BT474 cells upon treatment with PEVs (Fig. 16A).

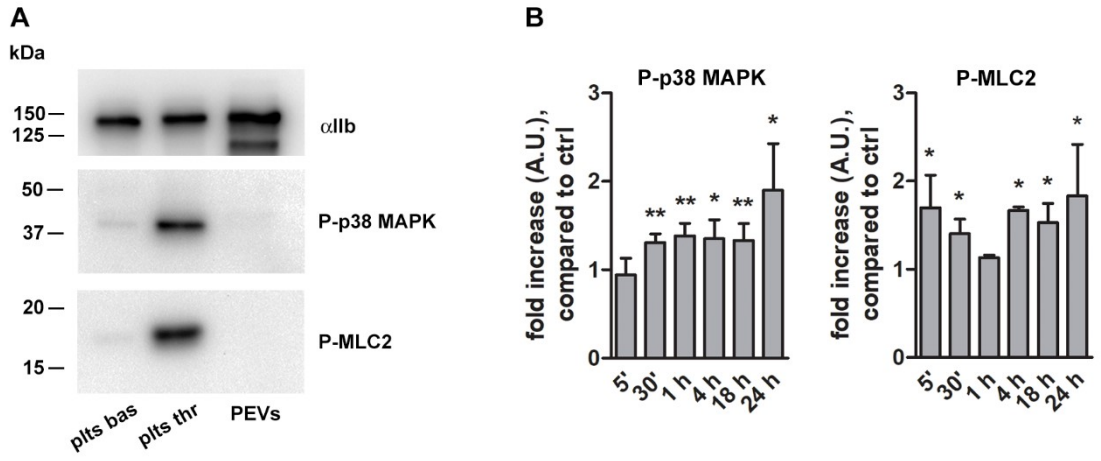

FIGURE 17.

(A) p38MAPK and MLC2 are not phosphorylated in PEVs.

Immunoblotting analysis of the phosphorylation of p38MAPK and MLC2 in basal platelets (plts bas), thrombin-stimulated platelets (plts thr) and released PEVs was performed with specific antibodies. Integrin αIIb staining was performed for equal loading control.

(B) Time-course of p38MAPK and MLC2 phosphorylation induced by PEVs.

*Phosphorylation of (A) p38MAPK and (B) MLC2 in MDA-MB-231 cells incubated with 30 µg/ml of PEVs from 5 minutes to 24 hours was analysed by immunoblotting. The densitometric analysis is reported as fold increase of phosphorylation compared to untreated control samples analysed at the same time points. Results are the mean ± SD of three different experiments. * $p < 0.05$ and ** $p < 0.01$.*

MLC2 is phosphorylated downstream of the Rho-associated protein kinase (ROCK) pathway. Interestingly, we observed that ROCK inhibition by selective Y27632 inhibitor did not alter the internalization of PEVs (Fig 18A) but the migration of MDA-MB-231 cells induced by PEVs was reduced (Fig. 18B).

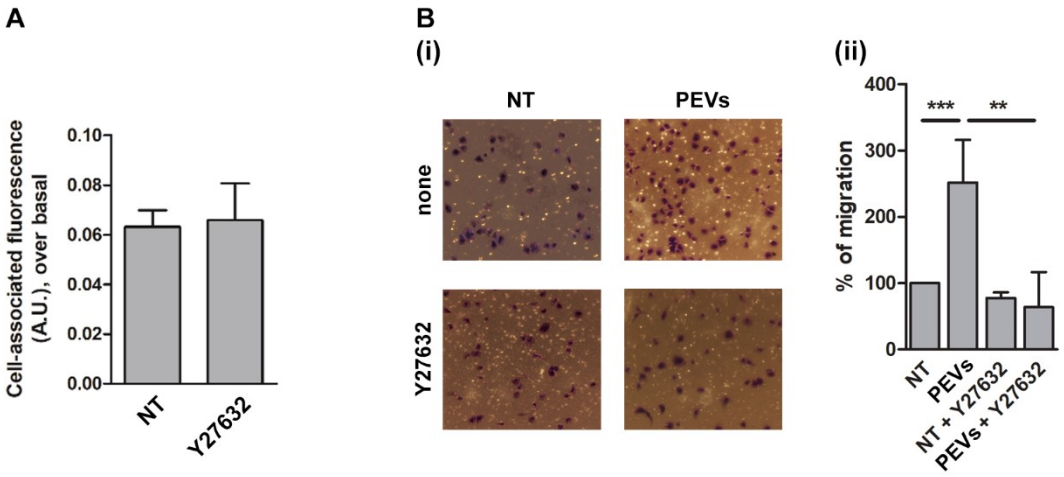

FIGURE 18. ***Y27632 inhibitor did not alter the internalization of PEVs but inhibits PEVs-induced migration of MDA-MB-231 cells.***

(A) *CFSE fluorescence labelled-PEVs (30 µg/ml) internalization by MDA-MB-231 cells was analysed by confocal microscopy and it was not inhibited by the selective ROCK inhibitor Y27632. The internalization analysis is reported as cell-associated fluorescence over basal (A.U). Results are the mean ± SD of three different experiments.*

(B) *Effect of the ROCK inhibitor Y27632 on PEVs-induced migration of MDA-MB-231 cells. Cells were let untreated (NT) or were treated with 30 µg/ml of PEVs in the absence or presence of 10 µM Y27632, as indicated.* ***(i)*** *Representative images of cell migration are reported.* ***(ii)*** *Quantification of the results expressed as percentage of cell migration. Data are the mean ± SD of three experiments. ** $p < 0.01$ and *** $p < 0.005$.*

Discussion and Conclusions

The most common malignance worldwide is breast cancer and despite forward steps in diagnosis and treatment, it remains the major cause of mortality [308]. A growing field in the cancer biology research is the investigation of the interplay between cancer cells and host microenvironment. It is fundamental understand the impact of host components to cancer progression, in this make possible design efficient and customized therapies. However, the complexity of this topic is increased by the large kind of subtypes of tumour. In 1968, Gasic et al. shown that platelets have been implicated in the regulation of cancer spreading [149], but only in 2014 Mezouar et al. proposed that PEVs are important mediators of this event [231]. Anyway, the effects of PEVs on cancer behaviour are not clearly understood and our current knowledge is still largely incomplete and partially controversial.

Our research group previously demonstrated that cancer cell lines are able to trigger the activation and aggregation of platelets and also stimulate the shedding of PEVs [168, 296]. These observations provide a possible explanation for the increased levels of circulating PEVs found in breast cancer patients [232]. Interestingly, we have demonstrated that PEVs generated by platelet-cancer cell interaction, may increase the metastatic phenotypes of cancer cells exploiting a novel positive feedback mechanism that can significantly improve the metastatic spread. However, *in vivo*, the PEVs release is also triggered by the stimulation with physiological agonists and a significant part of PEVs are not generated by directly interaction between platelets and metastatic cancer cells. PEVs are important procoagulant factors which support prothrombotic events [59].

In this work we have focused our attention on the effects of PEVs, which are generated by physiological stimulus, on cancer cells. We provided the evidence that these extracellular vesicles can regulate specific responses in different types of breast cancer cell lines.

PEVs were obtained after platelet stimulation with the strongest pro-thrombotic agonist, thrombin. We have analysed and compared PEVs functional interaction with four different breast cancer cell lines: BT474, MCF-7, SKBR3 and MDA-MB-231. They are characterized by different aggressiveness and were selected to represent distinct cancer subtypes [309]. MCF-7, SKBR3 and MDA-MB-231 cells are derived from metastatic site, whereas BT474 cells were from ductal carcinoma. MCF-7 cells and BT474 cells display strong cell–cell adhesions and a relatively low invasiveness in vitro, whereas the triple negative MDA-MB-231, and HER2-positive SKBR3 possess a more invasive phenotype [309, 310].

The doses of PEVs used in these experiments are set to microvesicles found in human plasma [304].

We have demonstrated, using different experimental approaches, that the four cell lines shown an ability to interact with thrombin-induced PEVs which is cell line-dependent. PEVs are internalized more efficiently by MDA-MB-231 and SKBR3 cells compared to BT474 cells, whereas MCF-7 cells were completely unable to interact with vesicles. These findings are consistent with previous observations indicating that morphological, molecular heterogeneity and different origin of breast cancer cells are fundamental differences in cell ability to bind extracellular vesicles [309, 311]. The understanding of precise molecular mechanism responsible for PEVs internalization by cells is a challenging task and further specific studies will be necessary to throw light on this event. Preliminary experiments focused on MDA-MB-231 cells, which are not reported in this work, indicated that vesicles binding to cancer cells was efficiently inhibited by heparin. However, our study clearly denotes a precise observation: different types of breast cancer cells interact differently with PEVs and, importantly, the interaction evolves in cell-specific functional responses. In fact, our results revealed that MDA-MB-231, SKBR3 and BT474 cell lines developed cell-specific responses upon exposure to PEVs.

PEVs evoked an increase in $[Ca^{2+}]_i$ in MDA-MB-231, SKBR3 and BT474 cells, which is dependent to Ca^{2+} influx. The effect was very quick and evidently anticipate PEVs internalization, as also revealed by time-course uptake experiments. It is likely, that this effect is triggered by the initial transient surface contact between PEVs and cancer cells, as shown upon the physical contact between exosomes and NIH3T3 cells [312]. As previously demonstrated, the $[Ca^{2+}]_i$ increase was associated to heavy changes in cancer cell phenotype [313-315]. MTT assay shown that PEVs significantly reduced the activity of mitochondrial dehydrogenases in SKBR3 cells. The reduction of MTT signal after PEVs incubation was less pronounced in BT474 cells, whereas MDA-MB-231 cells were unaffected. The inhibition of mitochondrial function by PEVs was previously observed in murine lung and colon cells [300]. The activity of mitochondrial dehydrogenases is often used to evaluate cell viability. However, by flow cytometry upon cell staining with propidium iodide, we found that the exposure to PEVs did not alter the viability of living SKBR3 and BT474 cells and the results were confirmed during cell cycle analysis. The study of cell cycle did not reveal any effect of PEVs on the accumulation of cells in the pre-G1 phase, which is an indication of cell death. Although these results seem controversial, they were not completely unexpected. In fact, it was previously demonstrated that mitochondrial disfunctions are not necessarily associated to a reduction of cell viability [316].

It was demonstrated that PEVs released by platelets stimulated with fatty acids caused important alterations in the cell cycle of triple-negative BT549 breast cancer cell line [299]. Conversely, our results show that thrombin-induced PEVs did not cause any changes in cell cycle progression or proliferation of MDA-MB-231 cells. Moreover, the same vesicles induced only minor changes in cell cycle progression of SKBR3 and BT474 cells, without affecting the overall proliferation rate. The first explanation for these different results is the different cellular models adopted. The other one is the composition of PEVs which is closely related by the stimulus that induced their generation [59]. It cannot be excluded that thrombin-induced PEVs and fatty acid-induced PEVs can elicit radically different responses.

The strongest functional response raised by thrombin-induced PEVs observed in our study was the potentiation of migration and invasiveness of MDA-MB-231 cells. Conversely, PEVs did not alter the migration of BT474 and SKBR3 cell lines. Therefore, although the three cell lines displayed the ability to internalize PEVs, only the migration of MDA-MB- 231 cells was significantly influenced.

The mechanism involved in this cell-specific event need to be elucidated, but we observed that the intracellular signalling pathways involving P38MAPK and the ROCK-MLC2 axis is stimulated. The functional interaction between PEVs and cancer cells is highly complex and this is documented by the fact that MDA-MB-231 migration is also inhibited by aspirin, suggesting that cancer cell response to PEVs may be regulated by prostanoids.

The results clearly show that the interaction of PEVs with MDA-MB-231, SKBR3, and BT474 cells equally triggered an initial $[Ca^{2+}]_i$ increase, but subsequently evolves toward functional responses that are cell-specific. The mechanism involved in these different responses is not understood, but it is possible to speculate that the responses may be related to the ability of cells to internalize PEVs.

In this respect, MDA-MB-231 and SKBR3 cells, which displayed a stronger efficiency in PEVs internalization, shown the strongest functional responses upon co-culture with PEVs. However, although the two events are concomitant, we did not collect any direct evidence to prove that the functional responses observed are consequent to PEVs internalization.

The diverse effects evoked by PEVs on cell cycle progression in different cell lines is probably due to several factors which required further investigations for precise characterization. However, in SKBR3 cells, the decrease of mitochondrial dehydrogenases activity and the slower rate of progression through S phase induced by PEVs may be related, as the energetic metabolism is known to regulate cell cycle [317].

Interestingly, MDA-MB-231 cells displayed a pronounced increase in $[Ca^{2+}]_i$ compared to SKBR3 and BT474 cells and this finding suggests a correlation between the magnitude of the initial Ca^{2+} peak and the downstream responses of protein phosphorylation and cell migration. The initial Ca^{2+} response reflects the typical IP3-dependent Ca^{2+} mobilization from the endoplasmic reticulum after extracellular stimulation in cancer cells [314, 315] and pharmacological manipulation confirmed that this the case also upon exposure to PEVs. Notable, it was demonstrated that IP3-dependent Ca^{2+} release controls cancer cell migration by involving p38MAPK[318] and MCL2 [319].

Our work demonstrated that the nature of recipient cell strongly influences the effects of endogenously generated PEVs. Within the same type of cancer model, different cancer cell lines displayed radically different responses elicited by to the same population of PEVs (Fig. 19). In conclusion, further studies are necessary to understand the complexity of the interplay between platelets and cancer and caution should be used before proposing a pro-tumour versus an anti-tumour function of PEVs.

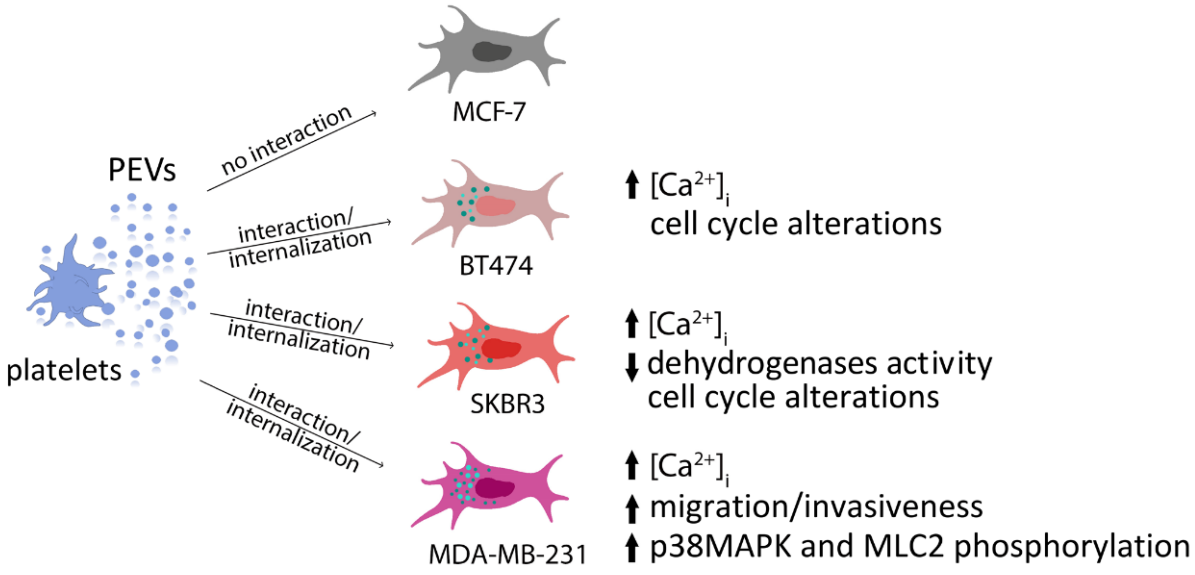

FIGURE 19. ***Schematic summary of the main effects of PEVs.***
PEVs released by activated platelets regulate selective responses in different types of breast cancer cell lines (MCF-7, BT474, SKBR3 and MDA-MB-231).

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