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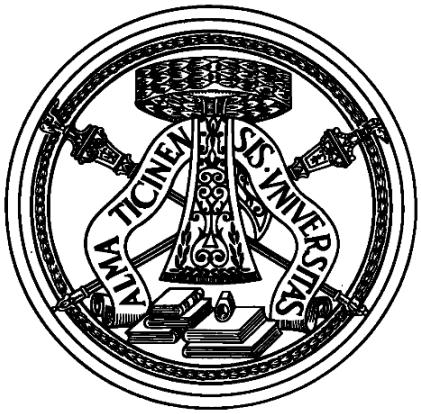

The crosstalk between blood platelets and cancer: molecular mechanisms and functional effects

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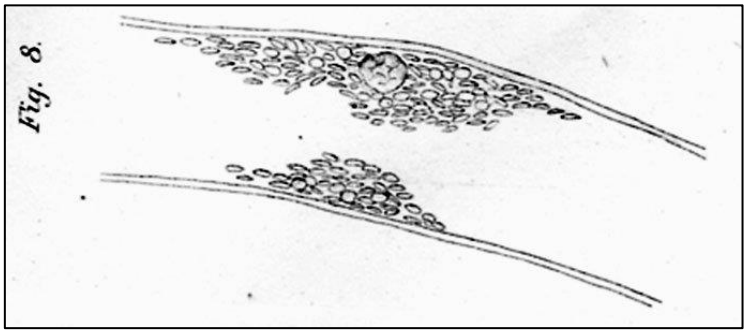
Introduction

The platelet-cancer interaction: ancient roots of modern science

The major role of blood platelets in preventing bleeding upon vascular injuries has known since the late nineteen century. In 1882, only a few years after their first identification as normal constituents of the blood, the Italian medical researcher Giulio Bizzozero demonstrated that platelets were involved in the formation of blood clots, and that they were able to promptly bind the site of endothelial damage and support the accumulation of fibrin. The original illustrations included in the work of Bizzozero (see figure 1) are profoundly suggestive and perfectly summarize the central role of platelets in hemostasis and thrombosis (Brewer 2006).

Figure 1



Thrombi observed in mesenteric artery of a guinea pig from the publication: Bizzozero (1882) Ueber einen neuen Forrnbestandteil des Blutes und dessen Rolle bei der Thrombose und Blutgerinnung. Archiv für pathologische Anatomie und Physiologie und für klinische Medicin, 90, 261–332.

Starting from Bizzozero's discoveries, platelet structure and function have been deeply analyzed using different scientific and technical approaches, and this effort allowed to obtain a rather precise description of the mechanism supporting the contribution of platelets to hemostasis and thrombosis. The investigation of platelet biology, however, clearly suggested that the function of these cells goes far beyond their role in limiting hemorrhage and supporting thrombotic events. It is now clear that circulating platelets participate to several physiological processes, including inflammation, blood/lymphatic vessel separation in the embryo, and host defense against microbial infections. Importantly, blood platelets are also involved in different pathological conditions and, in particular, play critical roles in cancer evolution (Leslie 2010, Meikle et al., 2017).

The first evidence of the existence of a strong link between the hemostatic system and cancer precedes the discovery of platelets and dates back to 1860, when French physician Armand Trousseau noted that cancer patients were often characterized by a generalized hypercoagulable state. Now it is very clear that venous thromboembolism (VTE) might be a clinical manifestation of an occult tumor. However, thrombosis is not merely a marker for the presence of a malignancy, rather it can represent a relevant cause of death for cancer patients. The mechanisms that support

thrombosis in cancer are only partially known, but it has been proposed that cancer-dependent platelet activation plays a central role in this process (Falanga et al., 2017).

Interestingly, the cross-talk between tumor and hemostasis is bidirectional: as mentioned above tumor can cause thrombotic events, but at the same time the components of the hemostatic system, and platelets in particular, also support cancer development (Xu et al., 2018). In the last few years, the cancer-platelet interplay has gained a great interest in the scientific community, which aims to fully understand the molecular mechanisms of platelet-mediated support to cancer, in order to identify novel adjuvant oncological therapies.

The following sections of this chapter will describe the biology of platelets and the complex relationship existing between these cells and cancer progression.

The biology of blood platelets

Platelet structure

Blood platelets are small anucleated cells that, in humans, have a diameter ranging from 2 to 4 μm and circulate in blood at a concentration of 150.000-400.000/μl. Platelets are produced in the bone marrow by polynucleated progenitors named megakaryocytes and survive for approximately 10 days in the circulation, before being destroyed in spleen and liver (Broos et al., 2011).

In resting conditions platelets have a discoid shape and are surrounded by an asymmetric plasma membrane that contains negatively charged phospholipids, as phosphatidylinositol and phosphatidylserine, in the inner leaflet. The exposure of negatively charged phospholipids to the outer leaflet during platelet activation is fundamental for the correct assembly of the activating complex of the coagulation cascade (Tenase and Prothrombinase), which culminate with thrombin production and fibrin accumulation. The platelet plasma membrane also contains several glycoproteins, including receptors for soluble agonists and adhesion molecules which are critical for platelet adhesion and activation (Gear and Polanowska-Grabowska, 2002).

Two different intracellular membrane systems are found in platelets: the open canalicular system (OCS) and the dense tubular system (DTS). OCS originates from invaginations of the plasma membrane and increases the platelet available surface area. The DTS is a closed system that derives from the endoplasmic reticulum of megakaryocytes and serves primarily as a store for calcium ions (Ca^{2+}) (Smyth et al., 2012).

In addition to mitochondria, lysosomes and peroxisomes the platelet cytosol contains two types of platelet-specific granules: α-granules, that store membrane receptors, growth factors and pro-aggregating soluble proteins, and dense-granules, which contain platelet–activating soluble factors such as Ca2+, ADP, ATP and serotonin (Reed, 2004; McNicol et al., 1999). The release of of α-granules and dense granulesin the extracellular environment during platelet activation leads to the amplification of platelet-dependent responses by autocrine/paracrine mechanisms (Broos et al., 2011). Platelets are also characterized by a complex cytoskeletal system composed by actin- and tubulin-based fibers that also provide a contractile system that, during the different phases of platelet activation, governs platelet shape change and spreading, emission of filopodia and lamellipodia, and granule secretion (Rendu and Brohard-Bohn, 2002).

Platelets in hemostasis and thrombosis

Under physiological conditions, platelet activation is triggered by injuries of the vessel wall and their main function is to support primary hemostasis. The precise mechanisms promoting hemostasis and the relative contribution of platelets to the coagulation cascade are strongly influenced by the type of injured vessel and the local rheological conditions. In any case, upon a vessel injury platelets arrest at the site of an endothelial damage and, through a process of cell-cell

interaction named platelet aggregation, create platelet plug that acts as the first barrier that prevents excessive bleeding. This initial process, named primary hemostasis, is then followed by secondary hemostasis, where the activation of the coagulation cascade culminates with the production of thrombin, a serine protease that converts soluble fibrinogen into insoluble fibrin and, in addition, 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 hemostasis are characterized by the retraction of the clot, which largely depends on the contractility of platelet cytoskeleton, and vessel repair.

Platelet function is therefore essential for preventing blood loss during hemostasis, but on the other hand, platelet hyper-activation caused by pathologic events, such as the rupture of an atherosclerotic plaque or atrial fibrillation-associated turbulent flows, can trigger undesired intravascular platelet aggregation. This may lead to the formation of an occlusive plug, or thrombus, in an intact vessel, thus reducing blood flow and preventing the correct supply of oxygen and nutrients to downstream tissues. Thrombosis occurring in heart and brain can cause dramatically severe conditions as myocardial infarction or stroke (Varga-Szabo et al., 2008).

The molecular mechanisms supporting hemostasis and thrombosis are largely overlapped, and the understanding of these processes opened the way to the identification of important antithrombotic targets.

The biochemistry of platelet activation

The mechanism of platelets activation that supports hemostasis has been deeply investigated for several years and a rather precise map of the major molecular players of platelet biology is now available. As also often reported in the literature, the complexity of platelet function will be here described by considering three main consecutive and interconnected phases of primary hemostasis: platelet adhesion, platelet activation and platelet aggregation.

Platelet adhesion

Upon a vessel wall damage due to a trauma or a pathological condition, platelets are arrested at the site of injury, in a process known as platelet adhesion. This process is mediated by the binding of platelet membrane receptors to several adhesion macromolecules that compose the exposed subendothelial matrix, in particular collagen, von-Willebrand factor (VWF), laminin, fibronectin and thrombospondin (Farndale et al., 2003).

In small arteries, which are characterized by fast blood flow and high shear rates, the initial platelet tethering to the site of injury is mediated by the interaction of VWF with the platelet membrane glycoprotein complex Ib-IX-V (GPIb-IX-V). VWF is a multimeric, adhesive glycoprotein secreted by endothelial cells and platelets, that accumulates at site of endothelial damage through the binding to fibrillar collagen and becomes able to recruit platelets from the circulation. The reversible binding of GPIb-IX-V to VWF 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 and specific platelet receptors (Broos et al., 2011).

At low shear rates, typical of the slow flows in large vessels, the GPIb-IX-V/VWF binding is not required and platelet are arrested at the site of injury by the direct interaction with collagen, fibronectin and laminin. Collagen is the major subendothelial adhesion molecule for which platelets express two main receptors: glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$. GPVI displays a low-affinity for collagen, is unable to mediate firm adhesion, but it is responsible of initiating a potent ITAM-based signal transduction cascade. Integrin $\alpha_2\beta_1$ supports the stable binding of platelets to collagen in a Mg^{2+}-dependent manner, but also triggers tyrosine phosphorylation of several signaling molecules, supporting initial platelet activation (Varga-Szabo et al., 2008; Nuyttens et al.,2011). Platelets also express integrin $\alpha_5\beta_1$ that binds fibronectin, integrin $\alpha_6\beta_1$ that interacts with laminin, and integrin $\alpha_V\beta_3$ that binds with vitronectin. However, the most important platelet receptor is integrin $\alpha_{IIb}\beta_3$ which is critical for platelet aggregation, but also mediates platelet adhesion to VWF, fibronectin and immobilized fibrinogen.

Platelet adhesion, supported by the different membrane receptors, allows platelet arrest at the site of vascular damage, but also initiates platelet activation that, through complex signal transduction pathways, sustains important platelet responses such as granule secretion, cell spreading and platelet aggregation (Broos et al., 2011).

Platelet activation

As mentioned above, platelet activation is initially mediated upon the interaction between membrane receptors and subendothelial matrix components. 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 signaling pathway is well characterized (Dütting et al., 2012). Upon interaction of GPVI with collagen, Src kinases phosphorylate the ITAM-bearing FcRγ-chain physically associated to the receptor. FcRγ-chain phosphorylation stimulates the assembly of a multiprotein complex that includes LAT, SLP-76 and the tyrosine kinase Syk, which promotes a signaling cascade that eventually culminates with the activation of phospholipase C γ2 (PLCγ2) (Watson et al., 2001). PLCγ2 is activated downstream most receptors, including GPIb-IX-V and integrins and it represents a critical intersection of adhesion-mediated platelet activation (Broos et al., 2011).

Active PLCγ2 hydrolyzes the phosphatydilinositol 4,5-bisphosphate (PIP_2) producing two important second messengers: inositol 1,4,5-trisphosphate (IP_3), which induces the release of Ca^{2+} from platelet DTS, and 1,2-diacylglycerol (DAG), that stimulates effectors that contain a C1 domain, including classical and novel protein kinase C (PKC) isoforms and the guanine nucleotide exchange

factor CalDAGGEFI. PKC phosphorylates different substrates on serine and threonine residues and regulates several aspects of platelet activation including granule secretion, whereas CalDAGGEFI directly supports platelet aggregation (Varga-Szabo et al., 2009). Also phosphatydilinositol 3-kinases (PI3Ks), that phosphorylate phosphoinositide lipids at the 3 position of the inositol ring, contribute to platelet activation. In particular class I PI3Ks, that preferentially phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) producing phosphatidylinositol 3,4,5-trisphosphate (PIP3), plays a key role in platelet function. Indeed, PIP3 recruits at the level of plasma membrane several proteins containing a PIP3-binding domain, including 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) (Laurent et al., 2014).

Among the consequences of platelet activation induced by adhesion receptors there is the release of different soluble agonists. Through an autocrine and paracrine mechanism, these molecules potentiate the activation of adherent platelets and also recruit additional circulating platelets to the site of injury.

Activated platelets synthesize and release thromboxane A2, which is produced through the sequential enzymatic activities of phospholipase A2 (PLA2), that liberates arachidonic acid from the plasma membrane, cyclooxygenase (COX) and thromboxane synthase. The release reaction is also largely supported by granules secretion, which liberates several pro-aggregating molecules in the proximity of the vascular damage. Among these molecules, ADP released from dense granule is crucial for hemostasis as it triggers signals required for full platelet aggregation. Also α-granule secretion contributes to release pro-aggregating factors, such as fibrinogen and VWF, in the intracellular environment and, importantly, it also increases the exposure of membrane receptors, including integrin $\alpha_{IIb}\beta_3$ and P-selectin on the platelet surface (Reed et al., 2004).

Platelet activation initiated by adhesion receptors and potentiated by soluble agonists is accompanied to the exposure of negatively charged phospholipids on the outer leaflet of plasma membrane. Platelet negative surface offers a support for the catalytic conversion of the zimogen prothrombin into the active serine protease thrombin by the prothrombinase complex. In turn, thrombin acts as a potent platelet soluble agonist and simultaneously supports the process of blood clotting by converting soluble fibrinogen into insoluble fibrin (Kaplan and Jackson, 2011).

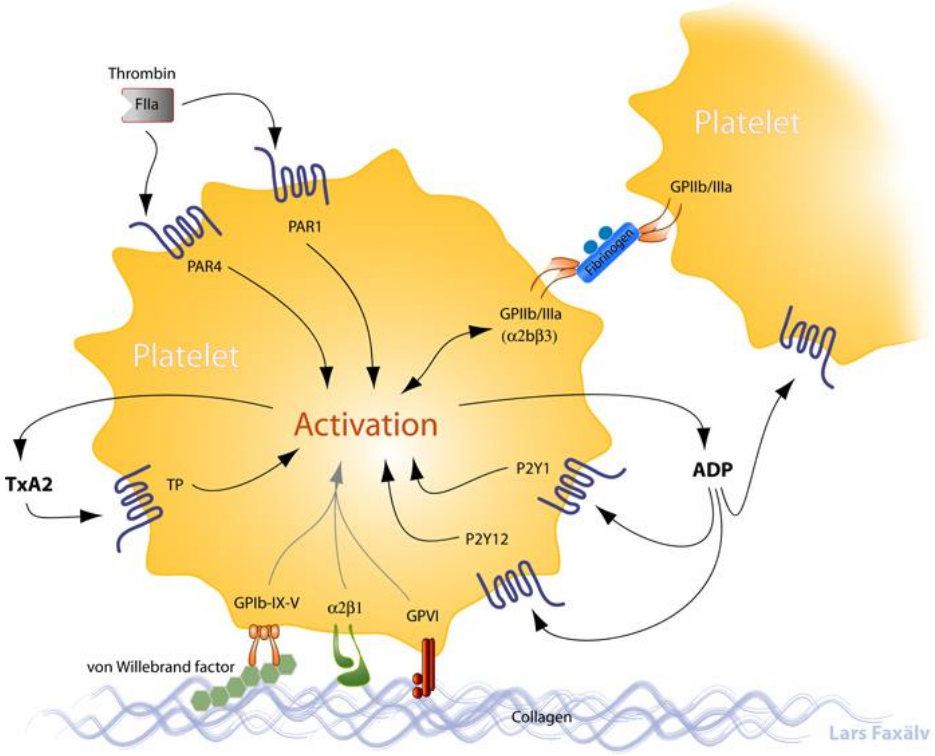
All the major soluble agonists (thrombin, thromboxane A2 and ADP) stimulate specific membrane G protein-coupled receptors (GPCRs), which are seven-span transmembrane receptors associated to heterotrimeric G proteins, composed by a guanine nucleotide-binding subunit named Gα and a βϒ dimer. The binding of GPCR to the specific ligand replaces GDP bound to Gα with GTP, and induces the dissociation of Gα from the βγ dimer. Both Gα and βγ dimer are able to support signal transduction and regulate different effectors. Different subtypes of activatory heterotrimeric G proteins are expressed in platelets (G_q, G_{13} and G_i) and they are associated to different receptors for

soluble agonists. G_q and G_{13} can be coupled to thrombin protease-activated receptors PAR1 and PAR4, and the TxA_2 receptors. G_q can also be associated to ADP P2Y1 receptor, whereas ADP P2Y12 receptor is coupled to G_i. (Broos et al., 2012)

Stimulation of G_q-coupled receptors leads to activation of phospholipase Cβ (PLCβ), that, as seen before about PLCγ2, induces consequent Ca^{2+} increase and PKC activation by releasing IP_3 and DAG. Upon binding to specific agonists, G_{13}-coupled receptors activate the Rho/Rho kinase pathway, the phosphorylation of Myosin Light Chain (MLC) and control the reorganization of cytoskeleton. The activation of G_i downstream P2Y12 ADP receptor is critical to obtain a stable platelet aggregation. Active Gαi inhibits adenylyl cyclase and reduces the cytosolic concentration of cyclic adenosine monophosphate (cAMP), a potent intracellular inhibitor of platelet activation. βγ dimer coupled to P2Y12 receptor contributes to full platelet aggregation by activating a PI3K-dependent pathway which controls the conversion of integrin $\alpha_{IIb}\beta_3$ into the active state.

In addition to GPCRs that mediate activation, platelets also express prostaglandin (PG)-specific GPCR which are responsible of maintaining platelet quiescence. *In vivo*, PGI_2 and PGE_2 released by undamaged endothelial cells bind Gs-coupled PG receptors on platelet surface. Gαs-mediated stimulation of adenylyl cyclase increases the intracellular concentration of cAMP. cAMP is an inhibitory second messenger that activates a cAMP-dependent protein kinase A (PKA) (Offermanns, 2006; Broos et al., 2011). Platelet are maintained in the resting conditions also by the action of nitric oxide (NO) released in the bloodstream by the action of endothelial nitric oxide-synthase (eNOS). NO is cell-permeable and directly stimulates the soluble intracellular enzyme guanylyl cyclase (sGC), which synthesizes cGMP that then induces the activation of protein kinase G (PKG). PKA and PKG phosphorylate and inhibit many critical enzymes involved in platelet activation, including PLCβ and CalDAGGEFI, and therefore cooperate to keep platelets in a quiescent state (Roberts et al., 2009, Guidetti et al.,2013).

Figure 2

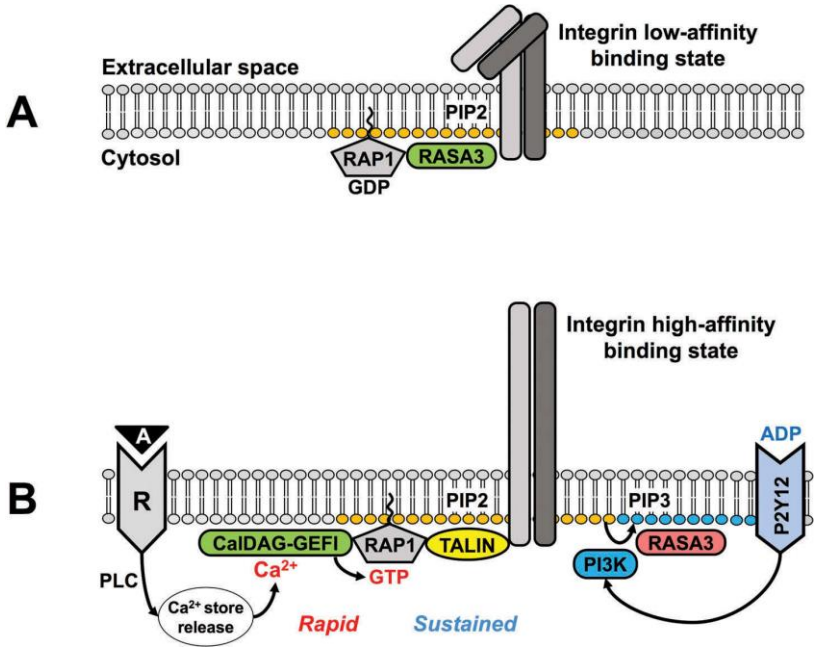


Summary of the main receptors that induce platelet activation (Scheme by Lars Faxälv, Linköping University)

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. Aggregation is supported by platelet-platelet interactions mediated by the binding of soluble fibrinogen to integrin $\alpha_{IIb}\beta_3$ expressed on adjacent cells. In quiescent platelets integrin $\alpha_{IIb}\beta_3$ is expressed in a conformation with low affinity for soluble fibrinogen (Vinogradova et al., 2002). Platelet activation promoted by the mechanisms described above, eventually culminates in the conversion of integrin $\alpha_{IIb}\beta_3$ into the active state with a high affinity for soluble fibrinogen, that acts as a bridge between platelets. This process is supported by a specific signal transduction pathway defined as integrin “inside-out” signaling (Broos et al., 2011). The central signaling event for platelet aggregation is the stimulation of PLC isoforms by adhesion receptors and GPCRs which leads to IP3-mediated Ca^{2+} increase. As mentioned above, Ca^{2+} directly stimulates CalDAGGEFI, which in turn induces the small GTPase Rap1b to exchange of GDP with GTP leading to its full activation. The active state of Rap1b is maintained by the PI3K-dependent inhibition of the Rap GTPase activating protein Rasa3 downstream of the stimulation of Gi-coupled P2Y12 receptor by ADP (Bergemeier and Stefanini 2018).

Figure 3



Scheme of integrin $\alpha_{IIb}\beta_3$ inside-out activation (Stefanini and Bergmeier (2018): RAP GTPases and platelet integrin signaling)

GTP-bound Rap1b, through the contribution of the cytoskeletal protein talin, triggers the conversion of integrin $\alpha_{IIb}\beta_3$ into the active state and the binding of soluble fibrinogen that mediates aggregation. The occupation of integrin $\alpha_{IIb}\beta_3$ by fibrinogen stimulates "outside-in" signaling pathways that further potentiate platelet activation and strengthen the platelet-platelet interaction, therefore avoiding thrombus embolization. Integrin $\alpha_{IIb}\beta_3$-dependent pathways also regulate the later phases of hemostasis, such as clot-retraction and vessel healing (Broos et al., 2011).

Platelets as therapeutic targets

Thrombosis has an important impact on worldwide morbidity and mortality and platelet activation and aggregation have a central role in development of arterial thrombosis. The use of drugs that prevent platelet activation, the so-called antiplatelet therapy, can limit the risk of thrombosis with overall limited side effects.

Antiplatelet drugs interfere with key events of platelet activation and aggregation and several platelet enzymes and receptors are considered potential candidate targets for novel antithrombotic therapies. Current antithrombotic therapies are based on three major classes of compounds: inhibitors of COX (aspirin), P2Y12 receptor antagonists and inhibitors of integrin $\alpha_{IIb}\beta_3$. Aspirin irreversibly inhibits COX1 and blocks the formation of prostaglandin H2, thus preventing TxA2 synthesis and its contribution to platelet stimulation.

Antagonists of P2Y12, such as clopidogrel, prasugrel, ticagrelor, and cangrelor, chemically block the interaction with ADP and prevent Gi-dependent signaling, resulting in strong impairment in

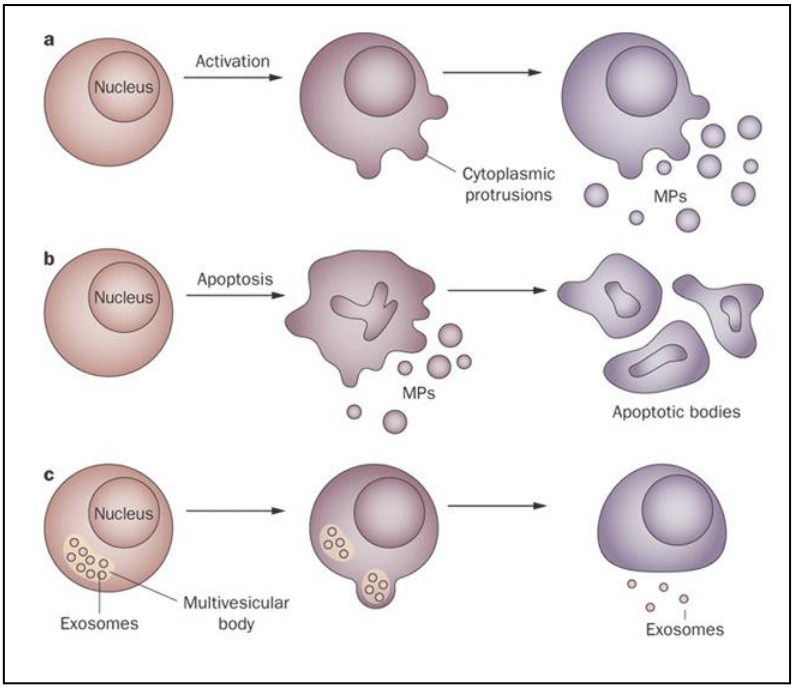
platelet responses including shape change, aggregation, and promotion of coagulation. P2Y12 antagonists and aspirin are often prescribed in conjunction in the dual antiplatelet therapy (DAPT). Inhibitors of integrin $\alpha_{IIb}\beta_3$ block the interaction between activated platelets and fibrinogen preventing aggregation. Currently used integrin $\alpha_{IIb}\beta_3$ inhibitors are the humanized moloclonal antibody abciximab, the small molecule triofiban, and the eptapeptide eptifibatide and they all must be administered intravenously (Koenig-Oberhuber and Filipovic 2016).

Platelet-derived microparticles (PMPs)

The formation and the composition of PMPs

Eukaryotic cells, but also plant and prokaryotic cells, can release in the extracellular environment different types of extracellular vesicles, including exosomes, microparticles (often referred to as microvesicles) and apoptotic bodies. Exosomes range between 50 and 150 nm in size, are stored multivesicular bodies and released by exocytosis. Microparticles have a larger diameter, approximately from 100 nm to 1 μm and are released through a mechanism involving plasma membrane budding and shedding. Apoptotic bodies are 1–5 μm in diameter, rich in phosphatidylserine on the outer membrane leaflet and are released by cells undergoing apoptosis. Extracellular vesicles are involved in intercellular communication and play important role in physiological and pathological processes including the control of cellular proliferation and differentiation, inflammation and blood coagulation, vascular function, and and mediate cell–cell communication (György et al., 2011).

Figure 4



Summary of the release of microparticles (a), apoptotic bodies (b) and exosomes (c). (from The role of microparticles in the pathogenesis of rheumatic diseases. Christian Beyer & David S. Pisetsky. Nature Reviews Rheumatology. 2010;6:21-29)

Blood contains extracellular vesicles of different origin and microparticles, in particular, are released by platelets, erythrocytes, endothelial cells, and leukocytes. Noteworthy, platelet-derived microparticles (PMPs) are the most abundant in blood, where they represent approximately 70% to 90% of circulating microparticles (Arraud et al., 2015, Mezouar et al., 2014). Moreover, several

diseases including heparin-induced thrombocytopenia, arterial thrombosis, sickle cell disease, rheumatoid arthritis and cancer, are associated to elevated PMPs levels (Italiano et al., 2010).

Platelet activation induced by physiological stimuli, such as thrombin, collagen, and lipopolysaccharides, as well as by non-physiological agonists such as the Ca^{2+} ionophore, is accompanied by the release of PMPs. Additional processes, including platelet storage, exposure to shear, exposure to complement proteins, and platelet apoptosis can be associated to increase of PMPs release (Boilard et al., 2015). Importantly, the size of PMPs strongly depends on the environmental conditions that promoted their production (Italiano et al., 2010). PMPs released in response to platelet activation by thrombin or Ca^{2+} ionophore range from 100 to 500 nm in size, whereas two rather distinct groups of PMPs (that measure between 80 and 200 nm, and between 400 and 600 nm, respectively) can be recovered upon platelet stimulation with collagen. In response to complement-induced activation, platelets release PMPs with a size up to 1 μm in diameter (Dean et al., 2009).

In general, PMPs contain different families of proteins (membrane receptors, signaling proteins, enzymes, proteases), nucleic acids (including mRNA and miRNA), cytokines, lipids, growth factors, and other components. As mentioned for the size, also the precise composition of PMPs in term of protein and lipid content, as well as of molecular surface exposure, depends on the different experimental conditions adopted to induce platelet activation (Plantureux et al., 2018, Shai et al., 2012).

Platelet membrane receptors GPIb-IX-V, P-selectin and integrin αIIbβ3 are commonly expressed on the PMPs surface. Depending on the nature of the stimulus that induced PMPs release, integrin αIIbβ3 can be present either in the inactive or active conformation, and the latter state facilitates the interaction of PMPs with activated platelets or with the endothelium. It has been proposed that the expression of active integrin αIIbβ3 on PMPs may be important for the development of thrombotic conditions (Owens & Mackman, 2011). The ability of PMPs to participate to thrombotic disorders may also derived by the expression of negatively charged phospholipids, such as phosphatidylserine, on the outer surface. As previously described about platelets, the exposure of negatively charged phospholipids on PMPs supports a strong procoagulant activity, as it catalyzes the assembly and activation of enzymatic complexes, eventually potentiating the production of thrombin and fibrin formation (Owens and Mackman, 2011).

Moreover, PMPs contain bioactive lipids that act as second messengers, as sphingosine 1-phosphate or second messengers like arachidonic acid (Plantureux et al., 2018). Phospholipase A_2 promotes the release of arachidonic acid from PMPs membrane, which then can be converted to TxA_2 promoting the transactivation of platelets and endothelial cells (Barry et al.,1997).

PMPs in intercellular communication

PMPs interaction with target cells represents an important mechanism of intercellular communication by exchanging biological signals and information. The mechanisms that allow the

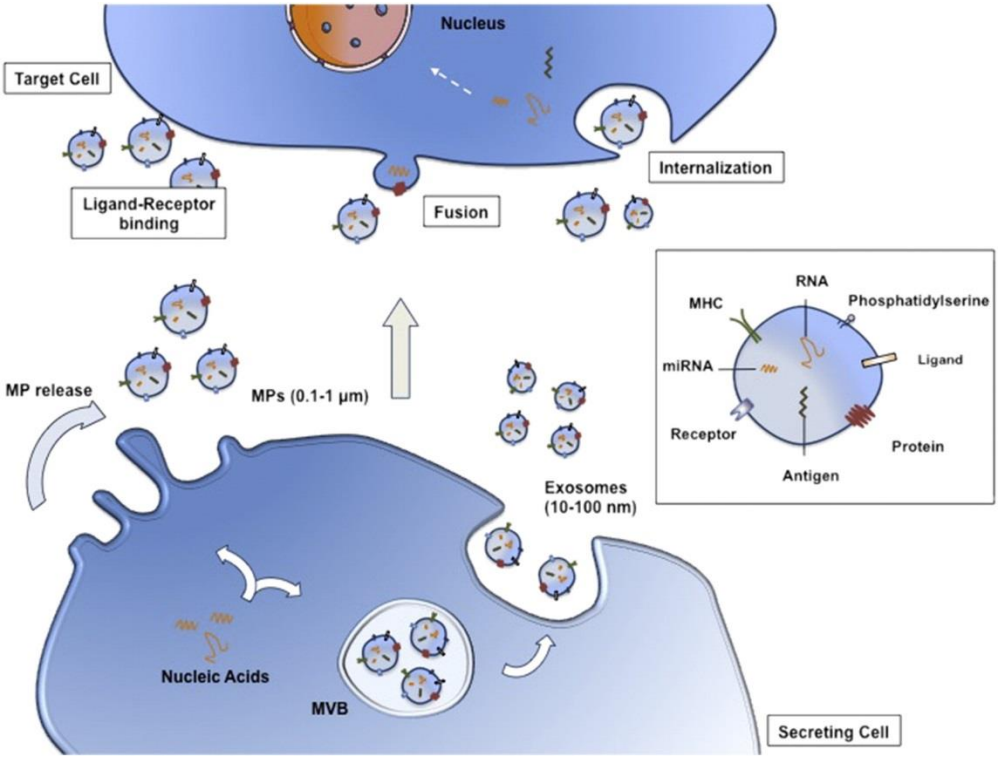
interaction between PMPs and target cells and support intercellular communication are only partially understood, but several important aspects have been investigated.

Molecules expressed on the surface of PMPs, such as lipids and membrane-associated proteins can directly stimulate functional responses to target cells, but PMPs can also be internalized, or membrane fusion between PMPs and recipient cell can occur, resulting in an active transfer of receptors, proteins, miRNAs and other molecules to recipient cells (Edelstein et al., 2017).The mechanism of interaction between PMPs and target cells is not well understood, but this process is considered particularly important, as it can promote relevant changes in target cell behavior.

If membrane fusion takes place, it results in the transfer of surface receptors from platelets, through PMPs, to recipient cells, thus providing part of the molecular machinery required to respond to extracellular stimuli that normally would have no effects on these cells (Mack et al., 2000; Morel et al., 2004). In this frame, the transfer of integrin αIIbβ3 to hematopoietic cells has been shown to facilitate the adhesion of these cells to fibrinogen (Baj-Krzyworzeka eta al., 2002) and the same receptor delivered to neutrophils enhances inflammation by enabling NF-κB signaling in response to Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) (Salanova et al., 2007). In addition, this process of plasma membrane fusion can confer a new immunological reactivity to target cells (Edelstein et al., 2017).

Membrane fusion and vesicles internalization also allow the transfer of PMPs content into target cells. In particular, the delivery of miRNA be extracellular vesicles appears to play a pivotal role in cell-to-cell communication and opened the way for novel therapeutic strategies (Edelstein et al., 2017). It has been demonstrated that, through the release of PMPs, activated platelets can deliver regulatory miRNA (miR-233) associated to the protein Argonaute 2 to endothelial cells, resulting in the regulation of gene expression in these recipient cells (Laffont et al., 2013). PMPs are also involved in the regulation of angiogenesis and support different proangiogenic responses by stimulating endothelial cell proliferation, survival, migration, and tube formation, eventually resulting in the promotion blood vessels sprouting (Kim et al., 2004; Varon & Shai, 2009).

Figure 5



Summary of the mechanisms of vesicle interaction with recipient target cell (from Extracellular vesicles: Pharmacological modulators of the peripheral and central signals governing obesity Edward Milbank, M. Carmen Martinez, Ramaroson Andriantsitohainav. Pharmacology & Therapeutics 2016;157:65-83)

Several observations indicate that platelets can regulate immunity and PMPs were shown to activate adaptive immune cells triggering antibody synthesis. PMPs stimulate antigen-specific B cells resulting in IgG production and, in cooperation with CD4+ cells, modulate germinal-center formation. Therefore, at site of platelet activation, the release of PMPs can stimulate an immune response (Sprague et al., 2008).

The clinical relevance of PMPs

By transfer highly functional biomolecules that influence cell activation, inflammation, immunomodulation, and other processes PMPs are believed to play important role in the development of several pathological conditions and, as already outlined, many clinical disorders have found to be associated with elevated PMPs (Burnouf et al., 2014). As expected, PMPs were found to be involved in thrombosis and hemostasis, but they are also key actors in the development of atherosclerosis, infectious diseases, immune thrombocytopenic purpura (ITP), rheumatoid arthritis, systemic Lupus Erythematosus (SLE), and cancer (Burnouf et al., 2014, Italiano et al., 2010). The importance of platelets and PMPs in cancer evolution and spread will be thoroughly discussed in the following chapter.

As mentioned above, PMPs are important for hemostasis as they bear platelet receptors and pro-coagulant negatively charged phospholipids on their surface. Defects in PMPs generation were

shown to be associated to coagulation defects and bleeding diathesis (Castaman et al., 1996, Italiano et al., 2010). Considering their procoagulant properties, PMPs can also contribute to thrombotic diseases. Accordingly, patients that experienced myocardial infarction and coronary diseases display increased levels of circulating PMPs that are believed to potentiate coagulation and thrombus formation (Boulanger et al., 2006). Moreover, the levels of PMPs are considered clinical indicators of cerebral atherothrombosis (Kuriyama et al., 2010) and reflect the severity of platelet activation during myocardial ischemia (Jung et al., 2012).

Platelets and PMPs play important roles in atherosclerosis, which is a major cause of arterial thrombosis and, ultimately, a leading cause of death worldwide. PMPs may have important effects on vasculature as they can interact with endothelial cells, as well as with the subendothelium at the level of the growing plaque. In turn, bound PMPs recruit activated platelets and leukocytes to the area of endothelial injury, and modulate monocyte–endothelial interactions, resulting in the exacerbation of atherogenesis (Forlow et al., 2000, Burnouf et al., 2014). In addition, PMPs may facilitate smooth muscle proliferation, completing the formation of atherosclerotic plaque (Fressynet 2003).

Recently, growing evidence have suggested a possible contribution of PMPs to inflammatory and autoimmune diseases, including rheumatoid arthritis, ITP, and SLE (Italiano et al., 2010, Burnouf et al., 2014.

Rheumatoid arthritis is systemic inflammatory disease involving both adaptive and innate immune system, that mostly affects synovial joints. Patients suffering of rheumatoid arthritis present inflammation of the joint synovium and blood vessels dilation, resulting in joints swollen, pain and stiffness. Platelets accumulated in the joints of patients are crucial for the development of inflammatory arthritis. PMPs generated locally through a GPVI/FCRγ-chain-dependent mechanism can activate the fibroblast-like synoviocytes, stimulating inflammation (Boilard et al., 2010).

ITP is an autoimmune disease characterized by low platelet count often due to the production of antibodies directed against platelet surface, including integrin αIIbβ3 and GPIb-IX, which accelerate platelet clearance (Semple 2002). The contribution of PMPs to the evolution of ITP is controversial as some patients may have an increased thrombotic risk that appear to correlate with higher levels of PMPs (Sewify et al., 2013, Jy et al., 1992), whereas elevated PMP concentration may be associated to protection against severe bleeding events in pediatric ITP patients (Tantawi et al., 2010).

Platelets have emerged as major actors in SLE and they appear to contribute to the disease through different mechanisms. SLE is characterized by lymphocyte dysregulation, production of autoantibodies and defective clearance of circulating immune complexes and apoptotic cells, resulting in chronic inflammation and organ damage. The high levels of circulating PMPs found in SLE patients opened interesting opportunities of considering PMPs themselves as potential marker of the disease, however conclusive evidence about this possibility is still missing. It is also poorly understood whether or not PMPs can actively participate to the development of SLE, and recently it

has been proposed that the transfer of miRNAs from platelets to immune cells might contribute to the generalized pro-inflammatory state (Scherlinger et al., 2017). PMPs have been associated also to infectious diseases, including malaria, Dengue virus, prion disease and HIV (Burnouf et al., 2014), but the information available about the mechanism of PMPs contribution to these pathologies is still largely incomplete.

The platelet-cancer interplay

The close relationship of cancer and thrombosis

Thrombosis is a common complication in cancer patients and it critically contributes to morbidity and mortality associated with cancer (Elyamany et al., 2004; Khorana et al., 2007; Young et al., 2012; Falanga et al., 2017). The relationship between cancer and thrombosis was first reported in 1865 by the French doctor Armand Trousseau, who described migrans trombophlebitis as a forewarning of occult malignancy (Trousseau, 1865). After Trousseau discovery, multiple studies have provided evidence for a clinical association between cancer and higher risk of developing venous and arterial thrombosis (Blom et al., 2006). Subsequently, the term "Trousseau's syndrome" was coined to describe thrombotic event preceding or appearing concomitantly with cancer; today the syndrome is well known to clinicians and its definition was refined and extended to any thrombotic complications associated with cancer (Varki, 2007).

Clinical evidence suggests that cancer cells induce a prothrombotic switch of the host hemostatic system, and in turn, this tumor-induced activation of coagulation is intrinsically involved in tumor growth and metastasis (De Cicco, 2004). Cancer is widely considered a risk factor for VTE and accounts for 20-30% of VTE events. Cancer patients display a significantly higher risk of death because of VTE than the general population and they have worse prognosis and survival than cancer patients without VTE (Heit et al., 2002; Young et al., 2012). Cancer is associated to 4- to 7-fold increase in the risk of VTE and the majority of cancer patients, even without overt thrombotic events, present hypercoagulable state and coagulation abnormalities (Young et al., 2012; Falanga and Russo, 2012, Blom et al., 2006). However, the risk of VTE is not uniformly distributed across cancer types and it strictly depends on cancer site, cancer stage, and use of chemotherapy. Cancers with the highest rates of VTE include pancreas, kidney, ovary, lung, and stomach. Among hematologic malignancies, myeloma, non-Hodgkin lymphoma and Hodgkin disease have the highest rates of VTE (Silverstein et al., 1998; Heit et al., 2000; Khorana et al., 2007). Noteworthy, the association between idiopathic VTE and the subsequent development of clinically overt cancer is statistically significant (Prandoni et al., 1992; Carrier et al., 2008).

Pathogenesis of cancer-associated thrombosis

The pathogenesis of cancer-associated thrombosis is multifactorial and not fully understood. The prothrombotic phenotype of cancer patients results from the concomitant activity of factors belonging to the so called "Virchow's triad": stasis of blood, vascular injury and blood hypercoagulability (Boccaccio and Comoglio, 2009). Blood stasis may be caused by blood vessel compression exerted by the growth of the tumor mass. In addition, patients with advanced-stage cancer often have reduced performance status and are more predisposed to bed rest, thus allowing relative venous stasis and reduced clearance of activated clotting factors (Lip et al., 2002; Young et al., 2012). Vascular injury may result from direct tumour invasion, drugs, therapeutic devices, and

other causes (Boccaccio and Comoglio, 2009; Young et al., 2012). Finally, tumour cells can activate blood coagulation through multiple mechanisms, including expression of procoagulant proteins, release of proinflammatory and proangiogenic cytokines, and direct interaction with host cells, such as endothelial cells and platelets (Noble and Pasi., 2010). Moreover, extrinsic factors (e.g. patient-related characteristics, surgery, chemotherapy, central venous catheter, radiotherapy) can contribute to the enhancement of the prothrombotic process (De Cicco, 2004; Falanga et al., 2012; Young et al., 2012)

Cancer-associated pro-thrombotic factors

Cancer cell may release procoagulant substances able to activate the coagulation system, as well as inflammatory cytokines (e.g. Tumour Necrosis Factor, Interleukin-1) and proangiogenic factors (e.g. Vascular Endethelial Growth Factor) which can stimulate the prothrombotic feature of vascular cells (De Cicco, 2004; Young et al., 2012; Falanga et al., 2017).

Tissue Factor (TF) is the best characterized procoagulant molecule expressed by cancer cell and it is widely considered as the major molecular driver of cancer-associated thrombotic disorders. TF is a transmembrane receptor constitutively expressed by cells surrounding blood vessels and, through its extracellular domain, binds and activates FVII thus initiating the extrinsic pathway of coagulation cascade (Mackman, 2009; Milsom and Rak, 2008). Several solid and hematologic tumors express TF, whose level of expression correlates with thromboembolic complications and poor prognosis (Kakkar et al., 1995; Rickles et al., 1995; Young et al., 2012; Mitrugno et al., 2016). Several studies have proposed that the upregulation of TF in cancer cells is driven by the genetic events of neoplastic transformations (e.g. activation of MET, loss of PTEN, induction of K-ras, and loss of p53) (Rak and Klement, 2000; Milsom et al., 2007). For example, in colorectal cancer cells (CRC), TF expression is regulated at transcriptional and translational level by the proto-oncogene kRAS and the tumor suppressor p53 (Milsom et al., 2007). Similarly, in glioblastoma cells, TF upregulation is associated to the loss of the tumour suppressor PTEN (Rong et al., 2005). In addition, in a mouse model of tumorigenesis, procoagulant activity of cancer cells was enhanced by the oncoprotein MET (Boccaccio et al., 2005). TF expression is also upregulated by TGFβ through the induction of an epithelial to mesenchymal transition (EMT) in cancer cells. Finally, TF can be actively released by tumor cells in a cell membrane-associated form represented by TF-bearing MPs (Falanga et al., 2017). Patients with solid tumors (e.g. pancreas and breast adenocarcinoma, colorectal cancer), as well as in hematological cancers (acute leukaemias, multiple myeloma) present high level of circulating TF-positive MPs which correlates with high VTE rate (Tilley et al., 2008; Del Conde et al., 2007; Zwicker JI, et al., 2009). Cancer-derived MPs, in addition to TF, carry also high concentrations of procoagulant phosphatidylserine, thus representing another important mechanism of tumor-promoted clotting activation (Falanga et al., 2012).

Extensive studies on factors responsible for the hypercoagulation in cancer patients led to the discovery of Cancer Procoagulant (CP), a cysteine protease able to directly activate factor X

independently of coagulation factor VII (Gordon et al.,1975). CP has been detected in different malignant cells, both from solid and hematologic tumors, but not in normal tissues (Falanga et al., 1998). CP activity in bone marrow cells from patients with acute non-lymphoid leukemia was detected during the active disease and 25 months before recurrence of the disease suggesting that the expression of CP may precede the appearance of the malignant phenotype (Donate et al., 1990). Even though the relative contribution of this factor to the overall cellular procoagulant activity is still unknown, it could potentially be a good tumor marker thanks to its production restricted to malignant tissues, its expression before the appearance of the malignant phenotype, and its presence in the blood of cancer patients.

In the frame of procoagulant state in cancer, the clinical importance of the enzyme heparanase is gaining much attention. Heparanase is the unique and specific endoglycosidase capable of cleaving heparan sulfate chains of proteoglycans on cell surfaces and on the extracellular matrix. Apart of its well characterized enzymatic activity, the role of this enzyme in tumor progression is becoming clearer and it is upregulated in a wide range of solid tumors (e.g. carcinomas of colon, liver, pancreas, bladder, breast, gastric, prostate) as well as in hematological tumours (multiple myeloma, leukemia and lymphoma) (Falanga et al., 2017; Vlodavsky et al., 2016). The up-regulation of heparanase in primary tumors correlates with higher rate of local and distant metastases as well as reduced post-operative survival, thus providing a clinical support for the pro-metastatic function of this enzyme (Masola et al., 2014). Heparanase may affect the hemostatic system by two independent mechanisms, by up-regulating TF expression and interacting with the tissue factor pathway inhibitor (TFPI) on endothelial cells and tumor cells, thus resulting in increased cell surface coagulation activity. Moreover, heparanase may directly enhance TF activity which leads to increased factor Xa production and subsequent activation of the coagulation system (Nadir and Brenner, 2016).

Mucin-rich adenocarcinomas are the most commonly associated with thrombus formations and with a significantly higher incidence of VTE (Falanga et al., 2017; Buller et al., 2007). Mucins are large glycoproteins that become abnormally glycosylated in several carcinomas. Carcinoma cells frequently upregulate expression of mucins which are then inappropriately secreted into the bloodstream. Glycans displayed by mucins mediate the pathological interaction with selectins and these interactions have been implicated in the haematogenous phase of tumour metastasis (Borsig et al., 2001). It has been demonstrated that carcinoma mucins generate platelet-rich microthrombi *in vivo*, through bidirectional signaling in neutrophils and platelets (Shao et al., 2011).

Cancer-associated coagulation is enhanced also by suppression of fibrinolytic activity and/or decrease of anticoagulant factors. Fibrin is degraded by plasmin derived from plasminogen through the action of plasminogen activators. The major inhibitor of this pathway, plasminogen activator inhibitor type-1 (PAI-1), is released by platelets and endothelial cells but is also expressed by several cancer cells. PAI-1 activity is therefore increased in cancer and this may contribute to decreased fibrinolysis and enhanced coagulation (Hajjar, 1996). Protein C (PC) and anti-thrombin (AT) are the

most important, naturally occurring anticoagulant proteins and are produced by the liver. PC, activated by thrombin, inactivates the coagulation cascade and stimulates fibrinolytic activity by inactivating the PAI-1. AT inhibits thrombin, factors IXa, Xa, XIa, and XIIa. The levels of these two physiological anticoagulants tend to fall in cancer patients, and their hepatic synthesis is decreased in metastatic cancer, thus contributing to the procoagulant condition (Rubin et al., 1980; Honegger et al., 1981; De Cicco et al., 1995).

Tumour-specific antigens and tumour-procoagulant molecules stimulate the release of inflammatory mediators (i.e., TNF, IL-1) from macrophages and T-cells. Apart from their key role in inflammation, these cytokines promote coagulation by inducing TF expression, activating platelets, and by decreasing fibrinolytic and anticoagulant activities through the down-regulation of PC pathway and expression of PAI-1 (Rickles and Falanga, 2001; Gouin-Thibault et al., 2001; De Cicco, 2004).

Finally, platelets play a key role in cancer-associated thrombosis and they represent the most critical determinants of hematogenous tumor metastasis and thrombotic complications in cancer patients (Stegner et al., 2014). The activation of platelets by cancer will be further discussed in detail in following sections.

Extrinsic pro-thrombotic factors

Besides the tumour itself, there are some extrinsic factors (such as anti-cancer treatments and patient-related risk factors) that may contribute to the prothrombotic state of cancer patients (Young et al., 2012).

The use of anti-neoplastic drugs, both cytotoxic (e.g. Cisplatin, L-asparaginase, 5-Fluorouracil) and targeted agents (Tamoxifen, antiangiogenic agents, immunomodulatory agents) is associated with alterations in hemostasis and further increased VTE risk. Indeed, cancer patients undergoing chemotherapy have 6.5-fold increase in the risk of developing VTE and it decreases after chemotherapy is completed (Silverstein et al., 1998; Heit et al., 2000; Levine et al., 1988; Khorana et al., 2005; Heit et al., 2000; Blom et al., 2006). The impact of chemotherapy on VTE risk depends strictly on the therapy used, for example thalidomide given in combination with dexamethasone has been associated with a very high VTE rates, such as 12–28% (Palumbo et al., 2008; Opptel et al., 2016). The mechanisms through which anticancer drugs may induce a prothrombotic state include direct tissue toxicity, the imbalance of the physiological levels of endogenous procoagulants and anticoagulants, release of cytokines, platelet activation, expression of monocyte/macrophage TF (Young et al., 2012; Opptel et al., 2016).

The influence of radiation therapy has been less explored. Recently, the potential link between radiotherapy and VTE has been reported in case reports and small series of patients, suggesting that radiotherapy may be thrombogenic (Guy et al., 2017). Indeed, it has been demonstrated *in vitro* that ionizing radiation alters hemostasis and promotes prothrombotic responses by inducing inflammatory processes, alteration of PC pathway, endothelial activation,

platelet activation, TF and Von Willebrand activation and additional prothrombotic responses (Goldin-Lang et al.,2007a; Blirando et al., 2012; Halle et al., 2010; Geiger et al., 2012; Hauer-Jensen et al., 2004; Krigsfeldet al., 2013; Boermaet al., 2004; Farías et al., 1997; Verheijet al., 1994). During radiotherapy a direct endothelial damage likely leads to inflammatory response that may be the major mechanisms of coagulation activation in this context (Guy et al., 2017).

Of course, the risk of VTE further increases in cancer patients undergoing surgery, with an incidence of postoperative DVT and pulmonary embolism of about 7-fold higher than non-cancer patients (Heit et al., 2000). The hemostatic system becomes more active upon surgery because the surgical trauma causes an inflammatory responses with the subsequent release of procoagulant cytokines and, in addition, the limited postoperative mobility favors the venous stasis (De Cicco, 2004; Khalil et al., 2015)

Finally, other conditions such as prior thromboses, prothrombotic gene mutations, age, gender, performance status and mobility have all been found to influence the risk for VTE (Khorana et al., 2007; Young et al., 2012).

The contribution of platelets to cancer

Metastasis (from the Greek “change of place”) represents the major cause of cancer-associated death and it arises from the dissemination of cancer cells from a primary tumour to a new anatomical site. Cancer detected at early stages, before its spread, is often treated successfully by chemotherapy, surgery or radiotherapy. However, when cancer is detected after its dissemination to other organs, therapeutic options are limited, and treatments are much less successful. In spite of the clinical importance of metastasis, the knowledge of its biology is limited. Metastatic dissemination is a complex process consisting of multiple steps that must be successfully completed to allow tumor spread (Chamber et al., 2002, Hanahan and Weinberg, 2000).

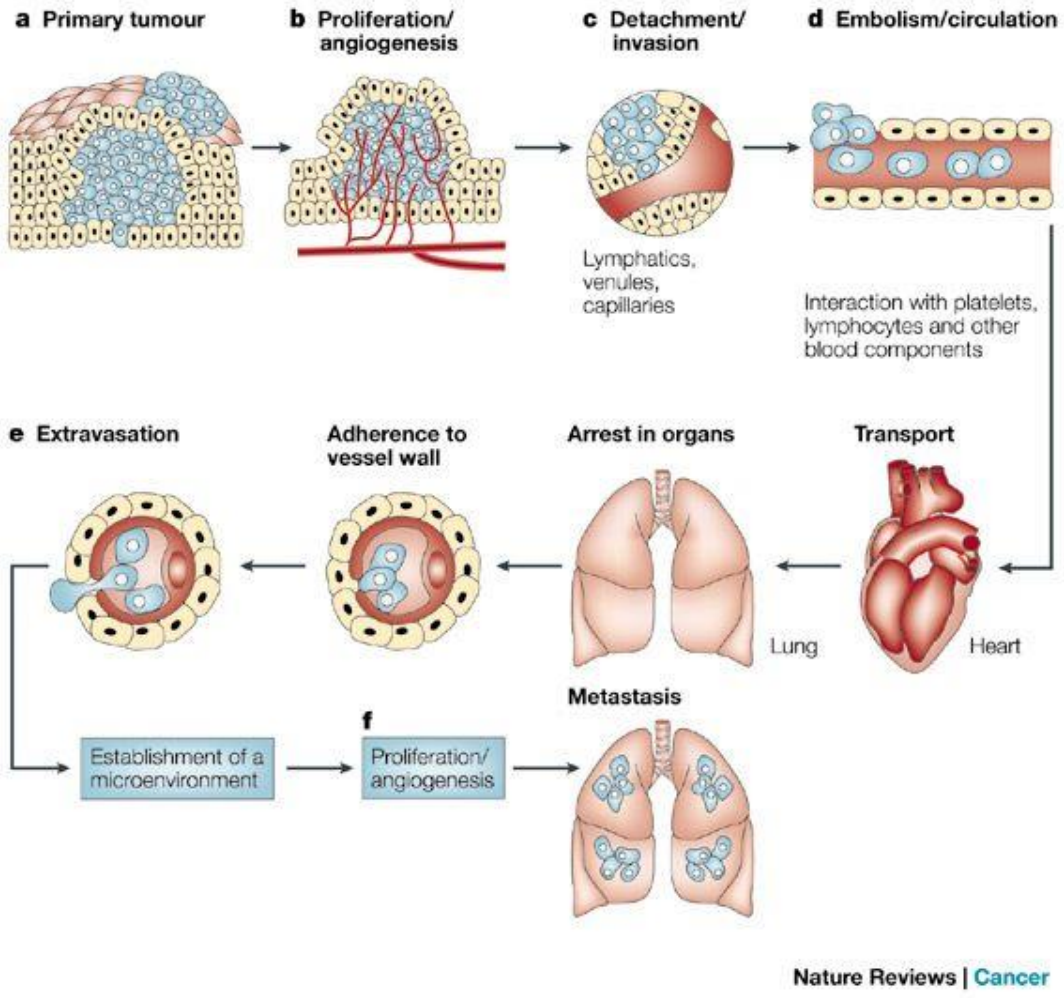
As a primary tumour grows, the formation of new blood vessels (angiogenesis) is fundamental for meeting the metabolic needs of the growing cancer (Folkman, 1971). However, tumour blood vessels display remarkable morphological abnormalities that increase vessel permeability and leakiness (Coussens and Werb, 2002). This abnormal vessel morphology allows cancer cells to enter the blood stream (a process known as intravasation), where they come in contact with platelets. Upon intravasation, platelets contribute to tumour cells survival in the circulation, until they can extravasate into the surrounding tissues. At anatomical site, possibly distant from the original tumour, platelets can also function as source of proangiogenic factors, thus regulating neo-angiogenesis (Walsh et al., 2015).

Despite the fact that large primary tumours can shed millions of cells in the vasculature every day, only few metastases eventually develop, demonstrating that, overall, metastasis is an inefficient process (Chambers et al., 2002). Tumor cells that succeed in forming metastases have acquired the necessary traits to complete all the required steps. However, it is now clear that the metastatic potential of a tumor cell is significantly modulated by the environmental conditions and by the interactions with the host cells, both in the circulation and at site of extravasation (Figure 6).

Circulating platelets play a key role in the frame of host-tumour interaction. The first experimental observation of platelet involvement in cancer metastasis dates back to 1968, when neuroaminidase-induced thrombocytopenia was found to be associated with reduced metastasis (Gasic et al., 1968). This result was further confirmed by later studies demonstrating that thrombocytopenia caused by platelet depletion with anti-platelet sera or by defective platelet production dramatically reduced the number of experimental metastases (Karpatkin et al., 1988; Mahalingam et al., 1988). In addition, besides the alteration of hemostatic system and platelet hyper activation, cancer is often associated with thrombocytosis (higher number of platelets) which usually is a marker of advanced-stage tumour and bad prognosis (Lin et al., 2014, Stegner et al., 2014). Subsequently, several *in vitro* and *in vivo* experimental models provided direct demonstrations of the pro-metastatic function of platelets, although the involved mechanisms are still largely unknown. Although much remains to be clarified, today it is clear that platelets fulfill their pro-metastatic activity essentially by prolonging the survival of circulating cancer cells (CTCs) in the circulation, supporting adhesion to the vascular endothelium and extravasation, and finally by enabling angiogenesis in the

new metastatic site and stabilizing tumour blood vessels (Labelle and Hynes, 2012; Ho-Tin-Noe et al., 2008; Gay and Felding-Habermann, 2001; Battinelli et al., 2011; Schumacher et al., 2013).

Figure 6



Summary of steps required for tumour growth and dissemination. (From: The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer. 2003 Jun;3(6):453-8).

Platelets increase cancer cell survival

Metastatic spread to distant sites relies on hematogenous dissemination and strictly depends on interactions with the host components. Metastasis is, overall, an inefficient process and despite millions of cancer cells from the primary tumour enter the blood stream every day, only few of them survive in the circulation and even fewer eventually create a metastatic lesion (Chambers et al., 2002).

During their migration through the bloodstream, CTCs are exposed to an unfavorable environment characterized by shear forces of the blood flow and immune cytotoxicity. However, CTCs are able to overcome these conditions by exploiting host cells, mainly platelets. While transiting in the bloodstream, CTCs induce the activation of platelets and initiate the coagulation cascade, thus causing the formation of platelet-rich thrombi around tumor cells which physically protect CTCs from shear stress and natural killer (NK) cells (Erpenbeck and Schon, 2010; Gay and Felding-Habermann, 2011; Egan et al., 2014; Degen and Palumbo, 2012). NK cells play a major role in anti-tumour immunity, but their action requires direct cell-cell contact. Apart from preventing the direct interaction between CTCs and NK cells, platelets impede NK cell–mediated elimination of tumor cells also by releasing TGFβ and PDGF which decrease NK cell's activity (Placke, 2011; Palumbo et al., 2005; Lipinski and Egyud, 2000; Egyud and Lipinski; 1991). The importance of platelet inhibition of NK cells is supported by the observation that the capacity of NK cells to lyse the tumour cells is inversely correlated with platelet density and that in the absence of NK cells platelet depletion only moderately affects cancer metastatic spread (Nieswandt et al., 1999).Moreover, the platelet coat enables a mechanism of molecular mimicry by which cancer cells acquire platelet-derived MHC-I, thus preventing recognition from immune system (Placke et al., 2012).

Platelets support cancer cell arrest and extravasation

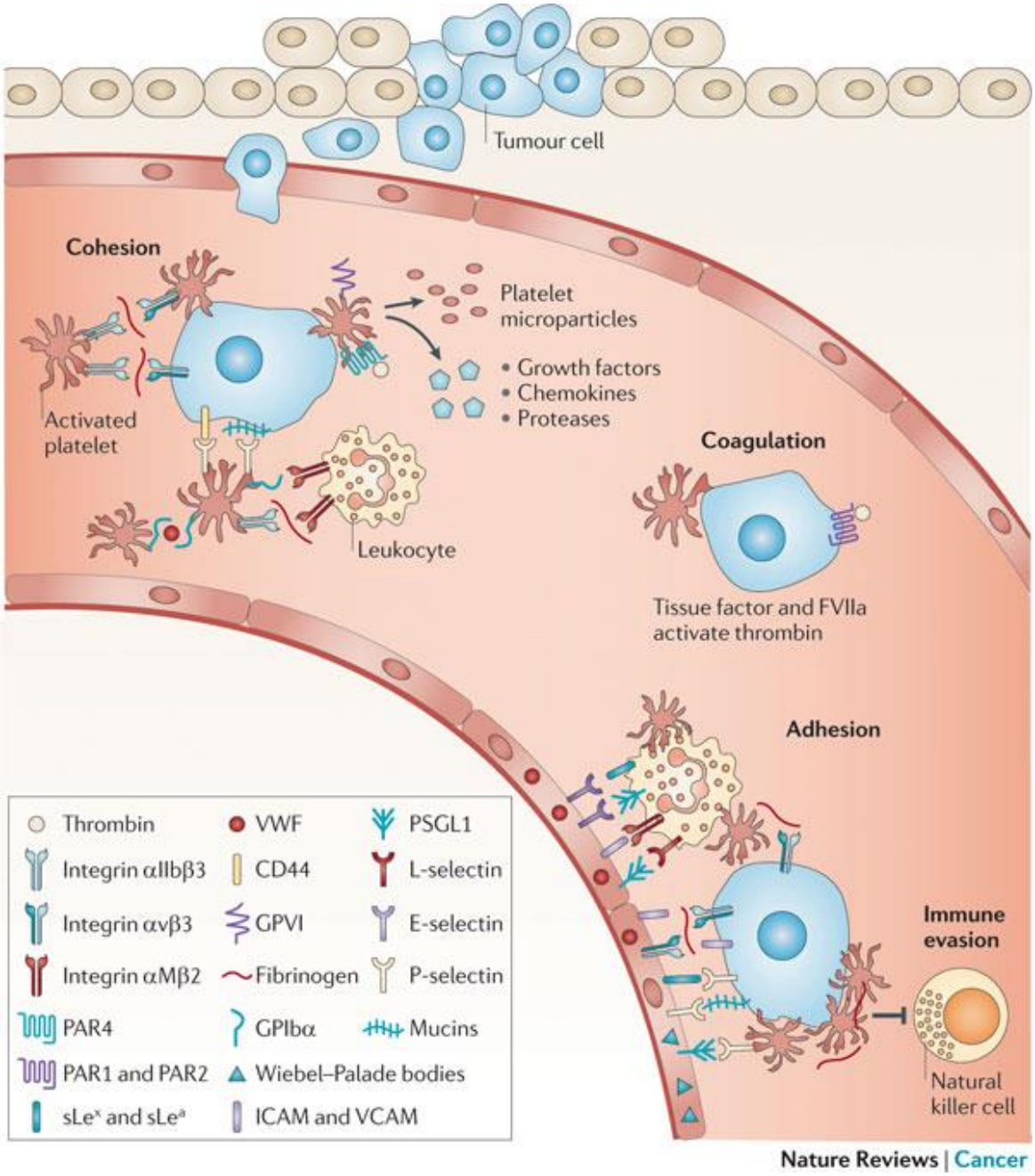
Once CTCs have managed to escape shear-mediated or immunological destruction, they have to arrest at the vessel wall and extravasate into a new tissue. This process involves an initial transient interaction with the vessel lining, that precedes firm adhesion and, eventually, extravasation. Adhesion to vessel wall is primarily mediated by adhesion receptors expressed on tumor and endothelial cells, but it is further facilitated by platelets and leukocytes (Labelle and Hynes, 2012; Stegner et al., 2014). Indeed, CTCs entrapped in platelets-leukocytes hetero-aggregates adhere to the endothelial cells of the vessel wall, allowing cancer cell rolling and tethering on the vessel wall. This initial interaction is mediated mainly by selectins expressed on leukocytes (L-selectin), platelets (P-selectin), and endothelial cells (E-selectin). Through a conserved lectin domain, selectins bind saccharides antigens expressed on other cells, such as P-selectin glycoprotein ligand 1(PSGL-1), mucins and CD44, which can be expressed at high levels by metastatic cancer cells (McCarty et al., 2000; Gay and Felding-Habermann, 2011). After the initial rolling on the endothelium, the firm arrest of cancer cell can be potentiated by adhesion receptors, in particular integrins, expressed by cancer cell-associated platelets. Integrin αIIbβ3 is the main

platelet receptor involved in this process and, together with integrin αvβ3 expressed by tumour cell, helps to overcome shear forces promoting the arrest of CTCs (Felding-Habermann et al., 1996; McCarty et al., 2000).

Once tumor cells-platelets aggregate firmly adheres at the vessel wall, platelets actively support extravasation through the modulation of vascular integrity and permeability. Growth factors and extracellular messengers released from platelet α granules (including PDGF, TGFβ, EGF, IGF-1, VEGF, S1P, and LPA) influence vascular integrity in the tumor microenvironment. In addition, through the release of TGFβ and by direct interaction with cancer cells, platelets activate the TGFβ/Smad and NF-κB pathways, thus promoting epithelial-to-mesenchymal transition and extravasation (Labelle et al., 2011;).

Moreover, ATP released from dense granules of activated platelets weakens the endothelial barrier by binding to its receptor P2Y2 on ECs, thus facilitating trans-endothelial migration of tumor cells. Indeed, in mice unable to secrete their platelet dense granules, as well as in mice with an EC-specific P2Y2-deficiency, tumor cell transmigration is strongly reduced (Schumacher et al., 2013). Serotonin, another soluble factor released from platelets dense granule, modulates the vascular tone, (Côté et al., 2004). Importantly, the levels of plasma serotonin are increased in murine models of experimental tumour metastasis and the blockade of serotonin receptors delays tumour dissemination (Skolnik et al.,1894).

Figure 7



Molecular coordination between platelets and tumour cells supports metastasis from the bloodstream. (From: Contribution of platelets to tumour metastasis. Nat Rev Cancer. 2011 Feb;11(2):123-34.)

Platelets promote tumour angiogenesis and maintain vascular integrity

Already in 1971, Folkman described the dependence of tumor growth on angiogenesis to prevent hypoxia-induced growth arrest and necrosis (Folkman, 1971). The assembly of a new vascular network is vital for the growth of solid tumours, both at the primary and at metastatic sites. The formation of new blood vessels is required to provide the tumor of nutrients and oxygen, to dispose waste products, and to sustain further tumour growth and invasion (Nash et al., 2002; Chambers et al., 2002; Kerbel, 2008).

Platelet are important regulator of angiogenesis as their granules contain compartmentalized pro-angiogenic (e.g. VEGF, LPA, IGF, PDGF, angiopoietin, and MMP-1, -2, and -9) and anti-angiogenic factors (TGFβ, thrombospondin-1, S1P, platelet factor-4, angiostatin, and tissue inhibitor of matrix metalloproteinases), which are differentially released depending on the stimulus. In particular, the potent pro-angiogenic factor VEGF is released by activated platelets and stimulates angiogenesis at the new metastatic site, but also stimulates endothelial cells to release vWF, which further supports platelet adhesion at metastatic sites (Brock et al., 1991).

Platelets play a key role also in stabilizing newly formed tumour vessels. Antibody-induced thrombocytopenia in mice was immediately followed by hemorrhage within tumours and this severe bleeding could be rescued by infusion of resting platelets, but not by infusion of platelets depleted of their granule content, demonstrating that tumour vascular integrity strongly depends on platelet granule secretion (Ho-Tin-Noe et al., 2008). Moreover, in platelet-depleted mice blood vessel density and maturation were strongly impaired at the tumour niche.

Tumor-educated platelets (TEPs)

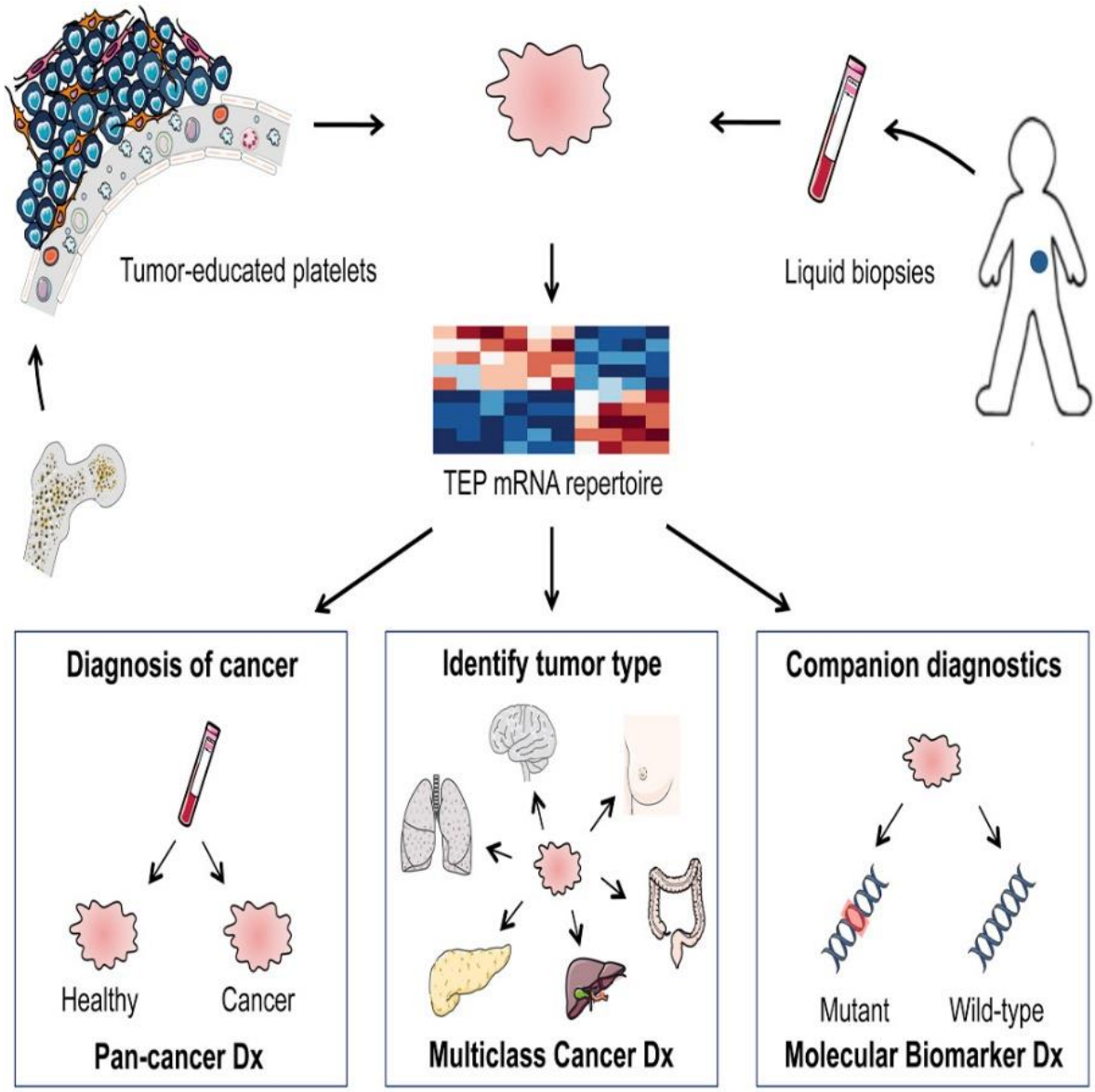
The critical role of platelets in cancer progression and metastates is longstanding and today platelets are considered as a potential diagnostic tool in oncology. In cancer patients, thrombocytosis is a strong predictor of poor survival demonstrating that a simple platelet count already harbors potentially relevant clinical information (Stone et al., 2012; Cho et al., 2012; Matowicka-Karna et al., 2013; Sol and Wurdinger, 2017). Apart from platelet count, cancer patients present alterations also in platelets size (Matowicka-Karna et al., 2013; Wang et al., 2015), expression of protein markers, (Matowicka-Karna et al., 2013; Mantur et al., 2002; Mantur et al., 2003; Dymicka-Piekarska et al., 2006; Osada et al., 2010; Peterson et al., 2012; Kamińska, et al., 2014; Meikle et al., 2017; Cooke et al., 2013; Angénieux et al., 2016; Balduini et al., 1999; Nilsson et al., 2010), and ratio of young versus total platelets (Angénieux et al., 2016). Importantly, platelets of cancer patients are typically hyper reactive (Meikle et al., 2017; Cooke et al., 2013).

Tumour-educated platelets (TEPs) is an emerging concept which is gaining attention for its potential as diagnostic tool in oncology (Best et al., 2015). Tumor cells release in the microenvironment biomolecules that are taken up by platelets, leading to their education (Klement et al., 2009, Kuznetsov et al., 2012, McAllister and Weinberg, 2014, Nilsson et al., 2011, Quail and Joyce, 2013). Through this mechanism, signals released by cancer cells and the tumor microenvironment may, directly and indirectly, impose changes on platelet RNA and protein content (McAllister and Weinberg, 2014; Power et al., 2009, Rowley et al., 2011, Schubert et al., 2014). The result of this education is an altered function of TEPs which become more efficient in promoting tumor cell survival and metastasis. The alteration in platelet RNA content induced by cancer is the aspect receiving more attention by the scientific community. Though platelets lack a nucleus, they are equipped with RNA processing machineries and most RNA transcripts are derived from megakaryocytes. However, platelets can dynamically modify their own RNA content by ingesting RNA molecules during circulation and/or interaction with other cell types (including cancer cells) or by modifying splicing events in response to external stimuli, such as signals from cancer cells (Best et al., 2015). The combination of these abilities provides TEPs with a highly specific mRNA profile that allows to distinguish between healthy donors and cancer patients (Nilsson et al., 2011; Best et al., 2015; Calverley et al., 2010). The exchange of nucleic acids and proteins with other cells, including cancer cells, occurs mostly through vesicle-mediated transport. In the last years, transfer of RNA from cancer cells to platelet has been demonstrated both *in vitro*, using glioma and prostate cancer cells, and *in vivo* in NSCLC (non-small cell lung carcinoma) patients and glioma patients (Best et al., 2015, Nilsson et al., 2011; Nilsson et al., 2015).

It has been demonstrated that mRNA sequencing of TEPs, coupled to advanced bioinformatic analytical tools, allows to distinguish cancer patients from healthy individuals with 96% accuracy and can provide specific information on the presence, location, and molecular characteristics of cancers. A total of 1453 out of 5003 mRNAs were increased and 793 out of 5003

mRNAs were decreased in TEPs as compared to platelet samples of healthy donors (Best et al., 2015).

Figure 8



Schematic overview of tumor-educated platelets as biosource for liquid biopsies. (From RNA-Seq of Tumor-Educated Platelets Enables Blood-Based Pan-Cancer, Multiclass, and Molecular Pathway Cancer Diagnostics. Cancer Cell. 2015 Nov 9;28(5):666-676.Best et al.)

Antiplatelet therapy in the treatment of cancer

In 1989 and 1993, Zacharski and colleagues, writing for the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis Subcommittee on Hemostasis and Malignancy, published an update of clinical trials using antiplatelet therapy and anticoagulants in cancer (Bambace and Holmes, 2011).

An inverse correlation between platelet count and disease-specific survival has been described for several types of cancer and several observations have demonstrated that platelets affect disease burden and treatment efficacy in cancer patients. Therefore, the use of antiplatelet agents as adjuvant cancer therapy or the exploitation of platelets as carriers of antitumor agents are considered attractive approaches.

Aspirin

Cyclooxygenase (COX) transforms arachidonic acid into the precursor of different prostaglandins and thromboxanes. COX-1 preferentially couples with TXA2 synthase in platelets, whereas COX-2 is associated mainly with prostaglandin I2 synthase in endothelial cells, and PGE synthase in several cell types. Aspirin irreversibly inactivates COX-1 and COX-2 through acetylation, with the consequent suppression of prostaglandin and thromboxane synthesis.

Early observations showed that aspirin treatment reduced tumor metastasis in rats suggesting a potential benefit of aspirin in cancer (Gasic et al., 1972). Supporting observations showed that aspirin significantly inhibited the incidence and number of colon adenocarcinomas in rats as well as the onset of lung tumors in mice (Reddy et al., 1993). Moreover, administration of aspirin at a dose equivalent to low-dose in humans (around 100 mg/day), induced apoptosis of colorectal cancer cells in mice (Tian et al., 2011).

The therapeutic use of aspirin has been extensively studied in colon cancer. A case-control study revealed that the chronic use of aspirin among CRC-patients was significantly lower than among controls (Kune et al., 1988). Later, a meta-analysis of several case-control studies in CRC patients, involving more than 30000 subjects concluded that regular use of aspirin was associated with a reduced risk of CRC (Algra and Rothwell, 2012). In patients carrying hereditary predisposition to non-polyposis colon cancer, aspirin was shown to prevent the development of cancer and to prolong survival (Burn et al., 2008; Cooke et al., 2015). Other subsequent meta-analyses of individual patient data from large aspirin studies of aspirin in cardiovascular prevention showed that aspirin reduced mortality also from non-gastrointestinal cancers, prevented metastasis from numerous cancer types and that the effect was only evident from 5 years of continued treatment onward (Rothwell et al., 2011; Rothwell et al., 2012; Rothwell et al., 2012).

In most of these trials the doses of aspirin used were sufficiently high to affect the activity of COX-2 systemically. Therefore, the observed antitumor activity of aspirin is considered to be largely mediated by the inhibition of COX-2 expressed in cancer cells, whereas the possible impact of platelet COX-1 inhibition to these beneficial effects of the drug is still unknown.

PDE inhibitors

Phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP and cGMP to inactive 5′AMP and 5′GMP, and over 60 different PDEs isoforms are known. Platelets express PDE2, PDE3, and PDE5 and their inhibition rises the intra-platelet levels of the two cyclic nucleotides, thus resulting in the inhibition of platelet activation.

Cilostazol, a specific inhibitor of PDE3, and dipyridamole, a PDE3 and PDE5 inhibitor, have been explored as possible adjuvant therapy in cancer, but is unclear whether their effects can be ascribed to inhibition of platelet enzymes. The effect of cilostazol on cancer has been only evaluated in preclinical studies, showing anti-tumor effect when used in association with cisplatin (Uzawa et al., 2013; Inufusa et al., 1995). Preclinical studies using dipyridamole have shown reduced metastases formation and potential benefits against cancer multidrug resistance (Tzanakakis et al., 1993; Desai et al., 1997; Shalinsky et al., 1990; Goda et al., 2008). Mice injected with doxorubicin-resistant melanoma cells showed a significant delay in the growth of tumors when dipyridamole was used as adjuvant drug compared to treatment with doxorubicin alone, thanks to a significant increase in intra-tumor accumulation of doxorubicin (Desai et al., 1997). The use of dipyridamole in melanoma patients for 11 years have resulted in an increased survival (Rhodes et al., 1985), and in patients with advanced gastric or pancreatic cancer, dipyridamole used in combination with anticancer drugs (such as 5-fluorouracil,and cisplatin) potentiated the cytotoxicity of chemotherapeutic agents and prolonged survival (Kohnoe et al., 1998; Todd et al., 1998). Despite these positive results, other trials examining the potential usefulness of dipyridamole to enhance chemotherapeutic efficacy in sarcoma, colorectal, breast, renal, and prostate cancers failed to show significant improvement in the therapyhic.

ADP receptor antagonists

Stimulation of ADP receptors (P2Y1 and P2Y12 receptors) by ADP released from platelet dense granules amplify the initial platelet activation and potentiate aggregation. P2Y12, the Gαi-coupled ADP receptor, is the target of all the currently used anti-platelet ADP antagonists. On the other hand, P2Y12 has also been shown to be expressed in several tumor cells (intestinal epithelial carcinoma cells, glioma C6 cells, ovarian cancer cells, etc.) where it regulates cell growth (Di Virgilio, 2012). In the Lewis Lung Carcinoma (LLC) spontaneous metastatic mouse model, P2Y12 deficiency significantly reduced pulmonary metastasis. In agreement with the LLC cell model, platelet P2Y12 deficiency also results in significantly less lung metastasis in the B16 melanoma experimental metastasis model (Wang et al., 2013).

Clopidogrel is an oral P2Y12 inhibitor that requires liver metabolic machinery for the conversion to its active form. Stimulation of P2Y12 receptor contributes to the release of angiogenic factor by platelets, therefore P2Y12 antagonists, beyond their antithrombotic activity, may affect tumor angiogenesis (Bambace et al., 2010). This hypothesis is supported by the observation that SR25989, an enantiomer of clopidogrel lacking anti-aggregatory activity, possesses anti-angiogenic

properties and inhibited pulmonary metastases in a mouse model of melanoma (Ma et al., 2001). However, a recent retrospective observational study of clopidogrel in a cohort of over 40000 newly diagnosed patients with colorectal, breast and prostate cancer reported no significant differences in cancer-related mortality (Hicks et al., 2015).

Ticagrelor is a P2Y12 antagonist that, differently from clopidogrel, does not require metabolic activation to exert its action. The potential antitumor effect of ticagrelor was explored in melanoma metastasis mouse models, demonstrating that treatment with ticagrelor markedly reduced lung and liver metastases and improved survival (Gebremeskel et al., 2015).

Platelets as drug carriers

The potential use of platelets as drug carriers in cancer therapy was explored using two different approaches: encapsulating an anticancer drug within intact platelets, or covering nanoparticles containing anti-cancer drugs with platelet membranes. The first approach was investigated by loading doxorubicin into platelets that were then infused in a mouse lymphoma model showing an enhanced therapeutic efficacy and reduced adverse effects (Sarkar et al., 2013). The second approach consists in coating nanoparticles containing anticancer drugs with membranes isolated from platelets. Nanoparticles are widely investigated as carriers for anticancer therapy, and their coating with platelet membranes may potentially provide some benefits, such as the prolongation of drug circulation time, reduced immunogenicity, and selective targeting (Li et al., 2016). Infusion of platelet-coated nanoparticles loaded with doxorubicin in breast tumor-bearing nude mice effectively eliminated circulating tumor cells *in vivo* and inhibited the development of tumor metastases. The effects observed were significantly increased compared to nanoparticles loaded with doxorubicin, but lacking the platelet membrane coating (Hu et al., 2015).

Another recent approach consists in conjugating platelets with a new therapeutic antibody against programmed-cell-death protein 1 ligand (PDL-1). Cancer cells express PDL1 that, upon binding with its receptor PD1 on T cells, induces lymphocytes inactivation allowing cancer cells to evade immune attack. Anti-PDL1 is an immune checkpoint blocker used in cancer immunotherapy that impedes the binding of PD1 with PDL1 thus preventing the inactivation of T cells. The antibody-conjugated platelets were injected in mice bearing partially removed primary melanomas or triple-negative breast carcinomas, demonstrating that platelets activated in the surgical bed shed PMPs bearing anti-PDL1 that significantly prolonged mouse survival after surgery (Wang et al., 2017).

Tumour cell-induced platelet aggregation – TCIPA

The mechanisms at the basis of pro-metastatic effects of platelets, described in the previous sections, imply that the metastatic potential of a cancer cell relies on its ability to interact with platelets and to induce their activation. Moreover, the prothrombotic condition of cancer patients indicates that the evolution of the malignancy is paralleled with an activation of platelets and the coagulation cascade. Few years later the observation that thrombocytopenia decreased experimental metastasis in mice (Gasic et al., 1968), the same group demonstrated for the first time that many tumors have the capacity to induce platelet aggregation and that this ability correlated with the metastatic potential of the cancer cell (Gasic et al., 1973). This original observation led to the hypothesis that the metastatic behavior of tumors is strongly influenced by their capacity to aggregate platelets. The ability of cancer cells to directly promote platelet aggregation, a process known as tumor-cell induced platelet aggregation (TCIPA), has been investigated in several early and recent studies but the mechanisms by which tumor cells aggregate platelets remain only partially known. As mentioned before, TCIPA confers several advantages to CTCs: aggregating around tumor cells, platelets create a shield that protects the tumor cell from immune cytotoxicity and mechanical stress of the blood flow. The platelet aggregate surrounding the tumor cell may also aid in the extravasation of the tumor cell by enhancing its adhesion to the endothelium. Therefore, TCIPA represents a critical process both for successful metastatic spread of the tumor and for the development of thrombotic complications in cancer patients (Jurasz et al., 2004; Erpenbeck and Schon, 2010; Yan and Jurasz, 2016).

Through the years, the ability of inducing aggregation of both human and mouse platelets, has been demonstrated by many different cancer cell lines including neuroblastoma (Bastida et al., 1986), small-cell lung cancer (Heinmoller et al., 1996), melanoma (Boukerche et al., 1994), breast carcinoma (Alonso-Escolano et al., 2004; Lian et al., 2013; Chiang et al., 1995), colon carcinoma (Mitrugno et al., 2014; Medina et al., 2006;), and fibroblastoma (Jurasz et al., 2001). It appears that the mechanism supporting TCIPA partly depends on intrinsic properties of the cancer cell lines and involves important differences in the relative contribution of both platelet and cancer cell receptors. Nevertheless, the molecular mechanisms and the signal transduction pathways involved in TCIPA are still poorly characterized. Importantly, several conflicting observations about the ability of determined cancer cell lines to induce TCIPA are reported in literature. This may depend on the different technical approaches adopted by different research groups, in particular concerning the anticoagulant used for blood withdrawal, the use of platelet rich plasma (PRP) or purified platelets, and the methods adopted for platelet purification.

Although a detailed and reliable map of the mechanisms involved in TCIPA by different cancer cell is still missing, some important observations about the involvement in this process of platelet soluble agonists and receptors have been reported. Several studies on TCIPA, performed using different cancer cell lines, investigated the contribution of extracellular messengers such as ADP and TxA2, as well as the role of tissue factor, thrombin and other components of the coagulation

cascade (Jurasz et al., 2004). Moreover, through the use of specific inhibitors, or genetically modified mouse models, several platelet receptors, including integrin αIIbβ3, GPIb-IX-V complex and P-selectin have been described to participate to TCIPA in different experimental contexts (Erpenbeck and Schon, 2010). Upon platelet activation, P-selectin is rapidly translocated to platelet surface and binds to a number of tumor cell lines via sialylated fucosylated glycans displayed on mucin and non-mucin structures. The crucial role of integrin αIIbβ3 in TCIPA has been confirmed both *in vitro* and *in vivo* by several studies (Karpatkin et al., 1988; Boukerche et al., 1989; Oleksowicz et al., 1995; Cohen et al., 2000; Amirkhosravi et al., 2003; Alonso-Escolano et al., 2004). Also integrin αvβ3, which is highly expressed on cancer cells, may mediate TCIPA by connecting cancer cells to platelet using fibrinogen and platelet integrin αIIbβ3 (Felding-Habermann et al., 2001).

Some tumor cells such as glioblastoma, neuroblastoma and pancreatic cancer cells display the ability to generate thrombin, which supports TCIPA by activating PARs on platelet surface. In addition to thrombin, other serine proteases such as cathepsin B and Cancer Procoagulant can be released by cancer cells and promote TCIPA (Jurasz et al., 2004). Metalloproteinases, such as MMP-2, can be released by both platelets and tumor cells (e.g. fibroblastoma HT-1080 and breast cancer MCF-7) and support TCIPA (Jurasz et al., 2001). Another important mediator of TCIPA is ADP, which can be released directly by some cancer cells, or by platelets themselves upon exposure to cancer cells. It has been shown that TCIPA promoted by melanoma and fibroblastoma cells strongly depends on ADP stimulation of platelet P2Y12 receptor (Alfonso-Escolano et al., 2004). Moreover, ADP scavangers, such as apyrase, can almost complete abolish TCIPA induced by several cancer cells (Jurasz et al., 2004).

Colon adenocarcinoma cells (Caco-2) and prostate cancer cells (PC3M-luc) are able to stimulate TCIPA and also secretion of dense granules (referred to as tumor cell-induced platelet secretion, TCIPS). In this context, a critical role for the immune receptor FcγRIIA in mediating platelet-tumor cell cross-talk was described (Mitrugno et al., 2014).

CLEC-2 is a novel platelet receptor that participate to thrombosis, as well as, to the organization of blood vessel connection to the lymphatic system during fetal development. CLEC-2 can interact with podoplanin, which is a transmembrane protein expressed by many types of tumors. It has been shown that inhibition of the interaction of podoplanin with CLEC-2 limited TCIPA and prevented metastasis *in viv*o (Lowe et al., 2012).

Role of PMPs in cancer spread

In the last decades, the importance of the host environment in the development and the spread of a malignancy potently emerged. We have already mentioned several effects that blood components, and platelets in particular, can mediate to support metastasis, including the stimulation of angiogenesis and the protection against the immune system (Labelle and Hynes, 2012; Bambace and Holmes 2011). During the transit from the primary tumor, through the bloodstream until the development of new metastatic niches, the cancer cell receives signals from the environment that may modulate its aggressiveness.

The contribution of platelets to cancer growth and dissemination remains an area of active investigation and, recently, the possible implication of PMPs in this frame gained a growing interest in the scientific community. The reactivity of PMPs and their ability to transfer molecules between cells suggested that they may play important roles in cancer spread. Importantly, several clinical reports clearly demonstrated that PMPs levels are significantly elevated in patients with cancer, in particular at the advanced stages (Mezoaur et al., 2014; Chaari et al., 2014). To date, most investigations about the potential role of PMPs in cancer spread have been focused on their involvement in tumor angiogenesis and cancer cells invasiveness.

PMPs show *in vivo* pro-coaugulative properties and they play a role in the normal hemostatic response to vascular injury, particularly because they provide a catalytic surface for the prothrombinase reaction. Similarly to platelets, also PMPs may have a role in angiogenesis, a this hypothesis is strongly supported by the correlation between the circulating levels of PMPs and angiogenic factor. In addition, PMPs were shown to regulate endothelial cell proliferation, migration, and chemotaxis, as well as the tubulogenesis *in vitro* (Kim et al., 2004). The ability of PMPs to regulate angiogenesis was further demonstrated *in vivo* by direct injection of microparticles in the heart of rats that resulted in the formation of new vascular structures (Brill et al., 2005). Lung cancer cells treated with PMPs show upregulation in mRNA expression for angiogenic factors involved in the vascularization of the tumour, such as MMP-9, VEGF, IL-8 and hepatocyte growth factor HGF, confirming the contribution of PMPs to tumor angiogenesis (Janowska-Wieczorek et al., 2005).

PMPs likely support metastasis also by mediating the adhesion of circulating cancer cells to the endothelium. Depending on the conditions that induced their release, PMPs may express P-selectin, thus supporting the interaction with glycoconjugates expressed by cancer cells. Moreover, PMPs can also bind platelets, through a process at least partially supported by the interaction between fibrinogen and integrin αIIbβ3, and leukocytes (neutrophils and monocytes) through P-Selectin-, PSGL-1-, and β2 integrin-mediated interactions (Janowska-Wieczorek et al., 2001; Nomura et al., 2000). PMPs have also been implicated in cancer cell survival, escape from the immune system and the support of matrix proteolysis required for the efficient invasion of target tissues by cancer cells (Mezouar et al., 2014).

There are only few works on this topic investigating the ability of PMPs to directly increase the metastatic potential of cancer cell both *in vitro* and *in vivo*. PMPs are able to transfer integrin

αIIbβ3 to lung cancer cells *in vitro*, thus potentially increasing their ability to interact with fibrinogen. Moreover, interaction of PMPs with lung cancer cells resulted in activation of signaling pathways involved in proliferative responses and in the upregulation of mRNA expression for cyclin D2, whose overexpression is associated with increased invasiveness and progression of various tumors *in vivo*. Moreover, PMPs stimulated the production of MMPs and invasion of lung cancer cell lines, along with the expression of angiogenic factors. The injection in mice of LLC cells preincubated with PMPs resulted in an increased formation of metastatic foci compared to control, confirming the ability of PMPs to enhance metastasis of lung cancer cells (Pearlstein et al., 1984; Janowska-Wieczorek et al., 2005). The same research group also analyzed the effect of PMPs on breast cancer cell lines and demonstrated that, also in this experimental context, PMPs were able to transfer integrin αIIbβ3 to cancer cells and increase their ability to interact with endothelial cells. PMPs also stimulated MAP kinase and Akt signaling pathways, potentiated the expression of metalloproteinases, and stimulate the invasive ability of breast cancer cells (Janowska-Wieczorek et al., 2006; Tesselaar et al., 2007). A study by Varon and collaborators demonstrated that PMPs are able to physically interact with prostate cancer cells, and to potentiate their ability to bind endothelial cells and subendothelial matrix components. A publication by the same research group also showed that PMPs stimulated the expression of MMP-2 from prostate cancer cells and increased their invasion (Dashevsky et al., 2009; Varon and Shai, 2009). In spite of these few investigations, the mechanisms involved in the prometastic effects of PMPs remain almost completely unexplored.

2

Aim of the work

Metastasis represents the major cause of death in cancer patients and anti-metastatic treatments are still lacking, mainly because of the limited knowledge about mechanisms supporting metastasis. Cancer dissemination depends on the intrinsic metastatic potential of cancer cells, however it is now clear that the interaction of cancer cells with the host environment is fundamental for an efficient metastasis (Chamber et al., 2002; Labelle and Hynes, 2012). It has been known for decades that circulating blood platelets are an important component of the tumor microenvironment, since they play a critical role in cancer biology by supporting tumor progression and systemic spread (Bambace and Holmes, 2010). Recently, the interest in the interplay between cancer and platelets has been renewed thanks to the finding that tumor-educated platelets might serve as novel potential diagnostic tool in oncology (Best et al., 2015).

Hyper-responsiveness of the hemostatic system usually correlates with tumor progression and metastasis, demonstrating that the evolution of malignancy is paralleled by the activation of platelets and coagulation cascade. Activated platelets support cancer dissemination mainly by prolonging cancer cell survival in the blood stream, supporting cancer cell extravasation and regulating vascularization of the new metastatic site (Bambace and Holmes, 2010; Gay and Felding-Habermann, 2011). Moreover, cancer patients often experience severe thrombotic events, that are largely driven by the ability of tumor cells of inducing platelet activation (Young et al., 2012; Falanga et al., 2017).

In this context, TCIPA represents a critical step for cancer cell survival in the blood circulation and for the onset of cancer-associated thrombosis. The ability of cancer cells to aggregate platelets has been known for a long time and this ability correlates with their metastatic potential. Over the past three decades, TCIPA has been investigated *in vitro* using several cancer cell lines, but despite of the huge number of works on TCIPA, the molecular mechanisms involved are still poorly characterized (Jurasz et al., 2004). In particular, information about the ability of breast cancer cells to induce platelet activation and aggregation is still limited mainly due to the conflicting results present in literature. Therefore, we decided to perform a thorough investigation on TCIPA induced by breast cancer cell lines and to analyse the possible differences related to the metastatic potential of cancer cells. To this purpose, we selected two widely used breast adenocarcinoma cells lines, MCF7 and MDA-MB-231 cells, which are characterized by low and high metastatic potential, respectively.

As already said, TCIPA is important in cancer biology since it confers several advantages to survival of circulating tumor cells. Since the aim of this work was to get further insights in the complex interplay of platelets and cancer, we have investigated another potential pro-metastatic consequence of TCIPA, namely the release of PMPs. The extreme reactivity of PMPs and their role in intercellular communication suggest that these platelet-derived cell fragments may modulate the metastatic potential of circulating cancer cells. To date, few studies on this topic demonstrated that PMPs can potentiate the aggressiveness of cancer cells, providing a preliminary proof for PMPs ability to modulate cancer cell potential. The hypothesis of PMPs involvement in the modulation of

cancer spread is supported by the observation that increased levels of circulating PMPs were detected in cancer patients, particularly in the advanced stages of the disease.

However, the mechanisms supporting cancer-associated increase of PMPs levels are not totally understood. Moreover, it has not been documented whether cancer cells can directly induce the release of PMPs, and if these PMPs may operate any feedback regulation on cancer cells phenotype. Therefore, after a deep investigation of the mechanisms of breast cancer cell-induced platelet aggregation, we focused on the study of cancer cell-induced release of PMPs and on the characterization of the effect of these PMPs on the metastatic potential of cancer cells. In particular, we examined the ability of PMPs to modulate invasiveness of breast cancer cells, studying *in vitro* cancer cell migration and invasion, two critical aspect for cancer dissemination.

In the last part of the study, we further investigated the effects of PMPs on breast cancer cells and attempted to identify novel possible consequences of PMPs release. To this purpose, as experimental model, we adopted PMPs released by platelets stimulated with the physiological agonist thrombin. The ability of thrombin-induced PMPs to alter cancer cells' viability, cell cycle progression, and invasiveness was investigated. Importantly, in search of the mechanisms of interaction of cells and microparticles, we have preliminary analyzed the effect of PMPs on the autophagic flux of breast cancer cells. The role of autophagy in cancer is complex, since it has a dual function: on the one side, autophagy is cancer-suppressive since eliminate potentially harmful components but on the other side it helps cancer cells to overcome the stressful conditions that they undergo during metastatic dissemination (Cicchini et al., 2014). Moreover, autophagy-associated processing of PMPs components might be a relevant mechanism adopted by cancer cells to exploit platelet-derived material to improve its own survival and aggressiveness.

3

Materials and methods

Materials

Thrombin, MRS2179, prostaglandin E1 (PGE1), acetylsalicylic acid (ASA), GPRP, RGDS and apyrase were from Sigma. FURA-2-AM was from Calbiochem. AR-C69931MX was provided by Astra-Zeneca. Wortmannin and U73122 were from Alexis Biochemicals. PP2 and PPACK were from Enzo Life Sciences. Thrombin Activity Fluorometric Assay Kit was from BioVision Incorporated. (^{14}C)serotonin and GSH-Sepharose 2B were from GE Healthcare. ATP determination kit was from Biaffin GmbH & Co. Matrigel was from BD. Hoechst 33342 was from Cell Signaling Technology. BCA was from Euroclone.

The following antibodies were used in this study:
Anti-Rap1 (121), anti-α2-macroglobulin (H-8), HRP-conjugated anti-GAPDH (V-18), anti-Tissue Factor (H-9), anti-tubulin (DM1A), anti-integrin αIIb (B-10) from Santa Cruz Biotechnology; anti-Phospho(Ser)PKC substrates, anti-phospho-Akt (Ser473), anti-phospho-Erk (Tyr202/204), anti-phospho-MLC (Ser19), anti-phospho-p38MAPK (Thr180/Tyr182), anti-LC3B from Cell Signaling Technology; anti-p62 and anti-pleckstrin were from Abcam; PE-anti human CD41 from Biolegend;Anti-P-Selectin (CLB/thromb/6 (c2)) was from Novus Biologicals.

Cancer cells maintenance

section 3.1

MDA-MB-231 and MCF7 cells were kindly provided by Dr. Livia Visai (Department of Molecular Medicine, University of Pavia) and Dr. Maria Grazia Bottone (Department of Biology and Biotechnology, University of Pavia), respectively. MCF7 and MDA-MB-231 cells were maintained in a humidified 5% CO_2 atmosphere at 37°C, in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin. Cells were split every 2 days, in any case avoiding to reach complete confluence, and used for the experiments within 1 month.

For platelet stimulation experiments, cells were washed twice with PBS and detached by incubation for 15 min at 37 °C with 5mM EDTA in PBS and gentle pipetting. Cells were recovered by centrifugation and finally resuspended in HEPES buffer (10mM HEPES, 137mM NaCl, 2.9mM KCl and 12mM NaHCO3, pH 7.4) containing 5.5mM glucose. The count of vital cells was determined by trypan blue staining and phase contrast microscopy analysis, and finally adjusted to the concentration of $1 \cdot 10^7$ cells/ml.

Platelet purification and analysis of cancer cell-induced platelet activation

section 3.2

1-Platelet purification procedures

Platelet-rich-plasma (PRP) was obtained by centrifugation at 120 g for 15 min from blood withdrawn from healthy volunteers using 0.3% sodium citrate as anticoagulant.

Washed and isolated human platelets were prepared from buffy-coat bags collected the same day of the experiment. 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. Diluted blood was divided in 5 ml aliquots and centrifuged at 120 g for 15 minutes at room temperature, and a volume corresponding to one third of the upper platelet-rich supernatant was recovered. Apyrase (0.2 U/ml) and PGE1 (1 μM) were added and platelets were recovered as a pellet by centrifugation at 750 g for 15 minutes. Isolated platelets were obtained by directly resuspending the platelet pellet in HEPES buffer plus 5.5mM glucose, 1mM $CaCl_2$, and 0.5mM $MgCl_2$ at the concentration of $0.3 \cdot 10^9$ platelets/ml, skipping any washing step.

Conversely, in order to obtain washed platelets, the pellet was resuspended in 5 ml of PIPES buffer (20 mM PIPES and 136 mM NaCl, pH 6.5). Upon an additional centrifugation at 750 g for 15 min, the pellet was finally resuspended in HEPES buffer in presence of 5.5mM glucose, 1mM $CaCl_2$, and 0.5mM $MgCl_2$, at the concentration of $0.3 \cdot 10^9$ platelets/ml.

A small aliquot (typically 1 ml) of buffy-coat was directly centrifuged at 5000 g for 5 minutes to precipitate all the blood cell types, thus allowing the recovery of autologous platelet-free plasma. Serum was obtained by collecting whole blood in Vacutainer Serum tube and allowing the blood to clot by leaving it undisturbed at room temperature for 1 hour. The clot was removed by centrifuging at 2000 g for 10 minutes and then the serum was transferred.

2-Analysis of TCIPA

Analysis of TCIPA was performed in a light transmission Born aggregometer from Chrono-Log Corporation, under constant magnetic stirring at 37 °C. Platelet samples (0.3 ml at $0.3 \cdot 10^9$/ml), left untreated or preincubated with different inhibitors, were incubated with cancer cells (typically $1 \cdot 10^5$ cells/ml) in the absence or in the presence of different doses of platelet-free plasma or serum. The concentration of cancer cells used for platelet stimulation was selected through preliminary dose-response experiments. The extent of platelet aggregation was continuously monitored for 30 min.

3-Analysis of dense granule secretion

Dense granule secretion was assessed as the release of both serotonin and ATP. For the analysis of serotonin release, platelets resuspended in PIPES buffer at the concentration of $0.5 \cdot 10^9$/ml were metabolically labeled with (^{14}C)serotonin for 30 minutes at 37°C. Platelets were then washed in a large volume of PIPES buffer, centrifuged at 750 g for 15 min, and finally resuspended in HEPES buffer in presence of 5.5mM glucose, 1mM $CaCl_2$, and 0.5mM $MgCl_2$, at the final concentration of $0.3 \cdot 10^9$ platelets/ml. Platelets were incubated with 5 µM imipramine to prevent serotonin reuptake and a 10 µl aliquot was collected to evaluate the total incorporated radioactivity. (^{14}C)serotonin-labelled platelets (0.1 ml samples) were then stimulated with cancer cells in the presence of plasma (0.05% v/v). Stimulation was stopped by addition of 0.1 ml of 2% formaldehyde and 100mM EDTA and cooling on ice. Platelets were recovered by centrifugation at 10000 g for 3 min, and the radioactivity of (^{14}C)serotonin released in the supernatant was determined by liquid scintillation counting.

For ATP quantification, washed platelets ($0.3 \cdot 10^9$/ml) were stimulated with cancer cells (10^5/ml) and the reaction was stopped at different time points by adding 2mM EDTA. Supernatant was collected by centrifugation at 10000 g for 5 min and the amount of ATP in the cell- and platelet-free supernatant was quantified by using the luminescence-based ATP determination kit (Biaffin, Kassel, Germany), following the manufacturer's instructions.

4-Measurement of thrombin generation

Washed platelets ($0.3 \cdot 10^9$/ml) were stimulated with cancer cells (10^5/ml) in the presence of plasma (0.05% v/v), under stirring for different periods of time. Platelets and cancer cells were removed by centrifugation at 10000 g for 5 min and thrombin in the supernatant was quantified using the Thrombin Activity Fluorimetric Assay Kit (BioVision) following the manufacturer's instructions.

5-Analysis of Rap1 and PKC activation

Rap1b activation was evaluated through a pull-down assay, by selective precipitation of the GTP-bound active form of the protein, followed by immunoblotting analysis with an anti-Rap1 antibody, whereas PKC activation was determined directly by immunoblotting using an anti-Phospho(Ser)PKC substrates antibody.

For both analyses, 0.5 ml samples of washed platelets were stimulated with cancer cells (10^5/ml) in the presence of autologous plasma (0.05% v/v), under stirring at 37 °C for different time points. Stimulation was stopped using 0.5 ml of ice-cold lysis buffer 2X (100 mM Tris/HCl, pH 7.4, 400 mM NaCl, 5 mM $MgCl_2$, 2% Nonidet P-40, 20% glycerol, 0.2 µM aprotinin, 2 µM leupeptin, 2mM PMSF, 2mM Na_3VO_4 and 2mM NaF). Samples were clarified by centrifugation at 18000 g for 10 minutes at 4°C and 0.2 ml aliquots were immediately dissociated with 0.1 ml of SDS sample buffer 3X (37.5 mM Tris/HCl, pH 8.3, 288 mM glycine, 6% SDS, 1.5% DTT, 30% glycerol and 0.03% Bromophenol Blue) and heated at 95°C for 3 min for the total immunoblotting analyses.

Active Rap1 was precipitated from 0.8 ml of clarified lysate samples using 20 µg of purified recombinant GST-tagged Rap binding domain of RalGDS (GST-RalGDS-RBD) coupled with 30 µL GSH-Sepharose 2B by incubation for 2 hours at 4°C. Precipitated active Rap1b was washed 3 times with 1 ml ice-cold lysis buffer, dissociated with 25 µL of SDS sample buffer 2X and revealed by immunoblotting with anti-Rap1 antibody. Aliquots of the total cell lysates were also analyzed by immunoblotting with anti-Rap1 antibody to prove that the activation assay had been performed with samples containing the same amount of proteins.

6-Analysis of calcium movements

Fura-2-AM-loaded platelets were stimulated with cancer cells, in the presence of 1mM $CaCl_2$, 0.5mM $MgCl_2$, and 0.05% (v/v) of autologous plasma under gentle stirring at 37 °C. The Fura-2 emission fluorescence at 500 nm was monitored every 5 minutes using a Perkin Elmer Life Sciences LS3 spectrofluorometer.

Purification and characterization of PMPs

Section 3.3

Purified platelets ($3 \cdot 10^8$ platelets/ml) resuspended in HEPES buffer in the presence of 1mM $CaCl_2$ and 0.5mM $MgCl_2$, were incubated with breast cancer cells ($5 \cdot 10^4$ cells/ml) and 0.05% (v/v) of autologous plasma, or with the physiological agonist thrombin (0.2 U/ml) for 30 minutes at 37°C under constant stirring. In selected experiments, platelets were labeled with 3 μg/ml of carboxyfluorescein succinimidyl ester (CFSE) for 10 minutes, before stimulation.

Platelets and cancer cells were pelleted by centrifugation (750 g, 15 minutes), the supernatant was recovered and directly analyzed by flow cytometry and fluorescence microscopy or further centrifuged at 20000 g for 90 minutes at 10 °C to collect PMPs, which were quantified for protein content by bicinchoninic acid assay (BCA) and used for subsequent experiments.

Flow cytometry quantification of PMPs release was performed with a three laser-equipped BC Navios flow cytometer (Beckman Coulter), upon staining with PE-labeled anti-CD41 antibody by using reference counting beads (Flow-Count Fluorospheres BC).

Fluorescent CFSE-labeled PMPs were visualized by fluorescence microscopy using a BX51 Olympus microscope equipped with a 100 W mercury lamp.

Analysis of the effects of PMPs on cancer cells

Section 3.4

1-Analysis of PMPs-cancer cell interaction

MCF7 and MDA-MB-231 cells ($5 \cdot 10^4$ cells/well) were grown for 24 hours on glass coverslips placed in 12-well plate and then incubated with 30 µg/ml of PMPs from CFSE-labeled platelets for 4 or 18 hours. Cells were subsequently washed, fixed and finally treated with 1 µg/ml of Hoechst-33342. Glass coverslips were mounted on microscope slides and examined by fluorescence microscopy using BX51 Olympus microscope. The interaction between PMPs and cancer cells was quantified at the microscope as the percentage of cells associated with fluorescent PMPs, and as the average incorporated fluorescence using ImageJ software.

The interaction between CFSE-labeled PMPs and MDA-MB-231 cells was also evaluated by flow cytometry. Upon incubation with PMPs for 18 hours, cells were harvested as described above, fixed in 1% PFA for 10 minutes and analyzed with the flow cytometer Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, USA).

The internalization of cell-associated PMPs was demonstrated through the analysis of the samples described above by confocal microscopy at the Centro Grandi Strumenti (University of Pavia) using a Leica DM IRBE Inverted Microscope and LAS AF software.

The internalization of PMPs was further investigated by transmission electron microscopy (TEM) upon 3,3' diaminobenzidine (DAB) photoconversion. Cancer cells were incubated with CFSE-labeled PMPs for 18 hours and then fixed with 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer, pH 7.4, at 4°C for 1 h. Cells were then washed and incubated with DAB (20 mg/10 ml in Tris HCl 0.05 M, pH 7.6) under irradiation with two 8W Osram Blacklite 350 UV lamps for 2 h at room temperature, causing FITC excitation associated to the generation of highly reactive singlet oxygen, which in turn induces the oxidation of DAB into an electron-opaque osmiophilic precipitate. Upon photoconversion, cancer cells were then post-fixed with 1% OsO4 and 1.5% potassium ferrocyanide at room temperature for 1 h, dehydrated with acetone and embedded in Epon. Ultrathin sections were weakly stained with uranyl acetate and observed in a Philips Morgagni transmission electron microscope operating at 80 kV and equipped with a Megaview II camera for digital image acquisition.

2-Analysis of cell viability

Cell viability was assessed by using the colorimetric MTT assay. Cancer cells, seeded in 96-well plate at a density of $5 \cdot 10^3$ cells/well the day before the experiment, were incubated with increasing amounts of PMPs for 24 hours at 37°C. MTT solution was added at the final concentration of 0.5mg/ml and incubated at 37° for 3 additional hours. Violet crystals were dissolved by incubation with dimethyl sulfoxide for 10 minutes at room temperature, and finally the plate was read using a test wavelength of 570 nm and a reference wavelength of 650 nm.

3-Analysis of cell cycle

Cancer cells were left untreated or treated with PMPs for 24 hours and then harvested and fixed in cold 70% ethanol for 30 min at 4°C. Cells were spun at 850 g washed twice in PBS and resuspended in PBS containing 100µg/ml RNase, 0.05% NP-40 and 5µg/ml propidium iodide PI and incubated for 30 minutes at room temperature in the dark and then left overnight at 4°. Cells were then analyzed by flow cytometry using the Attune™ NxT Software v2.7 (Thermo Fisher Scientific, USA).

4-Assessment of migration and invasion

Cancer cell migration and invasiveness was analyzed using Falcon cell culture inserts (8-µm pore size) positioned in a 24-well plate. For the invasion assays, the upper side of the insert was coated with 0.1 ml of Matrigel (50 µg/ml) following the manufacturer's indications. Cells were serum starved for 6 hours and then resuspended in DMEM containing 0.5% FBS plus 2 mM L-glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin. Cell samples were either left untreated or treated with increasing amount of the different PMP preparations and then transferred inside the inserts. Complete medium, containing 10% FBS, was added to lower chamber as a chemotactic stimulus. Upon 18 hours, cells that migrated through the porous membrane were stained with 0.5% crystal violet and counted at the microscope.

5-Study of autophagy: flow cytometry, western blotting and immunofluorescence

The effect of PMPs internalization to the autophagic flux of MDA-MB-231 cells was evaluated using different approaches. MDA-MB-231 cells were left untreated or incubated with PMPs for 4 and 24 hours either in the absence, or in the presence of 50µM chloroquine for the last 3 hours of incubation.

For western blotting analysis, adherent cells were lysed with ice-cold lysis buffer 1X (100 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.1% SDS, 0.25% deoxycholic acid, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1mM PMSF, 2mM Na_3VO_4 and 10mM NaF) and recovered using a cell scraper. The samples were clarified by centrifugation at 18000 g for 10 minutes at 4°C, dissociated and heated at 95°C for 3 min. Total lysates were separated on 15 % polyacrylamide gel, blotted on PDVF membranes and analyzed with appropriate primary and secondary antibodies.

For immunofluorescence analysis MDA-MB-231 were grown for 24 hours on glass coverslips placed in 12-well plate and then treated with PMPs from CFSE-labeled platelets for 24 hours either in the absence, or in the presence of 50µM chloroquine for the last 3 hours of incubation. Cells were subsequently washed twice with PBS, fixed with 3% PFA for 10 minutes and permeabilized by a 10 minutes incubation with ice-cold 0.25% TRITON X-100 in PBS. Permeabilized cells were then incubated with anti-LC3 or p62 antibodies for 2 hours, washed three times with PBS and finally incubated with a PE-conjugated anti-rabbit secondary antibody for 1 hour. Samples were further

washed with PBS and incubated for 5 minutes with 1 µg/ml Hoechst 44432 before being mounted and visualized under BX51 Olympus microscope.

In order to determine the total amount of LC3-II cells were harvested and analyzed by a flow cytometry-based approach using the FlowCellect Autophagy LC3 Antibody-based Assay Kit (Merck) following the manufacturer's instructions.

Statistical analysis

All the reported figures are representative of at least three experiments and data were analyzed by t-test (for comparisons of two groups) or one-way ANOVA with the Bonferroni post-test (for multiple comparisons).

4

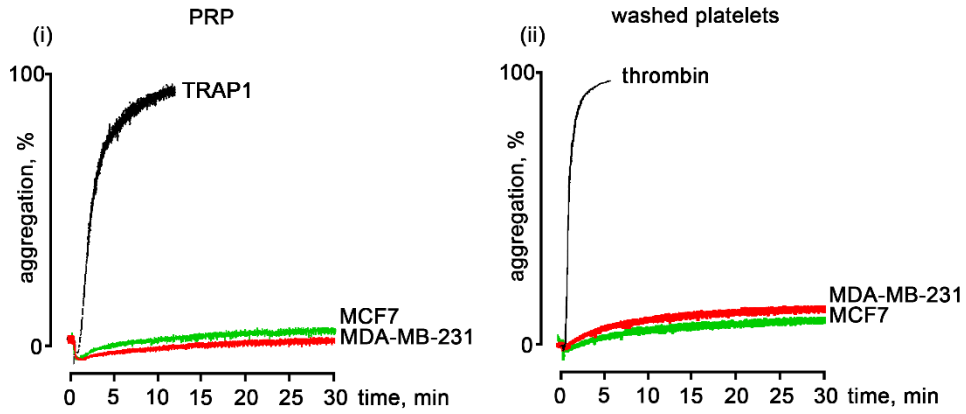
Results: Analysis of TCIPA

The contribution of plasma to TCIPA

In the first part of this study, the molecular requirements and mechanisms of TCIPA were investigated using two breast cancer cell lines with a different metastatic potential: the mesenchymal-like, high-metastatic MDA-MB-231 cells, and the epithelial-like, low-metastatic MCF7 cells. Both cancer cell lines had been analyzed in some previous studies (Belloc et al., 1995; Wei et al., 2015; Lian et al., 2013), but a direct comparison and complete characterization of their effects on platelets have never been reported.

We found that both cancer cells at a final concentration of 10^5 cells/ml were unable to induce aggregation of citrated PRP, or of washed platelets resuspended in Hepes buffer in the presence of $CaCl_2$, over a period of 30 minutes (figure 9A). No platelet aggregation was detected over a wide range of cancer cells concentration (10^4-10^6 cells/ml) or when the analysis was prolonged up to 60 minutes (data not shown). These results are in agreement with previous findings (Wei et al., 2015), but are in clear contrast with some other reports documenting cancer cell-induced aggregation of washed platelets (Mitrugno et al., 2014; Lian et al., 2013).

Figure 9A

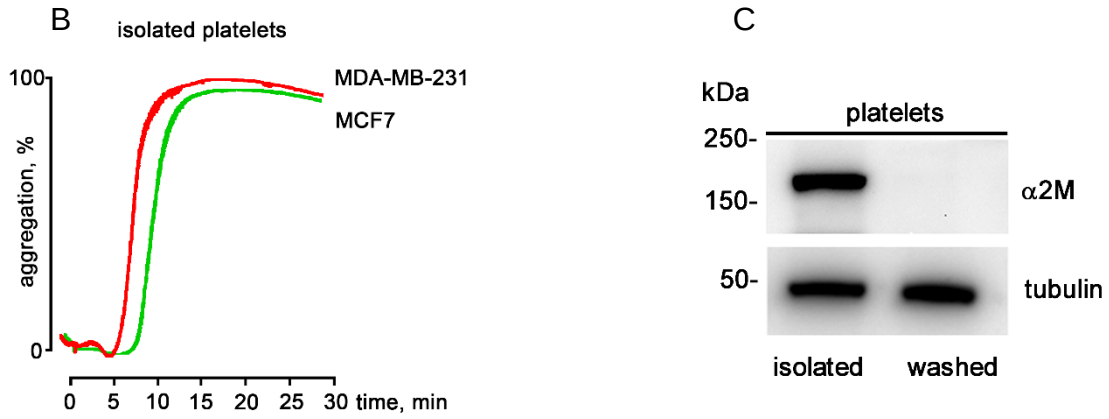


PRP (i) or washed platelets (ii) were stimulated in an aggregometer at 37°C under constant stirring with 10^5/ml of MCF7 and MDA-MB-231 cells, as indicated. Representative aggregation traces are reported.

Platelet responses are strongly influenced by the purification procedure and some previous investigations available in literature, where aggregation of washed platelets was documented, lack detailed reference to the platelet washing procedure. Therefore, we analyzed TCIPA using isolated platelets, obtained by omitting the washing step. As reported in figure 9B, isolated platelets fully aggregated in response to MDA-MB-231 and MCF7 cells, with a lag time of about 5 minutes and the aggregation was maximal 15 minutes after stimulation. Importantly, if $CaCl_2$ is omitted from the resuspension Hepes buffer, TCIPA of isolated platelets was completely prevented suggesting an essential role for Ca^{2+} in TCIPA (data not shown).

The different responses observed in the two platelet preparations may be reasonably due to the presence of residual plasma contamination in isolated platelets, which were not subjected to a thorough washing procedure. This hypothesis was confirmed by immunoblotting analysis that revealed the presence of the plasma protein α2-macroglobulin in the isolated platelet preparation, but not in washed platelets (Figure 9C).

Figure 9B – C

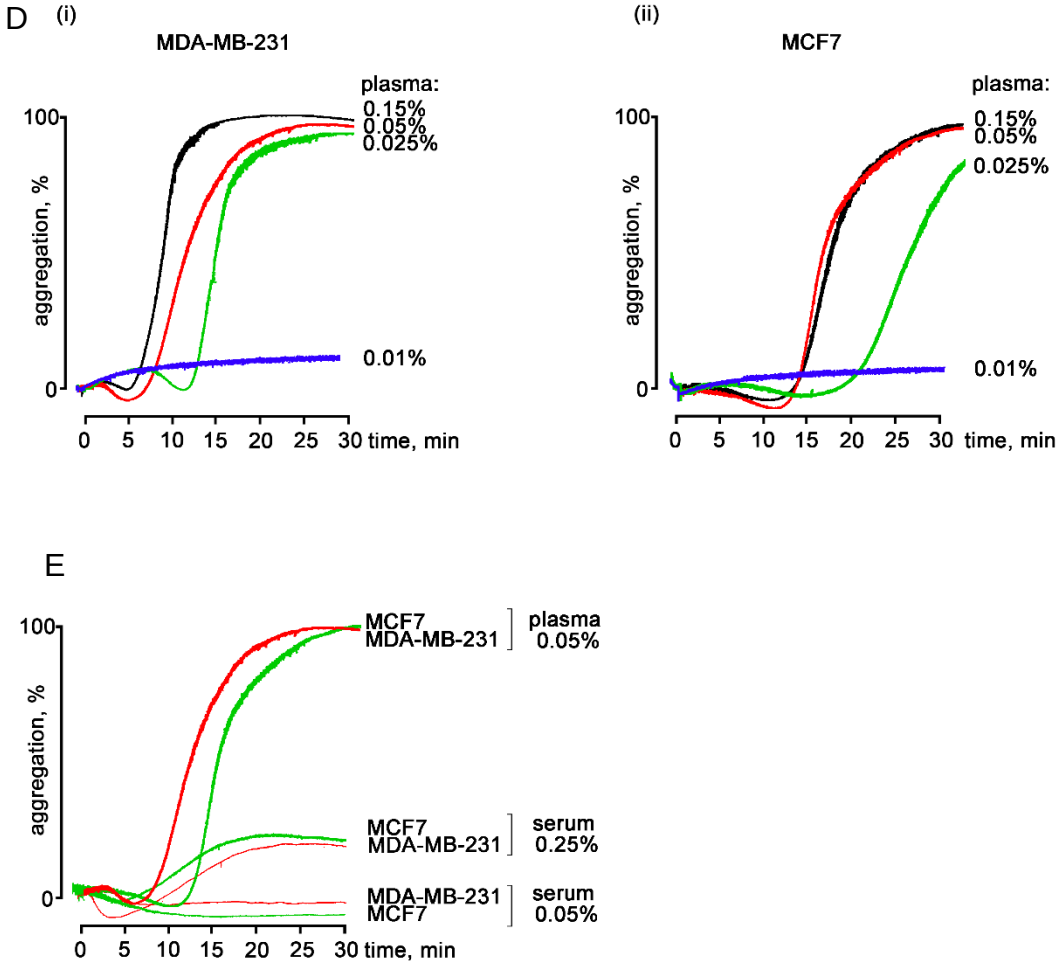


B: isolated platelets were stimulated in an aggregometer at 37°C under constant stirring with 10^5/ml of MCF7 and MDA-MB-231 cells, as indicated. Representative aggregation traces are reported.C: Immunoblotting analysis of protein expression from total lysates of isolated and washed platelets (10 µg) with anti-α2-macroglobulin antibody (α2M, upper panel). Subsequent reprobing with anti-tubulin antibody was performed for equal loading control.

To confirm the contribution of plasma to TCIPA, the aggregation of washed platelets induced by MDA-MB-231 and MCF7 cells was investigated in the presence of increasing amounts of autologous plasma. Figure 9D shows that the addition of small amounts of plasma was sufficient to allow a full aggregation of washed platelets induced by both tumor cells. This effect was dose-dependent and maximal aggregation in response to both cell types was observed in the presence of 0.05% (v/v) autologous plasma. Further increase in the amount of added plasma caused a progressive shortening of the lag time; however, amounts of plasma higher that 0.5% (v/v) often induced spontaneous aggregation (data not shown). Therefore, all the subsequent analysis of TCIPA were performed using washed platelets in the presence of 1 mM $CaCl_2$ and 0.05% (v/v) autologous plasma.

As reported in figure 9E, the ability of low amounts of plasma to allow TCIPA could not be reproduced by serum, as washed platelets failed to aggregate in response to both cancer cell lines in the presence of 0.05% (v/v) serum, nevertheless TCIPA was partly restored only when doses of serum as high as 0.25% (v/v) were used.

Figure 9D-E

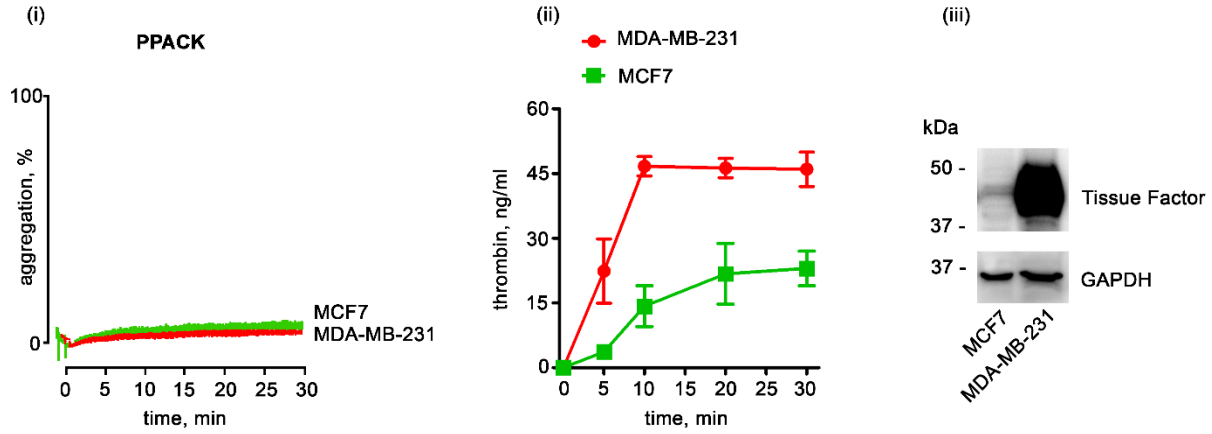


D: Washed platelets were incubated with increasing amounts of autologous plasma, (from 0.01 to 0.15% v/v, as indicated) and then stimulated with 10^5/ml of MDA-MB-231 (i) or MCF7 (ii) cells. E: Representative traces of platelet aggregation induced by cancer cells in the presence of 0.05 or 0.25% of serum. Aggregation was monitored for 30 minutes at 37°C under constant stirring and representative traces of 3 different experiments are reported.

Role of thrombin in TCIPA

Since serum is depleted of coagulation factors, we hypothesized that fibrinogen or serine proteases present in plasma may support TCIPA. In this regard, we found that the irreversible serine protease inhibitor PPACK totally prevented TCIPA in the presence of 0.05% plasma (Figure 10A), suggesting that generation of thrombin or other proteases of the coagulation cascade may occur in the samples. By analyzing thrombin generation in the course of platelet aggregation, we found that both cancer cell lines were actually capable of promoting thrombin formation, albeit with different efficiency. In particular, the amount of generated thrombin was higher in samples stimulated with MDA-MB-231 rather than MCF7 cells. In all samples, however, a significant increase in thrombin activity was detected within 5 to 10 minutes after stimulation, a kinetics that well correlates with aggregation (Figure 10A). Importantly, no thrombin generation was detected when either cancer cells, plasma or washed platelets were omitted in the samples (data not shown), indicating that the presence of all the three components is strictly required for thrombin generation. Several tumor cells express tissue factor (TF) which may activate the coagulation cascade. We thus analyzed the presence of TF on these 2 breast cancer cell lines. As shown in figure 10A, TF was actually present in both cancer cells, but the level of expression was highly variable. In particular, TF is expressed at high levels in MDA-MB-231 cells, but only barely detectable in MCF7.

Figure 10A

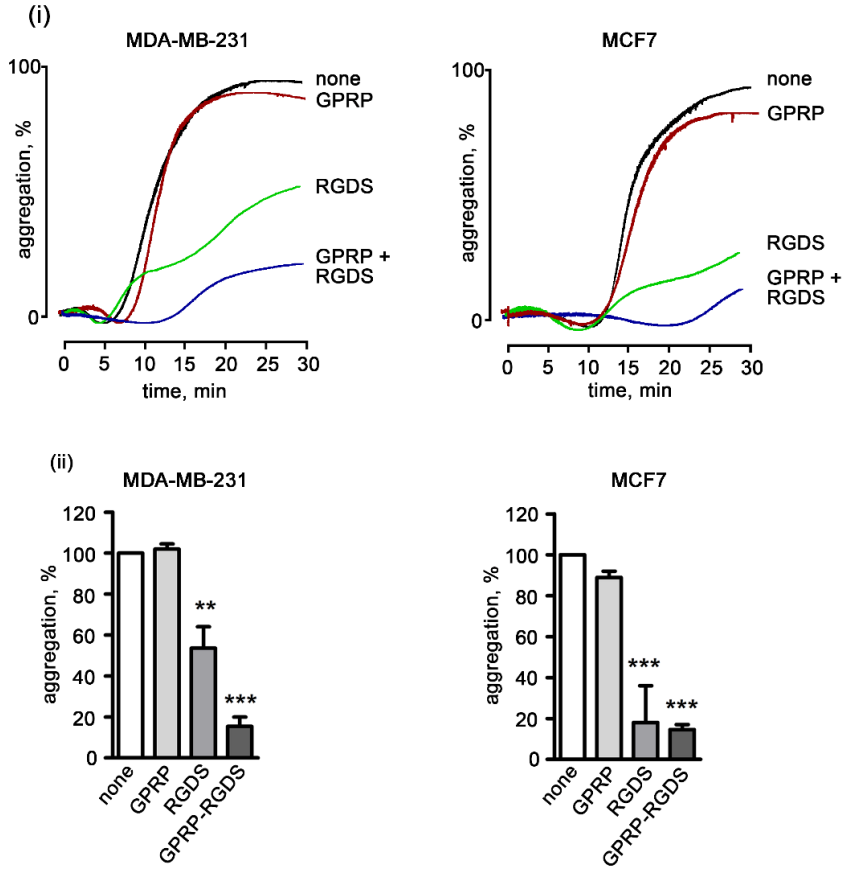


(i) the effect of 2 µM of the Ser-protease inhibitor PPACK on aggregation of washed platelets induced by MDA-MB-231 or MCF7 cells in the presence of autologous plasma. Aggregation traces representative of three different experiments are reported. (ii) Washed platelets (0.3x10^9/ml) were incubated for increasing times with 10^5/ml of the different cancer cells in the presence of 0.05 % (v/v) of autologous plasma. The amount of thrombin in the supernatant of the different samples at different time points was determined using a Thrombin activity fluorometric assay kit. Results are expressed as mean ± SD of 3 different experiments. (iii): Immunoblotting analysis of MCF7 and MDA-MB-231 cells whole lysate (25 µg) with anti-TF antibody. Control for equal loading was performed by reprobing the membrane with anti-GAPDH antibody.

We next investigated the mechanisms supporting TCIPA focusing on the contribution of the binding between integrin αIIbβ3 and fibrinogen. Preincubation of platelets with the integrin antagonist

GRGDS caused a delay in the light transmission increase induced upon addition of both breast cancer cells and markedly reduced the maximal aggregation. The contribution of fibrinogen binding to integrin αIIbβ3 to TCIPA appeared to be particularly evident in MCF7 cells-stimulated samples, however RGDS failed to completely suppress aggregation induced by both cell lines (Figure 10B). Since TCIPA was associated to thrombin formation and since this serine protease is responsible of fibrinogen conversion to fibrin, we analyzed the possible contribution of platelet entrapment into the fibrin network to the formation of aggregates. As shown in figure 10B, when used alone the peptide GPRP, which inhibits fibrin polymerization, had no effects on TCIPA induced by both cancer cell lines. However, GPRP used in combination with RGDS almost completely suppressed aggregation triggered by MDA-MB-231 and MCF7 cells, indicating that both fibrin formation and fibrinogen binding to platelets contribute to TCIPA.

Figure 10B

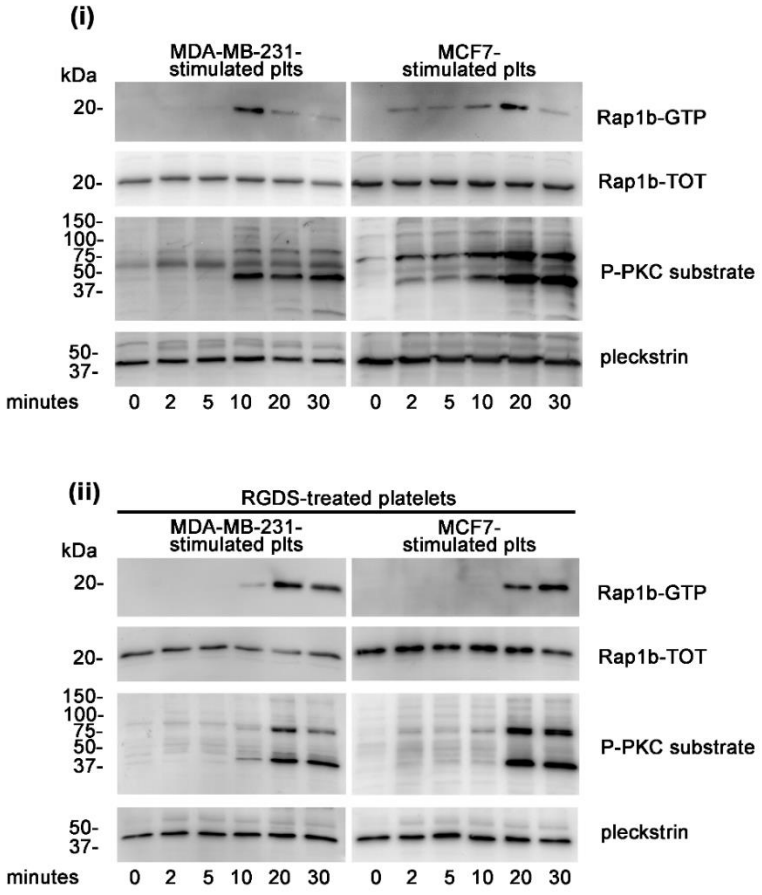


*Washed platelets were preincubated for 5 minutes with 0.5 mM of RGDS or 0.5 mM GPRP, either alone or in combination and then stimulated with MDA-MB-231 or MCF7 cells in the presence of 0.05 % (v/v) of autologous plasma. Representative aggregation traces are reported in (i) and quantification of maximal aggregation at 30 minutes is reported in (ii) as percentage of aggregation measured in the absence of any added inhibitors. Data are the mean ± SD of 4 independent experiments. **: p<0.01; ***: p< 0.005.*

Mechanism of platelet activation induced by cancer cells

To evaluate whether TCIPA was actually associated to effective platelet stimulation, we evaluated some typical biochemical responses which play key roles in platelet activation. In particular we have analyzed the activation of protein kinase C (PKC) by measuring the phosphorylation of its substrates and the stimulation of Rap1b, a known key regulator of integrin αIIbβ3 *(Materials and methods-section 3.2.5)*. As reported in figure 11A, the stimulation of platelets with cancer cells caused a delayed and transient accumulation of active Rap1b-GTP which was maximal between 10 and 20 minutes. PKC activation occurred with a similar kinetics and a marked phosphorylation of several PKC substrates, including the 47 kDa protein pleckstrin, was observed in response to both cancer cell lines. Importantly, no activation of PKC and Rap1b was detected when washed platelets were stimulated with cancer cells in the absence of added 0.5% autologous plasma (data not shown).

FIGURE 11A



Washed platelets were treated either in the absence (i), or in the presence of RGDS (ii). Active GTP-bound Rap1b was precipitated (Material and methods-Section 3.2.5) and visualized by immunoblotting using an anti-Rap1 specific antibody. Aliquots of total lysates were also analyzed using an anti-phospho PKC substrates antibody, as indicated on the right.

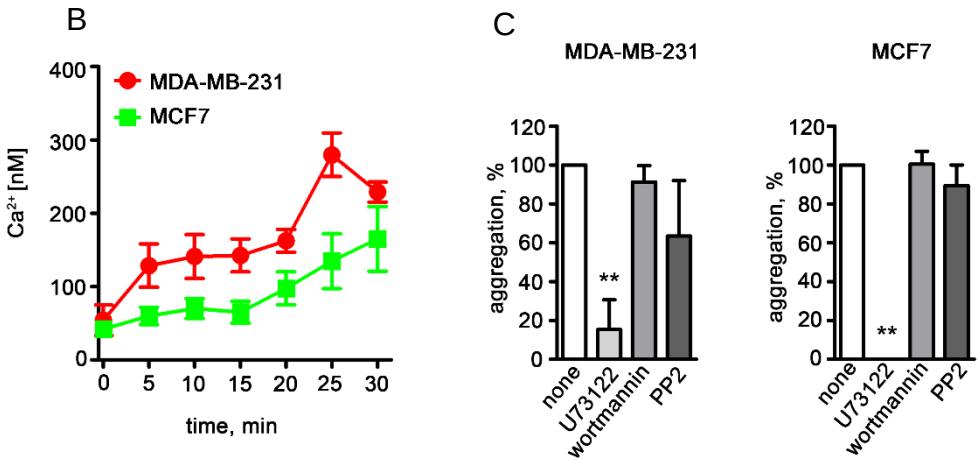
We also found that integrin αIIbβ3 outside-in signaling contributes to platelet activation induced by cancer cells, as in the presence of the peptide RGDS was observed a delay in the kinetics

of Rap1b and PLC activation, that became evident only after 20 min of stimulation (Figure 11A). Moreover, when integrin binding to fibrinogen was prevented by RGDS, Rap1b activation was no longer transient and remained sustained even after 30 minutes from stimulation (Figure 11A). These results indicate that integrin αIIbβ3 outside-in signaling during TCIPA accelerates platelet activation by sustaining both Rap1b and PKC stimulation.

The activation of PKC and Rap1b typically occurs downstream of phospholipase C (PLC), which is also required for IP3-mediated release of Ca^{2+} from intracellular stores. The analysis of Fura-2 loaded platelets *(Materials and methods-section 3.2.6)* demonstrated that both cancer cells induced the increase of cytosolic Ca^{2+} concentration, which was more pronounced upon stimulation with MDA-MB-231 rather than MCF7 cells (Figure 11B).

These observations indicate that exposure to breast cancer cells induced PLC activation, which then leads to the stimulation of PKC and Rap1b. The contribution of PLC-dependent signaling to TCIPA was investigated by using the selective PLC inhibitor U73122. These analyses confirmed the central contribution of PLC-driven intracellular events for TCIPA, indeed, in the presence of PLC inhibitor U73122 TCIPA was essentially abolished. By contrast, we found that TCIPA occurred independently of PI3K and did not require activation of SRC family kinases, as it was not affected by the specific inhibitors wortmannin or PP2 (Figure 11C).

Figure 11B-C

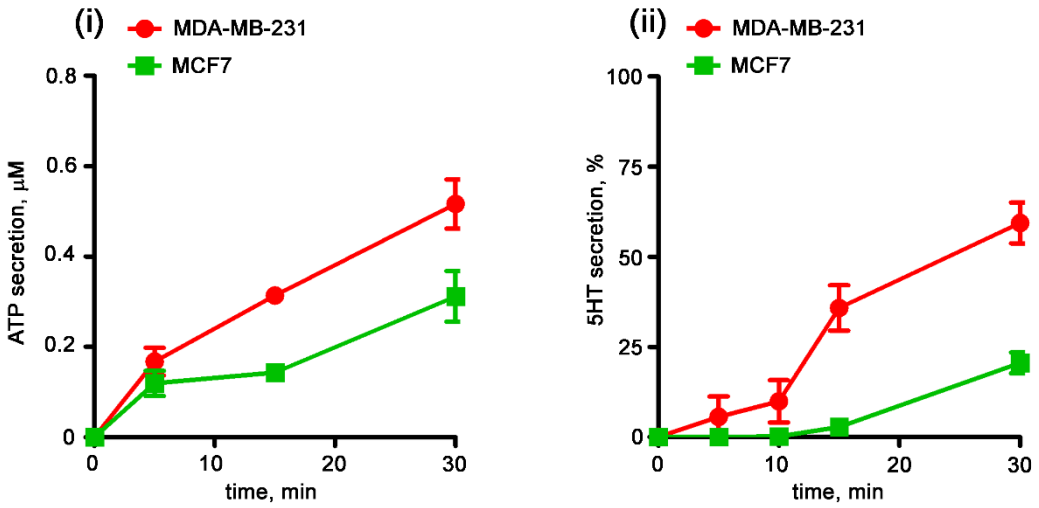


*B: FURA2-loaded platelets were incubated for increasing times with MDA-MB-231 or MCF7 cells, in the presence of 0.05 % (v/v) of autologous plasma. The cytosolic Ca2+ concentration was determined by measuring FURA-2 fluorescence at different times after stimulation. Results are expressed as the mean ± SD of 3 different experiments. C: Effect of 10 µM U73122, 100 nM wortmannin, or 10 µM PP2 on platelet aggregation stimulated by breast cancer cells. Platelet aggregation in the absence of inhibitors is reported as 100%. Data are the mean ± SD of 3 independent experiments. **: p<0.001*

Role of ADP secretion and TxA2 generation in TCIPA

Previous studies have demonstrated that prostate cancer cells can stimulate platelet granules secretion (Mitrugno et al., 2014). Here the release of dense granules was investigated by measuring the release of both ATP and serotonin *(Materials and methods-section 3.2.3)*. Figure 12A shows that both cancer cells were able to promote platelet dense granules secretion (Figure 12A), albeit with different efficiency. Indeed, platelets stimulated with MDA-MB-231 had a more pronounced serotonin, as well as ATP, secretion compared to MCF7 cells. Interestingly, ATP accumulation in the medium occurred earlier than serotonin secretion. In particular, ATP was detected as early as 5 minutes after stimulation, and thus clearly proceeded the markers of platelet activation such as PKC sand Rap1b stimulation. These results may be indicative of a contribution of cancer cells themselves in early ATP release.

FIGURE 12A

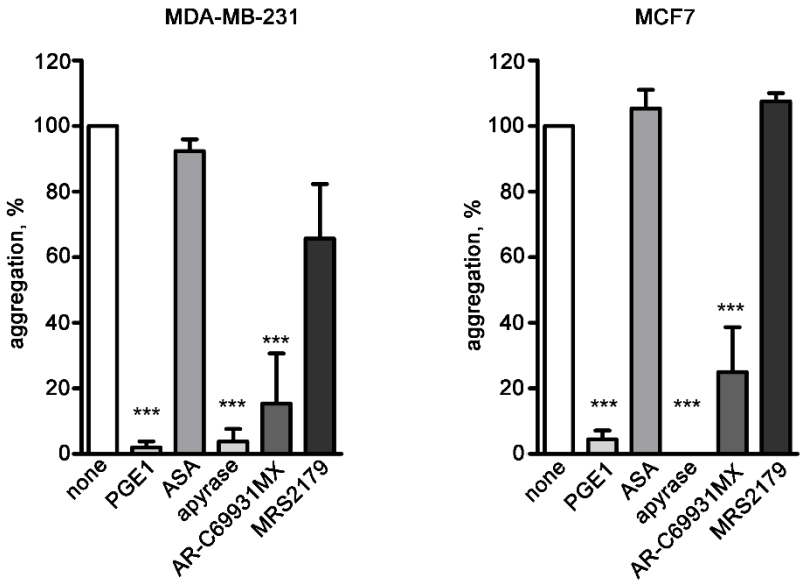


Analysis of platelet secretion induced by MDA-MB-231 or MCF7 cells. Secretion was determined as accumulation of ATP (i) or as release of ^{14}C serotonin form prelabeled platelets (ii). Results are reported as mean ± SD of 3 independent experiments.

To further characterize the role of platelet activation and granules secretion in TCIPA we analyzed the effect of selective platelet inhibitors. TCIPA induced by both cancer cells was completely suppressed upon the increase of intracellular cAMP induced by prostaglandin E1, confirming that the activation of platelet intracellular signaling pathways is absolutely mandatory to detect any aggregation. Moreover, platelet aggregation induced by MDA-MB-231 and MCF7 cells was insensitive to pretreatment of platelets with aspirin, which prevents TxA2 synthesis, but was completely abolished by the ADP scavenger apyrase (figure 12B). The contribution of ADP to TCIPA occurs through the stimulation of P2Y12 receptor as demonstrated by the use of the specific

antagonists of the two ADP receptors. Indeed, the P2Y12 specific inhibitor ARC69931MX, but not P2Y1 inhibitor MRS2179, strongly reduced TCIPA induced by MDA-MB-231 and MCF7 cells. Therefore, ADP-mediated positive feedback, but not TxA2 generation is essential for platelet aggregation induced by cancer cells.

FIGURE 12B



Quantification of tumor cell-induced platelet aggregation of platelets pretreated with 2 µM PGE1, 0.5 mM ASA, 2 U/ml apyrase, 1 µM AR-C69931MX, or 200 µM MRS2179, as indicated. Results are reported as percentage of aggregation compared to sample of untreated platelets, and are expressed as mean ± SD of 3 independent experiments.

5

Results:

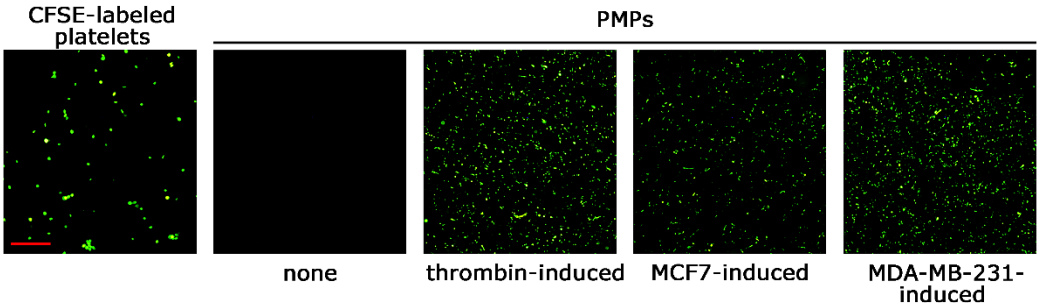
Analysis of cancer cell-induced release of PMPs

Cancer cell-induced release of PMPs

Cancer is often associated to an increased level of circulating PMPs (Mezoaur et al., 2014; Chaari et al., 2014) and elevated levels of circulating PMPs correlate with the advanced stages of cancer, thus suggesting a possible role of PMPs in cancer dissemination. This hypothesis is supported by few works (Janowska-Wieczorek et al., 2005; Janowska-Wieczorek et al., 2006) that demonstrated, *in vitro* and *in vivo*, a pro-metastatic potential of PMPs. The mechanisms responsible for the increase of PMPs observed in cancer patients are not known, therefore we have evaluated whether cancer cells are able to directly promote the release of microparticles from platelets.

Since platelets release PMPs mainly upon activation, to investigate the mechanism of cancer-associated increase of PMPs, we evaluated whether TCIPA induced by breast cancer cells was accompanied by the release of PMPs (*Materials and methods-section 3.3*). Fluorescently-labeled platelets were stimulated with MDA-MB-231 or MCF7 cells in the presence of 0.05% of autologous plasma under stirring and, after 30 minutes, PMPs released in the supernatant were analyzed. Fluorescence microscopy analysis revealed that both cancer cell lines were actually able to induce the release of microparticles from platelets (Figure 13A). Indeed, the supernatant of platelets stimulated with MDA-MB-231 or MCF7 cells clearly contained fluorescent microparticles, and although platelets have a small diameter of 2-4 µm, the size of fluorescent microparticles was clearly smaller and consistent with that expected for PMPs (up to 1 µm). As a positive control is reported the analysis of the supernatant of thrombin-stimulated platelets which is strongly enriched in PMPs, whereas the supernatant of unstimulated platelets contained no detectable particles.

Figure 13A

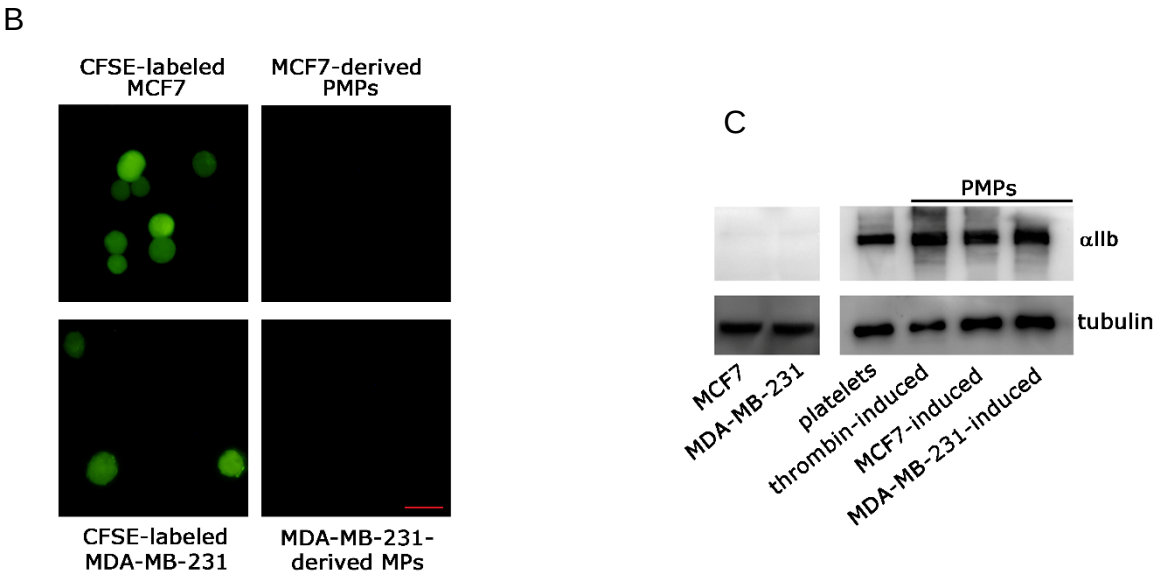


CFSE-labelled platelets were left untreated (none), co-cultured with either MCF7 or MDA-MB-231 cells or stimulated with thrombin for 30 minutes at 37°C under stirring (Materials and methods-section 3.3). Upon low-speed centrifugations performed to remove platelets and cells, labeled PMPs in the supernatant were visualized by fluorescence microscopy. Representative images of labeled platelets and released PMPs are reported. Scale bar: 50 µm.

Conversely, when unlabeled platelets were incubated with fluorescently-labeled breast cancer cells, no fluorescent microparticles were detected in the supernatant (Figure 13B),

demonstrating that co-culture of platelets and cancer cells do not induce the release of microparticles from cancer cells. Accordingly, the western blotting analysis of released microparticles, upon recovery by ultracentrifugation, clearly indicate that they all were CD41-positive (Figure 13C), further confirming the platelet origin of the microparticles collected.

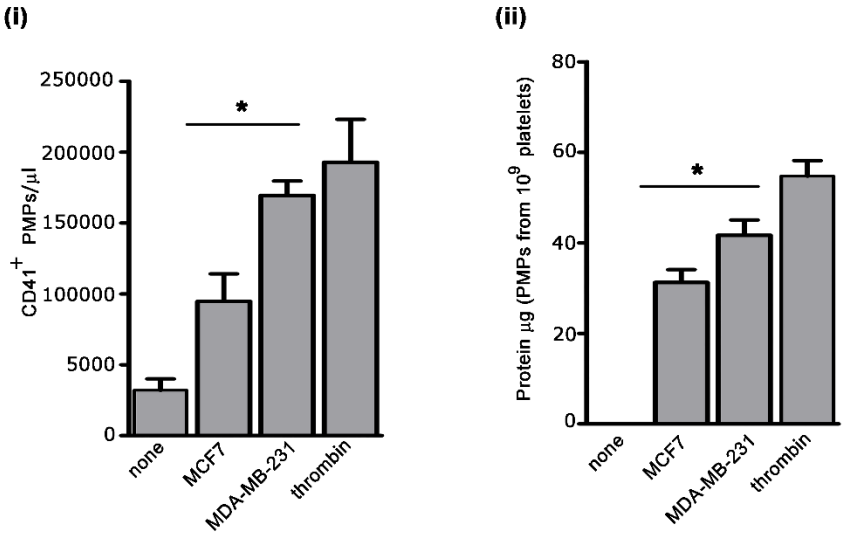
Figure 13B-C



B: Unlabelled platelets were stimulated with CFSE-labelled cancer cells and the microparticles released in the supernatant were analyzed by fluorescence microscopy. Scale bars: 50 µm. C: Immunoblotting analysis of total lysates of cancer cells, platelets and PMPs.

The quantification of tumor cell induced-release of PMPs was performed both by flow cytometry and by protein content quantification (*Materials and methdos-section 3.3*) and, interestingly, it revealed that the two cells lines were differently potent in inducing the release of microparticles from platelets (Figure 13D). Indeed, PMPs release induced by MDA-MB-231 cells was significantly higher both in terms of particles number (169,533±11,763 versus 95,000±22,584 PMPs/µl) and protein content (41.6±3.4 µg versus 31.2±2.8 µg of protein contained in PMPs released from 10^9 platelets) compared to MCF7 cells. Noteworthy, the amount of PMPs released upon stimulation with MDA-MB-231 cells was similar to that induced by the physiological agonist thrombin (190,000 ± 30,292 PMPs/µl) (Figure 13D).

Figure 13D



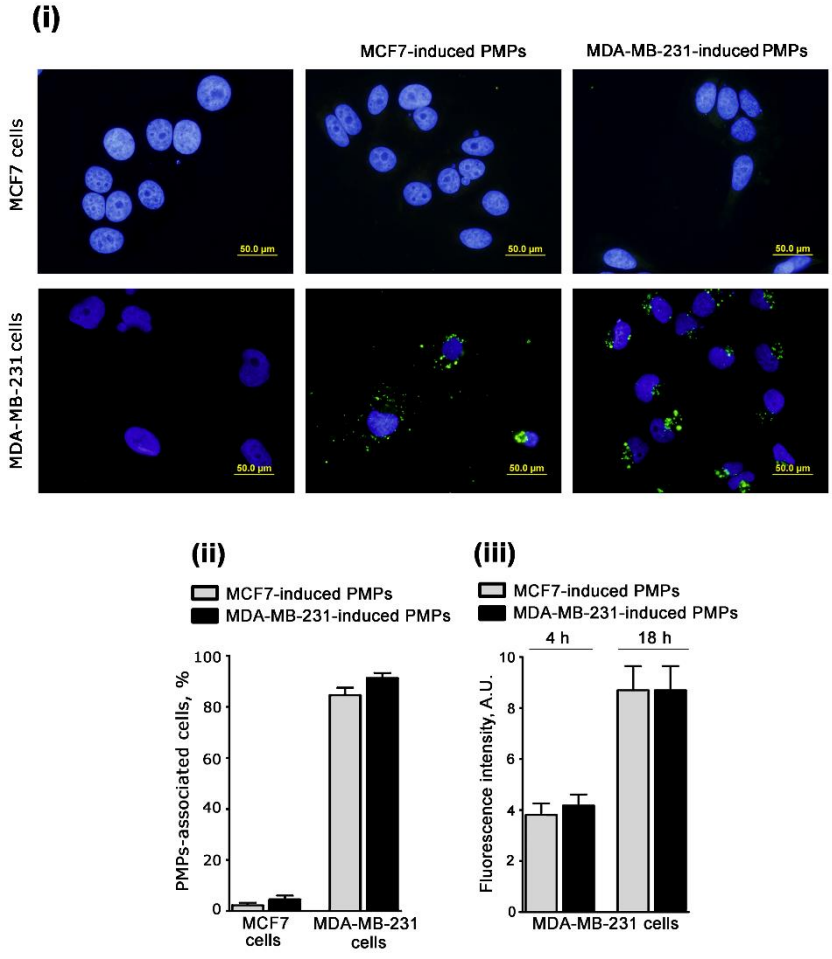
*(i) PMPs release was quantified using a Navios flow cytometer (Beckman Coulter), upon staining with PE-labeled anti-CD41 antibody (Bio Legend). The instrument was set using Megamix-Plus (BioCytex, Marseille, France) fluorescent calibrated beads (0.1–0.9 µm range). Data were analyzed with the FCS Express 6.0 software (De Novo Software),and are reported as mean ± SEM (n = 3). *p < 0.05. (ii) PMPs in the supernatant of the different samples were recovered by ultracentrifugation at 20,000 g for 90 minutes, resuspended in HEPES buffer, and analyzed by BCA protein assay. Results report the protein content of PMPs released from the same number of stimulated platelets (10^9) and are mean ± SEM (n = 7). *p < 0.05.*

Interaction of cancer cell-induced PMPs with cancer cells

PMPs released upon platelet activation with physiological agonists interact with several types of cells, including cancer cells (Janowska-Wieczorek et al., 2005; Janowska-Wieczorek et al., 2006), therefore we wondered whether cancer cell-induced PMPs had the ability to interact with the same cell types that promoted their release.

To address this question, cultured MDA-MB-231 and MCF7 cells were incubated for 18 hours with fluorescent PMPs recovered from the supernatant of platelet samples stimulated with either MCF7 or MDA-MB-231 cells (indicated as MCF7-induced and MDA-MB-231-induced, respectively) *(Material and methods-section 3.4.1)*.

Figure 14A

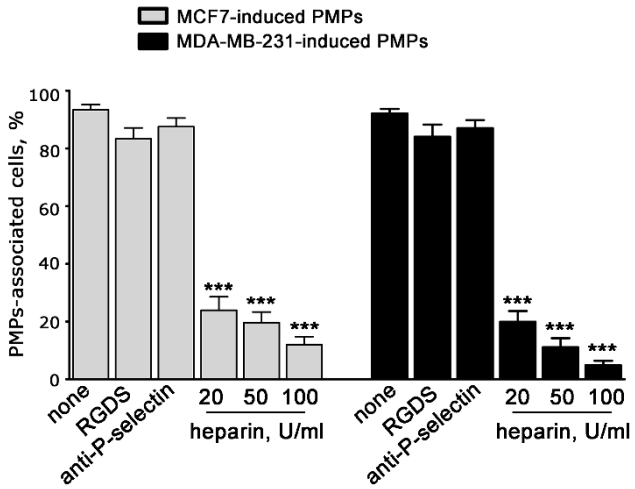


MCF7 and MDA-MB-231 were incubated with 30 µg/ml of MCF7-induced or MDA-MB-231-induced PMPs obtained from CFSE-labeled platelets for 4 or 18 hours. (i) Representative fluorescence microscopy images upon 18 hours of incubation. Green: CFSE-labeled PMPs; blue: cell nuclei. (ii) Quantification of the percentage of MCF7 orMDA-MB-231 cells (as indicated on the bottom) associated with fluorescent PMPs, induced by either MCF7- or MDA-MB-231 cells (gray and black bars, respectively, as indicated on the top). Data are the mean ± SEM (n=3). (iii) Comparison of MDA-MB-231 cells' ability to interact with MCF7- or MDA-MB-231-induced PMPs after 4 or 18 hours of incubation. Data are expressed as average green fluorescence intensity associated with each cell and are the mean ± SEM of three independent experiments.

As reported in figure 14A, only MDA-MB-231 cells were actually able to interact with both types of PMPs. Indeed, more than 90% of MDA-MB-231 cells was found to interact with both PMPs preparations and, interestingly, MDA-MB-231 cells were equally able to bind MCF7-induced and MDA-MB-231-induced PMPs as shown by the analysis of the cell-associated fluorescence. By contrast, no interaction was detected between MCF7 cells and either of cancer cell-induced PMPs (Figure 14A).

Integrin αIIbβ3 and P-selectin were shown to play a key role in the interaction of platelets with several cancer cell lines, therefore to verify their possible involvement in the interaction of MDA-MB-231 with PMPs we exploited αIIbβ3 antagonist peptide RGDS and a P-selectin blocking antibody (CLB/thromb/6, NOVUS Biologicals). Figure 14B shows that interaction of MDA-MB-231 cells with either type of PMPs was influenced by neither integrin αIIbβ3, nor P-selectin inhibition. Inetrestingly, we found that heparin caused a dose-dependent inhibition of this binding, suggesting the implication of a mechanism of interaction dependent on still unidentified carbohydrate moieties (Figure 14B).

Figure 14B

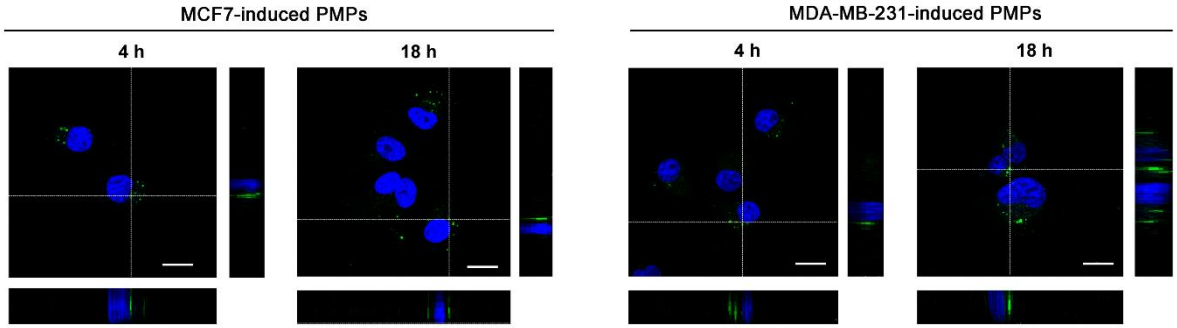


*MDA-MB-231 cells were incubated with 30 µg/ml of MDA-MB-231-induced PMPs (black columns) or MCF7-induced PMPs (gray columns) obtained from CFSE-labeled platelets in the presence of buffer (none), 0.5 mM RGDS, 10 µg/ml anti-P-selectin antibody CLB/thromb/6, or increasing amount of heparin, as indicated. The percentage of PMPs-associated cells was determined as described before and the results are expressed as the mean ± SEM (n=3). ***p < 0.001.*

Confocal microscopy analysis revealed that the detected association between MDA-MB-231 cells and PMPs was not due to a simple interaction between the plasma membrane of the cell and the surface of the microparticle, rather it involved a full vesicle internalization. Indeed, as shown in figure 14C, both MCF7- and MDA-MB-231-induced PMPs are localized in the cancer cell boundaries on the same focal plane of the nuclei, demonstrating that they were actually internalized by MDA-

MB-231 cells. Importantly, this process occurred rather rapidly as was detectable already after 4 hours of incubation for both PMPs preparations (Figure 14C).

Figure 14C



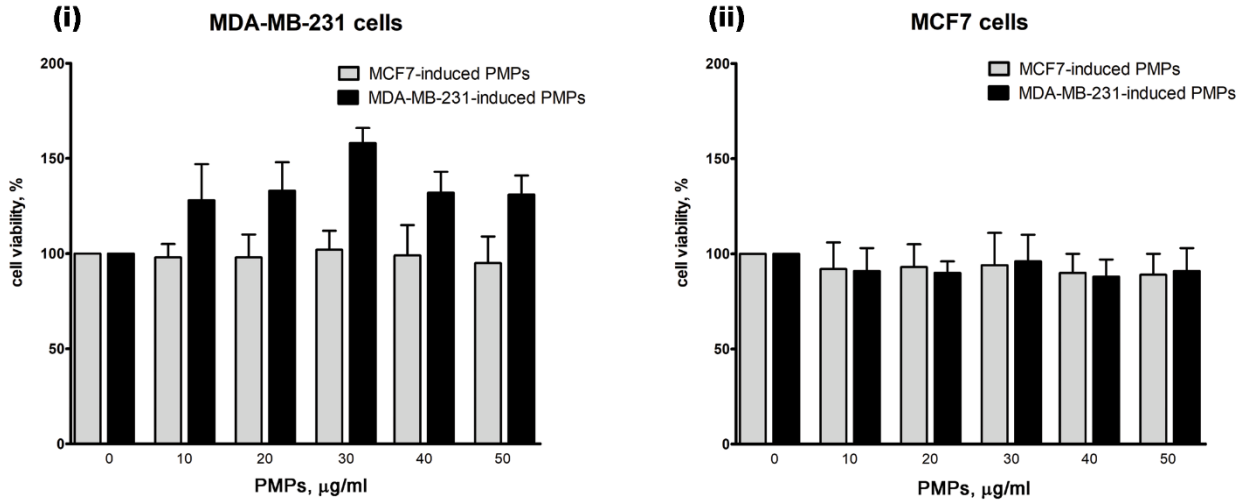
Confocal microscopy analysis of MDA-MB-231 cells interacting with MCF7- or MDA-MB-321-induced PMPs for the reported times of incubation. Representative confocal middle z-sections and orthogonal views are reported. Scale bars: 20 µm.

Altogether, these results indicate that the two different breast cancer cell lines, in spite of being both able to induce the release of PMPs by platelets, are differently targeted by PMPs themselves.

PMPs modulate migration and invasiveness of MDA-MB-231 cells

To investigate the functional consequence of PMPs on breast cancer cells, we initially analyzed their possible cytotoxic effect *(Materials and methods-section 3.4.2)*. Incubation of MDA-MB-231 or MCF7 with increasing amounts of PMPs (10-50 µg/ml) for 24 hours did not affected cell viability of both cancer cells (Figure 15A). MDA-MB-231-induced PMPs induced a slight, but still not statistically significant increase in viability of MDA-MB-231 cells.

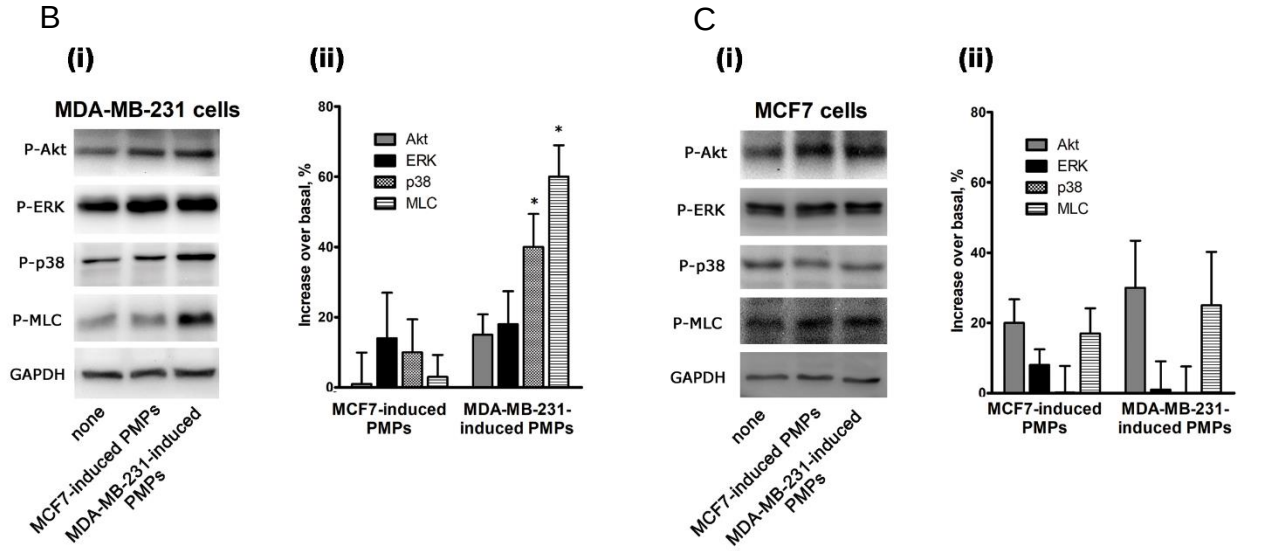
Figure15A



Viability of MDA-MB-231 (i) or MCF7 (ii) cells incubated with the indicated amounts of PMPs for 24 hours was assessed by a colorimetric MTT assay. Results are reported as the mean ± SEM (n=3).

In search of possible functional effects of PMPs on breast cancer cells, we have analyzed the phosphorylation of a panel of selected signaling proteins after cancer cells treatment with 30 µg/ml of PMPs. The levels of phosphorylation of different signaling proteins (including Akt and Erk, as reported in figure 15B) was found to be unaffected in both cell lines, upon incubation with either preparations of PMPs. However, as reported in figure 15B, MDA-MB-231 cells showed a significant increase in the phosphorylation of p38MAPK upon incubation with MDA-MB-231-induced PMPs, but not with MCF7-induced PMPs (Figure 15B). Consistent with their inability to bind PMPs, MCF7 did not display significant changes of protein phosphorylation upon addition of both types of PMPs (Figure 15C).

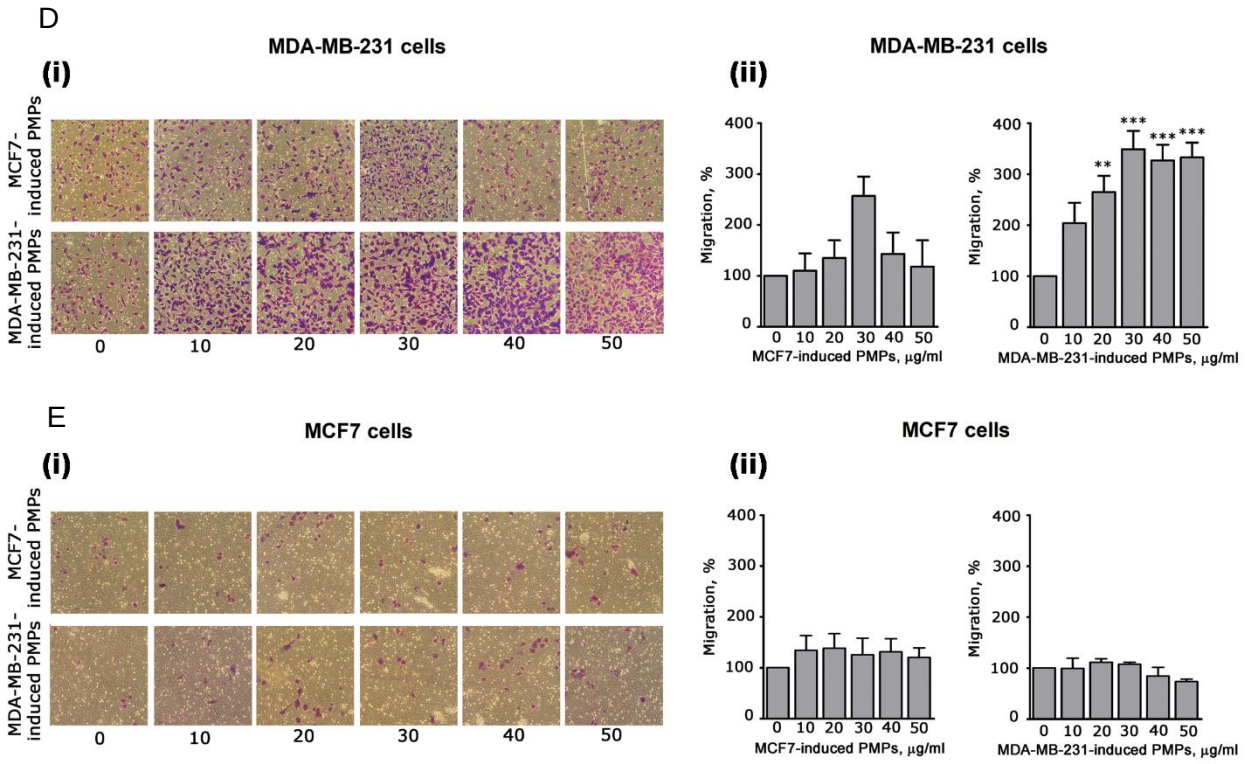
Figure 15B-C



Phosphorylation of selected signaling proteins in MDA-MB-231 (B) or MCF7 (C) cells incubated with MCF7- or MDA-MB-231-induced PMPs for 18 hours, as indicated on the bottom. (i) representative immunoblot with specific anti-phosphoprotein antibodies directed against the protein indicated on the right is reported in, where GAPDH staining is for equal loading control. (ii) quantification of the results by densitometric scanning is reported in, as % of phosphorylation increase over the level of untreated cells. Results are the mean ± SEM (n=3). $p < 0.05$.

Since p38MAPK and MLC are involved in the control of cell motility (Huang et al.,2004), we evaluated the effect of PMPs on the migration ability of both types of cancer cells *(Materials and methods-section 3.4.4)*. Interestingly, MDA-MB-231-induced PMPs, but not MCF7-induced PMPs, strongly and dose-dependently potentiated the migration of MDA-MB-231 cells (Figure 15D). Interestingly, this effect was significant at doses of PMPs which are in the range of those found in the plasma of cancer patients, that are lower than 50 µg/ml (Kim et al., 2003). Both preparations of PMPs had no effect on the migration of MCF7 cells (Figure15E).

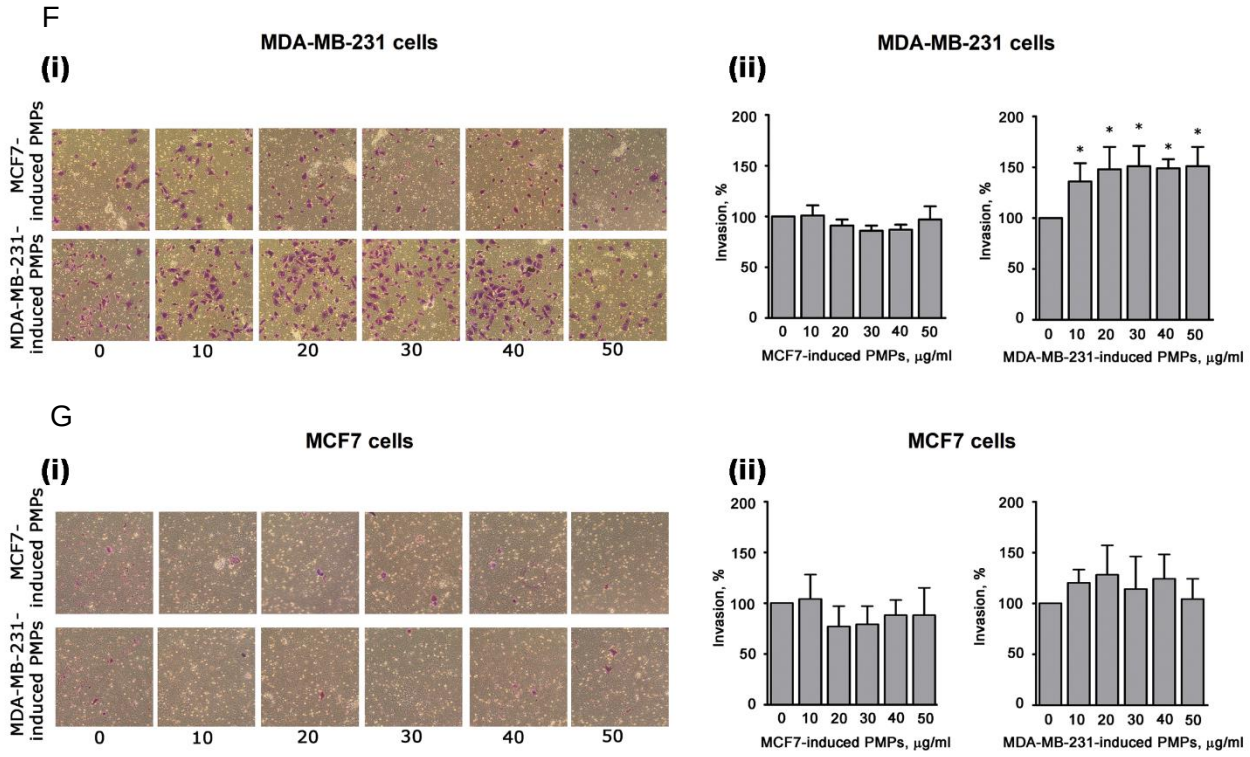
Figure 15D-E



*Effect of MCF7- or MDA-MB-231-induced PMPs (as indicated on the left) on migration of MDA-MB-231 cells (panel D) or MCF7 cells (panel E), as indicated. Cancer cells were treated with increasing amounts of the two different types of PMPs preparations (0–50 µg/ml, as indicated on the bottom) and then transferred inside cell culture inserts. Incubation was prolonged for 18 hours and the cells that moved through the porous membrane were stained and counted. In all the panels, representative images are reported in (i), while quantification of the results is shown in (ii) as mean ± SEM of three experiments. Statistical significance of the difference was calculated between treated and untreated cells (sample 0). **p < 0.01; ***p < 0.001*

Finally, using matrigel-coated transwell assay *(Materials and methods-section 3.4.4)*, we found that, as for migration, the invasion ability of MDA-MB-231 was potentiated upon addition of MDA-MB-231-induced PMPs, whereas MCF7-induced PMPs had no effect (Figure 15F). By contrast, the invasiveness of MCF7 cells resulted unaffected upon treatment with both types of PMPs (Figure 15G).

Figure 15F-G



*Effect of MCF7- or MDA-MB-231-induced PMPs (as indicated on the left) on invasiveness of MDA-MB-231 cells (panel F) or MCF7 cells (panel G), as indicated. Cancer cells were treated with increasing amounts of the two different types of PMPs preparations (0–50 µg/ml, as indicated on the bottom) and then transferred inside cell culture inserts. coated with 0.1ml of Matrigel (50 µg/ml). Incubation was prolonged for 18 hours and the cells that moved through the porous membrane were stained and counted. In all the panels, representative images are reported in (i), while quantification of the results is shown in (ii) as mean ± SEM of three experiments. Statistical significance of the difference was calculated between treated and untreated cells (sample 0). *p < 0.05.*

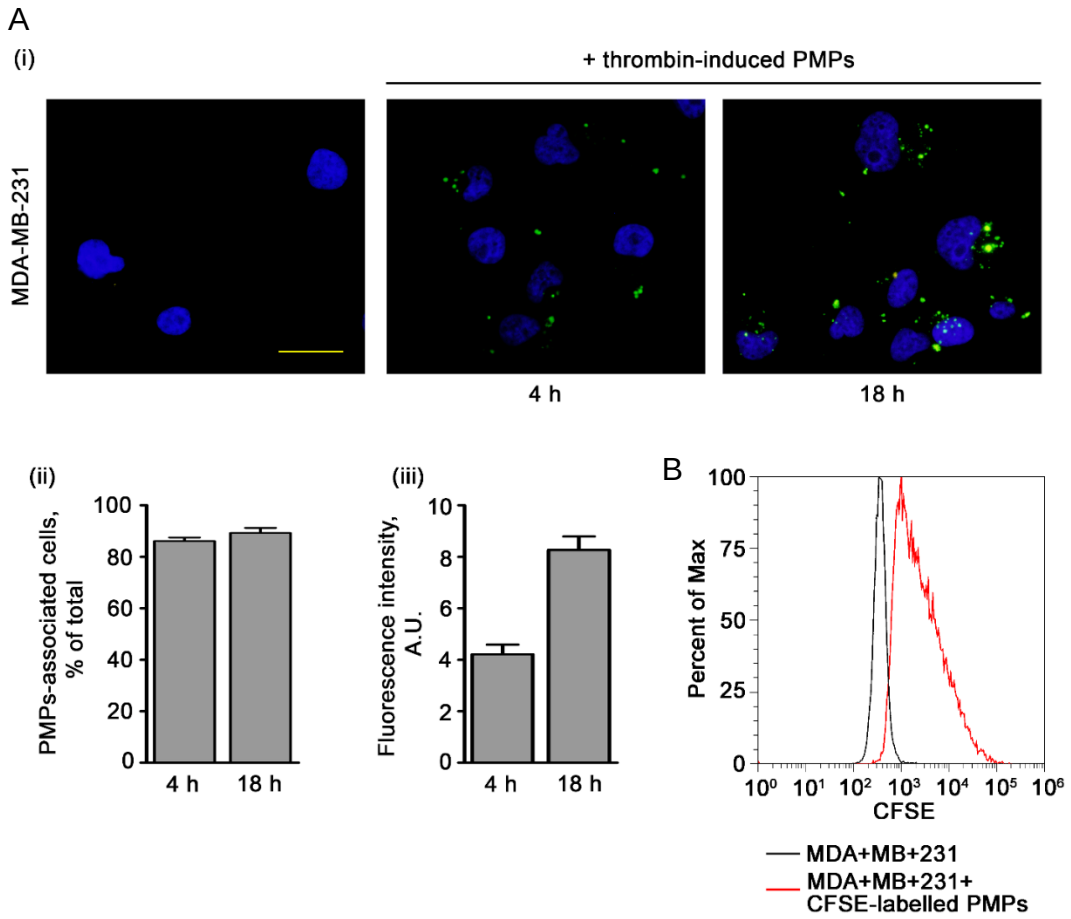
6

Results: Further investigations on the crosstalk between PMPs and cancer cells

Interaction of thrombin-induced PMPs with MDA-MB-231 cells

In the last part of the work we aimed to further investigate the importance of platelets in cancer focusing our attention on novel possible functional effects of PMPs on cancer cells. As experimental model, we adopted PMPs released upon platelet stimulation with the physiological agonist thrombin, limiting the study on their interaction with MDA-MB-231 cells. The ability of physiological agonist-induced PMPs to interact with other cell types has been already demonstrated (Janowska-Wieczorek et al., 2005; Janowska-Wieczorek et al., 2006), however we decided to verify this interaction in our experimental setting and using MDA-MB-231 cells.

Figure 16A-B

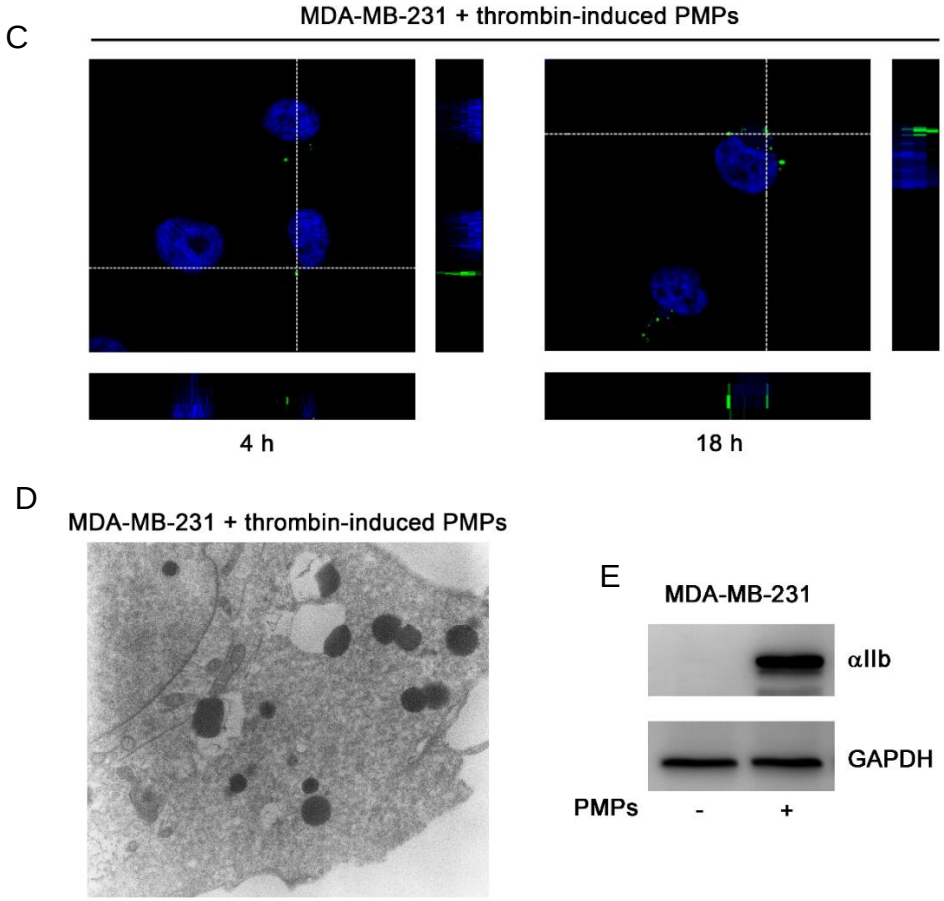


A: MDA-MB-231 cells (5 x 10^4 cells/well) were grown on glass coverslips and then incubated with 30 µg/ml of thrombin-induced PMPs obtained from CFSE-labeled platelets for 4 or 18 hours. (i) Representative fluorescence microscopy images upon 18 hours of incubation. CFSE-labeled PMPs are in green, whereas cell nuclei were stained with 1 µg/ml of Hoechst (blue). (ii) Quantification of the percentage of MDA-MB-231 cells associated with fluorescent PMPs. Data are the mean ± SEM (n=3). (iii) Comparison of MDA-MB-231 cells' ability to interact with thrombin-induced PMPs after 4 or 18 hours of incubation. Data are expressed as average green fluorescence intensity associated with each cell and are the mean ± SEM of three independent experiments. B: Upon incubation with PMPs for 18 hours, cells were harvested, fixed in 1% PFA for 10 minutes and analyzed with the flow cytometer Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, USA).

PMPs were isolated from CFSE-loaded platelets upon stimulation with the thrombin and then incubated with MDA-MB-231 cells. Thrombin-induced PMPs were able to interact with MDA-MB-231 cells either at short (4 hours) or long (18 hours) times of incubation, nevertheless, cell-associated fluorescence increased progressively with the incubation time (figure 16A). MDA-MB-231 interaction with PMPs was tested also by flow cytometry. As seen in figure 16B, incubation of MDA-MB-231 cells with CFSE-labelled PMPs resulted in an evident increase in green fluorescent signal associated to cells (Figure 16B).

The nature of this interaction was further characterized by confocal microscopy. Similarly to what was shown before for cancer cell-induced PMPs, also thrombin-induced PMPs were actually internalized by MDA-MB-231 cells (Figure 16C), both at short and long time of incubation, with most of microparticles accumulated in perinuclear position. The internalization was further confirmed using a transmission electron microscopy-based assay. To examine the interaction of CFSE-loaded PMPs with cancer cells by TEM, the fluorescent signal associated to PMPs was turned into electron-dense signal by photoconversion of DAB (*Materials and methods-section 3.4.1*). As reported in figure 16D, after DAB photoconversion, PMPs were labelled with homogeneously distributed, dark reaction product which made them unequivocally recognizable. Several dark particles were detected inside the cell cytoplasm confirming that, as already suggested by confocal microscopy, PMP were actually internalized by MDA-MB-231 (Figure 16D). Finally, the immunoblotting analysis of the platelet marker CD41, clearly revealed that this antigen was undetectable in untreated MDA-MB-231 cells, whereas it was present at high levels in the same cells upon incubation with thrombin-induced PMPs (Figure 16E).

Figure 16C-D-E

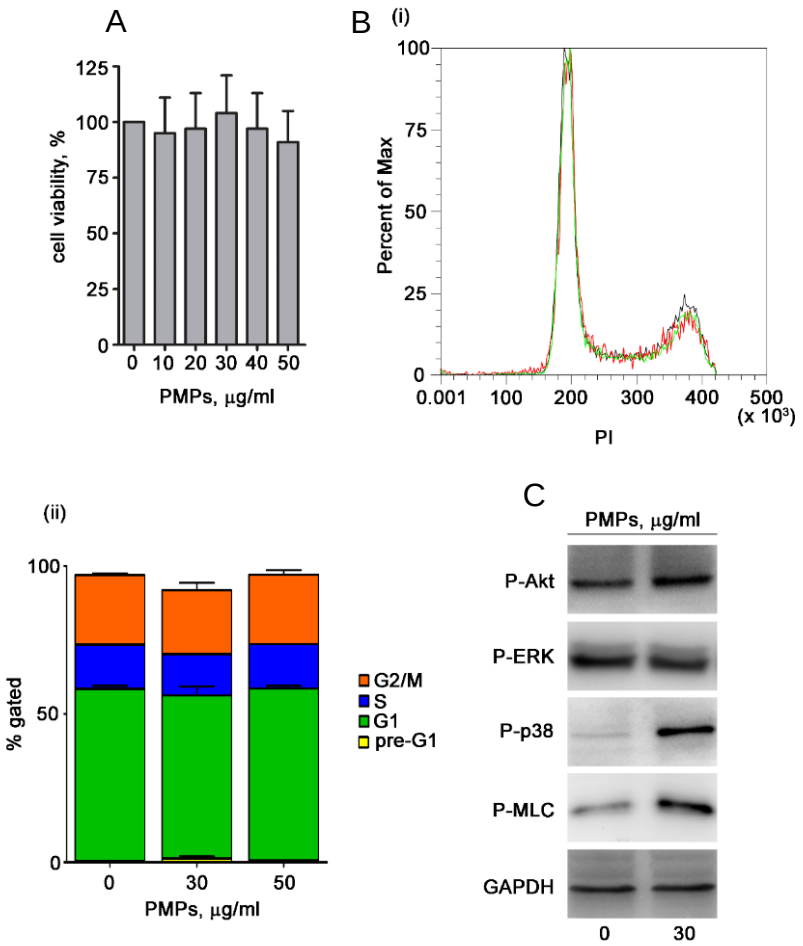


C: Confocal microscopy analysis of MDA-MB-231 cells interacting with thrombin-induced PMPs for the reported times of incubation. Representative confocal middle z-sections and orthogonal views are reported. D: TEM analysis of MDA-MB-231 cells interacting with thrombin-induced PMPs. E: Immunoblotting analysis of total lysates of MDA-MB-231 cells untreated or incubated with PMPs.

Effect of thrombin-induced PMPs on MDA-MB-231

To assess the effect of thrombin-induced PMPs on breast cancer cells viability, MDA-MB-231 cells were incubated with increasing amounts of PMPs and subsequently analyzed by the MTT assay. As shown in figure 17A, the viability of cancer cells did not show any significant change upon incubation with increasing amounts of PMPs. We then investigated whether PMPs can influence cell cycle progression of targeted cancer cells *(Materials and methods-section 3.4.3)*. As reported in figure 17B, PMPs used either at 30 µg/ml or 50 µg/ml were unable to cause significant alterations in the distribution of cell cycle phases (Figure 17B). We previously shown that cancer cell-induced PMPs caused an increase in the phosphorylation of selected signalling proteins, here we observed that also thrombin-induced PMPs stimulated the phosphorylation of MLC and p38MAPCK, while not affecting phosphorylation of other proteins, such as Akt and ERK (Figure 17C).

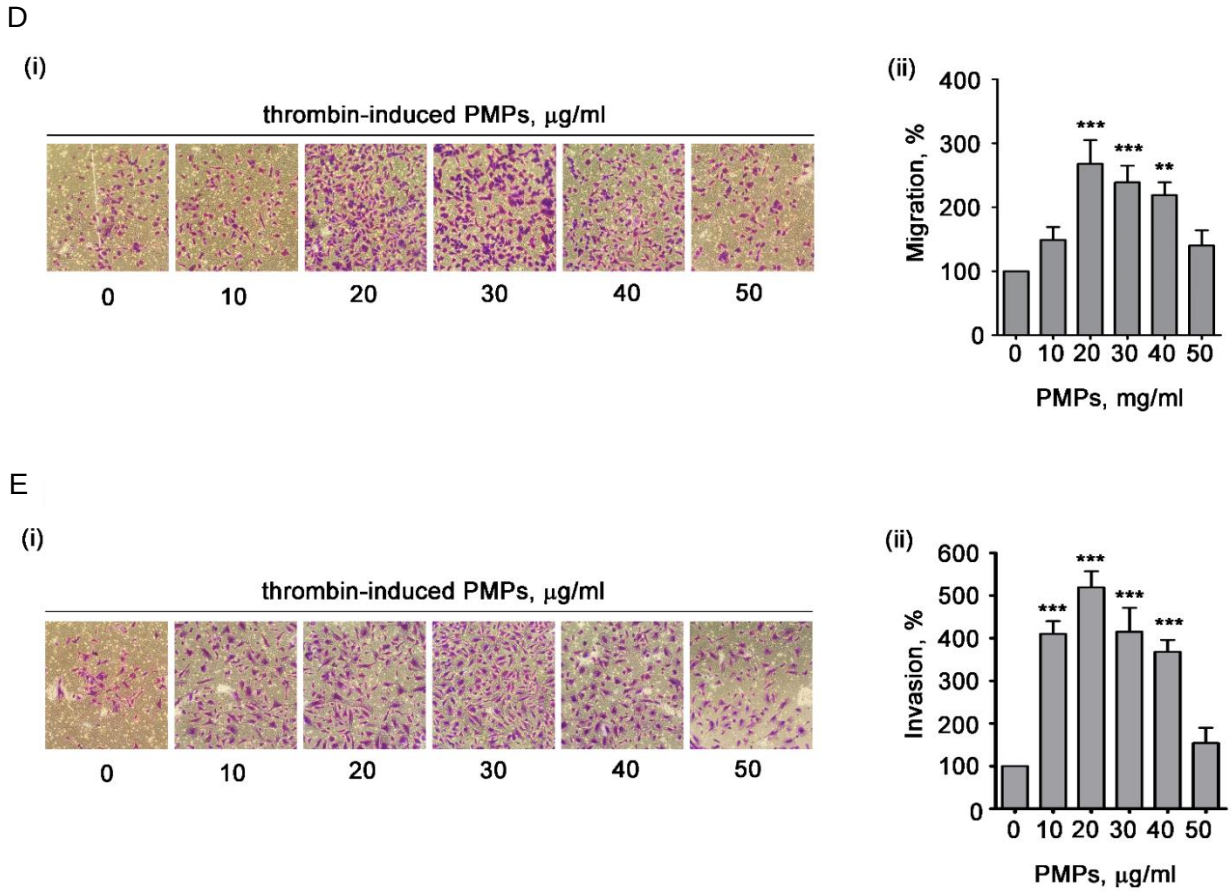
Figure 17A-B-C



A: Viability of MDA-MB-231 (i) or MCF7 (ii) cells incubated with the indicated amounts of PMPs for 24 hours was assessed by a colorimetric MTT assay. Results are reported as the mean ± SEM (n=3). B: Effect of thrombin-induced PMPs on cell cycle distribution of MDA-MB-231 cells.(i) Representative cell cycle analysis, (ii) Summarized flow cytometry data. C: Representative immunoblot of phosphorylation of selected signaling proteins in MDA-MB-231 cells incubated with thrombin-induced PMPs for 18 hours.

MLC and P38 are involved in the control of several cellular responses, but they play a pivotal role in the regulation of cell motility. Therefore, we analyzed the effect of thrombin-induced PMPs on migration and invasion of MDA-MB-231 cells, which are both critical steps for metastatic dissemination *(Materials and methods-section 3.4.4)*. Similarly to what observed with MDA-MB-231-induced PMPs, here we found that also thrombin-induced PMPs stimulate a strong potentiation of both migration and invasion ability of MDA-MB-231 cells (Figure 17D–E). Both events are particularly evident when doses of PMPs in the range of 20-30 µg/ml were used. Thrombin-induced PMPs stimulated about a three-fold increase of cell migration, which is in line to that observed or MDA-MB-231-induced PMPs (see figure 15D); interestingly, they are far more efficient in sustaining cell invasion. Indeed, thrombin-induced PMPs potentiated matrix invasion up to about five-folds, whereas MDA-MB-231-induced PMPs promoted only a 1.5-fold increase.

Figure 17D-E



*Effect of thrombin-induced PMPs on migration (Panel D) and invasion (Panel E) of MDA-MB-231 cells. Cancer cells were treated with increasing amounts of thrombin-induced PMPs (0–50 µg/ml, as indicated on the bottom) and then transferred inside cell culture inserts. For the invasion assays (panel E), the upper side of the insert was coated with 0.1ml of Matrigel (50 µg/ml). Incubation was prolonged for 18 hours and the cells that moved through the porous membrane were stained and counted. In all the panels, representative images are reported in (i), while quantification of the results is shown in (ii) as mean ± SEM of three experiments. Statistical significance of the difference was calculated between treated and untreated cells (sample 0). **p < 0.01; ***p < 0.001*

Effect of thrombin-induced PMPs on cancer cell autophagy

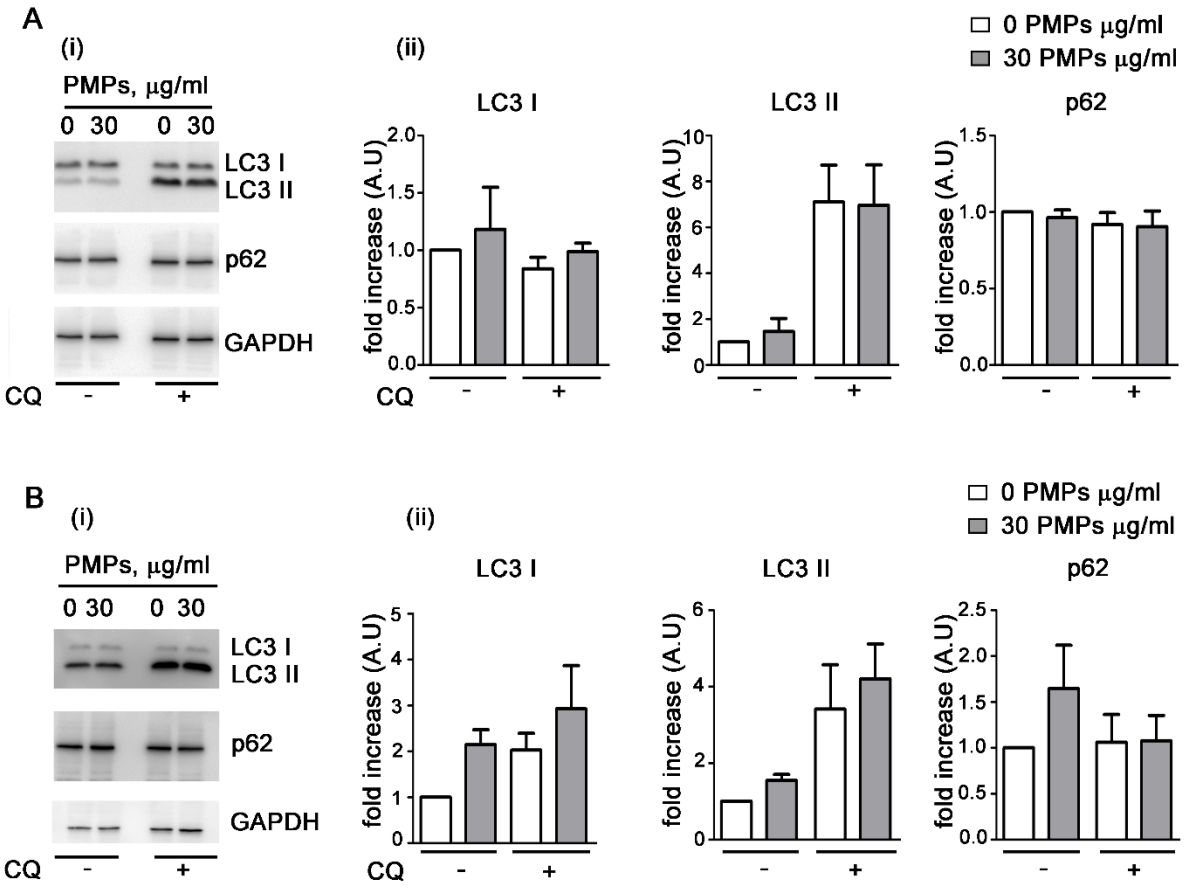
In the last section of this work, we focused on the possible effects elicited by PMPs on autophagy of cancer cells. This is an aspect that has never been investigated before, but it may be extremely important in the context of the platelet-cancer cross-talk. Depending on the context, autophagy is believed to act either as a pro-, or an anti-cancer process. It may prevent cancer initiation by eliminating potentially detrimental components. By contrast, it may help cancer cells to overcome the stressful conditions that they undergo during cancer progression thus supporting cancer dissemination (Cicchini et al., 2014). LC3 is a ubiquitin-like protein that represents the most widely monitored autophagy-related protein. Upon autophagy induction, LC3 is conjugated to phosphatidylserine (PE); the non-lipidated and lipidated forms are usually referred to as LC3-I and LC3-II, respectively. The amount of LC3-II reflects the amount of autophagosomes, therefore an increase in LC3-II usually correlates with an increased autophagic flux. While studying autophagy, however, it is important to note that the LC3-II amount at a given time point does not necessarily reflect an increased autophagic activity, because not only autophagy activation but also inhibition of autophagosome degradation greatly increases the amount of LC3-II. To correctly measure the autophagic flux, it is essential to use a lysosomal inhibitor to determine how much LC3-II (as a model substrate of autophagy) is degraded during a certain time period. To estimate the lysosome-dependent degradation of LC3-II, chloroquine (CQ) or other lysosomal enzyme inhibitors are commonly used. CQ impairs autophagosome fusion with lysosomes thus inhibiting the last phase of autophagy. In this way, the difference in the amount of LC3-II between samples with and without CQ is indicative of the real level of autophagic flux. The two forms of LC3 are easily detectable by immunoblotting as LC3-II, although larger in mass, shows faster electrophoretic mobility in SDS-PAGE gels, creating a two-band pattern easily distinguishable. Moreover, autophagosomes can be visualized in immunofluorescence by staining LC3 (Klionsky et al., 2016).

The analysis of the effect on the autophagic flux of MDA-MB-231 cells was limited to the dose of PMPs that showed meaningful effects in the other assays (i.e. 30 µg/ml). Initially, we assessed autophagic flux by western blotting analysis of MDA-MB-231 cells treated with thrombin-induced PMPs in the presence, or not, of chloroquine *(Materials and methods-section 3.4.5)*. After 4 hours incubation, no alteration of the autophagic activity was observed. Indeed, the level of LC3-II remained stable upon PMPs incubation (Figure 18A). An alternative method to detect autophagy is the measure of the enhanced degradation of p62, a long-lived scaffolding protein that directly binds to LC3 and is selectively degraded by autophagy (Klionsky et al., 2016). Consistently with the levels of LC3-II, expression of p62 in cancer cells treated with PMPs resulted unchanged (Figure 18A).

Assuming that the duration of treatment may influence the response, we extended the incubation time till 24 hours. As shown in figure 18B, here we found an increase of LC3-II levels that might suggest an increase of the autophagic flux; however, the difference detected was not statistically significant. Interestingly, the level of LC3-I also resulted increased.

With a higher autophagic flux, a decrease in the expression of p62 should be observed; however, here we found an increased level of p62 (Figure 18B).

Figure 18A-B



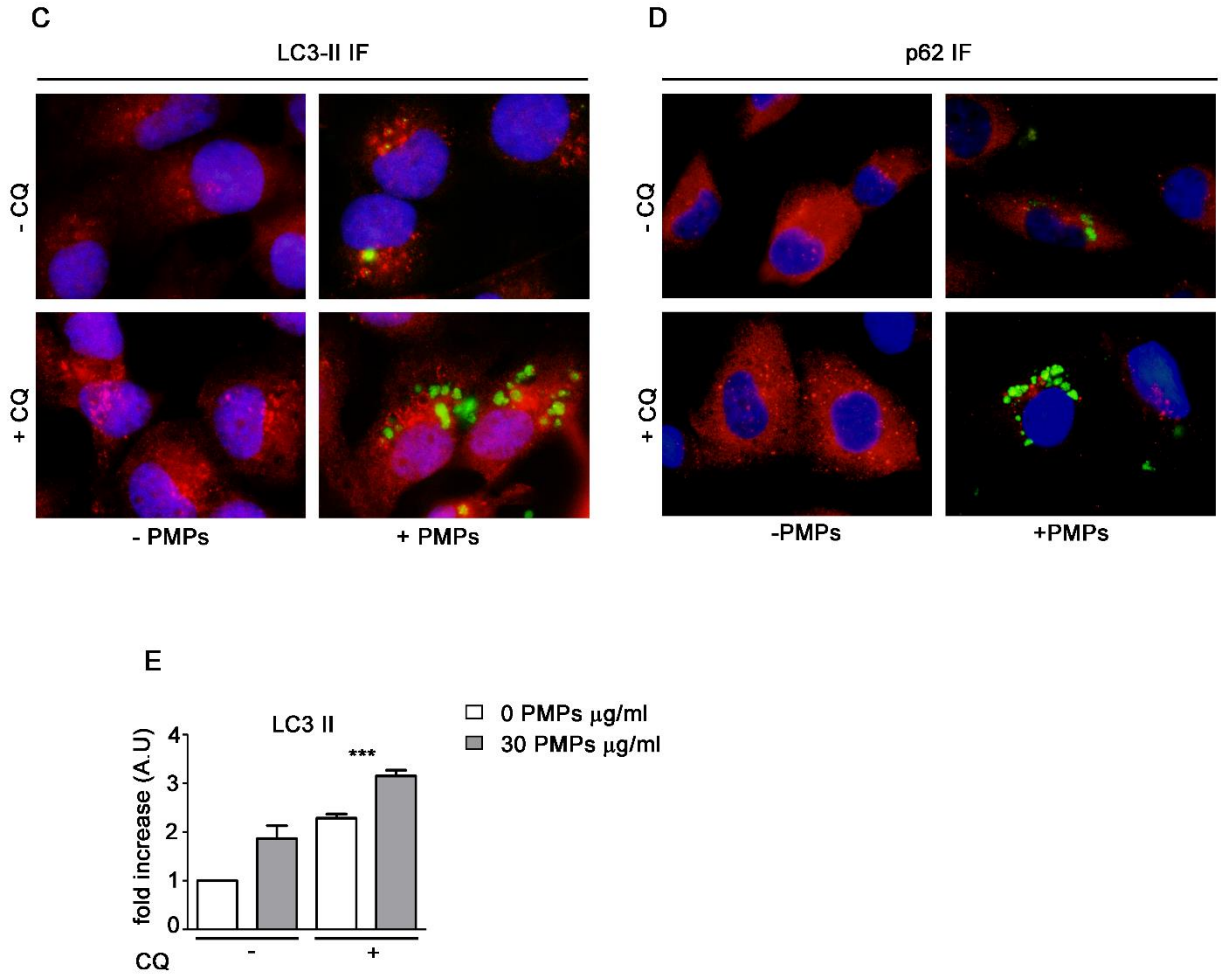
Immunoblot analysis of LC3 and p62 in MDA-MB-231 cells treated with thrombin-induced PMPs for 4 hours (panel A) or 24 hours (panel B). (i) representative immunoblot; (ii) quantification of the results by densitometric scanning. Results are the mean ± SD (n=3).

These conflicting results prompted us to analyse autophagy by immunofluorescence through immune-specific staining of LC3 and p62 *(Materials and methods-section 3.4.5)*. As demonstrated by the representative images in panel 18C, immunostaining with LC3 showed an increase in the number of autophagic vacuoles in cells treated with PMPs, which is compatible with an increase in the cell autophagic flux (Figure 18C). Noteworthy, PMPs (in green) appeared to be enclosed in the autophagosome. Differently from what observed in immunoblotting analysis, here p62 immunofluorescence showed an evident reduction of p62 levels upon treatment with PMPs, in line with an increased autophagic flux (Figure 18D).

Finally, to resolve the conflicting observations collected by western blotting and immunofluorescence analyses, and to provide solid evidence about the effect of PMPs on autophagy, we exploited the FlowCellect GFP-LC3 Reporter Autophagy Assay Kit *(Materials and methods-section 3.4.5)*. This assay allows to perform a more accurate quantification of LC3-II by a

flow cytometry-based approach. As reported in the histogram (Figure 18E), cancer cells treated with PMPs displayed a significantly accumulation of LC3-II upon 24 hours incubation with thrombin-induced PMPs, demonstrating the ability of PMPs to alter of the autophagic flux of MDA-MB-231 cells.

Figure 18C-D-E



*C-D: Immunofluorescence analysis of LC3 (panel C) and p62 (panel D) in MDA-MB-231 cells treated with thrombin-induced PMPs for 24 hours. Representative merged images are reported. Blue: nuclei; green: PMPs; red: LC3 (panel C) or p62 (panel D). Panel E: flow cytometry analysis of LC3-II expression in MDA-MB-231 cells treated with thrombin-induced PMPs for 24 hours. ***p < 0.001*

7

Discussion and conclusions

The relationship between cancer and platelets and its pathological implication are longstanding concepts. Upon they left the primary tumor and entered the blood stream, cancer cells exploit blood components to survive in the circulation. In this context, TCIPA, i.e. the ability cancer cells to induce the formation of platelet aggregates, is crucial to tolerate shear stress forces and overcome the action of immune cells (Bambace and Holmes, 2011). TCIPA thus increases the success rate of the metastatic process, but it also heavily contributes to the thrombotic complications often observed in cancer patients (Mitrugno et al., 2016). Given the importance of TCIPA, through the years a large number of studies have investigated the mechanisms supporting this process by characterizing, *in vitro*, the ability of different cancer cell lines to stimulate platelet activation and aggregation (Jurasz et al., 2004). These studies, however, are often characterized by significant discrepancies in the obtained results, likely due to important differences in the experimental conditions and cell lines used.

In the first part of this study, we performed a thorough comparative analysis of TCIPA induced by two widely used breast cancer cell lines characterized by different metastatic potential: the mesenchymal-like, highly metastatic MDA-MB-231 cells, and the epithelial-like, low metastatic MCF7 cells. These cancer cell lines had been used before in investigations on the platelet-cancer interplay (Alonso-Escolano et al., 2004; Belloc et al., 1995; Wei et al., 2015), however, a detailed characterization of the molecular mechanisms involved is still missing.

The collected results allow to delineate an interesting, partially unexpected scenario that is hereby summarized. First, differently from previously published data (Alonso-Escolano et al., 2004; Lian et al., 2013; Mitrugno et al., 2014) breast cancer cells are absolutely unable to induce aggregation of washed platelets, indeed plasma, even at very low amount, is crucial to induce TCIPA. The functional interaction of tumor cells and platelets promotes the formation of small amount of thrombin, which represents an important trigger for full platelet aggregation, which is mediated by fibrinogen binding to integrin αIIbβ3. Importantly, cancer cells actually induce platelet activation and TCIPA requires PLC activity, as well as the secondary autocrine signaling through released ADP. By contrast, thromboxane A2 synthesis appeared to be completely dispensable for TCIPA. Altogether, these results clearly indicate that there are no remarkable differences in the mechanisms by which the two breast cancer cell lines trigger platelet activation and aggregation, suggesting that TCIPA is likely less dependent on the type and metastatic phenotype of cancer cells than expected. In this context, explorative experiments, not included in this thesis, have been performed using colon adenocarcinoma and melanoma cell lines, and, once again, the overall molecular mechanism involved in TCIPA was found to be essentially the same.

A critical conclusion of our work is that previous studies largely underestimated the role of plasma and Ca^{2+} in TCIPA. Although previous investigations reported platelet aggregation induced by MCF7 cells in heparinized PRP (Yamamoto et al., 1986; Heinmoller et al., 1996; Meikle et al., 2016), we failed to detect TCIPA in citrated PRP. This evidence suggests the essential role of Ca^{2+}, which was confirmed by the observation that TCIPA in the presence of plasma was abolished when

$CaCl_2$ was omitted from the resuspension buffer (data not shown). On the other hand, we constantly failed to detect TCIPA in the absence of plasma contamination even when $CaCl_2$ was present. Indeed, cancer cells were able to induce TCIPA of washed platelets in the presence of $CaCl_2$ only upon addition of small amount of autologous plasma. Some previous studies have already reported the requirement of plasma addition to washed platelets in order to observe TCIPA, thus our results are in agreement with these observations (Belloc et al., 1995; Wei et al., 2015). Nevertheless, our results disagree with many other studies that documented TCIPA of washed platelets (Alonso-Escolano et al., 2004; Medina et al., 2006; Lian et al., 2013). Our explanation for this inconsistency points toward a possible, unaddressed contamination of plasma in the platelet preparations adopted in those studies. The presence of undesired contaminations of plasma is critical issue working with washed platelets, particularly since we found that the amount of plasma required to allow maximal TCIPA is actually very low (0.05% of the total volume). Our results thus stress the importance of isolation procedure of washing platelets and of a careful check for contaminant traces of plasma in the platelet preparations used for TCIPA studies. Interestingly, by increasing the volume of added plasma, the lag-time shortened thus accelerating TCIPA but without affecting the maximal aggregation.

The observation that serum was not able to replace plasma in sustaining TCIPA and that PPACK completely prevented aggregation clearly suggested the possible contribution to this process for serine proteases of the coagulation cascade. Neuroblastoma or pancreatic cancer cells were found to promote thrombin formation in the presence of platelets (Esumi et al., 1987; Heinmoller et al., 1995). Here, we have directly measured the amount of thrombin generated upon platelet stimulation with breast cancer cells, and we found that thrombin accumulation occurred earlier than aggregation. This thrombin generation may be due to TF expressed on cancer cells as we found that both cell lines expressed this protein, albeit at different levels. TF was found to be extremely abundant in MDA-MB-231 cells, but barely detectable in MCF7 cells. Noteworthy, the different levels of TF expression correlated to the different amount of generated thrombin. Nevertheless, in spite of the differences in TF expression and thrombin generation, both breast cancer cell lines caused a comparable final platelet aggregation. Importantly, preliminary experiments not reported in this thesis demonstrated that thrombin generation occurred only when cancer cells, platelets and plasma were concomitantly present (data not shown). We thus hypothesize that the initial interaction of cancer cells with platelets is essential to stimulate TF activity of cancer cells. Thrombin may in turn represent the initial trigger for subsequent platelet aggregation. However, further experiments, involving the inhibition of TF activity, will be required to fully demonstrate this hypothesis.

We found that TCIPA was associated to platelet activation and granules secretion, and that the increase of intracellular cAMP, a potent antagonist of platelet activation, completely prevented TCIPA. Both breast cancer cells promoted the activation of platelet PLC, led to increase of intracellular Ca2+, and stimulated the activation of the small GTPase Rap1b. Rap1b controls integrin αIIbβ3 inside-out signaling and its stimulation is consistent with TCIPA being largely sustained by

fibrinogen binding to platelets, as demonstrated by using the integrin antagonist RGDS peptide. We also found that PLC activation, but not PI3K or Src kinases activity, is required for TCIPA induced by both breast cancer cells. Therefore, tumor cells apparently exploit only selected components of the platelet activation machinery involved in physiological responses.

Previous works have suggested that TxA2 production is not essential in TCIPA (Alonso-Escolano et al., 2004; Lian et al., 2013; Jurasz et al., 2001; Bradley et al., 1997) and, in agreement with these studies, we confirmed that platelet aggregation induced by the tumor cells analyzed was not affected by aspirin. In addition, our results demonstrated the importance of secreted ADP as secondary agonist required to support complete TCIPA. These observations suggested that, although thrombin is the priming agonist for TCIPA, subsequent positive feedback mechanisms are required to compensate platelet response and to trigger maximal aggregation. The role of platelet contribution to cancer metastasis is also supported by the observation that anti-platelet agents decrease

tumor metastasis in mice. The use of aspirin in clinical studies resulted in reduced cancer spread (Rothewell et al., 2012). Our results, however, suggest that it is unlikely that these effects can be ascribed to the prevention of TCIPA. Conversely, platelet aggregation induced by breast cancer cells was significantly reduced upon inhibition of P2Y12 receptor. This observation, along with recent evidence demonstrating reduced cancer spread associated to deficiency of P2Y12 receptor (Cho et al., 2017; Wang et al., 2013), provide further evidence that pharmacological inhibition of this ADP receptor, in addition to beneficial effects on cancer-associated thrombosis, may display usefulness in anti-cancer therapy.

In agreement with previous observations (Mitrugno et al., 2014), using two different experimental approaches, we found that incubation with tumor cells induced the release of platelet dense granules. However, we believe that platelets may not represent the exclusive source of ADP. In fact, the release of serotonin induced by both cancer cells was delayed compared to the release of ATP. In control samples of cancer cells incubated with plasma in the absence of platelets, we failed to detect significant ATP release (data not shown). Therefore, it is possible to speculate that interaction with platelets leads cancer cells to rapidly release some ATP/ADP which may contribute to the initial platelet activation, thus inducing the subsequent release of platelet dense granules. Previous works have also suggested that cancer cells may release ATP (Raffaghello et al., 2006), therefore we propose that cancer cell-induced secretion of platelet dense granules should be more reliably evaluated using a different and more specific marker such as radiolabeled serotonin.

As already said, TCIPA is important in cancer biology since it confers several advantages to survival of circulating tumor cells. The aim of this work was to get further insights in the complex interplay of platelets and cancer, we thus investigated another potential pro-metastatic consequence of TCIPA, namely the release of PMPs. The extreme reactivity of PMPs and their role in intercellular communication suggest that these platelet-derived cell fragments may modulate the metastatic

potential of circulating cancer cells. To date, few studies on this topic demonstrated that PMPs can potentiate the aggressiveness of cancer cells, providing a preliminary proof for PMPs ability to modulate cancer cell potential (Janowska-Wieczorek et al., 2005; Janowska-Wieczorek et al., 2006; Dashevsky et al., 2009). In spite of the relatively limited information available on this topic, the hypothesis of PMPs involvement in the modulation of cancer spread is further supported by the increased levels of circulating PMPs that were detected in cancer patients, particularly in the advanced stages of the disease (Mezoaur et al., 2014; Chaari et al., 2014).

We focused on the possible ability of cancer cell to induce the release of microparticles from platelets, demonstrating for the first time that TCIPA is accompanied by a massive release of PMPs and that the released microparticles can signal back to cancer cells to alter their invasive properties. We found that both MDA-MB-231 and MCF7 cells are able to cause the release of PMPs. By contrast, TCIPA is not accompanied by the evident release of microparticles from cancer cells. Moreover, the two breast cancer cells lines, albeit able to induce comparable TCIPA, are differently potent in stimulating the release of PMPs. In particular the more aggressive MDA-MB-231 cells are more potent than MCF7 cells in inducing PMPs release. An additional important finding of this work is that PMPs, released upon cancer cells stimulation, may influence cancer cells themselves. Previous studies have demonstrated that PMPs released from platelet activated with classical agonists, such as thrombin, are able to activate cancer cells (Janowska-Wieczorek et al., 2005; Janowska-Wieczorek et al., 2006; Dashevsky et al., 2009). Here we show that cancer cells may be not only the target of PMPs, but also the inducers of their release. Interestingly, MDA-MB-231, but not MCF7 cells, are able to interact with, and rapidly internalize, both PMPs preparations. This observation suggests that the ability to bind PMPs depends on the cell type and it is not a general feature of cancer cells. The ability of MCF7 to induce platelet aggregation and PMPs release but not to bind them, suggests that receptors and surface components involved in the interaction of cancer cell with PMPs are likely different from those that mediate TCIPA. However, how MDA-MB-231 cells bind to PMPs is still unclear. As demonstrated by our results, the interaction of MDA-MB-231 cells with PMPs does not involve either integrin αIIbβ3 or P-selectin, but it is probably mediated by a still unknown carbohydrate-based recognition mechanism, as suggested by sensitivity to heparin. Further studies are certainly required for a precise characterization of the molecular mechanisms required for this interaction.

MDA-MB-231 cells internalize MDA-MB-231-induced and MCF7-induced PMPs with a similar efficiency. However, only PMPs generated upon platelet stimulation with MDA-MB-231, but not with MCF7 cells, stimulate the phosphorylation of selected signaling proteins and potentiate migration and invasion abilities of MDA-MB-231 cells. These findings suggest that different types of cancer cells may stimulate the release of PMPs which are intrinsically different. This hypothesis is in agreement with previous studies demonstrating that the composition of PMPs released from platelets stimulated with physiological agonists depends on the generating stimulus. Noteworthy, migration and invasion of MDA-MB-231 cells were potentiated by a range of PMPs concentrations comparable

to that detected in the plasma of cancer patients (Lansdorf et al., 2012), indicating that the effect described in our work may be physiologically relevant.

In the last part of the work, we preliminary investigated other possible functional effects of PMPs in the modulation of cancer cell metastatic potential. For these investigations, we used PMPs released upon platelet stimulation with the physiological agonist thrombin, which can be obtained with a higher yield, and we limited the study to MDA-MB-231 cells. Through a multiple approach exploiting confocal, as well as electron microscopy, flow cytometry and western blotting we clearly demonstrated that thrombin-induced PMPs are efficiently internalized by MDA-MB-231 cells. In addition, as already shown for MDA-MB-231-induced PMPs, thrombin-induced PMPs were able to stimulate phosphorylation of the signaling proteins P38-MAPK and MLC, and to increase the aggressiveness of MDA-MB-231 cells.

Finally, we intended to explore whether PMPs influence autophagy of cancer cells. Autophagy is a catabolic process that mediates degradation of unnecessary or dysfunctional cellular components. Through this mechanism cells remove damaged or potentially dangerous molecules or organelles, thus maintaining cellular homeostasis. However, autophagy is also exploited to overcome intracellular or environmental stresses, such as nutrient deprivation, hypoxia and drugs effect. This dual function makes autophagy a 'Janus-faced' player in cancer progression since it can be tumor-suppressive, or tumor-promoting in different contexts. Autophagy involves the formation of double membrane-bound vesicles called autophagosomes that engulf the material that needs to be degraded, such as cytoplasmic proteins and organelles; these autophagosomes are trafficked to lysosomes where they fused together (creating an autolysosome) and finally the sequestered contents are digested and recycled (Boya et al., 2013). A key player in autophagy is the protein LC3, that is actively recruited in this process by lipidation (i.e conjugation to phosphatidylserine), which mediates its binding to autophagosomes, on both outer and inner membranes. LC3-II then contributes to the closure of autophagosomes and enables the docking of specific cargos and adaptor proteins such as Sequestosome-1/p62. Following the fusion of autophagosome with a lysosome, LC3-II is retained on the vesicle's inner side and thus degraded within the autolysosome along with the cargo. LC3-II is currently the most widely used autophagosome marker since it is the only protein marker that is reliably associated with completed autophagosomes (Klionsky et al., 2016).

We initially assessed autophagy by immunoblotting analysis of LC3 and p62. Through this approach we found that the treatment with PMPs caused an increase in LC3-II which is further enhanced upon inhibition of lysosomal degradation by CQ, indicating a potentiation of the autophagic flux. Interestingly, PMPs also increase LC3-I levels, suggesting a possible upregulation of transcription and synthesis of LC3 itself. Noteworthy, PMPs treatment was associated to the accumulation of p62 in cells in the absence of CQ, which is in conflict with the accumulation of LC3-II, since it is expected that p62 levels decrease upon autophagy induction. The upregulation of both

LC3-II and p62 may suggest a delay in the final step of autophagosome degradation, that however cannot explain the further increase in LC3-II after CQ treatment. A possible explanation is that the increase of p62 observed upon PMPs treatment may be due to some compensatory response as already documented elsewhere. Previous work, indeed, have reported that p62 protein levels tend to recover in long-term treatment, because p62 transcription is compensatory up-regulated under prolonged starvation (Sahani et al., 2014). Additional experiments performed at different times of incubation will likely be required to obtain definitive information about this aspect.

Nonetheless, the use of immunoblotting analysis to assess autophagy presents several weaknesses, especially working with cells, such as MDA-MB-231, that are characterized by a high basal autophagic flux (Klionsky et al., 2016). Therefore, to clarify the previous observations, we performed immunofluorescence analysis with LC3 and p62 to visualize the autophagic structures and p62 cytoplasmic distribution. In response to PMPs, an increased number of LC3 punctate structures and a decreased cytosolic p62 signal were detected, suggesting an increased autophagic flux. Accordingly, the treatment with PMPs cause the formation of microparticle-associated vesicles rich in LC3, that became even more evident upon CQ treatment. Anyway, the hypothesis that PMPs may potentiate autophagy was further confirmed by exploiting a flow cytometry-based approach which allowed to reliably quantify the increase of LC3-II in response to PMPs. However, these results are only preliminary observations that need to be deeper investigated but they suggest another possible role of PMPs in metastatic spread. Interestingly, fluorescent PMPs are surrounded by LC3-positive structures, thus suggesting a possible autophagy-mediated processing of PMPs.

In conclusion, our study provides new evidence in the interplay between cancer and blood platelets. We have demonstrated that, differently from what is expected, the modality and molecular mechanisms supporting TCIPA are poorly related to the metastatic aggressiveness of cancer cells analyzed. Moreover, our study identifies a previously unrecognized positive feedback mechanism by which some cancer cells may increase their own aggressiveness while circulating in the bloodstream, by inducing the release of specific PMPs. Cancer cells by inducing platelet aggregation and release of PMPs, create a pro-metastatic niche in which high concentrations of PMPs may signal back to cancer cell to alter its phenotype. However, the nature of the cancer cell dictates the relevance of this pro-metastatic feedback. Finally, the preliminary observation that PMPs upregulate autophagy may represent an additional mechanism by which platelets support cancer dissemination. Further effort will be required to obtain a complete identification of the molecular determinants responsible for platelet-mediated support to metastasis, but the understanding of this cross-talk will be vital to design novel anti-cancer therapeutic strategies

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