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Preliminary investigation on the crosstalk between blood
platelets and a Glioblastoma Multiforme cell line

Studio preliminare dell'interazione tra le piastrine e una linea
cellulare di Glioblastoma Multiforme

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Experimental thesis by

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Academic Year 2025/2026

Il glioblastoma multiforme (GBM) è il tumore cerebrale primario più aggressivo negli adulti, con una sopravvivenza mediana di circa 12–15 mesi nonostante i trattamenti standard attualmente disponibili, tra cui la resezione chirurgica, la radioterapia e la chemioterapia. Le piastrine e le vescicole extracellulari piastriniche (PEVs, platelet-derived extracellular vesicles) sono emerse come importanti mediatori della progressione tumorale in diversi tipi di cancro, tra cui il carcinoma mammario, del colon e del pancreas, contribuendo all'evasione immunitaria, all'angiogenesi e alla disseminazione delle cellule tumorali. Tuttavia, il possibile ruolo delle piastrine e delle PEVs nella biologia del GBM non è ancora stato caratterizzato.

Nel presente studio, abbiamo utilizzato cellule U87MG come modello consolidato di GBM in vitro per caratterizzare, per la prima volta, l'interazione delle piastrine e delle PEVs con cellule di glioblastoma. Abbiamo innanzitutto dimostrato che le cellule U87MG sono in grado di indurre l'attivazione piastrinica e l'aggregazione (TCIPA, tumor cell-induced platelet aggregation) in maniera dose-dipendente, attraverso vie di segnalazione canoniche che coinvolgono l'attivazione della PLC e l'integrina $\alpha\text{IIb}\beta 3$. Tale attivazione piastrinica è accompagnata dal rilascio di PEVs in quantità comparabili a quelle indotte dalla stimolazione con il potente agonista fisiologico trombina. Mediante tre approcci differenti e complementari: microscopia a fluorescenza, citometria a flusso e immunoblotting, abbiamo dimostrato che sia le PEVs indotte dalla trombina sia quelle indotte dalle cellule GBM sono in grado di interagire con le cellule U87MG. Infine, la valutazione della vitalità cellulare mediante colorazione con Annessina V e ioduro di propidio ha rivelato che le PEVs non influenzano significativamente la proporzione di cellule vive, apoptotiche o necrotiche nelle condizioni sperimentali testate.

Nel complesso, questi risultati forniscono la prima evidenza di un asse funzionale piastrine–PEVs–GBM, suggerendo che tale interazione possa avere rilevanza sia nel contesto della trombosi associata al cancro, sia come potenziale piattaforma per la veicolazione di farmaci attraverso la barriera ematoencefalica, aprendo la strada a future indagini sul ruolo delle piastrine e delle PEVs nella biologia e nel trattamento del glioblastoma.



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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor in adults, with a median survival of approximately 12-15 months despite current standard-of-care treatments including surgical resection, radiotherapy, and chemotherapy. Platelets and platelet-derived extracellular vesicles (PEVs) have emerged as important mediators of tumor progression in several cancers, including breast, colon, and pancreatic cancer, contributing to immune evasion, angiogenesis, and tumor cell dissemination. However, whether platelets and PEVs play any role in GBM pathobiology is mostly unknown.

In the present study, we used U87MG cells as an established in vitro GBM model to characterize, for the first time, the interaction between platelets, PEVs, and glioblastoma cells. We first demonstrated that U87MG cells are capable of inducing platelet activation and aggregation (TCIPA) in a dose-dependent manner, through canonical signaling pathways involving PLC activation and integrin $\alpha\text{IIb}\beta 3$. This platelet activation was accompanied by PEV release at quantities comparable to those triggered by thrombin. Using three different and complementary approaches; fluorescence microscopy, flow cytometry, and immunoblotting, we then demonstrated that both thrombin-induced and GBM-induced PEVs efficiently associate with U87MG cells. Finally, assessment of cell viability by Annexin V/propidium iodide staining revealed that PEVs did not significantly affect the proportion of live, apoptotic, or necrotic GBM cells under the tested conditions.

These results provide the first evidence of a functional platelet-PEV-GBM axis, suggesting that this interaction may have relevance both for cancer-associated thrombosis and as a potential platform for drug delivery across the blood-brain barrier (BBB), paving the way for future investigations into the role of platelets and PEVs in glioblastoma biology and therapy.

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INTRODUCTION

1. Platelets

1.1. Platelet genesis and morphology

Platelets originate from megakaryocytes (MKs), which, in turn, develop from the differentiation of hematopoietic stem cells in the bone marrow (Italiano *et al.*, 1999; Mazzarello, 2020). The bone marrow represents the primary site of megakaryopoiesis and platelet formation (figure 1). However, accumulating evidence indicates that the lungs also contribute to platelet biogenesis by serving as a site for terminal platelet release from circulating megakaryocytes (Lefrançois *et al.*, 2017). Megakaryocytes migrate from the osteoblastic niche to the vascular niche of the bone marrow where they will eventually release platelets into the vascular system (Thon and Italiano, 2012). The movement of megakaryocytes is influenced by the dynamic movement of the components of the cytoskeleton consisting of microtubules, actin filaments and spectrin (Patel *et al.*, 2005).

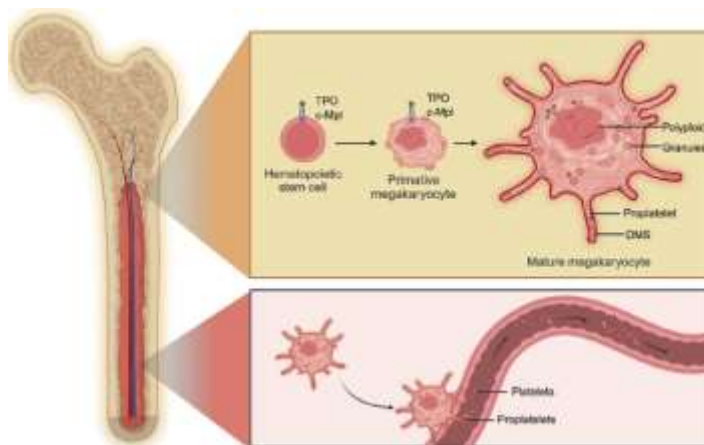


Figure 1. Platelet biogenesis in the bone marrow. Platelets develop from hematopoietic stem cells (HSCs) that differentiate into immature MKs. TPO induces MK maturation via c-Mpl receptor, during this phase a complex set of membrane structures, named the demarcation membrane system (DMS), is formed. The DMS serves as a reservoir for proplatelets formation that grow into bone marrow sinusoids. Once developed, platelets are released from MKs by fusion or bending of their membranes (Tian *et al.*, 2025).

During maturation, which is primarily induced by stimulation of c-Mpl receptor by thrombopoietin (TPO), MKs undergo polyploidization through a process known as endomitosis. In this process, DNA replication is not associated with cell division, resulting in polyploid cells with DNA content increasing from the normal 2N up to 64N. Polyploidization

is an essential phase in MKs maturation, promoting substantial cytoplasmic expansion and enhancing the metabolic capacity required for pro-platelet synthesis. Meanwhile, the cell membrane of MKs experiences significant actin-driven invagination, resulting in the formation of a delineated membrane system (DMS). Mature MKs emit pseudopod-like structures called pro-platelets, where platelets are formed and released at the level of their distal tips (Thon and Italiano, 2012). The released platelets enter the bone marrow sinusoids and reach the bloodstream (Tian *et al.*, 2025).

Circulating platelets are anucleate, discoid cells with a diameter of 2-3 micrometers. Platelets contain organized and complex structural components including a lipid bilayer membrane and a cytoskeleton composed of microtubules, actin filaments and spectrin. In addition, platelets possess specialized membrane system such as the open canalicular system (OCS) and the dense tubular system (DTS) which play important role in material transport, secretion and intracellular signaling (French *et al.*, 2020; Tian *et al.*, 2025). Platelets also contain several types of membrane-enclosed organelles, such as platelet-specific α -granules and dense granules, as well as common cellular organelles, mitochondria and lysosomes (figure 2) (Roka-Moia *et al.*, 2023; Thon and Italiano, 2012).

Platelet plasma membrane is a lipid bilayer, which contains phospholipids, cholesterol, glycolipids and glycoproteins, some of which function as receptors for mediating platelet adhesion, activation and aggregation (French *et al.*, 2020). Several family of receptors are expressed on the platelet plasma membrane, including integrins, G protein-coupled receptors (GPCRs) and ITAM-bearing receptors, which are essential for platelet adhesion, activation, and aggregation (Lu *et al.*, 2024; Tian *et al.*, 2025). Lipids distribution in the plasma membrane is asymmetrical, and this is essential to modulate platelet function in different contexts. The submembrane area of cytoskeleton provides platelets with the ability to adapt their shape dynamically throughout the course of their life cycle. These changes in shape also enable the redistribution of receptors across the platelet surface, altering their dynamic structural features and responsiveness (Tian *et al.*, 2025).

The OCS is a highly complex system of membrane channels within the cytoplasm of platelets. These channels are primarily located adjacent to the outer surface of the plasma membrane (Tian *et al.*, 2025). The primary role of the OCS is to facilitate the exchange of material between platelets and the extracellular environment, during both the uptake of extracellular compounds and the secretion of platelet-derived molecules. The OCS plays an essential role in platelet

activation by acting as a membrane reservoir which supports shape change and platelet spreading. Furthermore, it provides a preferential route for the secretion of granule content from the cell to the extracellular environment (Tian *et al.*, 2025).

The DTS is a membranous network derived from the smooth endoplasmic reticulum of MK, playing a crucial role in platelet activation. Differently from the OCS, the DTS is not associated with the plasma membrane. It primarily functions as the platelet storage for intracellular calcium ion (Ca^{2+}) (Tian *et al.*, 2025). Ca^{2+} is released from the dense tubular system into the cytoplasm during platelet activation and this initiates Ca^{2+} -dependent signaling pathways that regulate cytoskeletal reorganization, granule release, and phosphatidylserine (PS) exposure (Dolan and Diamond, 2014). The DTS also contains essential enzymes that facilitate the synthesis of prostaglandins and thromboxane, hence enhancing platelet activity and contributing to procoagulant responses (Mammadova-Bach *et al.*, 2019).

In addition to OCS and DTS, three main types of granules can be found in platelets: α -granules, dense granules, and lysosomes, which differ for both cargo composition and biogenesis/final feature (Tian *et al.*, 2025). Granule secretion supported by exocytosis is a key step in platelet activation, as this process leads to the release of cargo in the extracellular environment and to the exposure of granule's membrane molecules on platelet surface. The release of different compounds from platelet granules is important for platelet aggregation and thrombus stabilization, but also for many other platelet-driven processes (Tian *et al.*, 2025).

α -granules represent the most abundant type of granules in platelets, primarily responsible for storing proteins necessary for hemostasis, thrombosis, inflammation, and wound healing. Adhesion molecules such as fibrinogen and von Willebrand factor (vWF), membrane proteins (P-selectin), a mixture of growth factors, cytokines, chemokines, coagulation factors, complement components, and protease inhibitors are also found in α -granules (Tian *et al.*, 2025; Xu, *et al.*, 2016).

Dense granules are smaller than α -granules and they store a lower molecular weight, which includes ADP, ATP, serotonin, and a high concentration of Ca^{2+} (Chen *et al.*, 2018; Dupuis *et al.*, 2020). They rapidly get released post platelet activation to amplify both platelet recruitment and aggregation and signaling events.

Lysosomes contain high concentrations of hydrolytic enzymes, such as hydrolases, that contribute to platelet activation and clot formation which may participate in the remodeling of the injury site. Ca^{2+} is also present in the lumen of lysosomes (Tian *et al.*, 2025).

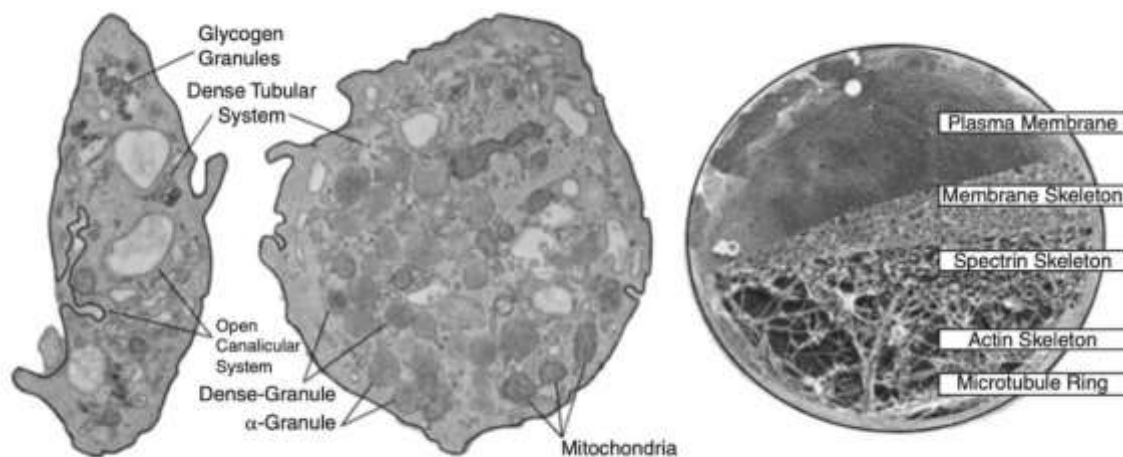


Figure 2. Platelet morphology. Ultrastructural features of platelets obtained by electron microscopy analysis (Thon and Italiano, 2012).

1.2. The roles of platelets in physiological and pathological conditions

1.2.1 Platelets in hemostasis and thrombosis

The primary function of platelets is to support and coordinate hemostasis. Hemostasis is an essential physiological process that prevents hemorrhage (i.e. blood loss) at sites of vascular damage and maintains the integrity of blood vessels. This complex process depends on the physical and functional interaction of platelets, coagulation factors, and endothelial cells. In intact vessels, platelets circulate in a resting inactive condition and do not interact with endothelium. This condition is maintained by the release of signaling molecules from intact endothelial cells, including nitric oxide (NO) and prostacyclin (PGI₂), which inhibit platelet activation and aggregation, thereby preventing unnecessary clot formation (Becker *et al.*, 2018). Upon vascular damage, platelets adhere to the exposed subendothelial extracellular matrix (ECM), which is a network of macromolecules that provides structural support and regulates vascular permeability, collagen and vWF are key components of the ECM and represent the most relevant molecules supporting platelet adhesion. Platelet adhesion is supported by specific platelet membrane receptors, including the collagen-binding receptors integrin $\alpha 2\beta 1$ and glycoprotein VI (GPVI), as well as the vWF receptor complex glycoprotein Ib–IX–V (GPIb–IX–V). Platelet adhesion is influenced by local hemodynamic conditions, particularly shear stress, which modulates the interaction between platelets, vWF and ECM (Scridon, 2022). Following adhesion, platelets undergo activation, spreading, and aggregation, eventually

leading to formation of a platelet plug, in the process referred to as primary hemostasis (Oshinowo *et al.*, 2023, Chaudhary *et al.*, 2022; Yeung *et al.*, 2018). During this process, platelets activated by adhesion to the ECM amplify the hemostatic response by releasing soluble agonists, that recruit additional platelets at the site of injury. Activated platelets further enhance thrombus formation by releasing secondary mediators such as ADP and thromboxane A2 (TxA2) via cyclooxygenase-1 (COX-1), thereby promoting platelet activation and aggregation through a positive feedback mechanism (Zou *et al.*, 2022; Holinstat, 2017).

Hemostasis is also supported by the activation of the coagulation cascade, leading to fibrin formation and deposition, with the consequent formation of more stable plug, in the process known as secondary hemostasis (Mereweather *et al.*, 2023). The coagulation cascade involves the sequential activation of clotting factors, primarily serine proteases, which circulate as inactive zymogens and are rapidly activated at sites of vascular injury. This culminates in thrombin generation, a key enzyme that converts the soluble form of fibrinogen into insoluble fibrin to stabilize clot formation and amplifies coagulation (Hoffman and Monroe, 2001; Tian *et al.*, 2025). It is important to mention that platelet activation is accompanied by translocation of negatively charged PS to the external leaflet of the plasma membrane. This process is primarily driven by elevated intracellular Ca^{2+} which controls the changes in the asymmetric distribution of membrane phospholipids, creates a negatively charged pro-coagulant surface (Broos *et al.*, 2012; Tian *et al.*, 2025). This negatively charged surface supports the assembly of coagulation enzyme complexes, including prothrombinase complex, which enhances prothrombin conversion to thrombin and the formation of the fibrin clot (Gremmel *et al.*, 2016; Xu *et al.*, 2016). In turn, thrombin is one of the most potent platelet agonists, that is essential for the amplification of the platelet-mediated responses and for platelet aggregation (Bye *et al.*, 2016). A schematic summary of the platelet involvement in hemostasis is reported in figure 3.

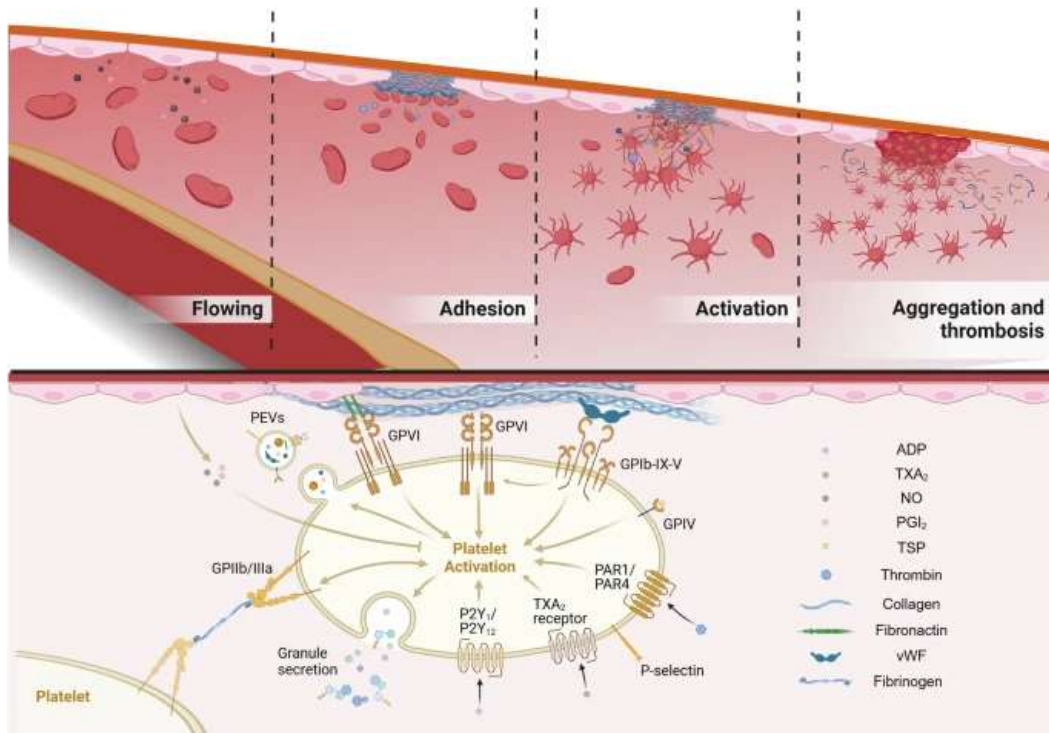


Figure 3. Roles of platelets in the biological process of hemostasis, illustrating key steps in platelet function and the underlying molecular mechanisms (Tian *et al.*, 2025).

Under physiological conditions, hemostasis is maintained in a delicate balance between coagulation and anticoagulation. However, in response to different triggers this balance may become dysregulated, leading to excessive thrombus formation. Pathological thrombosis is defined as the formation of a blood clot (thrombus) within a blood vessel, resulting in partial or complete obstruction of blood flow in arteries or veins (Mereuta *et al.*, 2023). Structurally, a mature thrombus consists of a dense core of activated platelets stabilized by fibrin.

Thrombus formation within an intact vessel represents a particularly critical pathological condition. As the thrombus enlarges, it progressively narrows the vascular lumen, impairing blood flow and reducing oxygen and nutrient delivery to downstream tissues. When luminal obstruction exceeds approximately 75%, tissue hypoxia develops, which may progress to anoxia when occlusion becomes more severe (>90%). This ultimately leads to irreversible tissue damage and necrosis. In addition, thrombi may undergo embolization, whereby fragments detach and travel through the circulation, causing occlusion of distant vessels and further exacerbating tissue injury (Furie and Furie, 2008).

Overall, thrombosis represents a central pathogenic mechanism underlying major cardiovascular diseases, including myocardial infarction, ischemic stroke, and venous thromboembolism. These conditions are leading causes of morbidity and mortality worldwide,

highlighting the importance of understanding thrombus formation, its consequences, and targeted therapeutic interventions.

The mechanism of formation and composition of thrombi differ significantly between arterial and venous systems. Arterial thrombosis typically occurs under conditions of high shear stress and is primarily driven by platelet activation. It is most commonly initiated by rupture or erosion of atherosclerotic plaques, which expose subendothelial components and triggers rapid platelet adhesion, activation, and aggregation. These thrombi are platelet-rich and can lead to acute vessel occlusion, resulting in severe clinical events such as myocardial infarction or ischemic stroke (Koupenova *et al.*, 2017).

In contrast, venous thrombosis develops under low shear conditions and is mainly associated with activation of the coagulation cascade. It is strongly linked to endothelial dysfunction, inflammation, and blood flow stasis. Activated endothelial cells increase the expression of adhesion molecules such as selectins, promoting the recruitment of platelets and leukocytes. This process enhances tissue factor (TF) expression and initiates fibrin formation. Venous thrombi are therefore rich in fibrin and red blood cells. Unlike arterial thrombi, they are loosely attached to the vessel wall and have a higher tendency to detach, leading to complications such as pulmonary embolism (Koupenova *et al.*, 2017).

The development of thrombosis is commonly explained by Virchow's triad, which includes three major contributing factors: endothelial injury, abnormal blood flow (stasis or turbulence), and hypercoagulability. Arterial thrombosis is primarily associated with endothelial damage and atherosclerosis, whereas venous thrombosis is more closely related to blood stasis (e.g., prolonged immobility) and hypercoagulable states, including cancer and genetic coagulation disorders (Koupenova *et al.*, 2017).

Given these mechanistic differences, therapeutic strategies for thrombosis are tailored according to the type and location of the thrombus. Arterial thrombosis is mainly managed with antiplatelet therapies that inhibit platelet activation and aggregation. Aspirin, an irreversible inhibitor of cyclooxygenase, remains a cornerstone of therapy for secondary prevention. In addition, P2Y₁₂ receptor inhibitors and newer agents such as PAR-1 inhibitors and factor Xa inhibitors are widely used to reduce thrombotic risk (Koupenova *et al.*, 2017).

In contrast, venous thrombosis is primarily treated with anticoagulant therapies targeting the coagulation cascade. These include heparins, vitamin K antagonists, and direct oral

anticoagulants (DOACs), which are increasingly preferred due to their predictable pharmacokinetics and improved safety profiles (Chen *et al.*, 2018).

In acute thrombotic events, rapid restoration of blood flow is critical. Thrombolytic therapy using tissue plasminogen activator (tPA), can dissolve clots and significantly improve clinical outcomes, although it carries a risk of bleeding. Long-term management also includes preventive strategies such as statin therapy to improve endothelial function and reduce inflammation, as well as strict blood pressure control to minimize the risk of recurrent vascular events (Chen *et al.*, 2018).

1.2.2 Platelet functions beyond hemostasis and thrombosis

Beyond their central role in hemostasis and thrombosis, platelets play many functions in physiological and pathological contexts. Importantly, platelets are increasingly recognized as key mediators of immune and inflammatory processes. Platelets express a diverse repertoire of surface pattern recognition receptors (PRR), most notably Toll-like receptors (TLRs), which enable them to directly sense pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Tian *et al.*, 2025). Through these receptors, platelets are able to recognize and respond to different classes of pathogens. (Yeung *et al.*, 2018; Guo and Rondina, 2019).

Once activated, platelets become potent regulators of the inflammatory environment. They release a broad spectrum of cytokines and chemokines that recruit and activate neutrophils, monocytes, and lymphocytes at sites of infection and tissue injury (Tian *et al.*, 2025). Platelet-derived mediators also promote the upregulation of adhesion molecules and selectins on the vascular endothelium, facilitating leukocyte trafficking from the circulation into inflamed tissue which represents a critical step to the progression of the immune response (Xu *et al.*, 2016).

Platelets also contribute to intercellular communication through the release of vesicles containing cytokines and surface proteins such as P-selectin. This vesicle-mediated signaling enables platelets to influence immune responses beyond direct cell-to-cell interaction (Tian *et al.*, 2025).

The interaction between platelets and leukocytes is an important step in the innate immune response. Platelets bind to neutrophils via P-selectin (expressed on platelets) and P-selectin glycoprotein ligand-1 (PSGL-1 expressed on neutrophils), as well as through platelet integrin $\alpha\text{IIb}\beta 3$ and neutrophil $\beta 2$ integrins (e.g., Mac-1) through fibrinogen bridges, facilitating the

formation of platelet-neutrophil aggregates (Tian *et al.*, 2025; Guo and Rondina, 2019, Weber and Springer, 1997).

The close functional overlap between platelet-mediated hemostasis and immune activation creates the conditions for a pathological feedback loop known as thromboinflammation. In this state, inflammation promotes a pro-thrombotic environment, while thrombosis in turn amplifies the inflammatory response, with the two processes mutually reinforcing each other (Yeung *et al.*, 2018; Guo and Rondina, 2019). Under physiological conditions, this interplay is tightly regulated and contributes to effective pathogen containment, for example through the formation of platelet aggregates that physically trap pathogens within small vessels, preventing their dissemination (Tian *et al.*, 2025). However, when dysregulated, as occurs in severe infections such as sepsis, this cycle can escalate beyond tissue containment and become systemically destructive. Uncontrolled platelet-leukocyte interactions, excessive neutrophil activation and formation of Neutrophil Extracellular Traps (NETs), and widespread thrombin generation can lead to microvascular occlusion, impaired oxygen delivery, and multi-organ failure. The organism's own defense mechanisms, initially deployed to combat a foreign threat, ultimately become a source of self-inflicted damage. This paradox lies at the core of the pathophysiology of sepsis and other severe inflammatory conditions, and it highlights why understanding platelet biology in the immunological context is of considerable clinical importance. Platelets have also been shown to contribute to the progression of neurodegenerative diseases. Since platelets are well-known drivers of inflammation, and inflammation plays a central role in neurodegeneration, their involvement in these diseases is not surprising. In Alzheimer's disease (AD), platelets carry and release proteins associated with amyloid accumulation in the brain, and share biological abnormalities with affected neurons, suggesting they actively participate in disease progression. In Parkinson's disease (PD), platelets show the same pattern of mitochondrial damage and increased oxidative stress that is observed in the affected areas of the brain, pointing to a shared disease mechanism. This makes platelets a potentially accessible window into the oxidative processes driving PD. In multiple sclerosis (MS), activated platelets damage the blood-brain barrier (BBB), making it easier for immune cells to enter the brain and cause inflammation and neuronal injury. Across all three diseases, a common theme emerges: platelets are not simply reacting to disease but appear to actively contribute to its development and worsening. As a result, they hold real promise as accessible biomarkers for early diagnosis

and as potential targets for new therapeutic strategies (Cadoni *et al.*, 2024; Ferrer-Raventós and Beyer, 2021).

Platelets are also considered as active contributors to cancer. In the tumor environment, platelets release factors that help cancer cells grow, move, and avoid the immune system. They also promote the formation of new blood vessels (angiogenesis), which supply the tumor with oxygen and nutrients. In the bloodstream, platelets surround tumor cells, protecting them from immune attack and helping them survive. Overall, platelets are believed to support both tumor growth at the original site and the spread of cancer to other parts of the body. Among the different mechanisms through which platelets exert these effects, platelet-derived extracellular vesicles (PEVs) have emerged as important mediators of platelet–tumor communication. The role of platelets and PEVs in cancer progression will be discussed thoroughly in section 4-2 (Tian *et al.*, 2025).

1.3. Platelet activation

Under physiological conditions, circulating platelets remain in a quiescent state and do not interact with the intact vascular endothelium. This resting state is maintained by endothelial inhibitory mediators, including nitric oxide (NO) and prostacyclin (PGI₂) (Broos *et al.*, 2012; Tian *et al.*, 2025).

Vascular injury leads to the exposure of ECM constituents, including collagen and vWF, which interact with platelet receptors. Collagen binding is supported by glycoprotein VI (GPVI) and integrin $\alpha 2\beta 1$, whereas the GPIb-V-XI complex interacts with vWF (Gauer *et al.*, 2023; Mangin *et al.*, 2023).

The adhesion supported by these and other important platelet receptors triggers complex signaling cascades, largely supported by Src family tyrosine kinases (such as Fyn and Lyn), which propagate intracellular activation through phosphorylation immunoreceptor tyrosine-based activation motifs (ITAMs). This leads to the recruitment and activation of Syk kinase, ultimately resulting in the phosphorylation and activation of phospholipase C γ 2 (PLC γ 2), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ induces Ca²⁺ release from the DTS, and the increased intracellular Ca²⁺ concentration is a key step in platelet activation, that supports platelet shape change, activates calcium-dependent enzymes, and triggers granule secretion. Meanwhile, DAG stimulates target enzymes, including protein kinase C (PKC) which controls

the cytoskeletal rearrangements, that lead to changes in the discoidal shape of platelets and to secretion of intracellular granules (Broos *et al.*, 2012).

This is an initial activation phase which leads to granule secretion and the release of secondary soluble agonists, which act in an autocrine and paracrine manner to amplify platelet activation. Among the many molecules present in platelet granules, Adenosine Diphosphate (ADP) plays a particularly important role in platelet activation. In addition, activated platelets synthesize and release Thromboxane A2 (TxA2), which recruits and activates adjacent platelets, thereby amplifying and extending the platelet response (Broos *et al.*, 2011; Yeung *et al.*, 2018). Platelet activation and intracellular Ca^{2+} increase are also accompanied by translocation of PS to the external surface of the plasma membrane (Broos *et al.*, 2012; Tian *et al.*, 2025). As mentioned above, the presence of PS on the outer leaflet of platelet plasma membrane creates a negatively charged pro-coagulant surface that supports the assembly of the coagulation enzyme complexes and leads to the activation of thrombin and the production of a fibrin clot (Gremmel *et al.*, 2016; Xu *et al.*, 2016).

Importantly, thrombin generated through this coagulation cascade, together with ADP released from dense granules and TxA2 synthesized by activated platelets, acts on platelets themselves as potent soluble agonists, further amplifying the activation response through a family of membrane receptors known as G protein-coupled receptors (GPCRs). GPCRs are a large family of seven-transmembrane cell surface receptors that transmit extracellular signals intracellularly via heterotrimeric G proteins composed of $\text{G}\alpha$, $\text{G}\beta$, and $\text{G}\gamma$ subunits. In the inactive state, the $\text{G}\alpha$ subunit is bound to GDP and forms a complex with $\text{G}\beta\gamma$. Upon ligand binding, the GPCR acts as a guanine nucleotide exchange factor (GEF), triggering GDP-to-GTP exchange on the $\text{G}\alpha$ subunit, which causes dissociation of $\text{G}\alpha$ -GTP from $\text{G}\beta\gamma$, allowing both to independently activate their downstream effectors. In platelets, GPCRs couple to three main G protein families with distinct functional outcomes: G_q , which activates phospholipase $\text{C}\beta$ ($\text{PLC}\beta$), leading to the generation of IP3 and DAG, intracellular Ca^{2+} mobilization, granule secretion, and platelet aggregation; G_i , which inhibits adenylyl cyclase and activates the PI3K/Akt pathway, thereby sustaining and amplifying the platelet activation response; and $\text{G}_{12/13}$, which signals through RhoA and its downstream effectors, including Rho-associated kinase, to drive platelet shape change (Tian *et al.*, 2025; Yeung *et al.*, 2018).

Thrombin is one of the most potent platelet agonists and plays a role in platelet aggregation and thrombus formation by through of protease-activated receptors (PARs) (Bye *et al.*, 2016).

Thrombin activates PARs through the proteolytic cleavage of the N-terminal domain and the formation of a tethered ligand that causes the beginning of intracellular signal transduction through PARs. The receptor PAR1, coupled to Gαq and Gα13, could activate platelets rapidly when there are low concentrations of thrombin that contribute to early platelet responses such as aggregation and granule secretion. In contrast, PAR4, which signals through the same Gαq and Gα13 subunits, requires higher thrombin concentrations for maximal activation and provides a longer-term stimulus for signal transduction; notably, PAR4 stimulation induces significantly higher TxA2 formation compared to PAR1, further contributing to sustained platelet activation (Broos *et al.*, 2012; Tian *et al.*, 2025; Xu *et al.*, 2016).

TxA2 is synthesized by activated platelets from arachidonic acid via cyclooxygenase-1 (COX-1) and thromboxane synthase, and acts as a potent positive-feedback amplifier of platelet activation. TxA2 signals through the TPα receptor, which couples to Gαq and Gα12/13, activating PLCβ and the RhoA signaling pathways, which, as described above, drive intracellular Ca²⁺ mobilization, platelet shape change and aggregation. Notably, Gα12/13 plays a critical role in TxA2-mediated signaling, as its absence significantly reduces TxA2 efficacy and impairs the formation of stable platelet aggregates under shear stress (Tian *et al.*, 2025; Yeung *et al.*, 2018).

ADP is a potent platelet agonist that signals through receptors P2Y1 and P2Y12. Binding of ADP to these receptors activates the Gαq and Gαi pathways, respectively, resulting in raised intracellular Ca²⁺, activation of further signaling enzymes, including PI3K, and enhanced platelet aggregation. Optimal platelet activation by ADP therefore requires the cooperative signaling of both receptors (Tian *et al.*, 2025).

The cascade of intracellular signals initiated by platelet adhesion and propagated by the action of soluble agonists converge to an inside-out signaling, which supports the conformational changes of integrin αIIbβ3 from a low- to a high-affinity state for its ligands. Activated integrin αIIbβ3 binds fibrinogen, enabling the cross linking between adjacent platelets, thus promoting platelet aggregation and plug formation at the site of vessel damage. This process is largely mediated by the Ca²⁺-dependent activation of the guanine nucleotide exchange factor CalDAG-GEFI that results in the conversion of the small GTPase Rap1b to its active, GTP-bound state, which, in turn, induces the conformational change of integrin αIIbβ3 that allows platelet aggregation (Offermanns, 2006; Broos *et al.*, 2012).

Fibrinogen is the principal soluble ligand that binds to active integrin $\alpha\text{IIb}\beta_3$ and is an essential component of platelet aggregation. Due to its symmetrical structure, fibrinogen can bind to integrins $\alpha\text{IIb}\beta_3$ expressed on adjacent platelets, acting as a bridge that allows platelet-platelet aggregation during the early stages of thrombus formation (Tian *et al.*, 2025).

The binding of integrin $\alpha\text{IIb}\beta_3$ to fibrinogen initiates an outside-in signaling that stabilizes the platelet aggregate by mediating a substantial cytoskeletal rearrangement and further granule secretion. Ultimately, this outside-in signaling culminates in the formation of a stable hemostatic plug, in which fibrin networks, generated through the coagulation cascade, crosslink and reinforce the platelet aggregate, ensuring clot stability at the site of vascular injury (Estevez and Du, 2017; Tian *et al.*, 2025).

An important consequence of platelet activation is the release of platelet-derived extracellular vesicles (PEVs), which are gaining growing interest for their involvement in many critical processes. PEVs will be discussed in detail in the following section.

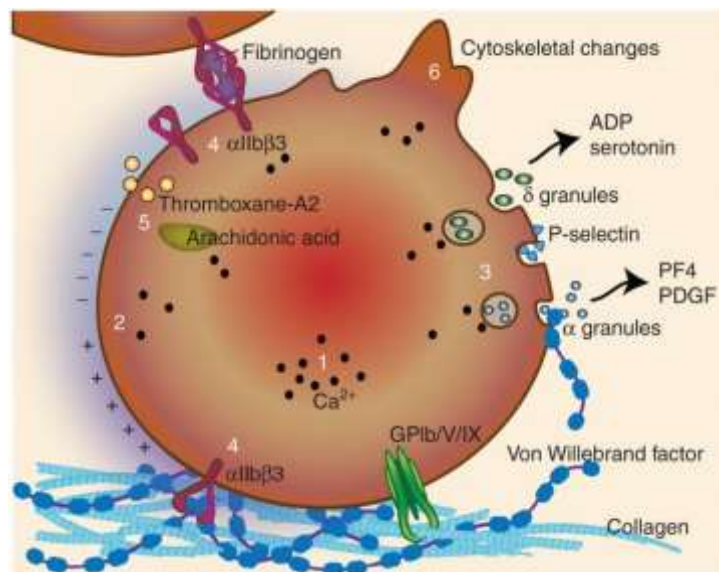


Figure 4. The mechanisms of platelet adhesion, activation, and secretion. Platelet binding to von Willebrand factor, collagen and other ECM molecules supports platelet adhesion to the damaged vessel wall. Subsequent platelet activation results in an increase in intracellular calcium (1), increased phosphatidylserine exposure to create a negatively charged surface (2), alpha and dense granule release (3), conversion of the Integrin IIb/IIIa receptor to a high-affinity state (4), thromboxane A2 generation (5), and cytoskeletal rearrangement (6) (Haley *et al.*, 2014).

2. Extracellular vesicles

2.1. Extracellular vesicle biology

Extracellular vesicles (EVs) are small membrane-bound vesicles ranging in size from 30 nm to few μm , released by numerous active or dying cells, including leukocytes, platelets, erythrocytes, and endothelial cells. In humans, EVs are important in both physiological and pathological conditions and they are found in several bodily fluids, including blood, urine, tears, and saliva (Zara *et al.*, 2019, Boulanger *et al.*, 2017).

EVs are composed of phospholipid bilayers and store lipids, proteins, nucleic acids (including microRNAs and noncoding RNA), and other substances that modulate several processes, such as blood clotting, inflammation, and communication between cells. In recent years, there has been considerable interest in EVs as modulators of inflammation, vascular dysfunction, and thrombosis. Because EVs express the same surface and cytosolic proteins found in the cells they originated from, they represent suitable candidates for a noninvasive liquid biopsy (Yuana *et al.*, 2013, Shahi *et al.*, 2024, Zarà *et al.*, 2019).

EVs are classified into three primary categories: apoptotic bodies, microvesicles, and exosomes, differentiated by their size and origin (Figure 5).

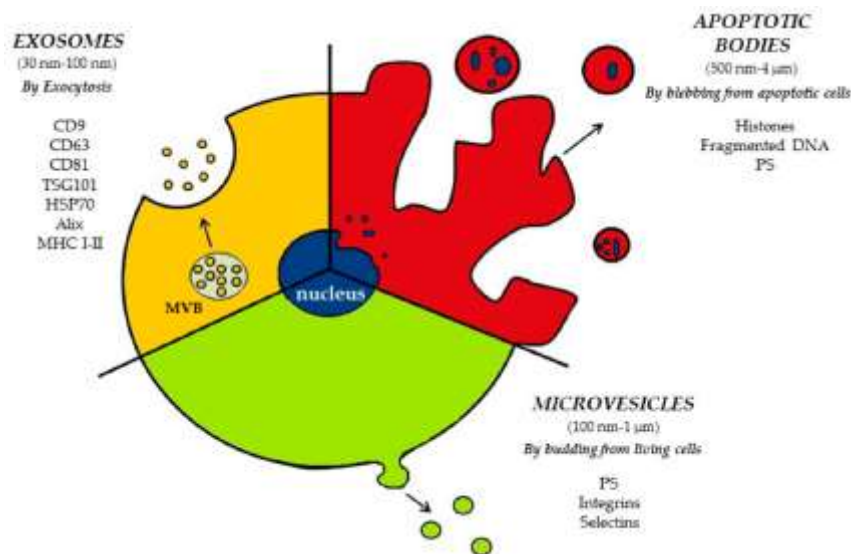


Figure 5. Mechanisms of extracellular vesicle (EV) release and their specific markers (Zarà *et al.*, 2019).

Diverse types of EVs demonstrate similarities in biophysical characteristics, including size range and protein expression. Selective molecular markers of exosomes, microvesicles, and

apoptotic bodies have been found, but their physical separation still represents a demanding task (van der Pol *et al.*, 2016).

Apoptotic bodies (ApoBDs) are membrane-bound vesicles formed during the final stages of apoptosis, with sizes ranging from about 500 nm to 5 μ m. As a cell undergoes programmed death, it begins to shrink and form membrane blebs, which then separate into smaller fragments known as apoptotic bodies. These vesicles contain portions of the original cell, including cytoplasm, organelles, and fragmented DNA, and are characterized by the exposure of PS on their surface. This signal allows phagocytic cells to recognize and engulf them efficiently, ensuring safe removal of cellular debris. After their release, ApoBDs can also interact with surrounding cells by transferring their contents, contributing to intercellular communication. In this way, they not only support cell clearance but can also influence immune responses, often leading to immunosuppressive effects and playing a role in conditions such as cancer and autoimmunity (Gyorgy *et al.*, 2011; Zara *et al.*, 2019).

Microvesicles (MVs) are smaller than apoptotic bodies, typically ranging from about 100 to 1,000 nm in diameter, and are generated by a different mechanism. Rather than resulting from cell fragmentation, MVs are formed directly from the outward budding of the plasma membrane of living cells, either constitutively or upon cell activation. This process is initiated by cellular stimulation that increases intracellular Ca^{2+} levels, leading to cytoskeletal rearrangement and changes in membrane lipid distribution. These changes promote membrane curvature and the formation of a vesicle that selectively incorporates proteins, lipids, and nucleic acids such as mRNA and miRNA. The final release of MVs is driven by actin–myosin contraction, which enables the vesicle to detach from the cell surface. MVs often carry surface markers and phospholipids, including phosphatidylserine, reflecting their cellular origin, most commonly platelets, red blood cells, or endothelial cells. Functionally, they act as mediators of intercellular communication and are involved in key processes such as coagulation, inflammation, and tumor progression, contributing to both physiological regulation and disease development (van Niel *et al.*, 2018; Zara *et al.*, 2019).

Exosomes represent a smaller class of Ev, typically 30–100 nm in diameter, with a distinct intracellular origin. Unlike vesicles formed by outward budding of the plasma membrane, exosomes are generated within the endosomal system. Their formation begins with inward budding of the membrane of late endosomes, producing intraluminal vesicles inside multivesicular bodies (MVBs). During this process, selected proteins, lipids, and nucleic acids

are incorporated into the vesicles. When MVBs fuse with the plasma membrane, these vesicles are released into the extracellular space as exosomes. Once outside the cell, exosomes interact with recipient cells through different mechanisms, allowing the transfer of their molecular cargo. In this way, they contribute to intercellular communication and play important roles in regulating immune responses and disease-related processes (Gyorgy *et al.*, 2011; Zara *et al.*, 2019).

2.2 Functions of extracellular vesicles

EVs are membrane-bound particles released into the extracellular space, where they influence recipient cell behavior in both physiological and pathological contexts. They function as mediators of intercellular communication by transferring regulatory RNAs, proteins, and bioactive lipids through paracrine and autocrine signaling mechanisms, thereby modulating diverse cellular processes including inflammation and thrombosis (Zara *et al.*, 2019).

EVs interact with recipient cells through a coordinated sequence of events involving membrane docking, receptor engagement, and intracellular trafficking. Target specificity is largely determined by molecular interactions between surface proteins enriched on EV membranes and complementary receptors on recipient cells. Key mediators of this recognition process include integrins, tetraspanins, lectins, heparan sulfate proteoglycans, and ECM molecules. These molecules facilitate selective adhesion and contribute to tissue-specific communication (van Niel *et al.*, 2018).

Following binding to the plasma membrane of target cells, EVs may activate intracellular signaling pathways directly through receptor-ligand interactions. In some cases, EVs fuse directly with the plasma membrane, allowing the transfer of transmembrane proteins and lipids. Internalized vesicles typically enter the endocytic pathway and are trafficked to multivesicular endosomes. While many are degraded in lysosomes, others release their cargo into the cytoplasm, thereby altering gene expression and cellular function (van Niel *et al.*, 2018).

Under normal physiological conditions, EVs contribute to the maintenance of tissue homeostasis and coordination of immune responses. Through the delivery of regulatory RNAs, proteins, and lipids, they regulate cellular signaling networks and support balanced inflammatory responses (Shahi *et al.*, 2024, van Niel *et al.*, 2018).

In pathological settings, dysregulation of EV production, cargo composition, or targeting can contribute to disease progression. Altered EV-mediated signaling has been implicated in

inflammatory disorders, thrombosis, tumor progression, and metastatic niche formation. Because their molecular cargo reflects the state of the parent cell, pathological alterations are often mirrored in circulating EV populations (Zara *et al.*, 2019).

MVs, particularly those derived from platelets, may expose PS and TF on their surface, thereby increasing their thrombogenic potential. As previously described PS provides a negatively charged membrane surface that facilitates the assembly of coagulation enzyme complexes, whereas TF initiates the extrinsic coagulation cascade, ultimately leading to thrombin generation, fibrin formation, and thrombosis (György *et al.*, 2011). Notably, platelets are among the most prolific producers of EVs in the bloodstream, and platelet-derived EVs play important roles in hemostasis and thrombosis, as will be discussed in detail in the following section.

The ability of EVs to retain surface and cytosolic components representative of their parental cells has positioned them as promising non-invasive diagnostic biomarkers (Zara *et al.*, 2019). Different subclasses of extracellular EVs exhibit distinct characteristics that influence their diagnostic and therapeutic applications. Exosomes, due to their endosomal origin and selective cargo packaging, are enriched in tumor-specific proteins, lipids, and regulatory RNAs, making them particularly attractive as tumor biomarkers in liquid biopsy approaches. Their relative stability in circulation further enhances their diagnostic utility. In contrast, MVs, which are generally larger in size and more readily detectable by conventional techniques such as flow cytometry, are extensively investigated as diagnostic tools. Their abundance in biological fluids, particularly in blood, and their frequent association with procoagulant and inflammatory markers contribute to their clinical relevance in disease monitoring (György *et al.*, 2011).

In addition to diagnostic potential, EVs demonstrate systemic distribution and organotropism, enabling their exploration as engineered nanocarriers for targeted drug and biomolecule delivery. However, despite their translational promise, clinical implementation remains limited by technical challenges related to EV isolation, quantification, and standardization (Zara *et al.*, 2019).

Among pathological contexts, tumor-derived EVs have received particular attention, contributing to cancer progression through multiple coordinated mechanisms including premetastatic niche formation, angiogenesis, and immune modulation. After being released into the circulation, these vesicles can reach distant organs and participate in the establishment of premetastatic niches by reprogramming resident stromal and endothelial cells. This process facilitates subsequent tumor cell colonization and metastatic outgrowth. In addition, tumor-

derived EVs promote angiogenesis by transferring pro-angiogenic factors and activating matrix metalloproteinases (MMPs), leading to ECM remodeling and activating endothelial cell migration. Through the delivery of regulatory mRNAs, miRNAs, proteins, and bioactive lipids, EVs can alter gene expression profiles in recipient cells, thereby modulating proliferation, invasion, and survival pathways (Shahi *et al.*, 2024).

Furthermore, EVs influence the immune microenvironment by modulating immune cell function, including the suppression or reprogramming of natural killer (NK) cells and macrophages. Collectively, these mechanisms underscore the multifaceted role of tumor-derived EVs in driving cancer progression (Shahi *et al.*, 2024).

Elevated levels of TF-positive MVs have been reported in cancer patients and are associated with an increased risk of venous thrombosis, with variability depending on tumor type. Furthermore, the procoagulant activity of circulating MVs has been proposed as a potential predictive marker for venous thromboembolism (VTE) in cancer patients (Zara *et al.*, 2019). Collectively, these findings highlight the diverse functional roles of EVs across physiological and pathological contexts, underscoring their emerging relevance as both disease biomarkers and therapeutic targets (Zara *et al.*, 2019; van Niel *et al.*, 2018).

2.3 Platelet-derived extracellular vesicles (PEVs)

Platelet-derived extracellular vesicles (PEVs) are the most abundant extracellular vesicles present in the blood of healthy individuals, around 70-90% of the circulating EVs (Italiano *et al.*, 2010). PEVs are released from platelets in response to various stimuli, including activation, shear stress, or apoptotic processes. In recent years, they have gained significant attention as potential tools for therapeutic applications, mainly due to their small size, low immunogenic profile, and relative ease of isolation. Based on their biogenesis, they can be broadly divided into two main categories: platelet microvesicles (PMVs), also known as platelet microparticles (PMPs) and medium/large PEVs, which are formed by outward budding of the plasma membrane and typically range from 100 to 1000 nm, and exosomes, or small PEVs, which arise from the endosomal pathway and are smaller, approximately 30 to 100 nm in diameter. Due to their nanoscale size, PEVs are able to circulate efficiently in biological fluids such as blood, lymph, and synovial fluid, enabling their involvement in both physiological and pathological processes (Fan *et al.*, 2024). PEVs represent a heterogeneous population whose composition is determined by the specific stimulus that activates platelets. In general, any stimulus capable of

platelet activation can induce PEVs formation, including shear stress, thrombin, calcium ionophores, ADP, and collagen. Importantly, the quantity, size, and structural characteristics of the released PEVs vary depending on the nature of the stimulus. Among these, thrombin is commonly used in experimental settings, as it induces the release of a relatively higher number of PEVs (Fan *et al.*, 2024).

PEVs contain a range of bioactive components, including proteins, glycoproteins, lipids, RNA, transcription factors, sugars, and coagulation factors. Notably, strong stimuli such as thrombin can also lead to the inclusion of organelles like mitochondria and mitochondrial DNA. As a result, vesicles generated under different conditions differ in both their surface characteristics and cargo, suggesting that their functions vary depending on the activating signal (Lou and Cai, 2025; Fan *et al.*, 2024).

PEVs also retain specific platelet-derived molecules, such as integrin $\alpha\text{IIb}\beta 3$ (CD41/CD61), reflecting their cellular origin. In the bloodstream, EVs are primarily derived from both platelets and MKs. While MK-derived vesicles are continuously released from the bone marrow under normal physiological conditions, PEVs are generated mainly in response to platelet activation. Consequently, MK-derived vesicles tend to predominate in healthy individuals, whereas PEVs levels increase under conditions associated with platelet activation, such as inflammation.

Although both PEVs and MK-derived vesicles share certain markers (e.g., CD41), they can be distinguished based on their surface characteristics. PEVs typically express activation-related markers such as P-selectin (CD62P) and often PS, whereas these features are generally absent in MK-derived vesicles (Puhm *et al.*, 2021; Melki *et al.*, 2017).

2.4 Biological functions of PEVs

PEVs participate in a wide range of physiological and pathological processes, functioning as versatile mediators of intercellular communication. By transferring proteins, lipids, nucleic acids, and other bioactive molecules to both adjacent and distant cells, PEVs can reprogram the metabolism and gene expression of target cells, thereby influencing diverse biological processes. Interaction with recipient cells occurs through direct surface receptor engagement, membrane fusion, or endocytosis, allowing PEVs to deliver their cargo and trigger downstream signaling responses (Puhm *et al.*, 2021; Italiano *et al.*, 2010).

One of the most well-established roles of PEVs is their contribution to hemostasis and coagulation. Due to the expression of PS and TF on their surface, PEVs display significant

procoagulant activity, supporting the assembly of coagulation enzyme complexes and promoting thrombin generation and fibrin clot formation. Notably, early studies demonstrated that PEVs membranes possess 50 to 100-fold greater procoagulant activity compared to activated platelets themselves (Fan *et al.*, 2024). In addition, PEVs carry surface integrins such as integrin $\alpha\text{IIb}\beta 3$, which bind fibrinogen and further support clot formation. Importantly, not all PEVs promote coagulation and, also depending on the conditions that induce their release, they may carry anticoagulant properties, contributing to the regulation of hemostatic balance by limiting excessive thrombin generation and activating anticoagulant proteins (Melki *et al.*, 2017; Puhm *et al.*, 2021).

Beyond coagulation, PEVs play an important role in modulating immune responses and inflammation. They support leukocyte recruitment to sites of infection and facilitate interactions between immune cells. Due to their nanoscale size, PEVs are able to cross endothelial barriers and accumulate in extravascular compartments that are inaccessible to platelets themselves, including synovial fluid, lymph, and bone marrow. Under inflammatory conditions, increased vascular permeability further facilitates this process. Once in these compartments, surface molecules such as P-selectin and integrin $\alpha\text{IIb}\beta 3$ mediate interactions with local immune cells, enabling PEVs to modulate immune responses at sites inaccessible to platelets. Notably, PEVs can exert both pro- and anti-inflammatory effects depending on the nature of the activating stimulus and the target cell type (Puhm *et al.*, 2021).

PEVs also demonstrate significant pro-angiogenic activity. They carry a range of growth factors including vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), which stimulate endothelial cell proliferation, migration, and tube formation (Fan *et al.*, 2024). Given their diverse biological roles and the involvement of platelets in tumor biology, the contribution of PEVs to cancer progression and their potential as diagnostic biomarkers will be discussed in detail in the following sections.

2.5 The therapeutic applications of PEVs

Beyond their physiological roles, PEVs have gained increasing attention for their therapeutic potential, both as biological agents and as drug delivery carriers, as summarized in Figure 6 (Fan *et al.*, 2024).

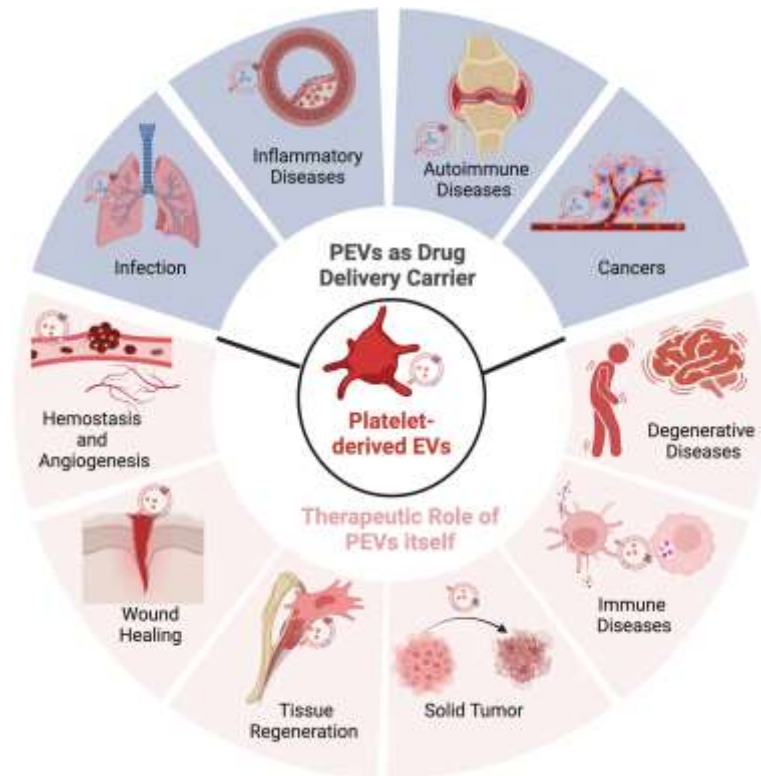


Figure 6. The therapeutic applications of PEVs (Fan *et al.*, 2024).

In the context of wound healing, PEVs have emerged as promising therapeutic agents. During the inflammatory phase of wound repair, PEVs attenuate excessive inflammation and promote the transition of macrophages from the pro-inflammatory phenotype to the tissue-regenerative phenotype, primarily through the delivery of TGF- β , IL-10, and miR-21. In the proliferative phase, PEVs stimulate fibroblast and endothelial cell proliferation and migration, supporting re-epithelialization and granulation tissue formation. During the remodeling phase, PEVs regulate MMP activity to maintain a balance between collagen synthesis and degradation, minimizing excessive scar formation. A first-in-human Phase I clinical trial of allogeneic PEVs for delayed wound healing demonstrated their safety and tolerability, representing an important step toward clinical translation (Lou and Cai, 2025; Fan *et al.*, 2024).

In the field of tissue regeneration, PEVs have shown osteogenic, chondrogenic, and musculoskeletal regenerative potential. Their cargo is enriched in growth factors associated with bone and cartilage repair, and different studies have demonstrated their ability to promote the osteogenic differentiation of mesenchymal stromal cells in a dose-dependent manner. PEVs have also been investigated in models of osteoarthritis, intervertebral disc degeneration, tendon

repair, and muscle injury, consistently showing beneficial effects on cell survival, proliferation, and functional recovery (Fan *et al.*, 2024).

PEVs have shown notable potential in the management of hemorrhagic shock and traumatic injury. Due to their high content of TF and PS, PEVs display strong procoagulant activity, enhancing fibrin polymerization and clot strength. Beyond hemostasis, PEVs improve post-traumatic outcomes by maintaining hemodynamic stability and mitigating the development of metabolic acidosis and cardiovascular shock, representing a promising alternative to conventional platelet transfusion therapies (Lou and Cai, 2025; Fan *et al.*, 2024).

PEVs exhibit significant immunomodulatory properties with therapeutic implications. They have been shown to downregulate macrophage reactivity and modify dendritic cell differentiation toward less inflammatory states. Additionally, PEVs selectively inhibit the differentiation of regulatory T-cell subsets into IL-17- and IFN- γ -producing proinflammatory cells through a P-selectin-dependent mechanism, suggesting a role in restraining excessive adaptive immune responses. In the context of autoimmune diseases, PEVs loaded with berberine have been shown to accumulate at inflamed joints following intravenous injection, effectively reducing joint swelling and suppressing bone erosion in preclinical models of rheumatoid arthritis (Fan *et al.*, 2024; Puhm *et al.*, 2021).

Emerging evidence supports a role for PEVs in the treatment of neurological conditions. PEVs have been shown to promote neural stem cell proliferation, survival, and differentiation, and to stimulate angiogenesis and neurogenesis by activating endogenous neural progenitor cells through the signaling pathways involving the kinases ERK and Akt. These findings suggest that PEVs may offer a novel cell-free therapeutic strategy for brain injury and neurodegenerative conditions, although the precise mechanisms of platelet-brain cell communication require further investigation (Fan *et al.*, 2024).

Furthermore, PEVs hold considerable promise as drug delivery vehicles. Their nanoscale size, low immunogenicity, natural biocompatibility, and ability to cross biological barriers, including the BBB, make them attractive candidates as natural nanocarriers. PEVs can encapsulate both hydrophilic and hydrophobic therapeutic molecules, as well as nucleic acids such as siRNA and miRNA, enabling targeted delivery to inflammation and tumor microenvironments (Fan *et al.*, 2024; Lou and Cai, 2025).

3. Tumor Disease

3.1 Tumor Biology

Cancer is a broad term encompassing more than 100 distinct disease types, each characterized by uncontrolled cell growth and the potential to invade surrounding tissues or spread to distant organs. Despite this diversity, all cancers share a common origin: the progressive transformation of normal cells into malignant ones through a series of genetic and epigenetic alterations. This process, known as tumorigenesis, is driven by the accumulation of mutations affecting oncogenes, which acquire dominant gain-of-function changes, and tumor suppressor genes, which undergo recessive loss-of-function alterations. In this sense, tumor development follows a logic comparable to Darwinian natural selection, where cells that gain a growth or survival advantage are selected and expand over time within the tissue (Cree, 2011). Although each cancer type presents with unique molecular and clinical features, virtually all malignant tumors share a common set of biological capabilities that are progressively acquired during tumor development. These capabilities, collectively referred to as the hallmarks of cancer, were first systematized by Hanahan and Weinberg (2000), who identified six fundamental traits shared across cancer types. A decade later, two additional hallmarks were proposed and incorporated into an updated framework, bringing the total to eight recognized capabilities (Hanahan and Weinberg, 2011). Together, these hallmarks provide a useful conceptual framework for understanding how tumors grow, survive, and spread. Before describing the individual hallmarks, it is important to acknowledge two enabling characteristics that create the conditions under which these traits can be acquired. The first is genomic instability and mutation. The accumulation of hallmark capabilities depends on an elevated rate of genetic change within tumor cells, which may result from defects in DNA repair mechanisms, increased sensitivity to mutagenic agents, or disruptions in genome maintenance pathways. In addition to direct mutations, epigenetic changes such as DNA methylation and histone modifications can silence tumor suppressor genes or activate oncogenes without altering the DNA sequence itself, further contributing to clonal evolution (Hanahan and Weinberg, 2011). The second enabling characteristic is tumor-promoting inflammation. Neoplastic tissues are almost universally infiltrated by immune and stromal cells, which release a variety of bioactive molecules into the tumor microenvironment (TME). Rather than eliminating the tumor, these signals often support its progression by providing growth factors (GF), survival signals, pro-angiogenic factors, and

enzymes that remodel the ECM, thereby facilitating the acquisition of multiple hallmark capabilities (Hanahan and Weinberg, 2011).

The first hallmark is self-sufficiency in growth signals. Under normal conditions, cell proliferation is tightly regulated and depends on external mitogenic stimuli. Cancer cells, however, are able to sustain proliferative signaling independently of these external cues. This can occur through several mechanisms, including autocrine production of GF or constitutive activation of signaling components (Hanahan and Weinberg, 2011). Closely related is the second hallmark, insensitivity to growth-inhibitory signals, often activated through inactivation of tumor suppressor genes play a central role in this regulation, and their inactivation is a recurring feature across many cancer types (Cree, 2011; Hanahan and Weinberg, 2000).

A third capability is the evasion of programmed cell death, or apoptosis. Apoptosis serves as a key protective mechanism in normal tissues, eliminating cells that have sustained irreparable damage or that carry potentially oncogenic alterations. Tumor cells frequently develop resistance to this process through a variety of strategies. One of the most common involves the mutational inactivation of the TP53 tumor suppressor gene, which encodes p53 protein and plays a central role in detecting DNA damage and initiating the apoptotic response. Loss of p53 function allows damaged cells to survive and continue dividing, thereby accumulating further mutations over time (Hanahan and Weinberg, 2000).

Tumor cells also acquire limitless replicative potential, primarily through reactivation of telomerase, allowing indefinite cell division beyond the limits imposed by telomere shortening. To sustain growth and require oxygen and nutrients, tumors activate a process known as angiogenesis, the formation of new blood vessels from pre-existing ones. Under normal conditions, angiogenesis is tightly regulated and occurs only transiently in specific physiological contexts. In tumors, angiogenic switch is activated, leading to the continuous formation of new vasculature. This neovascularization allows the tumor to maintain its growth and support further expansion (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

The sixth hallmark, tissue invasion and metastasis, represents one of the most clinically significant aspects of malignant disease and is responsible for the majority of cancer-related deaths. Primary tumor cells can acquire the ability to detach from the original tumor mass, invade the surrounding tissue, enter the bloodstream or lymphatic system, and ultimately colonize distant organs to form secondary tumors (Cree, 2011).

In addition to these six original hallmarks, two further capabilities have been identified and are now widely recognized. The first is reprogramming cellular metabolism. A characteristic feature of this reprogramming is aerobic glycolysis (referred as the Warburg effect), in which tumor cells preferentially convert glucose to lactate through glycolysis even in the presence of sufficient oxygen (Hanahan and Weinberg, 2011). The second evasion of immune destruction, whereby tumors suppress anti-tumor immune responses through multiple mechanisms (Cree, 2011).

Taken together, these hallmarks, enabling characteristics of genomic instability and tumor-promoting inflammation, offer a comprehensive framework for understanding the biological basis of cancer. It is worth noting that these capabilities are not acquired in a fixed order, nor through identical molecular mechanisms in every tumor type (Hanahan and Weinberg, 2011; Hanahan, 2022).

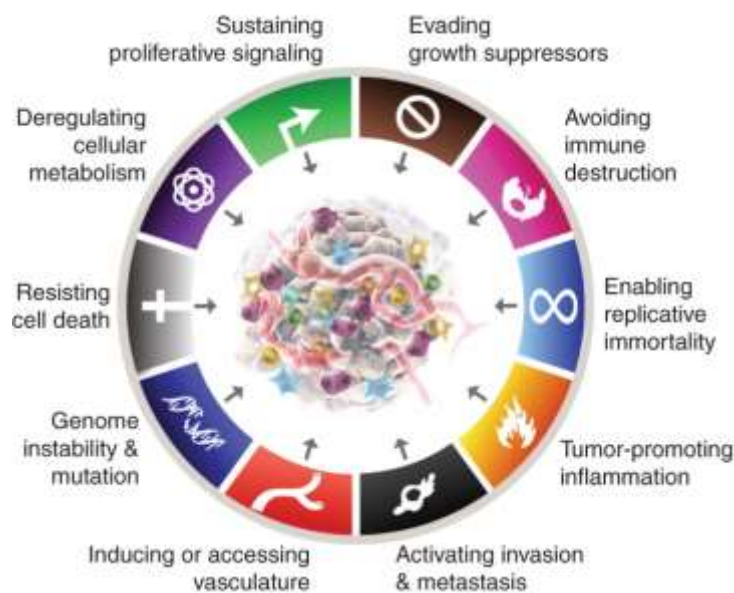


Figure 7. Hallmarks of Cancer. The diagram depicts eight essential cancer characteristics, enabled by two underlying features that drive tumor development (Hallmarks of Cancer: New Dimensions, Douglas Hanahan, 2022).

3.2 The Tumor Microenvironment

The description of tumor growth extends beyond the tumor cells themselves. As a tumor develops, it shapes and is shaped by the surrounding tissue environment, which undergoes progressive remodeling to accommodate and support malignant growth. This environment, referred to as the tumor microenvironment (TME), encompasses all non-malignant components

associated with the tumor mass, including immune cells, fibroblasts, endothelial cells, lymphocytes, the ECM, and a broad range of soluble signaling molecules. The TME is not a static passive compartment but a highly dynamic and evolving system in which continuous bidirectional communication occurs between tumor cells and surrounding stromal elements. While the composition of the TME can vary considerably between tumor types and even between individual patients, certain structural and functional features are shared across malignancies and have been shown to consistently support tumor progression (Arneth, 2020; Hanahan and Weinberg, 2011). An important cellular component associated with the TME is the population of cancer stem cells (CSCs). This is a subpopulation of tumor cells endowed with the capacity for self-renewal and the ability to give rise to the heterogeneous cell types that constitute the bulk of the tumor. CSCs are thought to play a central role not only in tumor initiation and growth but also in resistance to conventional therapies such as chemotherapy and radiotherapy, as they are often less sensitive to treatments that target rapidly dividing cells. Furthermore, CSCs have been implicated in disease recurrence and metastatic dissemination, making them subjects of considerable interest in both basic and translational cancer research (Arneth, 2020).

Among the stromal components of the TME, endothelial cells occupy a particularly important position due to their role in tumor vascularization, through angiogenesis and the secretion of pro-angiogenic factors, vascular endothelial growth factor (VEGF). Endothelial cells respond to these signals by proliferating and forming new blood vessels that penetrate the tumor mass, providing oxygen and nutrients while also offering a route for tumor cell intravasation and eventual metastatic spread. Notably, tumor-associated vasculature is structurally abnormal compared to normal vessels, often characterized by irregular architecture, increased permeability, and poor perfusion, which contributes to the development of hypoxic and acidic regions within the tumor (Arneth, 2020; Hui and Chen, 2015).

Immune cells represent another major cellular component of the TME, and their role in tumor biology is complex and context dependent. Although the immune system is capable of recognizing and eliminating malignant cells, tumors are frequently able to subvert immune responses to their own advantage. Lymphocytes, granulocytes, and macrophages are commonly found within the TME, where they participate in inflammatory processes that can paradoxically support tumor survival and progression rather than suppress it (Arneth, 2020; Anderson and Simon, 2020).

Cancer-associated fibroblasts (CAFs) represent another stromal cell population that contributes significantly to tumor progression. In normal tissue, fibroblasts are responsible for maintaining structural integrity and coordinating wound healing responses. Within the TME, however, fibroblasts become activated and reprogrammed, acquiring a phenotype that actively supports malignant growth (Arneth, 2020; Anderson and Simon, 2020).

The ECM constitutes the structural scaffold of the TME and exerts a profound influence on tumor cell behavior. In its normal configuration, the ECM is composed of collagens, glycoproteins, and proteoglycans that provide mechanical support and regulate cellular processes such as adhesion, migration, and differentiation. During tumor progression, however, the composition and physical properties of the ECM are substantially altered, largely as a result of enzymatic remodeling by MMPs and other ECM-degrading enzymes secreted by both tumor cells and stromal components. These changes reduce physical barriers to invasion, expose bioactive matrix fragments that promote cell proliferation and survival, and create permissive conditions for tumor cell migration toward blood and lymphatic vessels. The remodeled ECM thus actively contributes to the invasive and metastatic potential of the tumor (Arneth, 2020).

Overall, the TME represents a complex and highly integrated system in which tumor cells and surrounding stromal elements engage in continuous reciprocal interactions. The cellular and non-cellular components of the TME actively participate in shaping the course of tumor development, from early growth to systemic dissemination. Understanding the composition and functional dynamics of the TME is therefore of considerable relevance for the development of therapeutic strategies (Arneth, 2020; Hanahan and Weinberg, 2011).

3.3 Tumor Metastasis

As mentioned in the context of the hallmarks of cancer, the ability to invade surrounding tissues and spread to distant organs represents one of the most clinically significant features of malignant tumors. This process, known as metastasis, is responsible for the vast majority of cancer-related deaths and remains one of the most challenging aspects of oncological disease to treat (Zeeshan and Mutahir, 2017). Metastasis is not a single event but rather a sequential and highly coordinated process referred to as the invasion-metastasis cascade. This cascade encompasses a series of distinct biological steps: local invasion of the tissue surrounding the primary tumor, entry into blood or lymphatic vessels through intravasation, survival and

transport within the circulation, exiting from vessels at distant sites through extravasation, and finally the establishment of new tumor colonies in secondary organs (Lambert *et al.*, 2017).

The acquisition of metastatic potential is closely linked to both genetic and epigenetic alterations within tumor cells. Among the most relevant genetic changes is the loss of function of the TP53 tumor suppressor gene, which acts as a key regulator not only of cell cycle progression and apoptosis but also of cell motility and invasive behavior. Loss of p53 function has been shown to impair normal cellular polarity, stimulate migratory responses, and enhance the capacity of tumor cells to degrade the ECM, collectively facilitating invasion and dissemination (Zeeshan and Mutahir, 2017). In parallel, epigenetic mechanisms such as histone modifications also contribute to metastatic progression by modulating the expression of genes involved in invasion and cellular plasticity.

A central molecular program underlying the early stages of the metastatic cascade is the epithelial-mesenchymal transition (EMT). EMT is a developmental process originally associated with embryogenesis and tissue remodeling, but it is frequently reactivated in cancer cells to facilitate dissemination. Through EMT, tumor cells of epithelial origin undergo extensive reprogramming: they lose cell-cell adhesion structures, disrupt apical-basal polarity, and acquire a mesenchymal phenotype characterized by increased migratory and invasive capacity. This transition is driven by specific transcription factors and can be triggered by a variety of signals originating from both tumor cells and the surrounding stroma. Importantly, EMT is a reversible process, and disseminated tumor cells may subsequently undergo the reverse transition, known as mesenchymal-epithelial transition (MET), which is thought to support the re-establishment of epithelial growth characteristics at metastatic sites (Diepenbruck and Christofori, 2016).

The initial step of local invasion involves the detachment of tumor cells from the primary mass and their migration into adjacent tissues, either as single cells or as cohesive cellular cohorts that maintain epithelial markers such as E-cadherin. To penetrate surrounding tissue, tumor cells and associated stromal cells secrete proteolytic enzymes including MMPs, which degrade ECM components and create permissive conditions for cellular movement. Invading cells subsequently enter the vasculature through intravasation into either blood vessels or lymphatics. Once in the bloodstream, circulating tumor cells (CTCs) face considerable challenges including mechanical shear forces, hydrodynamic flow, and immune-mediated attack. Interactions with blood-borne cellular components such as platelets, monocytes, and endothelial cells can

however modulate CTC survival and support their eventual extravasation toward distant tissues (Lambert *et al.*, 2017).

CTCs that survive transit through circulation eventually arrest within the narrow microvessels of distant organs. At this point, tumor cells cross the endothelial barrier through trans endothelial migration, a process facilitated by tumor-derived factors including VEGF, which promotes vascular permeability and remodeling. Once disseminated tumor cells enter the parenchyma of a secondary organ, their fate varies considerably. Many are eliminated by local immune defenses or fail to adapt to the new microenvironment and enter a state of dormancy, persisting as single cells or small clusters for variable periods of time. Only a fraction of disseminated cells successfully proliferates and eventually form macroscopic metastatic lesions. The period between initial seeding and the emergence of overt metastatic disease is referred to as metastatic latency and can range from months to years depending on tumor cell properties and the characteristics of the secondary site (Lambert *et al.*, 2017; Zeeshan and Mutahir, 2017).

3.4 Glioblastoma multiforme (GBM)

Glioblastoma multiforme (GBM) is defined by the World Health Organization (WHO) as a grade IV astrocytic glioma, representing the most advanced and malignant form within the diffuse glioma spectrum. GBM originates from neuroglial stem cells or progenitor cells, a population that plays a supportive role in the central nervous system and is collectively known as glial cells. This cellular origin is reflected in the tumor's histopathological features, which exhibit strong astrocytic characteristics and highlight its close resemblance to astrocyte-like cells. Together, these features, along with their classification as the highest-grade glioma, emphasize the highly aggressive behavior and biological complexity of GBM (Wen *et al.*, 2020; Jo *et al.*, 2023).

Based on the 2016 WHO classification of Central Nervous System (CNS) tumors, GBM is divided into three subtypes according to the mutational status of the isocitrate dehydrogenase (IDH) gene. The most prevalent subtype is IDH-wildtype GBM, accounting for approximately 90% of cases. This form develops *de novo*, without an identifiable precursor lesion, and is more commonly diagnosed in older patients, typically above 55 years of age, following a more aggressive clinical course. IDH-mutant GBM represents approximately 10% of cases and is considered a secondary form, arising from the progression of a pre-existing lower-grade glioma.

It predominantly affects younger patients, with a median age at diagnosis of 44 years, and is generally associated with a relatively better prognosis. The third category, GBM not otherwise specified (NOS), is assigned when IDH mutation status cannot be determined due to incomplete molecular testing (Louis *et al.*, 2016).

The prognosis remains extremely poor regardless of subtype. The five-year survival rate is approximately 6.8%, and a median overall survival of only 12 to 15 months despite multimodal treatment, underscoring the urgent need for more effective therapeutic strategies (Wen *et al.*, 2020).

GBM exhibits distinct genetic alterations that differ depending on whether the tumor arises as a primary or secondary form. Understanding these differences is important, as they reflect the distinct biological pathways through which each subtype develops.

In primary GBM (IDH-wildtype), several key genetic alterations have been identified. TERT promoter mutation is among the most frequently observed changes and is considered a molecular hallmark of this subtype. Amplification of the epidermal growth factor receptor (EGFR) gene is another common alteration, which leads to excessive activation of downstream proliferative signaling and contributes to the aggressive growth behavior characteristic of primary GBM. Additionally, deletion of the PTEN tumor suppressor gene is frequently observed, resulting in loss of its regulatory function and further promoting uncontrolled tumor cell proliferation and survival (Lan *et al.*, 2024; Jo *et al.*, 2023).

In secondary GBM (IDH-mutant), the genetic landscape differs considerably. Mutations in the TP53 tumor suppressor gene, which as previously discussed play a central role in cell cycle regulation and apoptosis, are frequently detected in this subtype. Mutations in the ATRX gene are also commonly observed in secondary GBM, where they are thought to contribute to genomic instability and alterations in chromatin remodeling, thereby facilitating tumor progression (Lan *et al.*, 2024).

Epigenetic modifications, including DNA methylation, histone modification, and chromatin remodeling, represent another important layer of molecular dysregulation in GBM. These alterations contribute to tumorigenesis by disrupting key biological processes such as cell cycle regulation, cellular differentiation, angiogenesis, and apoptosis. Due to their reversible nature, epigenetic changes have gained attention both as potential biomarkers for patient stratification and as promising therapeutic targets, with epigenetic modulators currently being investigated in clinical trials (Lan *et al.*, 2024; Jo *et al.*, 2023).

Transcriptomic profiling has identified three main molecular subtypes of GBM: proneural, classical, and mesenchymal. These subtypes differ in their gene expression patterns and associated molecular alterations, although tumors frequently exhibit a mixture of subtypes, limiting the clinical utility of this classification (Jo *et al.*, 2023).

Three key signaling pathways are consistently altered in GBM: the receptor tyrosine kinase (RTK) pathway, which regulates cell growth and proliferation through surface receptor signaling, the TP53 pathway, which governs cell cycle control and apoptosis, and the retinoblastoma (RB) pathway, which acts as a critical regulator of cell cycle progression. These pathways frequently interact with one another and collectively drive tumor proliferation, survival, and progression (Lan *et al.*, 2024).

A defining feature of GBM is its remarkable intratumoral heterogeneity. The tumor is composed of multiple distinct cellular subpopulations that differ in their genetic makeup and behavior, contributing significantly to treatment resistance and tumor recurrence (Wen *et al.*, 2020).

GBM typically presents with a range of neurological symptoms, including headaches, focal neurological deficits, and alterations in mental status, reflecting the rapid growth and infiltrative nature of the tumor. Diagnosis is primarily established through contrast-enhanced MRI, which allows visualization of the characteristics enhancing necrotic mass surrounded by edema and infiltrative tumor regions, with advanced MRI techniques further supporting evaluation of the tumor's metabolic and physiological properties (Wen *et al.*, 2020).

The current standard of care consists of surgical resection followed by radiotherapy and chemotherapy with temozolomide. However, these treatment modalities are not curative, and tumor recurrence is almost inevitable. This is largely attributed to the significant heterogeneity of GBM, which drives resistance to therapy, as well as the presence of the BBB, which significantly limits the delivery of therapeutic agents to the tumor. Consequently, despite multimodal treatment approaches, GBM remains one of the most therapeutically challenging tumors in oncology, with no significant improvement in patient outcomes over the past decades (Jo *et al.*, 2023; Lan *et al.*, 2024).

4. Roles of Platelets and PEVs in Tumor Biology

4.1 The interaction between platelets and cancer cells

The functional interaction of platelets with cancer cells constitutes an important, yet poorly understood process in tumor biology. Far from being passive bystanders of the hemostatic system, platelets engage in a dynamic, bidirectional dialogue with tumor cells that begin the moment malignant cells enter the circulation and persist throughout every subsequent step of the metastatic cascade. This crosstalk is both contact-dependent and contact-independent, and its consequences, such as tumor cell survival, immune escape, EMT, angiogenesis, extravasation, and the establishment of pre-metastatic niches, collectively amplify the metastatic potential of CTCs (figure 8) (Li *et al.*, 2024; Josefsson, 2025).

An early defining event in platelet-tumor interaction is tumor cell-induced platelet aggregation (TCIPA), the rapid formation of platelet microaggregates around a CTC within minutes of its entry into the bloodstream. In experimental metastatic tumor models, microaggregates are detectable as early as one minute after intravenous injection of tumor cells, and this process has been shown to be mechanistically essential for successful hematogenous metastasis (Li *et al.*, 2024).

TCIPA is initiated through multiple redundant signaling pathways. Tumor cells can release classic platelet agonists such as ADP and TxA₂, which engage P₂Y and TxA₂ receptors on the platelet surface, triggering inside-out activation of integrin α IIb β 3 and granule degranulation. In parallel, tumor cell surface expression of TF and PS initiates thrombin generation via the extrinsic coagulation cascade; thrombin then provides a potent secondary stimulus through PAR1/PAR4 (Josefsson, 2025; Li *et al.*, 2024, Zara *et al.*, 2018).

Beyond classical agonists, several tumor-specific surface ligands engage dedicated platelet immunoreceptors. The lectin podoplanin, upregulated on the surface of glioma, brain, and ovarian cancer cells, binds C-type lectin-like receptor 2 (CLEC-2) on platelets, initiating Syk-dependent signaling and integrin α IIb β 3 activation (Josefsson, 2025; Li *et al.*, 2024).

Once activated, platelets expose P-selectin on their surface and shift integrin α IIb β 3 to its high-affinity conformation, enabling fibrinogen, fibronectin, and vWF to serve as molecular bridges between platelet integrin α IIb β 3 and tumor cell integrin α v β 3. P-selectin additionally binds tumor cell ligands including CD44, CD24, PSGL-1, stabilizing the platelet coat around the CTC. The resulting microaggregate is further consolidated by thrombin-generated fibrin networks that anchor platelets firmly to the tumor cell surface (Josefsson, 2025; Li *et al.*, 2024).

The platelet coat assembled via TCIPA confers upon the CTC a multi-layered survival advantage. At the physical level, the microaggregate shields the tumor cell from the hemodynamic shear forces of the bloodstream, which would otherwise induce lethal membrane damage. It has been estimated that fewer than 0.01% of tumor cells survive transit through circulation, and platelet adherence constitutes a primary determinant of this minority's survival (Josefsson, 2025).

Immune evasion through platelet coating operates at both physical and molecular levels. Physically, the platelet envelope occludes the tumor cell surface from recognition by cytotoxic NK cells, which constitute the principal immunological barrier to hematogenous metastasis. Molecularly, platelet-derived TGF- β downregulates NKG2D expression on NK cells, reducing their activating receptor density and cytotoxic capacity, while platelet-derived PDGF suppresses T-cell function (Josefsson, 2025; Li *et al.*, 2024).

Platelets also interfere with the adaptive immune response. Platelet surface P-selectin directly impairs T-cell receptor (TCR)-triggered T-cell activation and proliferation (Li *et al.*, 2024).

Furthermore, tumor cells can actively acquire platelet-derived lipids, nucleic acids, and surface proteins through direct phagocytosis or membrane fusion, a process described as 'platelet mimicry'. This uptake of platelet material endows tumor cells with platelet-specific surface biomarkers and anti-apoptotic signals, enhancing their stemness and proliferative capacity while further camouflage them from immune effectors (Li *et al.*, 2024; Tian *et al.*, 2025). A particularly elegant mechanism of immune mimicry involves the transfer of major histocompatibility complex class I (MHC-I) molecules from the platelet membrane to the tumor cell surface. By presenting these self-molecules, tumor cells are made indistinguishable from normal host cells, actively suppressing NK cell-mediated elimination (Josefsson, 2025; Li *et al.*, 2024).

Platelets do not merely protect tumor cells passively; they actively reprogram their phenotype towards greater invasiveness through the induction of EMT. This process, a hallmark of tumor cell metastasis, involves the loss of epithelial polarity and gain of migratory mesenchymal characteristics, and is critically dependent on sustained platelet-derived signals (Li *et al.*, 2024). Platelets reinforce EMT through multiple convergent signals beyond TGF- β , including integrin $\alpha 2 \beta 1$ -driven Wnt- β -catenin activation and PEVs-mediated transfer of 12-lipoxygenase, collectively programming tumor cells toward a pro-invasive mesenchymal phenotype (Li *et al.*, 2024).

Following survival in the systemic circulation, successful metastasis requires CTC arrest within blood vessels of a distant organ, adhesion to the endothelium, and subsequent extravasation into the tissue parenchyma. Platelets facilitate each of these steps through distinct mechanisms (Li *et al.*, 2024; Josefsson, 2025). CTC arrest at the vascular endothelium is promoted through platelet-mediated bridging, whereby the interaction between platelet GPIb-IX-V receptors and vWF displayed on activated endothelial cells anchors the platelet-CTC complex to the vessel wall. P-selectin on platelets within microaggregates additionally binds mucin-type glycoproteins on endothelial cells, facilitating rolling and firm adhesion of CTCs. Platelet activation also results in surface transfer of integrin $\beta 3$ (CD61) to the tumor cell membrane, directly increasing tumor cell adhesiveness to the endothelium (Li *et al.*, 2024).

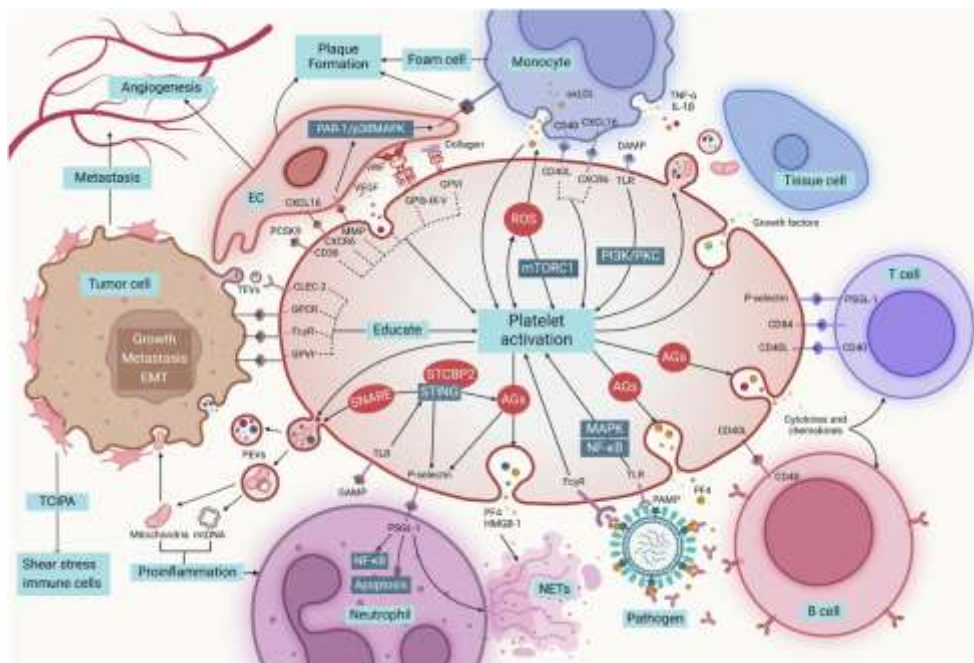


Figure 8. Role of platelets in pathological conditions. This image summarizes some of the major involvement of platelets in physiological and pathological conditions including cancer. The most relevant molecular details of the platelet-cancer cell interaction were discussed in this chapter (Tian *et al.*, 2025).

4.2 The role of PEVs in tumor

Building on the direct interactions between platelets and tumor cells described in the previous section, PEVs represent an additional key mechanism by which platelets extend their influence beyond direct cell-cell contact. PEVs function as an intracellular communication system with the ability to access tissue compartments that are largely inaccessible to intact platelets, including lymphatic system, bone marrow, and solid tumors. In cancer patients, elevated levels

of PEVs have been reported across multiple malignancies, including colorectal carcinoma, multiple myeloma, breast cancer, and pancreatic adenocarcinoma, where they directly contribute to the cancer-associated hypercoagulable state. Through their ability to transfer bioactive cargo and interact with diverse cell types, PEVs act as important mediators of tumor initiation, progression, metastasis, and immune evasion (Puhm *et al.*, 2021; György *et al.*, 2011).

One of the most paradigm-shifting observations in PEVs biology was that, while platelets are essentially absent within solid tumors, PEVs actively infiltrate tumor tissue where they transfer molecular cargo that reprograms tumor cell behavior. Upon infiltration, PEVs can promote apoptosis of tumor cells by delivering specific microRNAs, providing the first *in vivo* evidence that PEVs can function as endogenous tumor suppressors fundamentally challenging the exclusively pro-tumorigenic view of these vesicles (Puhm *et al.*, 2021). Conversely, PEVs also transfer microRNAs to breast cancer cells in complex with the RNA-induced silencing machinery, directly modifying recipient cell proliferative and invasive behavior. Beyond RNA, PEVs carry functional mitochondria that, upon delivery to recipient cancer cells, reprogram their bioenergetics and confer energetic advantages or contribute to chemoresistance (Puhm *et al.*, 2021). PEVs additionally transport transcription factors and mRNAs capable of regulating transcription, RNA stability, translation and metabolism in target cells. Internalization by macrophages and fibroblasts activates interleukin-1 β -mediated inflammatory cascades, and lipid mediators carried by PEVs further expand the repertoire of inflammatory signals deliverable to stromal and immune cells within the tumor microenvironment (Puhm *et al.*, 2021; György *et al.*, 2011).

In the tumor microenvironment, PEVs are also potent stimulators of angiogenesis. They harbor pro-angiogenic factors, including VEGF, FGF-2, and the lipid mediator sphingosine-1-phosphate, which act synergistically on endothelial cells to promote proliferation, tube formation, and vascular remodeling. Both protein and lipid growth factor fractions of PEVs cargo are required for full endothelial activation (Brill *et al.*, 2005; Kim *et al.*, 2004). Notably, PEV internalization by endothelial cells drives transcriptional upregulation of MMP-2 and MMP-9, enzymes absent from the vesicles themselves, illustrating an indirect mode of signaling through vesicle-delivered cues that remodels the ECM to facilitate tumor invasion and neovascularization (Sun *et al.*, 2017). PEVs support endothelial cell proliferation, survival, migration, and tube formation *in vitro* (Brill *et al.*, 2005). Through their highly adhesive surface,

bearing integrin $\alpha\text{IIb}\beta 3$ and GPIb, PEVs bind tumor-associated endothelial cells, leukocytes, and ECM molecules, and can transfer these surface glycoproteins to neutrophils, broadening the pro-tumorigenic stromal compartment (Lo *et al.*, 2006; Salanova *et al.*, 2007). At the systemic level, during states of inflammation and cancer-associated platelet activation, PEVs leave the circulation and penetrate the bone marrow, rapidly binding to MKs and their CD41⁺ progenitor cells and functionally reprogramming MK differentiation (French *et al.*, 2020). This represents a unique feedback mechanism by which signals from the peripheral tumor milieu are relayed back to the hematopoietic niche, potentially driving cancer-associated thrombocytosis and generating phenotypically altered PEVs populations that further support tumor progression. The organotropism of circulating EVs, governed by the integrin repertoire on their surface, enables them to preferentially engage specific organ-resident cells, conditioning pre-metastatic niches at distant sites by remodeling ECM and activating stromal cells prior to the arrival of circulating tumor cells (Hoshino *et al.*, 2015).

In addition to the mechanisms described above, PEVs-mediated signaling is closely associated with intracellular Ca^{2+} dynamics that contribute to tumor cell activation and migration. Experimental studies have further demonstrated that PEVs generated following thrombin-induced platelet activation can elevate intracellular Ca^{2+} levels in tumor cells through PLC activation and Ca^{2+} influx. This Ca^{2+} dependent signaling contributes to the activation of migration-related proteins and supports a self-reinforcing feedback loop in which tumor cells promote platelet activation and, in turn, receive signals that enhance their own metastatic potential (Zarà *et al.*, 2017; Vismara *et al.*, 2021).

The accessibility of PEVs in peripheral blood and lymph, combined with the fidelity with which their cargo reflects the originating pathological state, positions them as promising liquid biopsy candidates. Flow-cytometry-based quantification of CD41⁺/CD62P⁺ PEVs is feasible from minimally processed plasma, and PEVs levels, surface phenotypes, and miRNA profiles correlate with tumor stage, metastatic burden, and treatment response (Flaumenhaft *et al.*, 2009; Helley *et al.*, 2009).

Beyond their diagnostic potential, PEVs have also been explored as therapeutic agents and drug delivery vehicles. However, the immunological heterogeneity of PEVs cargo, including TF, immunoregulatory molecules, cytokines, and pro-coagulant phospholipids, raises legitimate concerns about coagulation risk and off-target immune activation upon systemic administration (Boilard *et al.*, 2010). Moreover, because the pro- and anti-tumorigenic, pro- and anti-

coagulant, and pro- and anti-inflammatory effects of PEVs are each determined by the specific agonist driving their generation and the resulting vesicle subtype, achieving therapeutic precision demands rigorous subtype separation and thorough characterization before any clinical deployment (Milioli *et al.*, 2015; Puhm *et al.*, 2021). Collectively, these findings underscore the remarkable functional versatility of PEVs and their broad significance across multiple stages of cancer biology (Shahi *et al.*, 2024; Puhm *et al.*, 2021).

AIM OF THE WORK

Glioblastoma multiforme (GBM), classified as WHO grade IV astrocytic glioma, is the most aggressive primary brain tumor in adults. Despite maximal treatment, surgical resection, radiotherapy, and temozolomide chemotherapy, median overall survival remains 12 to 15 months, with a five-year survival rate below 7% (Wen *et al.*, 2020; Jo *et al.*, 2023). Profound intratumoral heterogeneity, therapy resistance, and BBB-restricted drug delivery collectively account for this dismal prognosis, rendering GBM one of the most formidable challenges in modern oncology (Jo *et al.*, 2023; Lan *et al.*, 2024).

Beyond hemostasis, platelets are active modulators of tumor biology, supporting cancer cell survival, immune evasion, and metastasis through direct interaction with tumor cells and the release of bioactive mediators (Li *et al.*, 2024; Josefsson, 2025). Among these, platelet-derived extracellular vesicles (PEVs) have emerged as key intercellular communicators, with documented pro-tumorigenic roles across several malignancies including colorectal, breast, and pancreatic carcinoma (Puhm *et al.*, 2021; György *et al.*, 2011). Despite this growing evidence, the interplay between platelets and GBM cells remains entirely uninvestigated, representing a significant gap in our understanding of GBM pathobiology.

This work aims to provide a first, preliminary characterization of the platelet-GBM interaction, using U87MG cells as an established in vitro GBM model (Li *et al.*, 2017) and platelets isolated from healthy donor buffy coats. Three specific objectives were addressed:

1. To investigate the capacity of GBM cells to induce platelet activation, assessed through platelet aggregation as a functional readout (Li *et al.*, 2024; Zarà *et al.*, 2018).
2. To determine whether GBM-induced platelet activation is accompanied by PEVs release and to characterize the resulting vesicle population in comparison with thrombin-induced PEVs, used here as a reference condition (Fan *et al.*, 2024; Vismara *et al.*, 2022).
3. To provide an initial assessment of the functional effects of PEVs on GBM cells, exploring whether platelet-derived vesicles modulate cancer cell behavior upon interaction (Zarà *et al.*, 2017; Vismara *et al.*, 2022).

MATERIALS AND METHODS

1. Materials and solutions

Human glioblastoma multiforme cells U87MG were used in this study. U87MG cell line (purchased from ATCC), is characterized by an epithelial pattern and a high capacity of migration and invasion, derived from a male patient affected by malignant glioma, likely glioblastoma (Li *et al.*, 2017; <https://www.atcc.org/products/htb-14>). Fetal Bovine Serum (FBS), Dulbecco Modified Eagle's Medium (DMEM), 200 mM L-Glutamine, Penicillin-Streptomycin solution 100X, Dulbecco's Phosphate Buffered Saline (D-PBS), Trypsin-EDTA in PBS, and BCA protein assay kit were from EuroClone. Ethylene Diamine Tetraacetic Acid (EDTA) was from BDH. Trypan blue solution 0,4%, Apyrase grade I, Paraformaldehyde (PFA), Accutase solution, RGDS, Thrombin, 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE), Hoechst-33342, and ECL kit were from Sigma-Aldrich. Sodium Dodecyl Sulfate (SDS) and Bovine Serum Albumin (BSA) were from Serva. ProLong gold antifade was from Thermofisher. Prostaglandin-E1 (PGE1) was from Santa Cruz Biotechnology. U73122 was from Alexis Biochemicals.

The following primary antibodies were used in this study: anti-GAPDH (Santa Cruz Biotechnology, 1:1000), anti-P-selectin (cell signalling, 1:1000), anti-integrin α Ib (Santa Cruz, 1:1000), anti-TSG-101 (novus biologicals, 1:1000), anti-CD81 (novus biologicals, 1:1000), anti-CD9 (novus biologicals, 1:1000), anti-CD63 (cell signalling, 1:1000), anti- β -actin (Merck 1:5000).

In addition, below are reported the solutions that will be mentioned in the following protocols:

- Tyrode's buffer: 10 mM HEPES, 137 mM NaCl, 2,9 mM KCl, 12 mM NaHCO₃ (pH 7.4)
- ACD: 152 mM sodium citrate, 130 mM citric acid, 112 mM glucose
- PIPES buffer: 20 mM PIPES, 136 mM NaCl (pH 6.5)
- PBS: 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 140 mM NaCl (pH 7.4)
- Lysis buffer: 50 mM Tris, 150 mM NaCl, 0,1% SDS, 1 mM EDTA, 0,25% deoxycholic acid, 1% Nonidet P-40, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF, 2 mM NaF, 1 mM Na₃VO₄ (pH 7.4)
- Dissociation buffer: 6% SDS, 1,5% dithiothreitol (DTT), 0.03% bromophenol blue, 30% glycerol, 3% β -mercaptoethanol diluted in electrophoresis buffer
- Electrophoresis buffer: 25 mM Tris/HCl, 192 mM glycine, 0,1% SDS (pH 8.3)

- Blotting buffer: 20 mM Tris Base, 150 mM glycine, 20% methanol
- Immunoblotting Washing buffer: PBS-T: PBS + 0,1% Tween 20

2. Cell culture

The GBM cell line U87MG was cryopreserved in liquid nitrogen (-196°C) in fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO). After thawing, cells were resuspended in complete culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. The cell suspension was centrifuged at $120\times g$ for 10 minutes, the supernatant was discarded, and the cell pellet was resuspended in 10 mL of complete medium. The cells were then seeded in 100 mm Petri dishes and maintained at 37°C in a humidified incubator with 5% CO_2 .

To prevent over confluence, cell cultures were periodically diluted. For this purpose, the culture medium was removed, and the cells were washed with 10 mL of D-PBS. Subsequently, 1 mL of trypsin-EDTA was added, and the cells were incubated at 37°C for 5 minutes to facilitate detachment. The detached cells were resuspended by adding 9 mL of complete medium. Based on the growth characteristics of the cell line, an appropriate dilution was performed, and a portion of the cell suspension was seeded into new Petri dishes containing fresh complete medium, reaching a final volume of 10 mL.

For experimental use, cells were detached using Accutase instead of trypsin-EDTA, as it is gentler and better preserves cell surface proteins and cell viability. Briefly, the medium was removed, cells were washed with 10 mL of D-PBS, and 1 mL of Accutase was added. After incubation at 37°C for 5 minutes, the detached cells were resuspended in complete medium. Cell counting was then performed using the trypan blue exclusion method. Cell suspensions were mixed 1:1 with 0.4% trypan blue solution and loaded onto a hemocytometer. Viable and non-viable cells were counted to determine cell concentration.

3. Purification of human platelets

Human platelets were isolated from buffy coat preparations obtained from healthy donors. All procedures were carried out under sterile conditions using a laminar flow hood. The buffy coat was diluted by adding one-third volume of a 1:10 mixture of acid citrate dextrose (ACD) and Tyrode's buffer. The diluted sample was divided into 5 mL aliquots and centrifuged at $180 \times g$

for 15 minutes at room temperature to separate the platelet-rich supernatant from leukocytes and erythrocytes.

From each aliquot, platelet-rich supernatant was collected and transferred into new tubes. Prostaglandin E1 (PGE1, 1 μ M) and apyrase (0.2 U/mL) were added to prevent platelet activation and desensitization of ADP receptors. The samples were then centrifuged at 750 $\times$ g for 15 minutes at room temperature to pellet the platelets.

The supernatant was discarded, and the platelet pellets were resuspended and washed with 6 mL of PIPES buffer to remove residual plasma components. A second centrifugation at 750 $\times$ g for 15 minutes was performed, after which the platelets were resuspended in Tyrode's buffer.

Platelet concentration was determined using a Bürker chamber, and the suspension was adjusted to a final concentration of 3×10^8 platelets/mL in Tyrode's buffer supplemented with 1 mM CaCl_2 , 0.5 mM MgCl_2 , and 5.5 mM glucose. The platelets were allowed to rest at 37 °C for 30 minutes prior to use. For the analysis of PEVs interaction with cancer cells, platelets were labeled by incubation with 3 μ g/mL CFSE for 15 minutes at the concentration of 10^9 platelets/mL and then diluted to the concentration aforementioned.

4. Tumor Cell-Induced Platelet Aggregation

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 0.3×10^9 platelets/mL) left untreated or preincubated with different inhibitors, were incubated with cancer cells (5×10^4 - 5×10^5 cells/mL) in the presence of 0.05% v/v of platelet-free plasma. The extent of platelet aggregation was continuously monitored for 30 min.

5. Isolation of PEVs

Platelet activation for PEVs release was induced under two conditions: by stimulating platelet suspensions (3×10^8 platelets/mL in Tyrode's buffer) with 0.2 U/mL thrombin for 30 minutes at 37 °C under constant stirring, or by co-incubation with U87MG glioblastoma cells (1×10^5 cells/mL) in the presence of 0.05% v/v of platelet-free plasma for 30 minutes at 37 °C under constant stirring, following the TCIPA conditions described above.

Following stimulation, 10 mM EDTA was added to stop aggregation and promote recovery of free PEVs. The samples were centrifuged at 750 $\times$ g for 20 minutes at room temperature to pellet activated platelets, and the supernatant containing PEVs was collected. The supernatant was

divided into 2 mL aliquots and centrifuged at $18,500\times g$ for 90 minutes at 10 °C to pellet PEVs. At this speed, mostly medium-large PEVs are expected to be collected. After removal of the supernatant, the PEVs pellets were resuspended in Tyrode's buffer. The quantification of PEVs was performed by Nanoparticle Tracking Analysis (protocol 6) and their protein content was determined by BCA protein assay (protocol 8) upon addition of 1/5 volume of 12% SDS prepared in Tyrode's buffer and boiled for 3 minutes at 96°C.

6. Nanoparticle Tracking Analysis (NTA)

Purified PEVs were analyzed by Nanoparticle Tracking Analysis (NTA) using a NanoSight NS300 (Malvern Panalytical) to determine their concentration and dimension. For this analysis each sample of PEVs was resuspended with Tyrode and five videos of 60 seconds were recorded and analyzed using NTA software (version 3.4; NanoSight Ltd.). The NTA was carried out by Dr. Marta Zarà in collaboration with the Unit of Brain-Heart Axis: Cellular and Molecular Mechanism, Centro Cardiologico Monzino IRCCS, Milan.

7. Cell incubation with PEVs and cell lysis

GBM cells were seeded at a density of $2,5\times 10^5$ cells/mL in 6-well plates and cultured for 24 hours at 37 °C. Cells were then either left untreated or treated with different concentrations of purified PEVs and incubated for an additional 24 hours.

Following incubation, the culture medium was removed, and the cells were washed twice with D-PBS. Cells were lysed by adding 200 μ L of ice-cold RIPA buffer. The lysates were collected using cell scrapers, transferred to microcentrifuge tubes, vortexed, and incubated under gentle agitation for 60 minutes at 4 °C to ensure complete lysis. Samples were centrifuged at $13,000\times g$ for 10 minutes at 4 °C to remove cellular debris, and the supernatant was collected. Samples were then analyzed by BCA assay and immunoblotting as described below.

8. BCA protein assay

Protein concentration was determined using the bicinchoninic acid (BCA) assay, a colorimetric method based on the reduction of Cu^{2+} to Cu^+ by proteins in an alkaline environment. The reduced Cu^+ ions subsequently form a complex with BCA, resulting in a purple-colored solution with a maximum absorbance at 562 nm, proportional to the protein concentration.

Samples were prepared in duplicate by mixing 5 μ L of lysate with 50 μ L of Milli-Q water. A calibration curve was generated using bovine serum albumin (BSA) standard. Blank samples were included for baseline correction.

Each sample was incubated with 1 mL of BCA working reagent, vortexed, and incubated at 37°C for 30 minutes in the dark. Absorbance was measured at 562 nm using a spectrophotometer, and protein concentration was calculated based on the standard calibration curve.

9. SDS-page and immunoblotting

For electrophoresis, samples were mixed with dissociation buffer at a ratio of 1:2 (v/v), heated at 96 °C for 5 minutes under stirring, and centrifuged at 13,000 $\times$ g for 30 seconds. Samples were then stored at -20 °C until use. Prior to loading, samples were thawed, heated at 96 °C for 5 minutes at 550 rpm, and briefly centrifuged.

Equal amounts of proteins were loaded onto polyacrylamide gels of appropriate acrylamide concentration and separated by SDS-PAGE at a constant voltage of 150 V. Protein were then transferred onto PVDF membranes, previously activated in methanol, by immunoblotting at a constant current of 200 mA for 2 hours. Membranes were blocked with 5% BSA in TBS for 1 hour at room temperature under gentle agitation to prevent non-specific binding. Membranes were incubated overnight at 4 °C with primary antibodies diluted in TBS containing 5% BSA, 0.1% Tween-20, and 0.02% NaN₃. The following day, membranes were washed six times (5 minutes each) with PBS-T.

Subsequently, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 1 hour at room temperature under gentle agitation. After washing, protein bands were visualized using an enhanced chemiluminescence (ECL) detection system and imaged using a Bio-Rad ChemiDoc XRS system. Band intensities were quantified by densitometric analysis using Quantity One software (version 4.6.8).

10. Fluorescence microscopy analysis of PEVs interaction with GBM cells

The analysis of interaction of GBM cells with PEVs was conducted by culturing cells in complete medium. A 12-well plate was set up for each cell line positioning in each well a glass coverslip previously washed with 90% ethanol. Cells were seeded on glass coverslip into the multi-well plates at a concentration of 2.5×10^5 cell/mL by diluting them with complete medium

and let grow for 24 hours in incubation at 37 °C. Cell samples were then either left untreated or treated with 30 µg/ml PEVs released from platelets previously labelled with CFSE, in order to obtain fluorescently-labelled PEVs. Cell samples were then incubated with PEVs for 18 hours at 37 °C. Upon incubation, the culture medium was removed, and the wells were washed twice with 1 ml of D-PBS. Cells were then fixed with 1 ml of 3% PFA in PBS for 10 minutes. Two additional washes with 1 ml of D-PBS were performed and then the nuclei were stained by incubation with 1 ml/well of 1 µg/ml Hoechst-33342 in PBS for 10 minutes. After two additional washes with 1 ml of D-PBS, the coverslips were recovered, washed with MilliQ water to remove the residues of buffer and mounted onto glass slides, previously washed with ethanol, using Pro Long gold antifade mounting solution. Samples were then analyzed using an Olympus BX51 microscope. Fields acquired for the different GBM cell lines were analyzed quantify in the percentages of cells with CFSE-labelled PEVs.

11. Flow cytometry analysis of PEVs interaction with GBM cells

The interaction between PEVs and GBM cells was analyzed by flow cytometry. Cells were seeded in 12-well plates at a density of $2,5 \times 10^5$ cells/mL in complete medium and cultured for 24 hours at 37 °C. Following incubation, cells were either left untreated or incubated with 30 µg/mL CFSE-labeled PEVs for 18 hours at 37 °C. After treatment, the culture medium was removed, and cells were washed with 1 mL of D-PBS. Cells were detached by incubation with 100 µL of accutase for 5 minutes at 37 °C. Subsequently, 900 µL of DMEM was added, and the cells were collected into 1.5 mL tubes. Cell suspensions were centrifuged at $700 \times g$ for 7 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 200 µL of D-PBS. Cells were then fixed with 100 µL of 3% PFA in PBS for 10 minutes. After fixing, D-PBS was added to reach a final volume of 0.5 mL. PEV-cell interaction was analyzed using a BD FACSLyric flow cytometer (BD Biosciences). CFSE fluorescence was detected using the blue laser (488 nm excitation).

12. Analysis of apoptosis

Apoptosis was assessed using Annexin V/propidium iodide staining. GBM cells were seeded in 12-well plates at a density of $2,5 \times 10^5$ cells/mL and cultured for 24 hours at 37 °C. Cells were then treated with PEVs for 48 hours, or with 1 mM H₂O₂ for 48 hours as positive control. Following treatment, the culture medium was removed, and cells were washed once with 1 mL

of D-PBS. Cells were detached using 100 μ L of accutase for 5 minutes at 37 °C, resuspended in DMEM to a final volume of 1 mL, and collected into 1.5 mL tubes.

Cells were centrifuged at 1000 $\times$ g for 10 minutes, and the pellet was resuspended in 100 μ L of Annexin V binding buffer containing 5 μ L of Annexin V-APC and 1 μ g/mL Propidium Iodide (PI). Samples were incubated in the dark for 30 minutes at room temperature. Finally, 300 μ L of Annexin V binding buffer was added, and samples were analyzed using a BD FACSLytic flow cytometer (BD Biosciences). Annexin V-APC fluorescence was detected using the red laser (642 nm), while PI fluorescence was detected using the blue laser (488nm).

13. Statistical analysis

Quantitative data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism software (version 5.01). Comparison between samples was carried out using unpaired t test. A p-value < 0.05 was considered statistically significant.

RESULTS

1. U87MG GBM cells can stimulate platelet aggregation

To investigate the physical and functional interaction between blood platelets and GBM cells, we performed an in vitro analysis using the U87MG cell line as a model. As an initial readout, we focused on tumor cell-induced platelet aggregation (TCIPA), which represents the major functional response triggered during platelet activation. Platelet aggregation was assessed by light transmission aggregometry (Born aggregometer), as described in the Methods section (Method 4).

Purified platelets (Method 3) were co-incubated with increasing concentrations of U87MG cells under constant stirring at 37 °C in the aggregometer. In Figure 9 the representative aggregation traces are reported. The x-axis reports the time of analysis (up to 30 minutes), while the y-axis shows the percentage of light transmission, which is proportional to the degree of platelet aggregation.

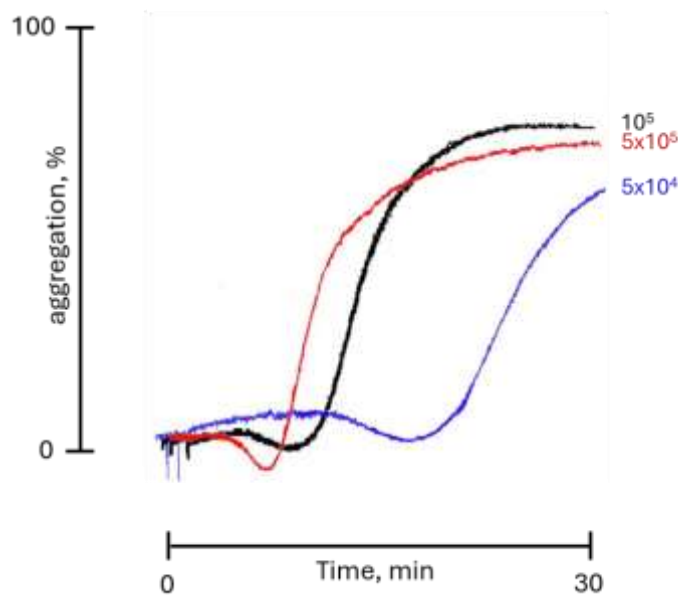


Figure 9. U87MG glioblastoma cells induce platelet aggregation in a dose-dependent manner. Representative light transmission aggregometry traces of platelets (3×10^8 platelets/mL) incubated with U87MG cells at indicated concentrations. Time is shown on the x-axis (0–30 min), whereas the percentage of light transmission, proportional to the degree of aggregation, is shown on the y-axis.

At the lowest cell concentration tested (5×10^4 cells/mL, blue trace), no significant change in light transmission was observed during the first 10 minutes. A transient, slight decrease of light

transmission was noted, likely attributable to the shape change that platelets undergo prior to aggregation, a transition from a discoid to a more spread and irregular morphology. From approximately 15 minutes upon stimulation a strong aggregation occurred, reaching an aggregation level of about 70% of maximal light transmission. A similar aggregation profile was observed using U87MG cells at the dose of 1×10^5 cells/mL. Under this condition, however, the lag time was shorter (approximately 5 minutes), and aggregation became evident by 10 minutes of incubation, reaching a final level of approximately 80% of aggregation. At the highest concentration tested (5×10^5 cells/mL, red trace), the lag time was further reduced; however, the final extent of aggregation was comparable to that observed at the intermediate dose. Taken together, these results demonstrate that U87MG cells induce platelet aggregation (TCIPA) in a dose-dependent manner, with increasing cell concentrations accelerating the onset of aggregation while producing a similar maximal response.

Having established that U87MG cells are capable of inducing platelet aggregation, we next performed a preliminary mechanistic analysis to determine whether canonical platelet activation pathways are engaged during TCIPA. Specifically, we investigated the involvement of integrin $\alpha\text{IIb}\beta 3$, the main platelet receptor that mediates fibrinogen binding and irreversible aggregation, and of PLC, a key signaling enzyme in the platelet activation cascade. For this set of experiments, a cell concentration of 1×10^5 cells/mL was selected based on the results described above.

The results are shown in Figure 10, panels A-C. Panel A shows representative aggregation traces; panels B and C show quantification of the maximal aggregation (%) and the area under the curve (AUC), respectively. As reported, U87MG cells at 1×10^5 cells/mL induced platelet aggregation reaching approximately 80% of maximal aggregation, with an AUC of approximately 1500 arbitrary units. Pre-incubation of platelets with the RGDS peptide; a competitive inhibitor of integrin $\alpha\text{IIb}\beta 3$ that blocks its interaction with fibrinogen, almost completely abolished the platelet aggregation response (green trace). Only a modest increase in light transmission, reaching approximately 10% of total aggregation, was observed at the end point of the 30 minutes analysis. Quantification confirmed that both maximal aggregation and AUC were significantly reduced compared to the untreated control (Figure 10, panels B and C). These results indicate that the physical binding of integrin $\alpha\text{IIb}\beta 3$ to fibrinogen is required for TCIPA.

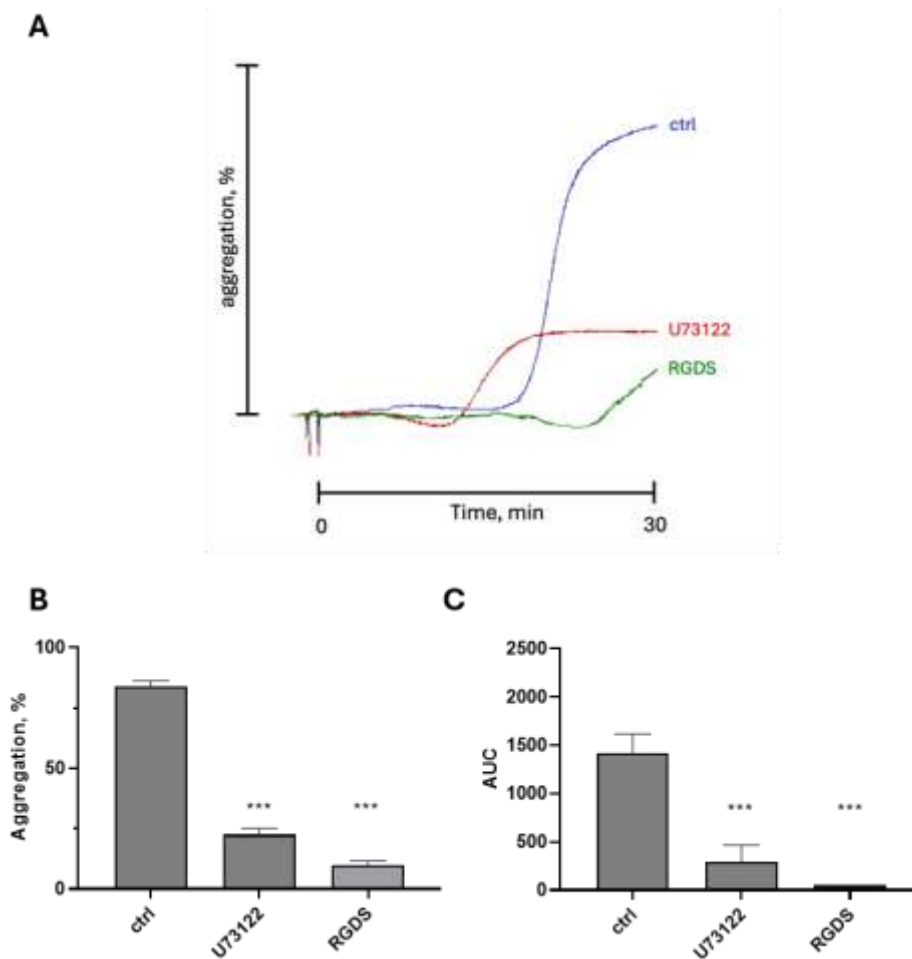


Figure 10. TCIPA depends on integrin α IIb β 3 and PLC activation. (A) Representative aggregation traces of platelets incubated with U87MG cells (1×10^5 cells/mL) in the absence or presence of 0.5 mM RGDS peptide (green) or 10 μ M of the PLC inhibitor U73122 (red). (B) Quantification of maximal aggregation (%). (C) Area under the aggregation curve (AUC, arbitrary units). (n=5; ***p<0.005).

To assess the role of intracellular platelet signaling, platelets were pre-incubated with U73122, a selective inhibitor of PLC. As shown in Figure 10 (panel A; red trace), pre-treatment with U73122 strongly inhibited platelet aggregation induced by U87MG cells. Although a minor shape change and a slight increase in light transmission were still observed, the total aggregation remained below 25%, with an AUC of approximately 200 arbitrary units, values significantly reduced compared to the untreated control.

Collectively these preliminary results indicate that TCIPA induced by U87MG cells is not the result of non-specific cell clumping but rather engages canonical platelet activation mechanisms. In particular, TCIPA appears to depend on: (i) the activation of PLC, a central enzyme in the platelet signaling cascade, and (ii) the engagement of integrin $\alpha\text{IIb}\beta 3$ and its interaction with fibrinogen.

2. U87MG cells induce the release of PEVs

Platelet activation encompasses a broad spectrum of functional responses that extend well beyond aggregation. Among these, the release of extracellular vesicles represents a particularly relevant event, given the established role of PEVs as mediators of intercellular communication and their documented involvement in tumor progression across multiple malignancies, as discussed in the Introduction (sections 2.3–2.4). Having demonstrated that U87MG glioblastoma cells are capable of inducing TCIPA, we next investigated whether platelet activation is also accompanied by release of PEVs. Platelets were therefore stimulated with U87MG cells, and the released vesicles were isolated by differential centrifugation and characterized by NTA, as described in Methods (5 and 6). As a reference condition, platelets were stimulated in parallel with thrombin, a potent physiological agonist that is known to induce a robust release of PEVs. The results are summarized in Figure 11. Panel A shows the quantification of vesicle concentration, whereas panel B, C and D report a representative NTA size distribution profile, the mode diameter, and the mean diameter, respectively.

Quantification of vesicle concentration (Figure 11, Panel A) revealed that U87MG cells induced the release of approximately 3×10^9 particles/mL, a value comparable to that obtained following thrombin stimulation. These results indicate that platelet activation induced by coincubation with U87MG cells was as effective as a physiological platelet agonist in triggering PEVs release.

Analysis of the size distribution by NTA (Figure 11, Panel B) showed that thrombin-stimulated platelets generated vesicles with peaks in the sub-200 nm region, consistent with the known size range of PEVs. Stimulation with U87MG cells produced a similar size distribution profile. While some sample-to-sample variation in peak position was observed, as expected given the use of platelets from different donors, the overall shape of the distribution was comparable between the two conditions.

Analysis of vesicle size parameters (Figure 11, Panels C and D) showed that both the mode and mean diameter of PEVs released upon U87MG stimulation were slightly but consistently lower than those observed after thrombin stimulation. While these differences were modest, they suggest that U87MG-induced PEVs may have a marginally smaller size distribution compared to those induced by thrombin. This observation may reflect differences in the specific activation pathways engaged by cancer cells versus a classical soluble agonist and warrants further investigation.

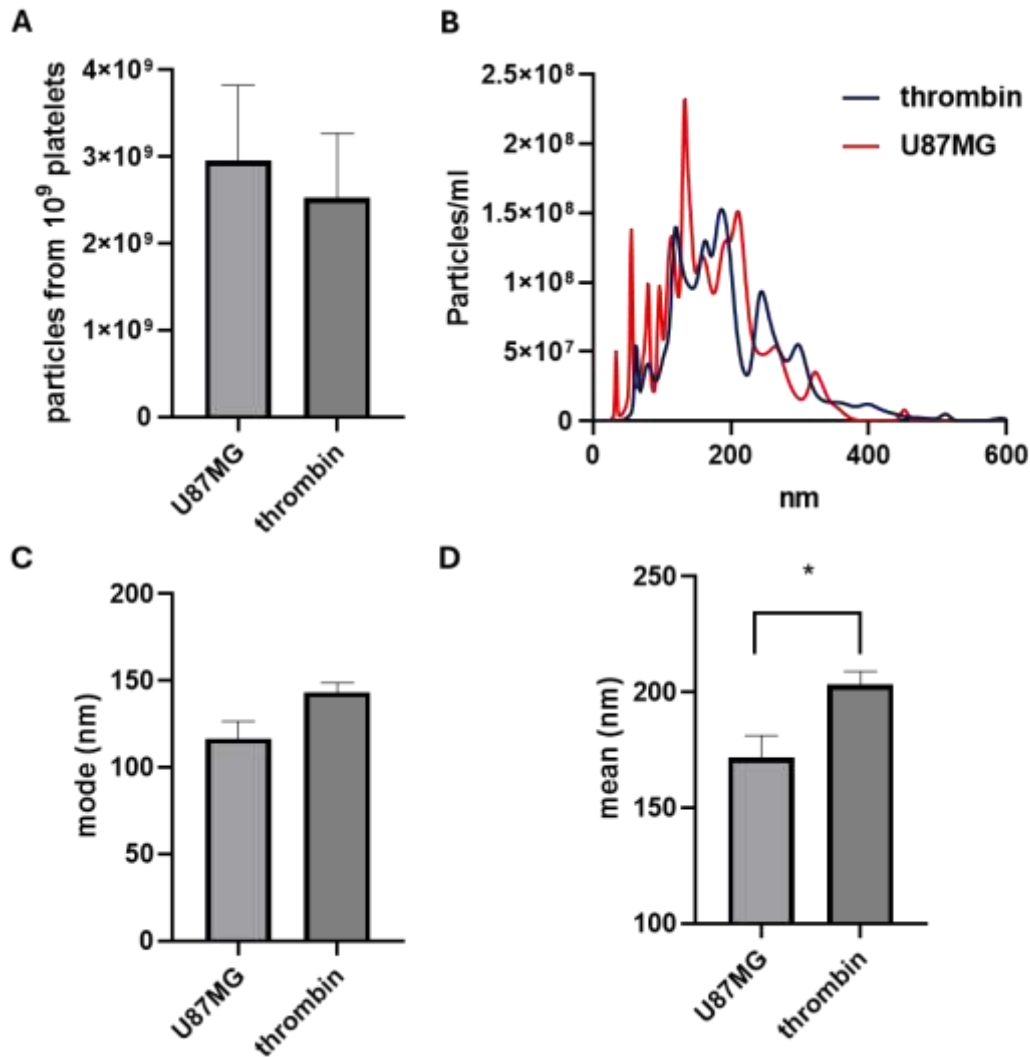


Figure 11. U87MG glioblastoma cells induce the release of PEVs. Platelets (3×10^8 platelets/mL) were stimulated by incubation with U87MG cells or thrombin as control, and PEVs were isolated by centrifugation. (A) Quantification of PEVs concentration expressed as number of particles released per 10^9 platelets. (B) Representative NTA size distribution profiles of PEVs released upon stimulation with thrombin (blue) or U87MG cells (red). (C) Mode diameter (nm) of PEVs. (D) Mean diameter (nm) of PEVs. (n=6; *p<0.05).

Taken together, these data demonstrate that U87MG glioblastoma cells are capable of inducing the release of PEVs in quantities and with a size profile comparable to those produced by the physiological agonist thrombin. This finding establishes PEVs release as a component of the platelet response to glioblastoma cells and supports the potential functional relevance of this interaction in the tumor microenvironment.

Having demonstrated that U87MG glioblastoma cells are capable of inducing platelet activation and aggregation (Section 1) and having established that this activation event is accompanied by the release of PEVs, we next sought to extend this characterization to the biochemical level. A preliminary assessment of the total protein content of the isolated vesicle populations was therefore performed using a BCA protein assay, as described in Methods (Method 8).

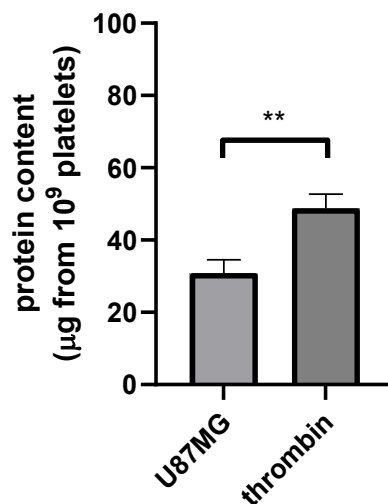


Figure 12. Protein content of PEVs released by U87MG-stimulated and thrombin-stimulated platelets. Total protein content of PEVs preparations isolated from 10^9 platelets stimulated with U87MG cells or thrombin was quantified by BCA protein assay. Data are expressed as mean $\pm$ SD from $n = 5$ independent experiments (** $p < 0.01$).

As shown in Figure 12, PEVs released from 10^9 platelets upon stimulation with thrombin contained approximately 50 μg of total protein. PEVs recovered from the same number of platelets activated by exposure U87MG cell contained a lower amount of total proteins, (approximately 30 μg of PEVs from 10^9 platelets). Statistical analysis confirmed that the protein content of U87MG-induced PEVs was significantly lower than that of thrombin-induced PEVs. These results suggest that the nature of activating stimulus, i.e. physiological agonist versus cancer cell contact, may influence the biochemical composition of the released vesicles.

To further investigate these aspects, immunoblotting analysis was performed to provide a preliminary characterization of the molecular composition of the isolated PEVs populations, as described in Methods (method 9). Equal amounts of protein from U87MG-induced and thrombin-induced PEVs were loaded onto polyacrylamide gels, separated by SDS-PAGE, and analyzed by immunoblotting using antibodies directed against a panel of selected markers. In

particular we focused our attention on two groups of proteins: platelet-specific markers and typical EVs-associated markers.

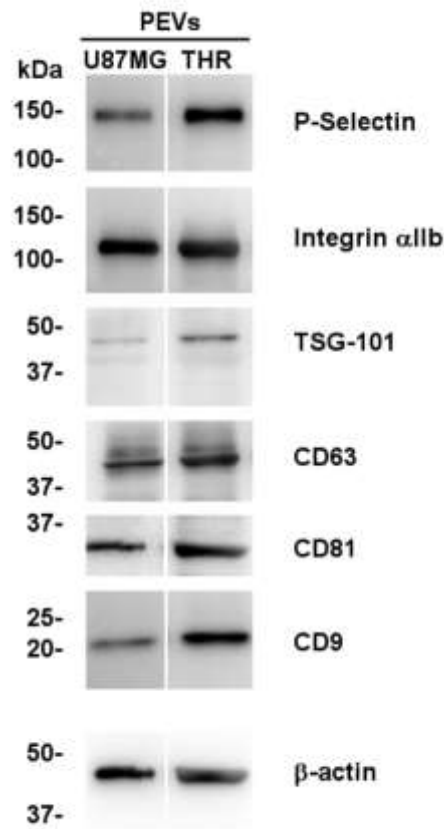


Figure 13. Molecular characterization of PEVs by immunoblotting analysis. Equal amounts of protein from PEVs isolated from thrombin-stimulated (THR) and U87MG-stimulated platelets were separated by SDS-PAGE and analyzed by immunoblotting. Membranes were probed with antibodies against the platelet markers P-selectin and integrin α IIb, the extracellular vesicle-associated markers TSG-101, CD63, CD81, and CD9, and β -actin as a loading control. Molecular weight markers (kDa) are indicated on the left.

As shown in Figure 13, all markers analyzed were detected in both PEVs populations, confirming that U87MG-induced platelet activation results in the release of vesicles carrying both platelet-derived and EV-associated proteins.

Regarding platelet-specific marker; P-selectin, a membrane protein stored in platelet α -granules and translocated to the platelet surface upon activation, where it mediates interactions with leukocytes and tumor cells, was detected in both samples, with a visibly stronger band in thrombin-induced PEVs. Conversely, integrin α IIb subunit, the platelet-specific component of integrin α IIb β 3, which is the main receptor mediating fibrinogen binding and platelet

aggregation, was present, with comparable expression levels in both vesicle preparations. Among the EV-associated markers, TSG-101, a component of the endosomal sorting complex required for transport (ESCRT) machinery and a widely used marker for the vesicular nature of EV preparations, was detected in both samples; however, its expression appeared notably more abundant in thrombin-induced PEVs, with only a faint band visible in U87MG-induced PEVs. The CD63, CD81, and CD9, transmembrane proteins enriched on EV membranes that contribute to vesicle biogenesis, target cell recognition, and intercellular communication, were all detected in both PEV populations. While CD63 and CD81 showed an overall comparable expression pattern between the two conditions, CD9 appeared more abundant in thrombin-induced PEVs. Finally, β -actin was included as a loading control and was detected at very similar levels in the two samples, confirming equal protein.

While the overall protein composition was broadly similar between the two vesicle populations, differences in the relative abundance of individual markers, particularly TSG-101, CD9, and P-selectin, were observed. As this analysis was performed as a single experiment, these findings should be considered quite preliminary, additional biological replicates will be required to determine whether the observed differences are reproducible and biologically meaningful.

3. PEVs interact with U87MG cells

Having demonstrated that U87MG glioblastoma cells are capable of inducing platelet activation, aggregation (Section 1), and the release of PEVs, characterized by biophysical and molecular features comparable to those of thrombin-induced PEVs (Section 2), we next aimed to investigate whether these vesicles are capable of interacting with cancer cells themselves. The interaction between PEVs and U87MG cells was investigated using multiple complementary approaches, including fluorescence microscopy (Method 10), flow cytometry (Method 11), and immunoblotting (Method 9). For fluorescence microscopy and flow cytometry experiments, PEVs were derived from platelets pre-labeled with CFSE, a membrane-permeable fluorescent probe that is incorporated into platelets and transferred to the vesicles they release upon activation (Method 3), enabling direct visualization and quantification of PEV association with target cells.

3.1 Fluorescence Microscopy Analysis

As reported in Figure 14, Panel A, untreated U87MG cells displayed essentially no detectable green fluorescence signal, with only the blue nuclear Hoechst staining clearly visible, confirming the absence of significant green autofluorescence under these analytical conditions. In striking contrast, cells incubated with CFSE-labeled thrombin-induced PEVs showed a strong and widespread green fluorescent signal indicating efficient association of PEVs to glioblastoma cells. Importantly, PEVs generated following platelet activation by U87MG cells displayed similar behavior. Indeed, upon incubation with U87MG-induced PEVs a strong and clear green fluorescence signal was detectable in target cells, comparable in intensity and distribution to that observed with thrombin-induced PEVs.

To obtain a quantification of the fraction of U87MG cells associated with CFSE-positive fluorescence a manual counting across multiple microscopy fields was performed. As shown in Figure 14, Panel B, the extent of interaction was remarkably high under both conditions, with approximately 80–100% of cells found to be physically associated with CFSE-labeled PEVs, regardless of whether the vesicles had been generated by thrombin or U87MG stimulation. These results demonstrate that both PEV populations interact efficiently and robustly with glioblastoma cells, and that the capacity to engage with target tumor cells is a shared feature of PEVs independently of the activating stimulus.

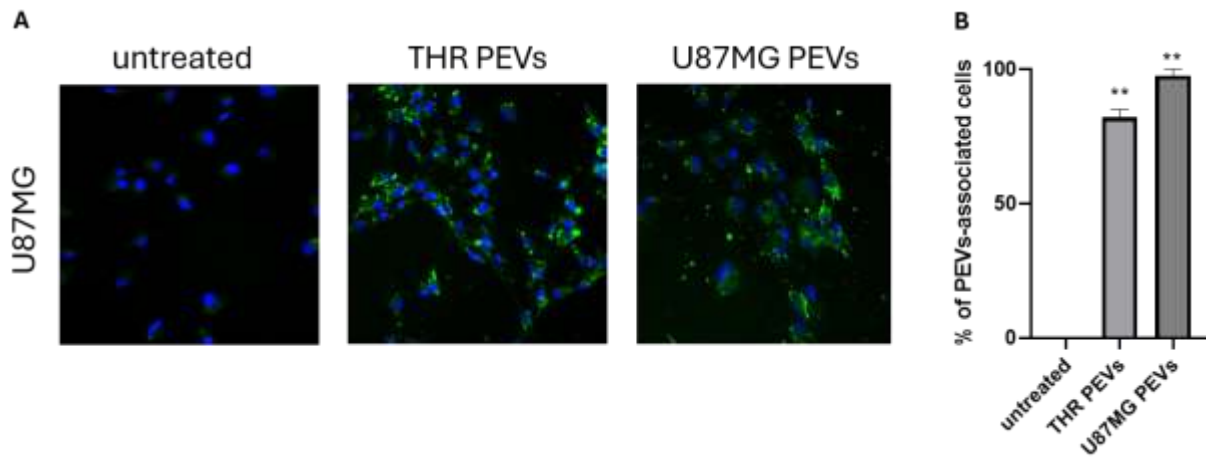


Figure 14. Fluorescence microscopy analysis. (A) representative images of untreated cells (left), cells incubated with thrombin-induced PEVs (middle), and cells incubated with U87MG-induced PEVs (right). Blue: Hoechst nuclear staining. Green: CFSE-labeled PEVs. (B) Quantification of the percentage of CFSE-positive cells across conditions. Data are expressed as the mean $\pm$ SD of 3 independent experiments (** $p < 0.01$ vs untreated).

3.2 Flow Cytometry Analysis

To obtain a more quantitative assessment of the interaction at the single-cell level, a flow cytometry analysis was performed (Method 11). U87MG cells were incubated with CFSE-labeled PEVs as described before. Following incubation, cells were washed to remove unbound vesicles, then were detached and recovered, and analyzed by flow cytometry. This approach allowed quantitative evaluation of both the percentage of CFSE-positive cells and the mean fluorescence intensity (MFI), which reflects the extent of vesicle-associated fluorescence per cell and therefore provides an indication of the amount of PEVs associated with individual tumor cells.

As shown in Figure 15, incubation with thrombin-induced PEVs resulted in approximately 60% of U87MG cells becoming CFSE-positive, whereas incubation with U87MG-induced PEVs yielded a slightly lower percentage of approximately 50% of positive cells.

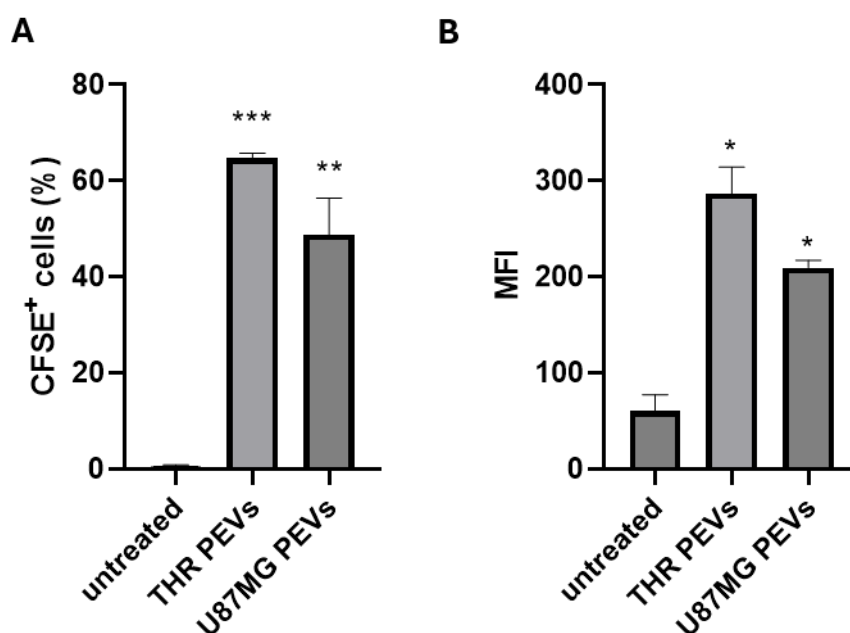


Figure 15. Flow cytometry analysis of the interaction between CFSE-labeled PEVs and U87MG glioblastoma cells. U87MG cells were incubated with 30 $\mu\text{g/mL}$ CFSE-labeled PEVs derived from thrombin-stimulated (light gray) or U87MG-stimulated platelets (dark gray) for 15 hours at 37 °C. Following incubation, cells were washed, recovered, and analyzed by flow cytometry using a BD FACSLytic flow cytometer (BD Biosciences). CFSE fluorescence was detected using the blue laser (488 nm excitation). Results are expressed as the percentage of CFSE-positive cells (left) and mean fluorescence intensity index (MFI, right). Data are expressed as mean $\pm$ SD of 3 independent experiments. (* $p < 0.05$ and ** $p < 0.01$ vs untreated).

However, this difference in the proportion of CFSE-positive cells between the two conditions did not reach statistical significance. When the extent of fluorescence per cell was considered, as reflected by the MFI, thrombin-induced PEVs displayed a higher mean fluorescence intensity compared to U87MG-induced PEVs, suggesting that, while a comparable proportion of cells interacted with both vesicle populations, thrombin-induced PEVs may associate with tumor cells to a greater extent at the individual cell level.

3.3 Immunoblotting Analysis

As a further independent and complementary approach, an immunoblot analysis was performed. Unlabeled PEVs were incubated with U87MG cells at a concentration of 30 $\mu\text{g/mL}$ for 24 hours at 37 °C, as described in Methods (Method 7). Following incubation, cells were thoroughly washed to remove any unbound vesicles and subsequently lysed for protein extraction (Method 7). Cell lysates were then analyzed by immunoblotting using an antibody

directed against integrin α IIb, as an indicator of PEVs association with tumor cells (Method 9). Since U87MG glioblastoma cells do not express this platelet-specific protein, the detection of integrin α IIb in cell lysates following incubation with PEVs was used as direct evidence of vesicle binding to tumor cells.

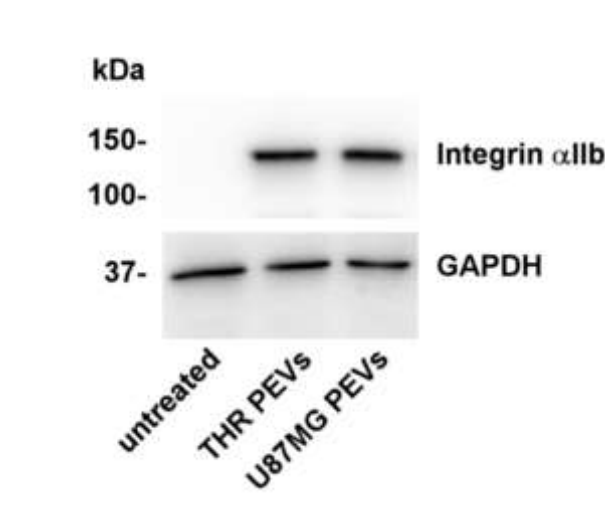


Figure 16. Immunoblotting analysis of PEVs association with U87MG glioblastoma cells. U87MG cells were left untreated or incubated with unlabeled PEVs derived from thrombin-stimulated (THR PEVs) or U87MG-stimulated platelets (U87MG PEVs) for 24 hours at 37 °C. Following incubation, cells were thoroughly washed and lysed for protein extraction. Cell lysates were analyzed by SDS-PAGE and immunoblotting. Upper panel: immunoblot probed with anti-integrin α IIb antibody (~130 kDa). Lower panel: immunoblot probed with anti-GAPDH antibody (~37 kDa) as a loading control. Molecular weight markers (kDa) are indicated on the left.

As shown in Figure 16, no detectable immunoreactive band corresponding to integrin α IIb was observed in untreated U87MG cells. This finding confirms that integrin α IIb is absent from GBM cells under basal conditions and that any signal detected following PEVs incubation can be unambiguously attributed to platelet-derived material.

In contrast, a strong and clearly detectable band corresponding to integrin α IIb was observed in lysates of U87MG cells incubated with either thrombin-induced PEVs or U87MG-induced PEVs, with comparable signal intensities under both conditions. The presence of this platelet-specific marker in thoroughly washed cell lysates provides biochemical evidence that both PEV populations efficiently associate with, or are internalized by, U87MG GBM cells.

The lower panel shows the immunoblot obtained using an anti-GAPDH antibody. GAPDH, a ubiquitous cellular housekeeping protein, was detected at comparable levels in all three samples, confirming the presence of cellular proteins and indicating that equal amounts of total

cell lysate were loaded in each lane. Taken together, these findings demonstrate that all samples contained comparable amounts of cellular material, whereas platelet-derived proteins were detected exclusively in cells that had been exposed to platelet-derived extracellular vesicles.

4. Analysis of the effect of PEVs in inducing apoptosis/necrosis of U87MG cells

Having demonstrated that both thrombin-induced and U87MG-induced PEVs efficiently interact with U87MG cells (Section 3), we next investigated whether this interaction may result in functional consequences for tumor cell biology. As a functional readout, we considered the possible ability of PEVs in modulating cell death using Annexin V/PI staining and flow cytometry analysis, as described in Methods (Method 12). As a positive control to validate the assay, samples of cells were treated with hydrogen peroxide (H_2O_2), a well-established inducer of oxidative stress and apoptosis.

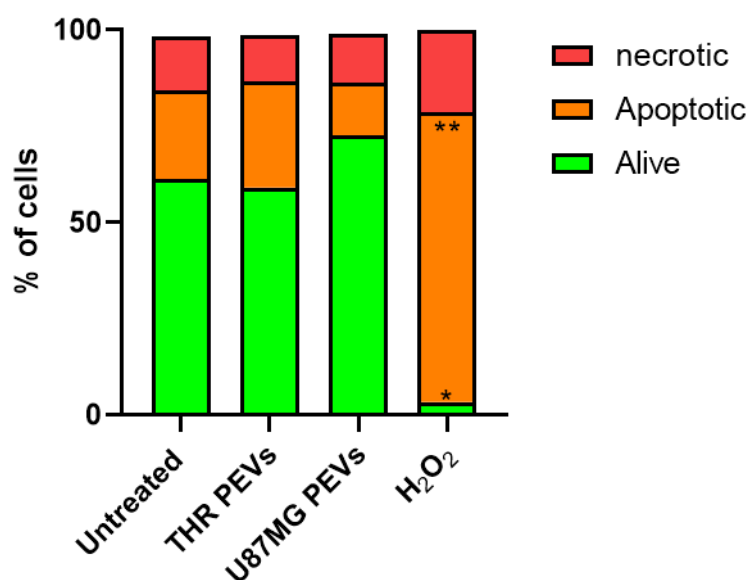


Figure 17. Analysis of apoptosis/necrosis of U87MG cells treated with PEVs. U87MG cells were left untreated (NT) or incubated with thrombin-induced PEVs (30 $\mu\text{g}/\text{mL}$), U87MG-induced PEVs, or H_2O_2 (1mM). Results are shown as the mean percentage of alive (green), apoptotic (orange), and late apoptotic/necrotic (red) cells from $n = 3$ independent experiments (* $p < 0.05$, ** $p < 0.01$).

The obtained results are reported in Figure 17 as stacked bar charts showing, for each condition, the mean percentage of cells alive (Annexin V⁻/PI⁻), apoptotic (early apoptotic Annexin V⁺/PI⁻ and late apoptotic Annexin V⁺/PI⁺), and necrotic (Annexin V⁻/PI⁺) cells. As shown in Figure 17, the distribution of cells across the different categories was broadly comparable between untreated cells and cells treated with either thrombin-induced or U87MG-induced PEVs, indicating that PEVs treatment does not induce any gross change in cell viability under the experimental conditions employed. Only a modest, statistically non-significant decrease of

apoptotic cells and increase in living cells was observed in samples treated with U87MG-induced PEVs. In contrast, H₂O₂ treatment resulted in a marked decrease in the fraction of living cells and increase of apoptotic cells compared to the untreated control ($p < 0.05$ and $p < 0.01$ respectively), confirming the overall reliability of the assay.

Taken together, these preliminary functional results suggest that, at the concentration tested, PEVs induced by thrombin or by U87MG cells, do not exert significant effects on viability and death of U87MG glioblastoma cells.

DISCUSSION

Glioblastoma remains one of the most aggressive and lethal brain tumors, and current therapeutic strategies remain insufficient for several reasons, including inefficient drug delivery across the BBB (Jo *et al.*, 2023; Lan *et al.*, 2024). Platelets and PEVs have emerged as important players in the biology of different types of tumors, contributing to angiogenesis, immune evasion, and tumor cell invasion (Puhm *et al.*, 2021; György *et al.*, 2011). However, whether platelets and PEVs play any role in GBM has not been thoroughly investigated. The present study therefore aimed to explore the functional interaction between platelets, PEVs, and GBM cells, using U87MG cells as an experimental model.

The main findings of this study can be summarized as follows:

- U87MG GBM cells induced platelet aggregation (TCIPA) in a dose-dependent manner;
- TCIPA engaged canonical platelet aggregation mechanisms, depending on integrin $\alpha\text{IIb}\beta 3$ and PLC;
- GBM cells triggered release of PEVs comparable to that observed upon stimulation with thrombin;
- Both thrombin-induced and GBM-induced PEVs efficiently associated with U87MG cells, as confirmed by fluorescence microscopy, flow cytometry, and immunoblotting;
- PEVs did not significantly affect GBM cell viability under the tested conditions.

The ability of U87MG cells to trigger TCIPA through canonical platelet activation pathways suggests that GBM cells may be capable of engaging platelets also *in vivo*, potentially leading to the formation of platelet aggregates in the proximity of tumors. In other cancer types, such platelet coating has been shown to physically shield tumor cells from NK cell-mediated cytotoxicity, thereby facilitating their survival during hematogenous dissemination (Josefsson, 2025; Li *et al.*, 2024). Whether a similar protective mechanism operates for GBM-derived cells remains to be investigated, but our findings provide the first evidence that the molecular machinery required for such an interaction is present and functional. Furthermore, the ability of GBM cells to trigger platelet activation *in vitro* raises the possibility that similar interactions may occur *in vivo*, potentially contributing to the prothrombotic state and increased risk of venous thromboembolism reported in cancer patients (Zarà *et al.*, 2019).

Beyond aggregation, platelet activation by U87MG cells was accompanied by the release of PEVs at quantities comparable to those induced by thrombin, a potent physiological agonist known to trigger robust vesicle release. This finding indicates that GBM cells are not only capable of triggering platelet aggregation, but also of inducing a broader activation response that includes vesiculation, a process increasingly recognized as a key mechanism through which platelets influence the tumor microenvironment (Fan *et al.*, 2024). Different activating stimuli are known to produce PEVs with distinct characteristics; the observation that U87MG-induced PEVs were slightly smaller and contained less total protein than thrombin-induced PEVs is consistent with this concept and may reflect differences in the specific activation pathways engaged by cancer cell contact compared with a soluble agonist. Furthermore, the PEVs generated upon GBM stimulation retained platelet-derived surface markers, including integrin α IIb and P-selectin, consistent with their platelet origin. The lower abundance of some of these markers compared to thrombin-induced PEVs represents a preliminary indication that the activating stimulus may shape PEVs composition, a possibility that will require confirmation through additional replicates and more comprehensive approaches such as tandem mass spectrometry. Taken together, these results establish PEVs release as an integral component of the platelet response to GBM cells and provide the biochemical basis for the subsequent analysis of PEV- GBM cell interactions.

Having established that U87MG glioblastoma cells are capable of inducing platelet activation, aggregation, and PEV release, the central question of this study was whether these vesicles are able to interact with the tumor cells themselves. This interaction was assessed using three different approaches: fluorescence microscopy, flow cytometry, and immunoblotting, each providing a different type of evidence and converging on the same overall conclusion. Fluorescence microscopy showed that the large majority of U87MG cells (80–100%) became associated with CFSE-labeled PEVs, regardless of whether the vesicles had been generated by thrombin or by U87MG stimulation, indicating that the capacity of PEVs to engage GBM cells does not depend on the specific activating stimulus. Flow cytometry provided a more quantitative, single-cell assessment, confirming that a substantial proportion of cells (50–60%) became CFSE-positive, although thrombin-induced PEVs displayed a somewhat higher mean fluorescence intensity, suggesting that, while comparable numbers of cells interacted with both vesicle populations, thrombin-induced PEVs may bind to a greater extent on a per-cell basis. These apparent discrepancies likely reflect the distinct parameters captured by each technique

rather than a biological inconsistency; fluorescence microscopy was primarily designed to assess the proportion of cells associated with PEVs rather than the amount of vesicle-derived signal per cell, whereas flow cytometry, through the MFI, specifically captures the latter. This distinction highlights the inherent complexity of quantitatively comparing vesicle-cell interactions across different methodologies, each of which probes a different aspect of the same biological phenomenon. Immunoblotting added a further, biochemical layer of evidence: the platelet-specific protein integrin α IIb, which is absent from untreated U87MG cells, was readily detected in cell lysates following incubation with either PEVs population, demonstrating that PEVs-derived material physically associates with, or is internalized by, GBM cells. Importantly, although this study cannot formally distinguish surface binding from internalization, the detection of platelet-derived protein in thoroughly washed cell lysates is consistent with genuine vesicle uptake. This will nonetheless need to be verified in future studies using confocal microscopy analysis.

Previous work from our laboratory has demonstrated that thrombin-induced PEVs can be internalized by breast cancer cells, where they trigger intracellular Ca^{2+} signaling and promote cell migration (Zarà *et al.*, 2017; Vismara *et al.*, 2021). To our knowledge, the present findings provide the first demonstration that PEVs are similarly capable of interacting with GBM cells, extending the relevance of the platelet/PEV–tumor axis to a malignancy in which it had not previously been explored. Whether this interaction in GBM leads to comparable functional consequences, however, remains to be determined. Furthermore, as U87MG represents only one of several molecularly distinct GBM subtypes, extending this analysis to additional cell lines or patient-derived models will be important to determine whether this interaction is a general feature of GBM.

Having established that PEVs efficiently associate with U87MG cells, we next analyzed whether this interaction results in functional consequences for tumor cell viability. The results showed that PEVs released upon stimulation with either thrombin or U87MG cells did not significantly change the proportion of living, apoptotic, or necrotic cells compared to untreated cells. In contrast, H_2O_2 , used as a positive control, significantly reduced cell viability, as expected, confirming the reliability of the assay. It can therefore be argued that, under the conditions and dose tested, the association of PEVs with GBM cells does not trigger an overt cytotoxic response, suggesting that PEVs may instead contribute to signaling or phenotypic changes rather than to programmed cell death. Future studies should explore other PEVs

concentrations and exposure times, as well as additional functional readouts such as cell migration or proliferation, to determine whether PEVs exert any protumoral or antitumoral effects on GBM cells.

CONCLUSIONS

PEVs are known to act as natural carriers, transferring proteins, lipids, and nucleic acids such as RNA and DNA between cells via the bloodstream (Fan *et al.*, 2024; Lou and Cai, 2025). Our finding that PEVs efficiently associate with U87MG cells (Result 3) suggests that this natural cargo-transfer property could in principle be exploited for therapeutic purposes. Considering PEVs nanoscale size and reported ability to cross the BBB (Fan *et al.*, 2024; Lou and Cai, 2025), one of the major obstacles to effective drug delivery in GBM, they could represent a vehicle capable of carrying therapeutic cargo, such as small molecules, siRNA, or miRNA, directly into GBM cells. Moreover, since PEVs derive from platelets, they may retain features that help circulating cells evade immune clearance, potentially improving the delivery efficiency.

However, this remains a speculative application, and substantial work would be required to design, load, and validate PEVs as drug carriers in a GBM-specific context. An important next step will be to test whether PEVs loaded with therapeutic cargo can actually cross BBB models and retain their ability to reach and associate with GBM cells, providing a first proof-of-concept for their potential use as drug carriers in this disease.

Several directions for future research emerge from this work. As mentioned above, confocal microscopy, including z-stack acquisition, could be employed to formally distinguish between surface binding and internalization of PEVs by GBM cells; clarifying this aspect would be particularly relevant for drug delivery applications, as definitive proofs of internalization would support the use of PEVs as intracellular drug carriers. Mass spectrometry-based proteomic analysis would allow a more comprehensive characterization of PEVs cargo, providing an unbiased map of the proteins transferred to GBM cells beyond the selected markers examined here. Future studies should also extend this analysis to additional GBM cell lines and patient-derived models, given the molecular heterogeneity that characterizes this tumor. Moreover, as already mentioned, before any therapeutic application of PEVs in GBM can be considered, it will be essential to exclude potential pro-tumoral effects beyond those assessed in this study.

In conclusion, this study provides pieces of evidence of a functional interaction between platelets, PEVs, and GBM cells, laying the groundwork for future investigations into the role of this axis in glioblastoma pathobiology and its potential therapeutic exploitation.

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