Platelets — Allies of Tumour Cells

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1. Introduction

1.1. History of platelets and their association with cancer

In 1865, Armand Trousseau observed several cases of severe blood clotting in patients with malignancy [1] but was unable to speculate on the underlying mechanism. In 1882, Bizzozero first demonstrated that blood platelets, or thrombocytes, adhered to damaged blood vessels and hypothesised that these blood components played a central role in haemostasis and experimental thrombosis [2]. Subsequently, Riess reported an association between thrombocytosis (defined as a platelet count of $>400\text{x}10^9$/ L of blood) and cancer death [3]. Almost a century later, these preliminary observations were revisited and confirmed [4, 5], initiating a renewed interest in a potential role for platelets in cancer metastasis, invasion and angiogenesis [6-10]. In this chapter, we will explore the evidence demonstrating a wider remit for platelets than simple haemostasis and thrombosis. We will review the data suggesting that platelets facilitate circulating cancer cells to traverse physiological and immunological obstacles and establish as solid tumours in remote places, where they enable a bespoke blood supply. In addition, we will explore the complex molecular mechanisms that underlie the platelet-tumour cell interactions.

2. Platelet biology in haemostasis and cancer

The role of platelets in haemostasis and thrombosis is well known. Platelets survey the blood vessels for evidence of damage. Upon detection, platelets undergo a rapid but highly regulated activation which results in dramatic shape change, adhesion to the exposed sub-endothelial matrix, secretion of important effectors from dense granules and/or alpha-granules, formation

of a haemostatic platelet aggregate and activation of the coagulation cascade. All of these processes lead to reinforcement of the platelet plug in closing the breech in the vessel wall [11]. Thus, the currently accepted role for platelets is to contribute to haemostasis by their physiological ability to maintain the structural integrity of the blood vessels.

The role of platelets in cancer metastases is less well known. However, numerous experimental studies have shown that thrombocytopenia (defined as a platelet count of $<100\times10^9$/L blood) induced in tumour-bearing mice, effectively reduces tumour dissemination and tumour growth [6, 17]. Moreover, thrombocytosis may be an indicator of an advanced stage of cancer and is often associated with poor prognosis. Thus, platelets appear to exert a pro-metastatic function, enabling tumour development.

Metastasis is the dissemination of cancer cells from the primary tumour mass to distant organs and occurs predominantly through the blood stream [12]. It is an intricate multi-step cascade during which cancer cells intravasate from the primary site into the blood vessels, circulate in the blood towards distant anatomical sites, adhere to the luminal wall of micro-vessels (arterioles and capillaries) and penetrate into the surrounding tissue (extravasation) to eventually colonize it [13]. Survival of the newly relocated tumour foci depends on the subsequent establishment of a novel blood supply to support tumour growth, a process termed angiogenesis [14]. Metastasis is responsible for as much as 90% of cancer associated mortality [15].

The pro-metastatic activity of platelets can be explained as the *pathological* capability to prolong tumour cell survival in the circulation and in the new metastatic location. Survival of circulating tumour cells (CTCs) in the circulation is achieved by protecting them from immune destruction, facilitating their adhesion to the vascular endothelium or enabling extravasation of the tumour cells to secondary sites. Moreover, platelets play a role in enabling angiogenesis to provide a necessary blood supply for a growing tumour mass [16-19, 20].

In normal circulation within intact vasculature, most platelets do not undergo significant interactions with the endothelial surface during their entire lifetime. The quiescent state of platelets is protected by the presence of extrinsic regulators, such as nitric oxide (NO) and prostacyclin (PGI$_2$), produced continuously by intact endothelial cells. Moreover, the presence of potential platelet activators such as collagen, adenosine diphosphate (ADP) and thrombin are tightly regulated in the bloodstream. Only strong biological signals such as the exposure of the sub-endothelial matrix components (VWF, collagen, fibrinogen, laminin, fibronectin) following endothelial damage or the generation of thrombin via the coagulation cascade will permit platelet activation to enable a haemostatic or a thrombotic response. Platelets adhere to sub-endothelial molecules via their surface receptors for von Willebrand Factor, VWF (GPIb/IX/V), collagen ($\alpha_2\beta_1$ and GPVI), fibrinogen ($\alpha_{IIb}\beta_3$), fibronectin ($\alpha_5\beta_1$) and laminin ($\alpha_6\beta_1$), resulting in platelet capture, activation, shape change and release of secretory granules. The relative contribution of each receptor-ligand interaction is influenced by blood flow condition, with GpIb-VWF interplay prevailing under high shear conditions observed in arterioles/stenotic arteries [21]. Activated platelets rapidly secrete and produce a number of soluble molecules, mainly ADP and thromboxane (TXA$_2$), triggering activation of surrounding platelets leading to the formation of a platelet-rich clot. Moreover, activated platelets express

negatively charged surface phospholipids that contribute to localised coagulation leading to thrombin generation. Thrombin then further activates platelets *via* protease-activated receptors -1 (PAR-1), -4 (PAR-4) and GPIb-IX-V [22-25] and also enzymatically converts fibrinogen into fibrin [26]. The binding of fibrin results in further reinforcement of the existing platelet plug and of its anchorage to the site of vascular injury [21]. Figure 1 shows an overview of platelet activation responses to wounding and cancer metastasis.
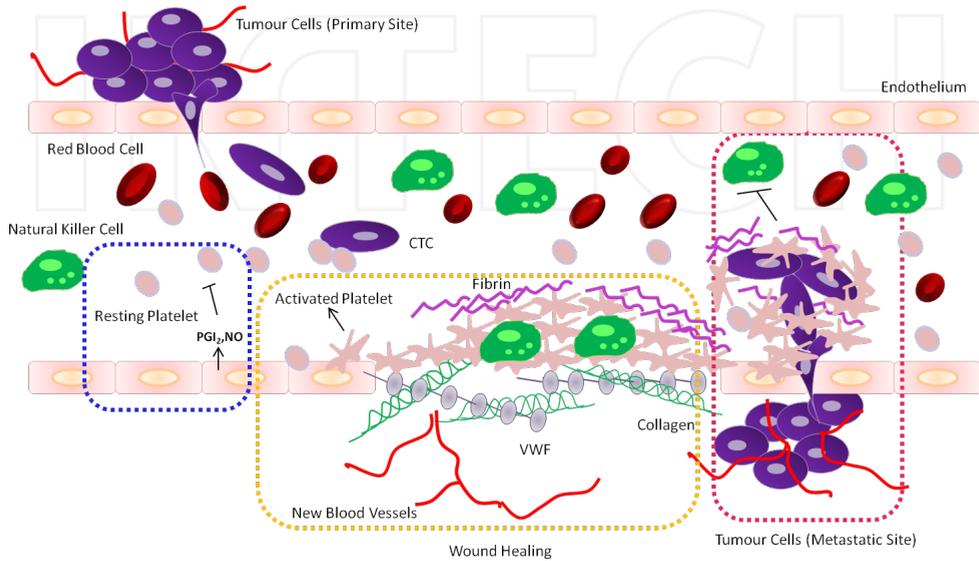

Figure 1. Differentiating platelet activity in haemostasis and cancer. *Blue Box*: In physiological condition platelets circulate in the blood stream in a quiescent state. Prostacyclin I_2 (PGI$_2$) and nitric oxide (NO) are the main soluble mediators through which the endothelium inhibits platelet reactivity. *Yellow Box*: In response to endothelial injury, highly thrombogenic proteins such as von Willebrand factor (VWF) and collagen are exposed, which synergistically induce platelet activation and haemostatic plug formation. Fibrin consolidates the platelet plug while newly formed blood vessels (angiogenesis) provide nutrients to inflammatory cells and clear cell debris. *Red Box*: Circulating cancer cells (CTC) activate and aggregate platelets *via* paracrine and/or juxtacrine signals to ensure their survival in the bloodstream, and to permit extravasation and proliferation at the metastatic site. Fibrin supports tumour inflammation and provides important scaffolding for tumour cell attachment to the endothelium. Newly formed vessels guarantee nutrients and oxygen for tumour growth and survival.

Importantly, platelet activation, aggregation and secretion, are also triggered in a carcinogenic microenvironment [6, 9, 27] and serve to enable tumour metastasis. The first experimental observation of platelet involvement in cancer metastasis dates back to 1968 when Gasic and colleagues demonstrated an anti-metastatic effect associated with thrombocytopenia in mouse models [6]. Subsequently, several *in vitro* and *in vivo* experimental models provided direct demonstrations of the profound pro-survival influence that platelet activation exerts on three critical stages during blood borne metastasis:

1. cancer dissemination through the blood (haematogenous spread);

2.   tissue invasion or extravasation from the vasculature at the metastatic site and

3.   Tumour angiogenesis and tumour blood vessels stability.

2.1. Platelets enable cancer cell dissemination during haematogenous spread

During their migration through the bloodstream, circulating cancer cells (CTCs) are exposed to an unfavourable environment, characterised by shear forces and innate immune cytotoxicity. In order to escape immune recognition and overcome shear forces, CTCs in the bloodstream attract an entourage of platelets and use them as a cellular shield for their survival [18, 28]. Evidence that platelets prevent natural killer cells (NK) from destroying CTCs, comes from both *in vivo and* in *vitro* studies. Platelet-depletion in mice causes a reduction in tumour colonization when compared to mice with normal platelet counts [29]. In addition, the ability of NK cells to lyse the tumour cells *in vitro* is directly correlated with platelet density [29]. Palumbo and colleagues subsequently demonstrated that platelet-fibrin deposits form a cloak around B16-F10 melanoma cells helping to camouflage the tumour cells to enable them to escape immune recognition and elimination [30]. The mesh of fibrin, induced by tissue factor on the surface of CTCs, envelops the cancer cells [18, 31] permitting them to evade recognition by NK cells [32, 33]. In addition, the coat of platelets enables a mechanism of molecular mimicry resulting in the acquisition of platelet-derived major histocompatibility complex (MHC-I) by cancer cells [34]. Moreover, platelet derived transforming growth factor β (TGF$_\beta$) and platelet derived growth factor (PDGF) can impede NK immune surveillance by down-regulating the NK cell activating immune-receptor (NKG2D) [35] and NK cell PDGF receptor- expression [36]. Thus platelets enhance CTC survival, permitting them to traverse the blood vessels to establish a secondary locations.

2.2. Platelets support cancer cell extravasation

In order to leave the circulation and metastasize, cancer cells must adhere to the microvasculature of a target organ and penetrate the surrounding tissue [37]. As early as 1985, electron micrographs showed B16 melanoma cells trapped in an intricate network of platelets and fibrin at the lung vasculature site [38]. Indeed, under *in vitro* flow conditions, platelets facilitate tumour cells adhesion to enable the cancer cells to tether and arrest to the subendothelium [39, 40] by using platelet P-selectin and $\alpha_{IIb}\beta_3$ as potential bridging molecules. Tethering and adhesion of colon carcinoma (LS174T, COLO205, and HCT-8) and melanoma cells (M21, M397, M501 and M537) to the subendothelium can be prevented by antagonism of P-selectin or $\alpha_{IIb}\beta_3$ on platelet surfaces [39-41]. Furthermore, platelets adhering to CTCs are activated to release growth factors (including vascular endothelium growth factor (VEGF), TGFβ, and PDGF) and proteases of the matrix metalloprotease (MMP) class at sites of adhesion to the endothelium [42-44]. Once released, platelet-derived cytokines enhance endothelial growth while MMPs will degrade specific component of the extracellular matrix (ECM) encouraging vascular permeability and extravasation of tumour cells, as well as the release of growth factors sequestered in the ECM [45].

Extravasation is also supported by platelet-derived nucleotides. Munc13-4 deficient mice, whose platelets lack the ability to secrete dense granule components such as ADP and ATP, or mice deficient in the P2Y$_2$-ATP receptor on endothelial cells, demonstrate a significant

reduction in metastasis in mouse models of melanoma (B16) and breast carcinoma (LCC) [20]. Thus, platelet-bound adhesive molecules and platelet derived soluble molecules synergistically help cancer cells to traverse the endothelial cell barriers, penetrate the parenchyma and establish new lesions.

2.3. Platelets promote tumour angiogenesis and safeguard vascular integrity

The assembly of a new vascular network (angiogenesis) is of central importance to the growth of solid tumours beyond 2-3 mm [46]. The role of platelets in angiogenesis and be found in recent comprehensive reviews [14, 47]. An intimate association with the circulation is required for the tumour to acquire necessary nutrients, to shed metabolic waste products and to sustain further tumour growth and invasion [43, 48]. Interestingly, tumour blood vessels display remarkable morphological abnormalities involving permeability and leakiness, and excessive and haphazard branching [45, 49]. This vessel morphology permits the access of CTCs to the circulation, favouring contacts with platelets which, in turn, can function as source of pro-angiogenic factors at the metastatic niche and contribute to tumour survival and progression [50]. Indeed, platelets can stimulate endothelial cell proliferation and augment the formation of capillaries-like tubes *in vitro* [51] and angiogenesis *in vivo* [17, 52, 53]. Brock's finding that tumour-derived vascular endothelial growth factor (VEGF), a molecule that enables angiogenesis by stimulating endothelial cells proliferation and migration, can stimulate endothelial cells to expose VWF is noteworthy [54]. It has in fact been speculated that the release of platelet binding or activating molecules, such as VWF, may favour platelet adhesion to the vascular wall with a consequent activation and release of further pro-angiogenic molecules [50].

Platelets are recognised as major physiological transporters of VEGF in blood of healthy subjects and patients with breast and colorectal cancer [50, 55-59]. The possible rationale underlying this phenomenon became apparent when the ability of platelets to actively and selectively sequester tumour-derived angiogenesis regulators in a carcinogenic microenvironment was shown [60, 61]. Therefore, it was not surprising to observe that platelets isolated from tumour-bearing mice and activated with ADP could induce angiogenesis more efficiently than platelets obtained from cancer-free mice [62].

In addition to VEGF, platelets are carriers of many crucial regulators of angiogenesis (pro- and anti- angiogenic factors), which can be released, in a selective fashion according to the nature of the stimulus [19, 63, 64], a concept that is still somewhat controversial. However in this context, MCF-7 breast cancer cells can orchestrate the preferential release of pro-angiogenic molecules (e.g. VEGF), but not of the anti-angiogenic counterpart (e.g. endostatin), from platelet α-granules, to induce angiogenesis *in vitro* [19]. Interestingly, treatment of human platelets with aspirin (COX-1 inhibitor) or antibodies against $\alpha_{IIb}\beta_3$ prior to exposure to breast cancer cells has been shown to inhibit the release of pro-angiogenic factors *in vitro* [19, 65, 66].

In addition to storage and release of angiogenic regulators, platelets can also contribute to maintaining tumour vessel homeostasis by protecting tumour-vasculature from haemorrhaging. In 2008, Wagner's group demonstrated that the metastatic rate of experimental tumours in a thrombocytopenic mouse model was restored when mice were retransfused with platelets from a littermate [17]. However, if the retransfused platelets were rendered incapable of a

secretion response, then the growth rate of metastatic tumours was again found to be low. Thus, platelet granular contents, released from activated platelets, was responsible for the greater metastatic potential in platelet-replete mice [17]. The authors noted a tendency for the metastatic tumours to have fragile blood vessels and also demonstrated evidence of haemorrhage in the thrombocytopenic mice. Transfusing these mice with fresh platelets rescued this effect, allowing robust angiogenesis at the secondary lesions and permitting more metastatic colonization of the mouse lungs[17, 53]. However, depletion of platelet granules prior to transfusion into the thrombocytopenic mice limited the angiogenic effect, without eliminating platelet's ability to blood clotting process. Overall, this research suggests that the platelet secretome is responsible for the establishment of a necessary blood supply to the tumour mass and that this ability occurs independently from thrombus formation. In addition to this, a recent study demonstrated impaired blood vessel density and maturation at the tumour niche in platelet-depleted mice [67]. Thus, the pathological role that platelets can play in cancer metastasis has now become more evident. However, the molecular mechanisms underlying these interactions have remained somewhat elusive.

Figure 2 summarises the proposed role for platelets in enabling (A) survival of CTCs, and (B) extravasation and metastatic growth as discussed above.
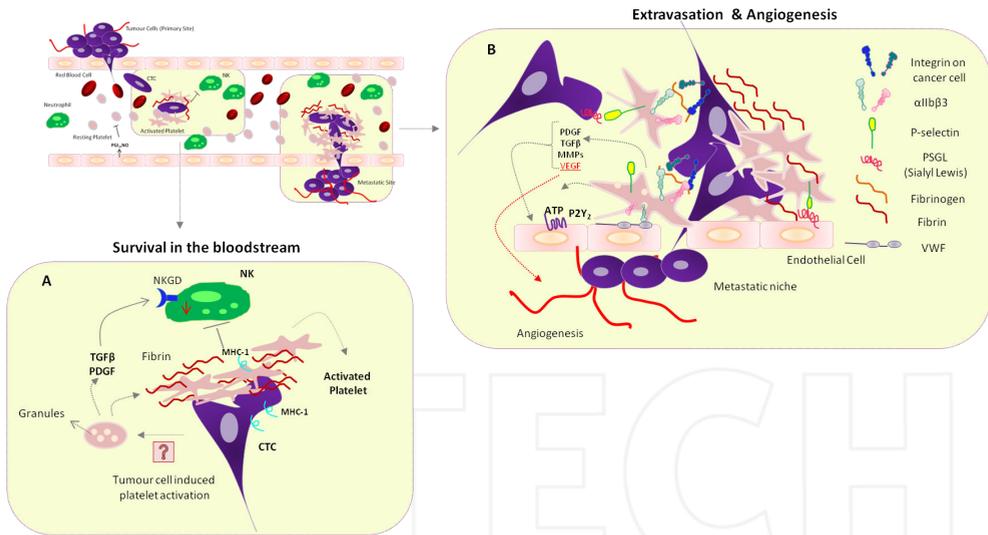

Figure 2. Simplified schematic model of platelet-mediated cancer progression. A. *Platelets support cancer cell survival in the bloodstream.* CTC induce platelet aggregation and secretion. Aggregated platelets surround and protect CTC from NK-mediated lysis via physical shielding. Platelets also release TGF$_β$ and PDGF, which function as downregulators of the NK-activating receptor NKGD. In addition, CTC can escape the immune surveillance through molecular mimicry of platelets (See Section 1.2.1 for further details). B. *Platelets contribute to tumour extravasation and angiogenesis.* Activated platelets interaction with tumour cells is thought to be mediated by P-selectin binding to PSGL-1 (P-selectin glycoprotein ligand-1) and integrin-fibrinogen-integrin bridges. Platelet release of growth and angiogenic factors as well as proteases contributes to cancer cell extravasation and formation of new stable capillaries at the metastatic niche (See section 1.2.2 & 1.2.3 for further details).

3. The identity of platelet surface receptors involved in cancer progression

The surface of human platelets contains multiple receptors that help platelets to react in response to a wide range of agonists and adhesive proteins. The roles of these receptors in thrombosis are well established. There is also ample evidence to support the involvement of some platelet receptors in the process of tumour cell induced platelet aggregation (TCIPA) and cancer spreading in the bloodstream [68-72].

3.1. G Protein-Coupled Receptors (GPCR)

The seven transmembrane receptors (7TM) are well represented in platelets (Table 1) and constitute both the major checkpoints that maintain platelets in a resting state (e.g.; PGI_2 receptor/IP) and the primary mediators of the second phase of platelet activation during thrombosis and haemostasis (e.g.; ADP receptors/$P2Y_1$ and $P2Y_{12}$; Thromboxane TXA_2 Receptor/TP) [73].

LIGANDS	RECEPTORS
Thrombin	PAR 1, 4
ADP	$P2Y_{12}$, $P2Y_1$
TXA_2	TP
PGI_2	IP
PGE_2	EP_1/EP_3/EP_4
Lipids	PAF
Lysophospholipids (LPL)	LPL-R
C-X-C Chemokines	CXCR-4
CC-Chemokines	CCR1,CCR3,CCR4
Epinephrine/dopamine	β2 Adrenergic
Serotonin	$5-HT_{2A}$

Table 1. Platelet GPCRs and Ligands

3.1.1. Protease-Activated Receptors (PARs): Thrombin receptors

In haemostatic conditions, PARs drive platelet activation in response to thrombin. Thrombin is a plasma serine-protease that is generated in response to activation of the blood coagulation system and converts fibrinogen into fibrin, forming a mesh that serves to strengthen the thrombus and support clot retraction. In addition, thrombin directly activates platelets causing platelet aggregation.

Thrombin is both a platelet agonist and a pro-coagulant molecule that is required for some of the molecular strategies used by cancer cells to progress in their lethal journey. Mice deficient in their primary thrombin receptor, PAR-4, have a reduced ability to support metastasis compared to wild-type mice [74].

3.1.2. Purinergic receptor $2Y_{12}$ and $2Y_1$: ADP receptors

$P2Y_1$ and $P2Y_{12}$ receptors are membrane-binding sites for ADP on platelets. ADP can activate platelets in an autocrine fashion, if released from platelet dense granules following a primary wave of activation, or in a paracrine fashion if secreted by damaged cells at sites of vascular injury. Some carcinomas, mainly neuroblastoma, melanoma and breast cancer cells, have been demonstrated to be capable of secreting ADP [75, 76]. ADP scavengers such as apyrase, efficiently impair *in vitro* platelet activation induced by 59M ovarian cancer cells [77], Caco-2 colon cancer cells [78], MCF-7 breast cancer cells, [79] and HeLa human cervical carcinoma cells [80]. Experiments in mouse models of cancer provided even more direct demonstration of the profound influence that ADP-triggered response exerts on tumour progression. When Lewis lung tumour cells were implanted into host mice lacking the expression of $P2Y_{12}$ receptors, their ability to metastasize to the lungs was disrupted [81]. In complementary experiments, ticagrelor, a $P2Y_{12}$ antagonist, succeeded in blocking tumour metastasis of B16-F10 melanoma- and 4T1 breast cancer cells in mouse models of carcinogenesis [82]. In the same study, ticagrelor prevent cancer cell adhesion to platelets and to endothelial cells, further supporting a role for $P2Y_{12}$-mediated platelet activation in tumour progression. Similarly, treatment with clopidogrel, an irreversible antagonist of $P2Y_{12}$ receptors protected the host mice from pathologic osteolysis and bone loss associated with B16-F10 melanoma tumour growth in bone [83]. Some controversy exists on whether tumour cell types generate ADP themselves or stimulate platelets to release ADP. However, Mitrugno et al, 2014 recently demonstrated that many tumour cells induce platelets to release ADP. Arguably, the most impactful evidence for a role for ADP as a secondary mediator of tumour cell-induced platelet activation comes from a study published by Battinelli and colleagues [19] who showed that both ADP and MCF-7 breast cancer cells were separately able to trigger the release of pro-angiogenic factors from platelet α-granules. Platelet ADP receptor antagonists reduced the platelet response to MCF-7, suggesting that the tumour activation operates to induce the secretory event. In addition, the impaired ability of cancer cells to transmigrate through the endothelial barrier in Munc 13-4 deficient mice is suggestive of a strong role for ADP in mediating this response [20]. Munc 13-4 deficient mice lack of the ability to secrete platelet dense granules, and thus ADP and ATP release is prevented. The inability of these mice to demonstrate detectable cancer extravasation and metastasis suggests that platelet dense granule release is vital to cancer development.

3.1.3. TP: Thromboxane A_2 receptor

Thromboxane A_2, the major TP ligand, is a prostanoid generated in platelets following agonist-induced mobilization of arachidonic acid (AA) from platelet membrane [84]. Similar to ADP, TXA_2 can activate platelets in an autocrine fashion, if released by platelets themselves, or in a paracrine modality, if liberated in the extracellular microenvironment by malignant cancer cells [85, 86]. Only a limited number of studies have addressed the issue of the role for platelet TP-TXA_2 molecular interplay in tumour cell induced platelet activation (TCIPA) and cancer metastasis. However, a role for TXA_2 in cancer metastasis is evident. SQ-29548, a TP antagonist, markedly inhibits osteogenic sarcoma cells- (MG-63) induced platelet aggregation [87]. In

addition, TP$^{-/-}$ mutant mice injected with B16F1 melanoma cells present with reduced lung colonization and mortality rate compared to wild-type littermates [88]. Finally, inhibitors of thromboxane synthesis such as Ozagrel, BM-567 or aspirin, impair tumour induced platelet aggregation and carcinogenesis in a number of diverse models [85, 89]. In this context, although aspirin fails to inhibit platelet aggregation elicited *in vitro* by various cancer cells [69, 72, 77, 78, 90] it significantly affects platelet α-granule release of pro-angiogenic factors in response to breast cancer cells [19].

Numerous studies from clinical trials have accumulating evidence to suggest that aspirin may represent a potential therapeutic strategy to reduce the risk of developing cancer metastasis and the consequent mortality [91, 92]. In a recent large meta-analysis of >17,000 patients, daily usage of low-dose aspirin has been shown to reduce the incidence, growth and metastasis of a number of cancers in a period of 5-6 years [93]. Overall, several platelet-dependent and independent mechanisms of actions of aspirin as a chemo-preventive agent have been proposed but their relevance in the treatment of cancer remains to be established [94].

3.2. Platelet Immunoreceptor Tyrosine-Based Activation Motif (ITAM) receptors

Immunoreceptors are ITAM or hemi-ITAM bearing receptors present on the platelet surface with a conserved double (ITAM) or single (hem-ITAM) YxxL-motif in their cytoplasmic tail. Upon receptor engagement or molecular cross-link, associated Src family kinases (SFKs) phosphorylate the tyrosine residues (Y) within the ITAM creating a docking site for Src homology 2 (SH2) domains of the tyrosine kinase Syk. Recruitment of Syk triggers its activation and the formation of a LAT (linker for activation of T-cells)-signalosome localised to lipid rafts. Among the proteins recruited to the signalosome there are cytosolic adaptors proteins (e.g.; Grb2: growth factor receptor bound protein 2; Gads: Grb2 related adaptor protein downstream of Shc; SLP-76: SH2 domain containing leukocyte protein of 76 kDa), and effector proteins (e.g.; PLCγ2 and PI-3 kinase). The molecular cross-talk culminates in PLCγ2 activation which will generate, via its lipase activity, IP$_3$ and DAG leading to calcium mobilization and PKC activation. Ultimately this pathway induces measurable platelet responses, including integrin activation, TxA$_2$ formation, and granule release (Reviewed in:[95]). The main ITAM receptors in platelets are glycoprotein VI (GPVI), FγRIIa and C-type lectin like receptor (CLEC-2) and their potential role in tumour cell-platelet cross-talk will be discussed below.

3.2.1. GPVI: Collagen receptor

GPVI, unique to platelets and megakaryocytes, is the primary signalling receptor for collagen. Its ability to activate an intracellular pathway relies on its constitutive association with the ITAM containing Fc receptor gamma receptor FcγR, [96]. GPVI is a binding-target for several diverse fibrillar types of collagen, such as types I and III, as well as synthetic collagen-related peptide (CRP) and snake venom proteins (e.g.; convulxin) [97]. Although GPVI is considered an essential receptor for platelet activation, there is only limited experimental evidence suggesting a potential involvement for this glycoprotein in tumour metastasis. In genetically altered mice devoid of GPVI, the number of metastatic foci to lungs, following challenges with B16F10.1 melanoma or D121 Lewis carcinoma cells, was dramatically reduced compared to

wild type littermates [98]. However, the molecular mechanism(s) that lead to this response remain unclear. Platelet pre-treatment with revacept, a GPVI blocker, or cancer cell treatment with inhibitor of galectin-3, an adhesive molecule with "collagen-like" domain, resulted in a dramatic inhibition of platelet-induced COX-2 over-expression in cancer cells [94]. Thus, GPVI may be involved in tumour cell-induced platelet secretion and induction of epithelial mesenchymal transition (EMT) in cancer cells.

3.2.2. FcγRIIa: IgG receptor

FcγRIIa is a low affinity Immunoglobulin receptor present in humans but not in mice. FcγRIIa in humans is primarily found in platelets [99, 100]. It binds to IgG immune-complexes, immunoglobulin-opsonised bacteria, and to auto-antibodies that target a subset of platelet membrane proteins [101, 102]. FcγRIIa also functions as an accessory receptor of GpIb-IX-V complex and integrin $\alpha_{IIb}\beta_3$ [103, 104]. Its physical association with the GpIb-V-IX complex has been shown to be responsible for tyrosine phosphorylation within ITAM motifs via Src-family Kinases (SFK) [104, 105]. Platelets from transgenic mice engineered to express FcγRIIa have an improved ability to respond to stimuli resembling haemostatic events, including spreading on fibrinogen, phosphorylation of Syk and PLCγ2, clot retraction and thrombus formation [106]. Mitrugno et al, recently demonstrated that this platelet receptor is responsible for mediating binding events between prostate cancer cells and platelets *in vitro* resulting in tumour cell induced platelet aggregation [72].

3.2.3. CLEC-2: Podoplanin receptor

In 2006, CLEC-2 was identified as a new hemi-ITAM (single YXXL motif) platelet receptor present as a homodimer on the platelet surface, able to activate molecular events resembling ITAM-bearing receptors through the snake venom toxin rhodocytin [107]. Importantly podoplanin, a sialylated transmembrane glycoprotein and the only-known physiological ligand for CLEC-2, has been found to be expressed on certain types of human glioblastoma (e.g.; LN319) and colon carcinoma (Colon-26) where it serves to enable TCIPA *in vitro* [108]. Metastatic events were found to be impaired in podoplanin knockout mice injected with kidney tumour cells [109]. In respect to this last observation, it has been proposed that the motile invasive behaviour of certain cancer cells relies in part on podoplanin-triggered signals. Indeed the ectopic expression of podoplanin in oral squamous cell carcinoma positively correlates with increased cell motility [110]. In addition, epidermal keratinocytes (MCA3D) acquire a malignant phenotype following transfection with podoplanin [110]. All together these observations reveal a potential role for podoplanin and podoplanin-CLEC-2 cross-talk in cancer progression, suggesting that inhibitors of this molecular program might represent an innovative therapeutic strategy for patients affected by certain types of cancer [44].

3.3. GPIb-IX-V Complex: VWF receptor

The GPIb-IX-V complex is a member of the leucine-rich repeats (LLRs) family of adhesive multimeric proteins consisting of two GPIbα subunits disulphide-linked to two GPIbβ subunits and associated non-covalently with GPIX and GPV protein on the platelet surface

[111]. This complex plays an essential role in mediating the initial adhesion of platelets to VWF at sites of vascular injury in high shear conditions (above $1000s^{-1}$), and in triggering activation of integrin $\alpha_{IIb}\beta_3$ [112, 113]. Upon ligand engagement, the GpIb-X-V complex recruits cytoplasmic SFKs and PI3Ks via the GpIbα cytosolic tail, leading to calcium release and integrin activation. Alternatively, it can couple with the FcγRIIa chain to trigger ITAM-like signalling [104, 114]. VWF, a major but not unique GpIbα ligand, is a large multimeric adhesive glycoprotein associated with collagen in the subendothelial matrix. Importantly, following activation, it can also be released by Weibel-Palade bodies in endothelial cells and α-granules in platelets [115].

The role of GpIbα in cancer metastasis has been subject of many studies that converge in contradictory results. GPIbα is present on some cancer cell surfaces and appears to play a role in cancer cell-induced platelet aggregation [116]. However, the expression of GpIbα on the surface of cancer cells is not common [70, 117]. Contradictory results have been reported in *in vivo* studies. Reduced melanoma metastasis to the lungs is observed in mice deficient in GpIb-IX-V complex [118]. In contrast, administration of anti-GPIbα monovalent antibodies in melanoma-bearing mice resulted in a strong enhancement of lung metastasis [119]. Similarly, an increased metastatic potential was observed in mice lacking VWF [120] but not in mice treated with anti-VWF antibodies, which were protected from metastasis [121]. Thus, the role of GpIb in the cancer cell-platelet loop remains unclear, making it object of further investigation.

3.4. Platelet integrins: $\alpha_{IIb}\beta_3$

Integrin $\alpha_{IIb}\beta_3$ is the most abundant cell adhesion molecule on the platelet surface (approximately 80,000 receptors per platelet), with an additional pool of protein that can be recruited from internal α-granule membranes upon platelet activation [122]. $\alpha_{IIb}\beta_3$ exists in a low affinity or inactive state in circulating, un-activated platelets and undergoes conformational changes following platelet stimulation by soluble agonists such as thrombin. Conformationally active integrin displays an increased affinity for its endogenous ligands: principally plasma fibrinogen and VWF. This interaction stabilises the adhesion of platelets to the extracellular matrix and permits cross-linking of activated platelets, causing aggregation and haemostatic plug formation. Importantly, ligand binding to $\alpha_{IIb}\beta_3$ enables "outside-in" molecular circuits, which lead to thrombus stability and fibrin clot retraction [123, 124]

Lacking enzymatic activity, integrins must cooperate with other cytoplasmic proteins to trigger intracellular signals. Importantly, recent investigation has revealed the capacity of $\alpha_{IIb}\beta_3$ to associate with molecular mediators of ITAM-bearing receptors. In this scenario, FcγRIIa has been proposed as a key molecular partner or accessory receptor for $\alpha_{IIb}\beta_3$ [102, 103]. FcγRIIa is recruited to activated $\alpha_{IIb}\beta_3$ microclusters enriched in SFK, leading to the phosphorylation of FcγRIIa-ITAM sequence that provides a link to Syk. As yet however, the mechanism and stoichiometry of the interaction between $\alpha_{IIb}\beta_3$ and FcγRIIa remains obscure.

Integrin $\alpha_{IIb}\beta_3$ is the adhesive molecule which has been most explored in *in vivo* models of haematogenous metastasis and in *in vitro* models of TCIPA [70]. A monoclonal antibody (10E5) directed against $\alpha_{IIb}\beta_3$ inhibits the ability of tumour cells to bind to platelets [121]. The same

antibody is also able to reduce the number of metastatic foci to lungs in a mouse model of tumour metastasis using colon carcinoma CT26 cells [125]. Similarly, a reduction in the extent of tumour cell colonisation of the lungs was also observed in integrin $\beta3^{-/-}$ mice injected with B16F10 melanoma cells [126]. These findings have then been confirmed in a number of additional studies [70]. In addition, $\alpha_{IIb}\beta_3$ inhibitors modulate TCIPA elicited by diverse human tumour cells, including colon carcinoma, cervical and vaginal melanoma and breast cancer [72, 90, 116, 127]. Moreover, abciximab (anti-$\alpha_{IIb}\beta_3$ monoclonal blocking antibody) inhibits the release of pro-angiogenic factors such as VEGF, from the platelet α-granules by MCF-7 breast cancer cells [66] indicating that integrin $\alpha_{IIb}\beta_3$ activation is necessary to support platelet activation and platelet secretion. Taken together these results highlight $\alpha_{IIb}\beta_3$ as attractive target for future anti-metastatic therapies.

4. Platelet granules and their role in cancer progression

Although considerable progress has been made in understanding how platelets interact with cancer cells to initiate an interdependent relationship, little is known about how that relationship develops. Following platelet association with tumour cells, platelet secretion of dense granules [72], and α-granules [19, 66] ensues and the resulting platelet releasates contribute to tumour progression. As yet, however, very little is known about the potential "intrinsic" ability of cancer cells to induce platelet granule release. Moreover, the precise molecular orchestrators of this phenomenon remain completely unknown. It is likely that, like platelet interactions with bacterial micro-organisms, different cancer cell types may interact in different ways with platelets [128]. Ultimately, however, the ability of tumour cells to engineer the release of platelet-stored cytokines and bioactive molecules appear to be a key component of the critical interaction that remains to be elucidated.

4.1. α-granules

α-granules are the most abundant granule population in platelets (50-80 per platelet) presenting a heterogeneous morphology, size and luminal content as well as protein repertoire [64, 129, 130]. Electron tomography of platelet cryo-sections revealed the presence of 3 major different subtypes of α-granules based on average size and contents: 1) 200-500 nm spherical α-granules featuring an electron dense core and eccentrically localised multimeric VWF tubules; 2) multivesicular 100-200 nm granules displaying a multitude of free luminal membrane vesicles; and 3) 50 nm- tubular α-granules rich in fibrin-like structures [129]. Importantly, there seems to be also two further dimension of complexity with regard to (i) α-granules protein content and (ii) speed of release.

Immunofluorescence and immuno-electron microscopy data from certain laboratories show distinct localizations for pro- and anti-angiogenic factors in platelet α-granules, indicating that proteins are segregated among α-granules during their initial synthesis, according to their biological function [60, 130]. This separate packaging then permits a differential secretory behaviour, in terms of type of protein released in response to different stimuli [19, 64, 130]. Ma

and colleagues first demonstrated the ability of platelets to release their content in a "thematic" way [131]. VEGF (pro-angiogenic), but not endostatin (anti-angiogenic) was selectively released upon platelet stimulation with PAR-4 agonist while an opposite response was observed following platelet exposure to a thrombin receptor ligand [131]. However, to date, the knowledge of the molecular mechanism(s) orchestrating the selective platelet release remains elusive [129]. It must be noted that recent studies show activation of different PARs elicit similar releasate patterns and cargoes within the α-granules are randomly packaged, however there are indications that spreading platelets are capable of sorting and separating α-granules subtypes [132-134]. Either way, it will be intriguing to address whether this classification of granules is physiologically meaningful and if it changes during pathological events such as cancer.

α-granules are functionally pleiotropic, meaning that they can serve different and significant functions [135]. Thus, the release of their contents can be pivotal in establishing the nature of the surrounding microenvironment [135]. More than 300 molecules have been shown to be released from activated platelets [136], which differ in their origin. Some components may be synthesized in the megakaryocyte whereas others are scavenged from plasma [135]. The contents of platelet α-granules also differ in their function [10, 135, 137]. Functionally, α-granule contents can be divided in the following categories: 1) adhesive molecules (e.g.; fibrinogen, fibronectin, vitronectin, thrombospondin, VWF, P-selectin, $\alpha_{IIb}\beta_3$) which mediate homotypic (platelet-platelet) and heterotypic (platelet-endothelial cells) interactions and subsequent clot formation (primary heamostasis) ; 2) coagulation factors (e.g.; fibrinogen, pro-thrombin, Factor V, Factor VII) which play a crucial role in the stabilization of the haemostatic clot; 3) fibrinolytic agents (e.g. plasminogen activator inhibitor-1 (PAI-1)) which are important for clot remodelling; 4) growth factors (e.g.; PDGF, VEGF, EGF, TGFβ) which can contribute to wound healing, angiogenesis, chemotaxis and cell proliferation; 5) pro-angiogenic and anti-angiogenic factors (e.g.; angiopoietin, VEGF, endostatin, angiogenin, angiostatin; and tissue inhibitors of metalloproteases 1 and 4 (TIMP1 and TIMP-4) which regulate the *de novo* formation of blood vessels; 6) tissue-remodelling factors (e.g. MMPs, TIMPs and disintegrin metalloproteinases (ADAMs)) which allow structural remodelling of the ECM and the solubilisation and activation of a variety of growth factors tethered in an inactive form to the proteoglycans of the ECM ; 7) pro-inflammatory factors which include a pool of chemokines (e.g.; CXCL1 (GRO-α), CXCL4, CXCL5 (ENA-78), CXCL7 (NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1a), CCL2 (MCP-1), CCL3 (MIP-1a), CCL5 (RANTES), CCL17 (TARC)), which induce recruitment, activation, chemokine secretion and differentiation of other cells, involved in wound healing, from the circulation [10, 135].

Importantly, all these molecules have the potential to contribute to the progression of cancer. Moreover, the platelet granule contents may change as a consequence of the host being infected with a cancer [61]. This leads to the intriguing possibility that certain cancer cells can prime circulating platelets with active cytokines, tailored to permit tumour cell survival at a meta-static site. Alternatively, tumour cells may upregulate the levels of select platelet cytokines to enable avoidance of an immune response and thus enhance CTC survival in a host. A more complete understanding of the platelet-tumour cell interdependence will be critical for the design of therapeutic interventions to supress cancer progression.

4.2. Platelet dense granules

Each platelet contains just 3 - 8 dense granules, which measure approximately 250 nm in diameter [138]. They concentrate a number of small molecules including: 1) ions (e.g.; calcium, magnesium, phosphate, pyrophosphate); 2) nucleotides (e.g.; ADP, ATP, GTP and GDP). 3) transmitters (e.g.; serotonin, epinephrine, histamine), and 4) membrane proteins which are also found in lysosomes (e.g.; LAMP-2). Serotonin and ADP function as secondary mediators, or positive enhancers of the platelet aggregation response. Interestingly, about 50% of platelet ADP is stored in platelet dense granules and cannot be refilled following platelet activation. In contrast, the metabolic pool of adenine nucleotides, housed in the platelet cytosolic compartment, can be synthesized but not released [139]. Serotonin, is instead sequestered by platelets from plasma and released into the circulation upon activation where it can act as both an autocrine and a paracrine mediator [140]. The autocrine function serves to amplify the platelet response through engagement of the Gq-coupled 5HT2A receptor on platelet surface, whereas the paracrine effect contribute to the modula-tion of the vascular tone, normally inducing vasoconstriction to limit the blood flow and reduce blood loss at sites of vascular injury [138]. Calcium found in dense granules constitutes from 60 to 70% of total calcium mobilised in platelets. [140-142]. The function of the calcium released by platelets into the extracellular space is uncertain, however it has been speculated that it may play a role in facilitating the binding of the extracellular adhesive proteins to their receptors on platelets [138].

4.3. Lysosomes

Lysosomes constitute a less characterised granule type present in platelets in marginal number (0-1 per platelet). They are packaged with cathepsins, carboxypeptidases, β-hexosaminidase, acid phosphatases, enzymes for hydrolyzing various sugars and aryl sulfatases. CD63 and LAMP2 are membrane-bound molecules normally used as markers of lysosomes [138]. Though the exact function of lysosomes in platelets is not known, they are thought to be involved in dissolution of the clot [10].

5. Platelet secretion and the progression of cancer

In 1984 Boneu et al., first reported the presence of degranulated platelets, characterised by low levels of ADP and serotonin, in patients with malignant solid tumours [143] suggesting that platelet secretion was a critical event in tumour growth. Wagner's research group subsequently showed that platelet secretions continuously support tumour vascular homeostasis by regulating the stability of tumour vessels [17]. However, the exact identity of platelet-derived factors responsible for tumour vessel protection remains unknown.

A potential role for ADP and serotonin, released by platelet dense granules, has been proposed [63, 72]. Platelet dense granule secretion of adenine nucleotides in a tumorigenic microenvir-onment may be key to tumour cell extravasation and to the formation of metastatic foci. One mechanism downstream from this phenomenon has been uncovered [20]. Platelet-derived

ATP binds to $P2Y_2$ receptors on endothelial cells triggering a signalling cascade that culminates in the disaggregation of the endothelial barrier. Moreover, platelet-derived ADP can also participate in tumour cell-induced platelet aggregation [144], a strategy adopted by several tumour cell lines to evade the immune system and overcome high arterial shear stress during the haematogenous journey [28].

Cancer cells can also induce platelet release of matrix metalloproteinases (MMPs) and cytokines from α-granules [145]. These bioactive molecules can destroy the extracellular matrix barrier and promote invasion and tumour extravasation. Among the MMPs, MMP-2 has been shown to be autocrine mediator of TCIPA elicited by human fibrosarcoma HT-1080, lung carcinoma A549, breast adenocarcinoma MCF7 and colon adenocarcinoma Caco-2 cells [68, 69, 78].

Platelet release of adhesive proteins such as $\alpha_{IIb}\beta_3$, P-selectin, and fibrinogen can also poten-tially support and stabilise adhesive interaction of tumour cells with both platelets (platelet-cancer aggregate) and endothelial cells, favouring tumour cells transmigration across the endothelial barrier. Similarly, the release of coagulation factors can prompt the conversion of fibrinogen into fibrin which will strengthen the heterotypic platelet-cancer aggregate and ensure its survival and safe docking at the metastatic site [146, 147]. As discussed previously, platelets contain both pro- and anti-angiogenic factors that can be differentially released to ensure optimal tumour angiogenesis [19, 63]. Moreover, in animals bearing malignant tumours, platelets can be conditioned to selectively store and release regulators of angiogenesis [60]. Importantly, it has been shown that platelets are not just simply transporters of tumour-derived pro-angiogenic molecules. In a mouse model of metastasis, Kerr and colleagues have demonstrated the ability of platelet to sequester diverse tumour-derived cytokines and to safely transport them to a distant metastatic niche for release [61]. This excellent strategy seems to be engineered by cancer cells to hijack important pro-metastatic and pro-inflammatory proteins and impede their degradation in plasma. It is noteworthy that this emerging concept of platelets as carriers of tumour-derived proteins can provide a window to the needs of cancer cells and guide the clinician towards an optimal therapeutic approach. Interestingly, in the study by Kerr and colleagues [61], granulocyte colony-stimulating factor (G-CSF) was found to be abundantly released in the plasma of tumour-bearing mice by both platelets and cancer cells. The same result was also separately observed by Wagner's group who subsequently uncovered a potential role for G-CSF in the formation of neutrophil extracellular traps (NETs) and cancer-associated thrombosis [148]. NETs are suggested to facilitate tumour metastasis by trapping circulating tumour cells [149].

Once recruited by the circulating tumour cells, platelets can release a plethora of growth factors including platelet derived growth factor (PDGF-BB) and transforming growth factor beta (TGFβ), which can contribute to the inflammatory state often associated with cancer [10]. In this context, TGF-β derived from platelet α-granules profoundly impacts tumour metastasis and survival by enhancing an epithelial-to-mesenchymal transition (EMT), *via* activation of the TGFβ/Smad and NF-KB signalling pathways in tumour cells, and suppressing tumour cell NK-mediated lysis [16, 35]. EMT is a program which enables cancer invasiveness and dissem-ination [150].

A summary flowchart of platelet secretion events in cancer is presented in Figure 3 summarizing 30 years of the causal evidence linking secretion and tumour survival characteristics. Despite these recent discoveries of platelet secretion affecting cancer cells, and vice versa, the precise molecular mediators at play between tumour cells and platelets are not yet fully understood. The use of anti-platelet agents is currently emerging as potential treatment of malignancy and tumour-derived thrombosis [63, 151]. However, this use of platelet inhibitors to improve cancer prognosis and reduce the risk of fatal metastasis must take into account the delicate balance between inhibiting platelet activation and the associated risk of bleeding. The identification of the precise molecular pathways in platelets in response to activation by cancer cells may permit greater progress in this area.
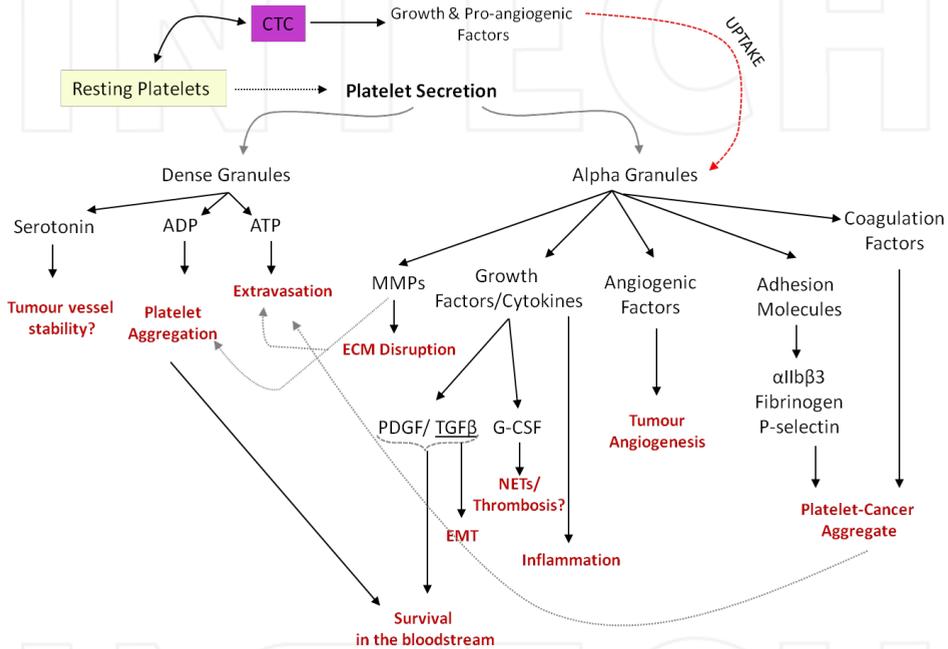

Figure 3. Flowchart highlighting the potential role of platelet-released molecular mediators in neoplastic progression.

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