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New horizons in platelet research: Understanding and harnessing platelet functional diversity

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Abstract. Recent years have been ripe with discoveries of non-haemostatic platelet functions. This led to the appreciation of the significant, previously unknown, role played by the platelets in various pathologies and regenerative processes. As a result, exciting opportunities for clinical applications in fields as diverse as regenerative medicine and cancer treatment are emerging. However, their realization depends on the understanding of the regulatory mechanisms governing these diverse platelet functions, so that particular platelet responses could be artificially tailored to specific clinical situations. Current understanding of the signalling pathways controlling haemostatic responses is rooted in the development of quantitative assays for measuring them and sensitive markers for their quantification. However, the existing assays and markers are not sufficiently sensitive for distinguishing between individual signalling pathways and unravelling inter-pathway connections. Moreover, entirely new approaches are needed for studying non-haemostatic platelet functions, since there are currently no assays or markers for quantifying them. We review the on-going efforts in these directions, including our own recent work on using lectins as sensitive probes for profiling platelet activation.

1. Platelets and their therapeutic potential

Last decade in platelet research has been ripe with exciting surprises. Originally thought of as clotting factories (Fig. 1), platelets turned out to be involved in a wide variety of physiological and pathophysiological processes [75]. They appear to function as a Swiss army knife, unsheathed as a first-response to injury, in processes ranging from coagulation, where they limit traumatic blood loss, to inflammation and the subsequent steps of the wound healing cascade [5, 88, 97], angiogenesis [88, 96], vascular remodelling and maintenance of vascular integrity [55], *de novo* blood vessel synthesis, initial response to pathogens [148], and other aspects of both innate and adaptive immune response (Fig. 2) [88, 121, 138]. Misfiring of these mechanisms is implicated in pathological conditions as diverse as atherosclerosis [13, 67, 78, 110] and the development of cancer metastases [33, 34, 127]. This diversity raises important questions but also presents therapeutic opportunities that are as unique as they are varied. The realization of these opportunities is predicated on the understanding of the regulatory processes that govern platelets' various functional roles.

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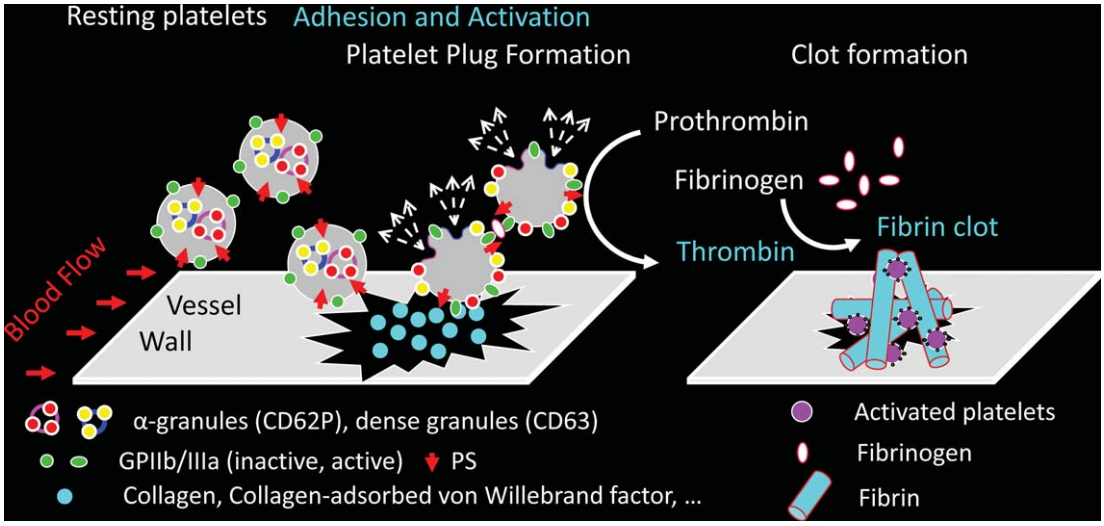


Fig. 1. Platelets in Haemostasis. Under normal physiological conditions, platelets (large grey circles) circulate in the blood in a resting state. They do not aggregate and their interactions with the healthy blood vessel endothelium or normally folded plasma proteins are limited. Exposure of collagen and collagen-adsorbed von Willebrand factor at wound sites (a star-shaped, black defect in the grey vessel wall) leads to platelet adhesion. This is followed by activation that involves a conformational change of GPIIb/IIIa, a constitutively expressed integrin receptor on the platelet surface (small green circles on the platelet surface). Activated GPIIb/IIIa (green ellipses on the platelet surface) becomes competent to bind fibrinogen. This leads to platelet aggregation and plug formation. Concurrent secretion of granule contents (dashed white arrows), that includes platelet agonists such as ADP, and production of thromboxane, causes activation and recruitment of additional platelets to the growing platelet plug. Phosphatidyl serine (PS, red arrowheads) that becomes exposed on the surfaces of the activated platelets catalyses the assembly of the clotting factor complexes such as tenase and prothrombinase (not shown). Their activity leads to thrombin production and ultimately fibrin formation from fibrinogen. Fibrin polymerizes forming the clot that seals the wound. It also becomes a natural scaffold for the subsequent wound healing process, in which platelets also play a leading role.

From a historical perspective, it is interesting to note that evidence of platelet functional diversity dates back to some very early studies. For example, their role in maintaining vascular integrity was noted as early as 1940s by Danielli [21], and their involvement in the early stages of atherosclerosis [67, 78] is somehow connected to this role. In 1960s, the effect of platelets on vascular integrity was essentially re-discovered and investigated more systematically (see, e.g., Gimbrone et al. [36]; similarly, in his review, written in 1964, T.H. Spaet refers to this platelet effect as “well-recognized”, without, however, mentioning Danielli’s work [125]). Subsequently, some of the underlying mechanisms began to be clarified. Most recent ideas on the subject are summarized in refs. [55] and [139]. Both direct interactions between the platelets and the endothelial cells (adhesion) and indirect effects mediated by the platelet secretion reactions appear to be needed for maintaining the integrity of the inflamed vascular endothelium [55], where platelet relevance has been demonstrated in thrombocytopenic mice [37]. Quiescent platelets were shown to adhere and roll on activated endothelium by intravital microscopy [29, 139]. This process is mediated by the endothelial P-selectin and vWF, stored in the Weibel-Palade bodies [141] of the endothelial cells, that interact with constitutively expressed P-selectin glycoprotein ligand (PGSL-1) and GPIb, respectively [139]. Until now, it appears that some form of activation and expression of P-selectin on platelets is required for attracting leukocytes, however [139]. Key signalling events that mediate platelet adhesion to the inflamed endothelium, the details of adhering platelet activation, and the transformation to the putative state in which adhering platelets promote leukocyte extravasation

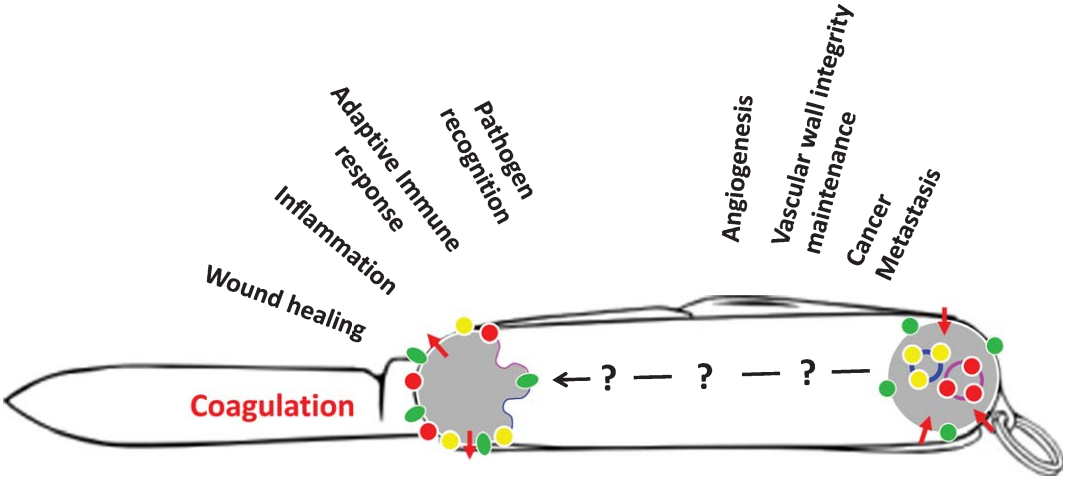


Fig. 2. Multifunctional platelets. Platelets appear to function as a Swiss army knife of first responses to injury, being involved in a variety of defensive reactions such as coagulation and subsequent wound healing processes, pathogen recognition and other aspects of the adaptive immune response, angiogenesis and vascular remodelling, and maintenance of vascular integrity. Pathologies such as thrombosis, atherosclerosis (not shown), and cancer metastases arise as a result of the malfunction of these responses. To explain these diverse functions of the platelets, it is supposed that platelets exhibit multiple activation states (indicated with question marks), as opposed to a unique procoagulant one (indicated on the left). Characteristics of these states are not yet understood. Colours and symbols are the same as in Fig. 1.

without initiating thrombus formation, remain obscure. This last process is thought to provide the link to the aetiology of atherosclerosis [67, 78]. *In vitro* studies of these processes continue to remain challenging [73].

Similarly, the link between platelets and cancer can be traced back to the work of Trousseau in 1860s; a very informative historical review of Trousseau's work can be found in ref. [70], while the molecular details of platelet-cancer links have recently been reviewed by Gay and Felding-Habermann [34]. Recent studies point to the relevance of their ability to assist extravasation of cells, their role in angiogenesis, and vascular remodelling. These platelet effects may or may not be directly linked to their procoagulatory function.

It is becoming clear that the procoagulant role of platelets may be divorced from their other functional roles, such as that of maintaining vascular integrity, angiogenesis, and vascular remodelling. All of this implies a multiplicity of different activation states. How these activities are regulated is not understood. Key questions surround relevant molecular triggers and specific responses, such as secretion and expression of specific signalling molecules, involved in the various roles platelets play. Understanding these mechanisms and harnessing them to stimulate the regenerative functions of the platelets while minimizing thrombotic and detrimental inflammatory effects would revolutionize prevention and treatment of cardiovascular disorders, the underlying atherosclerosis, healing of recalcitrant wounds, integration of implants, and possibly, even the treatment of cancers. The idea here is to tailor-manage platelet activity for different pathologies in individual patients. Such an approach to platelet-based therapy is quite different from the strategies for modulating platelet procoagulant activity constituting modern antiplatelet therapies [8, 60].

Current therapeutic approaches focus on taming the unwanted procoagulant activity of platelets. They entail targeting platelet surface receptors or modulating their autocrine loops. Aspirin remains the most

wide-spread and successful antiplatelet drug for chronic and prophylactic use [91, 100]. It is a cyclooxygenase inhibitor that blocks arachidonic acid metabolism into thromboxane A_2 (TXA2). TXA2 is an important platelet agonist, and, as discussed below, a key element of platelet autoactivation. The second most successful class of antiplatelet agents are ADP receptor antagonists ticlopidine and clopidogrel, prasugrel, and ticagrelor. Introduction of the dual aspirin/ticlopidine (now replaced by clopidogrel) antiplatelet therapy in the late 90 s has, without a doubt, revolutionized interventional cardiology by allowing the wide-spread use of stenting for treating coronary artery occlusion [16, 117]. Schömig et al. [118] and Holmes et al. [54] further discuss the combination of the dual antiplatelet therapy with oral anticoagulation, recommended, in particular, for patients with mechanical heart valves and other similar devices (e.g., LVADs), and a number of other conditions that lead to aggressive thrombosis, such as atrial fibrillation.

The efficiency of antiplatelet agents in modulating platelet activity is limited by the bleeding risk. For this reason, the development of oral GPIIb/IIIa antagonists met with some difficulties: they caused serious bleeding complications [60, 91]. This can be rationalized by the fact that GPIIb/IIIa represents the key point of intersection of many of the platelet signalling pathways (discussed in more detail below), is unique in promoting platelet aggregation by fibrinogen, and stabilizing clot formation. Its inhibition, therefore, works in a nearly all-or-none fashion, limiting the so-called therapeutic window of the drugs (the range of doses where platelet function modulation is maximized while the bleeding risk is minimized). GPIIb/IIIa antagonists are used in acute situations (e.g., during percutaneous coronary intervention), delivered by intravenous or intracoronary injections [42]. These, and other antiplatelet agents, together with the promising developments in this area, have been recently comprehensively reviewed by Jackson and Schoenwaelder [60], K. Schrör [119], Bhatt and Topol [8], and Papp et al. [91] The issues of resistance to aspirin and some of the other antiplatelet therapies are discussed by Fitzgerald and Pirmohamed [27], M. Cattaneo [11], and Papp et al. [91].

When discussing antiplatelet therapeutic agents, the topic of inflammation cannot be avoided. Firstly, platelets are themselves inflammatory mediators [8, 39, 88, 121, 138]. Secondly, aspirin is well-known for its anti-inflammatory and analgesic effects—in fact, originally this was its primary therapeutic use (see ref. [147] for a recent review). This has to do, of course, with its effect on both of the cyclooxygenase isoforms, COX-1 and COX-2. Its antiplatelet effects (mediated by COX-1 inhibition) are apparent at lower doses than the anti-inflammatory ones (mediated primarily by COX-2 inhibition) [91]. Similarly, GPIIb/IIIa antagonists such as Abciximab exhibit anti-inflammatory effects through cross-reactivity with integrin receptors expressed on macrophages [79]. Other antiplatelet agents, such as clopidogrel, appear to exhibit some anti-inflammatory properties, mediated by their inhibition of platelet activity—but this aspect is much less well understood [32].

Cardiovascular diseases (CAD) are currently responsible for more deaths than any other disease [123, 129]. Critical role of platelets in CAD is underscored by the success of antiplatelet therapies in mitigating the consequences of CAD, minimizing the risk of serious complications and death, and of the effects of the disease on patients' lifestyles [60, 91, 100, 123]. The severity of the CAD epidemic dictates the need for the development of new and improved antiplatelet agents [60, 123]. Current strategies, however, continue to focus on the haemostatic functions of platelets. Approaches harnessing their regenerative potential are yet to come.

Efforts to harness the regenerative potential of platelets are already under way in other areas. On one hand, Davies and colleagues observed a correlation between the extent, to which dental implant materials activated platelets, and osteoconductivity displayed by the implants [68, 92, 93]. On the other hand, spurred on by the surgical applications of fibrin gels as surgical glues [61], autologous

platelet-containing fibrin gels started to be used in dental, oral, maxillofacial, and plastic surgery [4, 10, 24, 88, 145]. Reports of their successful applications, however, remain somewhat anecdotal. The key hypothesis that appears to be driving these efforts is that growth factor secretion by the platelets enhances wound healing [6]. This hypothesis is difficult to reconcile with the fact that platelets secrete growth factors with contradictory functions (see below). Mechanistic studies are sparse and numerous open questions remain. In particular, the absence of leukocytes from the platelet-rich plasmas used in therapeutic applications may have an effect on the wound healing process initiated by these products. These doubts notwithstanding, the application of platelet-rich plasmas to wound healing and implant integration is an interesting example of a therapeutic application of platelets.

2. Platelets and their haemostatic activity

Under normal physiological conditions, platelets circulate in a quiescent state. The ability of quiescent platelets to interact with each other, with the normally folded plasma proteins, and with other cells, is limited (Fig. 1). On the contrary, activated, procoagulant platelets aggregate, adhere to other cells, and interact with plasma proteins such as fibrinogen and the factors of the coagulation cascade.

Their ability to aggregate arises from a change in the conformation of the GPIIb/IIIa integrin complex, which then becomes competent to bind fibrinogen that cross-links multiple platelets. As discussed above, this made GPIIb/IIIa an obvious target for antiplatelet medication, and several drugs targeting this receptor were developed. As already mentioned, they are used intravenously or intracoronary in acute situations, but carry with them too high a bleeding risk for prolonged use in chronic situations.

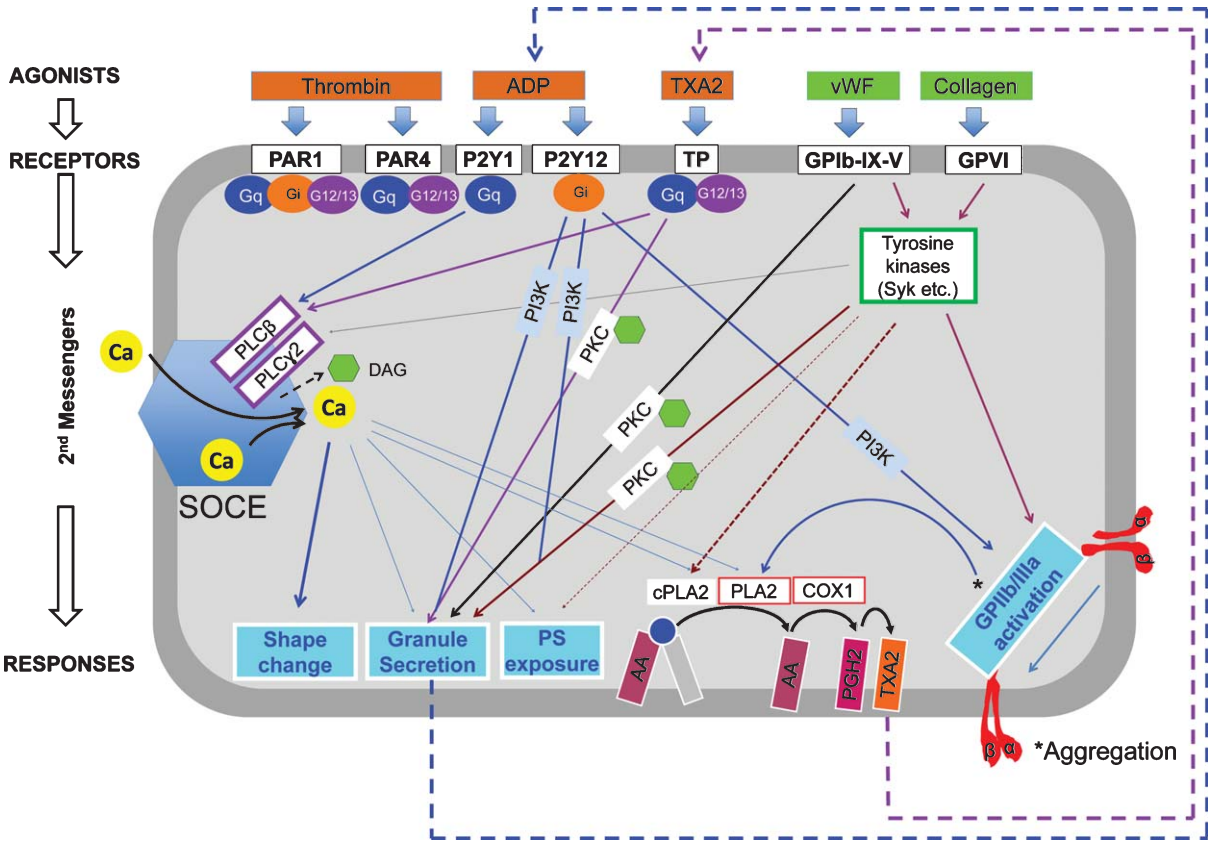
As a part of the activation process, platelets also increase in size, exposing the area previously stored in membrane folds of the open canalicular system (OCS), and change shape. Phospholipid phosphatidyl serine (PS), that activated platelets express on their surface, serves as an anchor for the assembly of clotting factor complexes (tenase and prothrombinase) that ultimately catalyse thrombin formation [7, 35, 85, 135, 151]. Thrombin cleaves fibrinogen into fibrin, and the latter polymerizes to form a clot. As PS expression is also associated with apoptosis, understanding the role apoptotic pathways play in platelet activation became an interesting avenue of investigation [46, 76]. Last but not least, activated platelets release the content of their secretory granules. Dense granules contain small molecules (ADP, serotonin, calcium, etc.) while alpha granules contain proteins, more than 300 growth factors, cytokines, adhesion mediators, and clotting factors [88, 95, 146, 150].

Details of these processes have been studied intently since the 1960s, in what B. S. Coller in his review [16] refers to as the "mechanistic period" in platelet research. The impetus for these studies was given by the development of quantitative assays for measuring platelet activity in coagulation and the discovery of reliable platelet activation markers. These included the platelet adhesion assay, aggregometry, aggregometry coupled with the luciferase assay to quasi-simultaneously measure granule secretion and aggregation, and subsequently—flow cytometry using monoclonal antibodies against platelet surface activation markers. These were later combined with intracellular calcium measurements [16]. Notwithstanding the impact of these tools on unravelling the molecular building blocks underlying platelet physiology, they do have limitations. Firstly, it appears that they are not sufficiently sensitive to distinguish between individual signalling pathways. Secondly, they are not sensitive to non-haemostatic platelet functions. In other words, both unravelling individual signalling pathways in platelets and understanding other aspects of platelet function will require the development of a new "toolbox" of assays and markers.

3. Platelet signalling pathways

The signalling circuitry that controls platelet activation processes is impressively complex and not completely understood. A separate review would be required to cover this topic, and indeed, several are available [16, 17, 22, 60, 77, 80, 90, 136, 137]. Information is also available in textbooks [84]. We will limit ourselves to a very much simplified sketch (Fig. 3) that serves to illustrate some key features.

The extent, to which platelet function is fine-tuned, can be appreciated from the number of different agonists and modulators that control it. These include molecules expressed on subendothelial tissues (collagen, collagen-adsorbed vWF, fibrin), and soluble substances that are secreted or produced by other cells (ADP [30], epinephrine, neuromediators [114], platelet activating factor (PAF) [23, 52]), the platelets themselves (serotonin, ADP, ATP, thromboxane), generated as a part of the coagulation cascade (thrombin, the most potent of platelet agonists), or constitutively present in the plasma (plasma vWF becomes an agonist under conditions of high shear [116]; the role of vWF in platelet aggregation and adhesion at different shear rates was described by Weiss et al. as far as 1978 in what appears to be one of the first reports on the subject [142]. See also ref. [58] for an interesting review). For several of the agonists, there is more than one receptor: P2Y1 and P2Y12 for ADP; [62, 90] protease activated receptors PAR1 and PAR4, [17] as well as GPIb, [109] for thrombin [22]. Some of these are shown in Fig. 3. These receptors have different binding affinities for their agonists and contribute to platelet responses in different ways [3, 18, 62, 128]. Current list of platelet agonists, and their receptors, is most likely incomplete—a point aptly illustrated by the recent account of the role galectins play in platelet activation [113].



Downstream of the soluble agonist receptors are G-proteins: Gq, Gi, and G12/13 (reviewed in refs. [14, 80, 89]). Of note, PRA1 receptor for the strongest platelet agonist thrombin, is connected to all three G-proteins, while the two ADP receptors are connected to one kind of G-proteins each [89]. Collagen and vWF receptors are kinases and act through the tyrosine kinase signalling pathways (Fig. 3) [77].

A key element of platelet signalling pathways is the intracellular calcium rise that follows the interaction of most of the agonists with their receptors. Calcium rise is initiated by the phospholipase C (PLC) hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) that generates IP3. IP3 induces Ca release from intracellular stores into the cytoplasm. The depletion of calcium in the stores triggers its entry from the extracellular space through the STIM1 (stromal interaction molecule 1)/Orai1 Ca-sensing and transport system. This process, called store-operated calcium entry (SOCE, Fig. 3), generates a rise in the intracellular calcium from nM range to uM range; it is described in detail in the reviews by Varga-Szabo et al. [136, 137] There are several forms of PLC; in particular, PLCβ is activated by a G-protein (Gq), while PLCγ2 is activated by the tyrosine kinase pathway (Syk) downstream of collagen and vWF receptors [136, 137].

It should be noted that platelet activation can proceed in the absence of extracellular calcium—as studies performed in the presence of citrate or EDTA indicate [38, 47, 57, 115]. EDTA is known to

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Fig. 3. Some aspects of platelet regulatory pathways are illustrated in this figure. Examples of soluble agonists shown are thrombin, ADP, and thromboxane. Their receptors are coupled to G proteins (GPCRs), the identity of which is indicated below each receptor. Agonists that are typically exposed at surfaces of cells or tissues are represented by vWF and collagen. Their receptors act through the tyrosine kinase pathways (e.g., Syk) [51, 69, 80]. (Collagen also binds to GPIa/IIa, also known as integrin $\alpha_{II}\beta_{I}$, and CD36; these are not shown). The fibrinogen receptor, GPIIb/IIIa ($\alpha_{II}\beta_{III}$ integrin), is a key point of intersection of all of the platelet regulatory pathways involved in procoagulant response (tyrosine kinase, G-protein mediated, etc.) This protein controls the aggregation of platelets into the platelet plug and the thrombus. A prominent second messenger system, Store-Operated Calcium Entry (SOCE), is indicated as a blue hexagon. It is reasonably well-understood and described in detail in Varga-Sabo et al. [136, 137] SOCE is accessed through PLC isoforms; PLCβ, activated by the G-protein Gq, and PLCγ, that is activated via the Syk pathway [136, 137]. Blue arrowheads follow the ADP-mediated pathway from its interaction with the P2Y1 and P2Y12 receptors to the Ca-mediated shape change, GPIIb-IIIa activation, aggregation- and Ca-dependent PLA2 activation and TXA2 production. It should be noted that ADP activates integrin $\alpha_{II}\beta_{I}$, making it competent for high-affinity collagen binding, in the same way it activates GPIIb-IIIa for fibrinogen binding [64]. Purple arrows follow the TXA2 pathways that lead to granule secretion (potentiated by ADP through the P2Y12 receptor). Some of the secretion events are Ca-dependent, while others are not. Black arrow illustrates the effect vWF binding to the GPIb-IX-V complex has on the secretion of ADP [83]. Full brown arrow indicates the calcium-independent pathway connecting GPVI with the granule secretion, and TXA2 production. It is modulated by ADP through its action on the P2Y12 receptor that is coupled to the Gi protein. Dashed brown arrow indicates a pathway terminating in the cytoplasmic PLA2 (cPLA2), another Ca-dependent phospholipase. Both vWF/GPIb-IX-V and collagen/GPVI directly activate GPIIb/IIIa. Grey arrow indicates the Syk activation of PLCγ2, which may be responsible for collagen-induced calcium rise in aspirin-treated platelets. A dotted brown arrow indicates collagen-induced, calcium-dependent loss in the PS asymmetry. This appears to be a synergistic pathway dependent on both the particular shape of the calcium rise signal and the activation of the tyrosine kinase pathway [51]. Isoforms of protein kinase C (PKC), activated by DAG and Ca, are involved in the regulation of the secretion events, independently of the pathway [49]. Gi proteins activate PI3K. G12/13 act through the rho proteins (not shown). Thrombin is the most potent of the platelet agonists, and achieves most of the effects of the other agonists—shape change, calcium rise, GPIIb/IIIa activation and aggregation, and secretion. Its PAR1 receptor is coupled to all three of the G-proteins, PAR4—to two of the three. In comparison, ADP receptors are coupled to one each, and TXA2 receptor is coupled to two. Furthermore, some of the effects of thrombin on the platelets are also exerted through GPIb, thus covering the entire set of the platelet activation pathways. This partially explains why thrombin covers most of the range of the responses induced by other agonists. The individual arrowheads corresponding to thrombin signals are not shown, as they would considerably complicate the diagram. The details of many of the pathways are still being worked out. This figure was adapted from ref. [43].

damage platelets; White et al. investigated which of its effects were reversible and which were not [143]. Similarly, extracellular calcium is not a platelet agonist; this appears to be a source of some confusion, especially in the biomaterials field, apparently because coagulation may be induced by recalcification of citrated blood or platelet-rich plasma. The point here is that platelet aggregation and coagulation cascade reactions depend on the calcium present in the plasma, not the platelet activation step itself. Therefore, platelets may very well become activated in citrate-anticoagulated blood (e.g., as a result of poor handling, blood drawing techniques, or prolonged storage). Nevertheless, it needs also to be remembered that the activation processes in the absence of extracellular calcium are not complete—especially if an ionophore is used as a stimulant [106].

The topic of platelet activation by anticoagulants deserves a separate mention. In particular, heparin was shown to activate platelets at least under some conditions [31, 40].

Hydrolysis of PIP2 also generates DAG (green hexagons in Fig. 3), which activates some of the protein kinase C (PKC) isoforms. Other isoforms are activated by Ca, yet others require both of these co-factors, while some of the more recently discovered isoforms depend on other factors for activation [49].

An important feature of the platelet signalling network appears to be the incorporation of extracellular events (such as secretion and aggregation) into the signalling pathways. This can be illustrated by following the effect of ADP (blue solid arrows in Fig. 3). Its binding to the P2Y1 receptor leads to the activation of PLCβ through the Gq protein and a transient rise in the intracellular calcium level through the SOCE mechanism, which drives platelet shape change. Its binding to the P2Y12 receptor drives GPIIb/IIIa conformational change. Platelet aggregation, which is initially reversible, [90] leads to the activation of PLA2 and thromboxane synthesis. TXA2 is released by the platelets (purple dashed arrow in Fig. 3) and interacts with own receptor, leading to the dense granule secretion and other responses (solid purple arrows in Fig. 3). Aggregation becomes irreversible [90]. Aspirin, which blocks TXA2 production, inhibits dense granule secretion and the second (irreversible) phase of aggregation, but not the calcium rise, the shape change, or the first (reversible) wave of aggregation caused by ADP (Fig. 2 in ref. [103]). In the absence of aggregation, ADP does not lead to thromboxane production or dense granule secretion [12]. On the other hand, aspirin had no effect on the expression of P-selectin or the secretion of β-thromboglobulin (both α-granule markers) by platelets stimulated with ADP in plasma, while inhibiting irreversible aggregation and dense granule secretion as measured by ADP and serotonin release [99, 101]. We also observed P-selectin expression in purified platelets stimulated with ADP [45].

Staged activation of platelets can also be illustrated with other examples. ADP is involved in activating the GPIa/IIa (integrin $\alpha_{II}\beta_I$) [64]. This process allows it to bind collagen with high affinity at low exposure levels (of collagen). Collagen then stimulates GPVI receptor, leading to other responses such as TXA2 production and PS expression (dashed brown arrow in Fig. 3) [51].

Similarly, ADP is involved in the transition from reversible to irreversible aggregates in platelets activated by vWF at high shear [83]. The signalling pathway from the vWF receptor to the secretion of dense granules is illustrated in Fig. 3 with a solid brown arrow. (The effect of aspirin was not investigated in that study, but unless GPIb/vWF interaction simultaneously leads to ADP secretion and TXA2 production, a three-stage process is actually involved: GPIb/vWF leading to ADP secretion which in turn leads to TXA2 production as described above). Furthermore, a similar effect is observed in the case of a non-physiological agonist, phorbol 12-myristate 13-acetate (PMA). PMA does not act via a receptor but directly activates protein kinase C (PKC), located downstream of the PLCβ-dependent calcium rise. It appears that the elevation of the intracellular calcium in PMA-treated platelets occur via an autocrine mechanism involving the secretion dense granules [48].

Such "staging" of the activation process appears to be a general feature of the platelet signalling pathways, as is the integration of extracellular events into the signalling pathways. This strategy serves an amplification function but also provides a key control mechanism for limiting platelet activation. One practical consequence of this is that platelet signalling is best studied with purified platelets, because various components of plasma may complete the signalling pathways without the control or knowledge of the investigator.

Ultimately, platelet signalling network funnels all of the incoming signals towards responses such as shape change, GPIIb/IIIa activation (and consequent aggregation), PS expression, and granule secretion through the amplifying autocrine steps and synergistic pathways. The response to each individual agonist, however, is different. Some of the differences between ADP and other agonists have already been illustrated above, as were some of the TXA2-specific effects. Differences in responses to individual agonists arise through several mechanisms. On one hand, there is evidence of different threshold calcium concentrations required for the different responses, such as shape changes, aggregation, and secretion [106]. In that study, intracellular calcium levels were measured with Ca-sensitive fluorescent dies in platelets treated with various agonists and a calcium ionophore ionomycin. The same study showed lower intracellular calcium levels accompanying responses in platelets treated with thrombin, implying the existence of Ca-independent signalling pathways. For detailed discussion of calcium-independent platelet activation pathways, the reader is referred to refs. [98, 102–105]. The two sets of pathways—calcium dependent and calcium-independent—act in synergy with each other.

On the other hand, the temporal profile of the calcium rise also plays a role. A well-documented example of this is the difference between PAR1 and PAR4 thrombin receptors in terms of the timing and duration of the calcium rise and in terms of the functions the two receptors play in early and late stages of thrombus formation [3, 18, 22, 128].

Ultimately, a platelet is akin to a piano (Fig. 4): its response is both carefully timed and spatially orchestrated to achieve a specific "melody" of the plug formation, clot formation, clot retraction, and subsequent wound healing processes, each initiated at the right location of the wound site and at the right time. This choreographed sequence of events is revealed, for example, at the level of the blood clot organization, with differences in platelet activation, packing density, fibrin formation, and transport of material through the clot, in the different areas of the clot [86, 126]. Some exciting work is now being done in this area, also in terms of linking clot organization to the way platelet signalling network integrates information from the agonist receptors [86, 126]. Indeed, differences in responses to individual agonists and their synergies hold the key to the spatiotemporal organization of the platelet response [86]. They also offer potential points for selective intervention, exploited by such agents as aspirin and P2Y12 receptor antagonists [60].

This kind of organization presupposes the ability of the signalling network elements to integrate the incoming signals over a period of time and over the different input channels (receptors for different agonists). Much remains to be learned about this sort of signal integration in the regulation of platelet activity even in the context of their haemostatic function, but especially—concerning the integration of cytokine receptors, pattern-matching toll-like receptors for microbial recognition, interactions with other cells, and other processes concerned with non-haemostatic platelet functions, [15, 107, 121, 148] into the signalling network.

To illustrate some of the limitations of the current approaches for studying differences between signalling pathways of the different agonists, we describe what is known about the secretion of α- and dense granules. These two sets of granules are different morphologically and in terms of their contents. Logically, the machinery that regulates their secretion also appears to be different: there are reports of

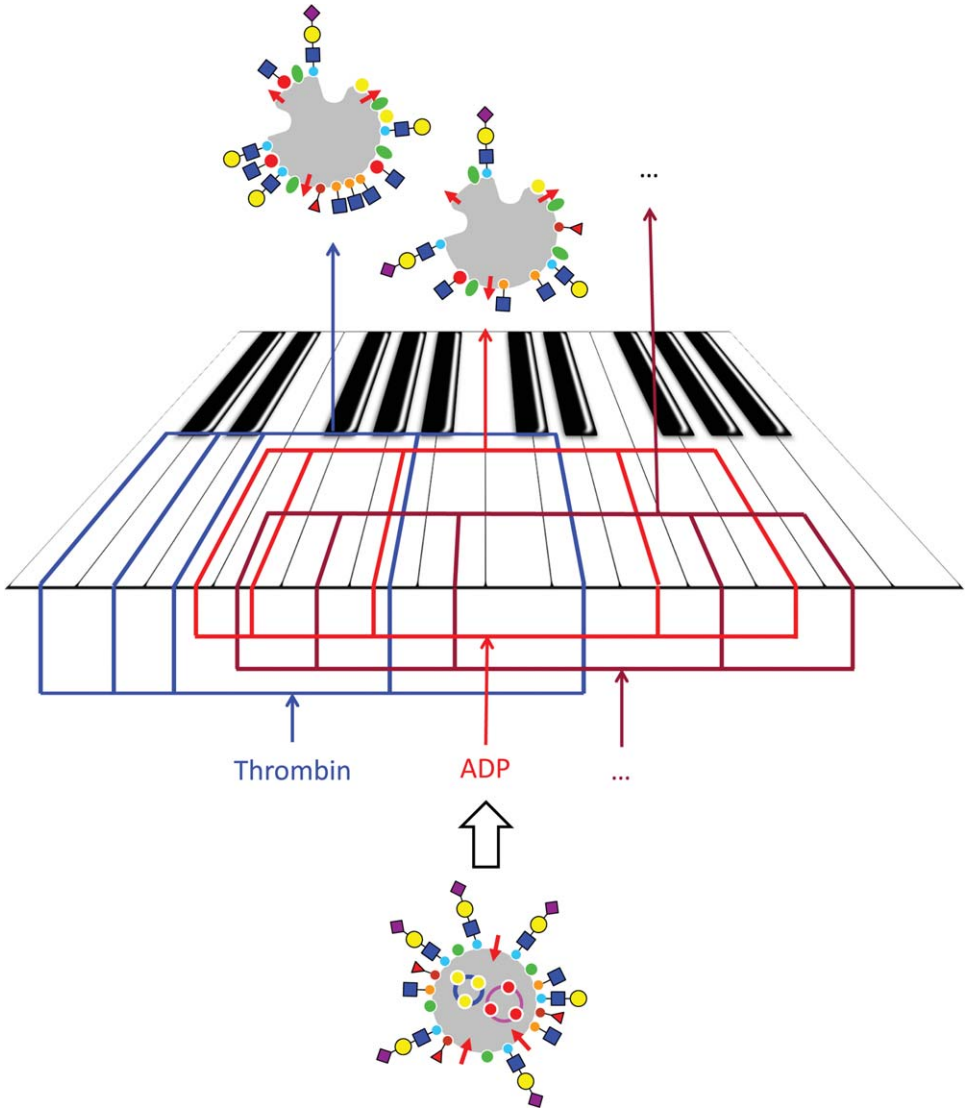

Fig. 4. Spectrum of platelet responses to various stimuli. This figure is meant to illustrate that platelet activation by different agonists is subtly different. The platelet signalling pathways are likened to a piano, with each agonist eliciting a unique "chord" of a response. Some of the keys overlap between agonists, but some are different. In this way, various combinations of agonists produce the melody of platelet responses. Platelets are depicted as grey circles, as in Fig. 1. Differences between platelets are illustrated in terms of different expression levels of CD62P (red circles) and CD63 (yellow circles) on platelets stimulated by thrombin and ADP, and in terms of differences in platelet surface glycosylation. The latter are based on our recent work on platelet surface glycosylation and its changes caused by agonist treatment [43, 45]. For example, thrombin activated platelets are shown to exhibit higher levels of expression of N-acetyl glucoseamine (blue squares) and lower levels of sialic acid (purple rhombi) than ADP-stimulated ones. N-acetyl glucoseamine is also shown to cluster on the thrombin-activated platelets. This figure is intended only as an illustration of the finding that platelets express different sets of carbohydrates, depending on how they were stimulated. The inspiration for this figure came from the piano analogy used by J. Tepperman and H. M. Tepperman in their endocrinology text to describe the role of sympathetic nervous system and adrenal medulla in the fight-or-flight response (Tepperman J, Tepperman HM. *Metabolic and endocrine physiology: an introductory text*. Year Book Medical Publishers, Chicago, 1987 (fifth edition).

the GTPase Rab4 being involved in regulating the α-granule secretion, and of Rab27b—in the secretion of dense granules [122, 131, 149]. There are several examples of differential secretion of α- and dense granules: [9, 28, 44, 66, 130].

- Investigating the effect of cytoskeleton rearrangements on granule secretion Flaumenhaft et al. [28] observed opposite effects: high concentrations (above $\sim$ 100 uM) of latrunculin A, which facilitates actin cytoskeleton disassembly, inhibited α-granule secretion but stimulate dense granule secretion in TRAP- or PMA-stimulated platelets. That study was done on platelets purified by gel filtration from citrate anticoagulated blood. Flow cytometry was used to monitor the expression of α-granule marker CD62P. Dense granule secretion was monitored by measuring levels of ATP and serotonin release.
- Taylor et al. [130] using citrate-anticoagualted platelet-rich plasma and flow cytometry assays for CD62P and CD63 expression examined the dependence of granule release on the type and concentration of various agonists. They found that the kinetics of the expression of the two markers was different and granule secretion events that did not correlate with platelet aggregation responses.
- Broberg et al. [9] using citrate anticoagulated platelet-rich plasma, identified surface-adsorbed vWF as a specific regulator of CD62P expression on adhering platelets; adsorbed vWF did not have such an effect on the release of other markers used in that study (PF4 and ATP for α-granules and dense granules, respectively). This study is noteworthy because it also indicates that CD62P expression and secretion of α-granule contents are not always correlated.
- Kang et al., [66] studying adhesion of platelets to surfaces of various peptides from platelet-rich plasma and whole blood, showed that serotonin release (dense granules) correlated with the number of adhering platelets but β-thromboglobulin release (α-granules) exhibited a maximum for a particular set of peptides. Unfortunately, in their study, the α- and dense granule release were not compared under identical conditions.
- In our own study of platelets, purified from citrate-anticoagulated blood by centrifugation, adhering on TiO_2 in the absence of extracellular calcium, we found that CD63 but not CD62P was expressed [44].

Although it is clear that secretion of these two sets of granules is regulated by separate pathways, [144] neither the physiological significance, nor the physiologically relevant triggers of such differential behaviour are known; individual signalling pathways connecting agonists to the secretion of each set of granules have also not been identified—and this is despite the fact that markers for the secretion of each of the sets of granules have been readily available for quite some time.

4. Need for new markers and functional assays to monitor platelet activation

The limitations of the existing repertoire of markers and assays for studying platelet activation are apparent already at the level of platelet haemostatic function. Considering their other functions, the situation grows more complex and the need for new assays and markers for studying platelets becomes more acute.

A reasonable place to start developing such assays is α-granule secretion. Given that α-granules contain active substances with contradictory functions, it is logical to suppose that their secretion is thematically controlled. Initial reports by Italiano et al. appeared to support this idea. These authors reported that pro-angiogenic vascular endothelial growth factor (VEGF) and thrombospondin-1 were packaged into a

different population of a-granules than the anti-angiogenic endostatin and basic fibroblast growth factor. Fibrinogen and von Willebrand factor (vWF) were also found in distinct α-granule subpopulations. Moreover, these authors also reported their thematic secretion in response to stimulation with different agonists [59].

Cryo-electron tomography confirms the notion, that different kinds of α-granules exist within the platelets [134]. It also shows that vWF and fibrinogen are localized to different granule subtypes. Another, earlier observation of differences in granule motion in adhering platelets that correlated with the distribution of vesicle-associated membrane protein (VAMP) isoforms, similarly confirms the notion that there are different α-granule subpopulations in the platelets [94].

However, other conclusions of Italiano have been challenged. Peters et al. [94] noted that VEGF co-localized with different VAMP isoforms (thought to be responsible for the secretion of different granule subpopulations). In a study of 15 different α-granule proteins, Kamykowski et al. reported no clustering patterns of pro- vs. anti-angiogenic proteins [65]. They did report that fibrinogen and vWF were spatially segregated, but concluded that α-granule content could be organized in different partitions, or zones, within the granules. Subsequently, Rönnuland et al. used super-resolution fluorescence microscopy (STED; one of the techniques the developers of which received a Nobel Prize in Chemistry in 2014) to examine the distribution of various α-granule cargo. They showed no evidence of thematic packaging, [108] suggesting that the resolution of confocal microscopy was not sufficient to discern such patterns. In the same study, Rönnuland et al. also showed that granule contents was organized into structures with sizes much smaller than the granules themselves, suggesting sub-granule zones, and that treating platelets with different agonists lead to distinctly different distributions of fluorescently labelled molecules. However, Jonnalagadda et al., analysing the release of 28 molecules known to reside in the α-granules by micro-ELISA arrays from platelet stimulated with different agonists as a function of time, did not observe agonist-specific release patterns. They concluded that the release was stochastic rather than thematic, and that agonist strength and kinetic effects played an important role in determining platelet secretion profile [63].

This survey of the latest attempts to identify markers of distinct platelet functional states through differential α-granule secretion shows, that this approach has so far not met with success. With the goal of expanding the range of markers that could be used to study various aspects of platelet physiology, we have recently examined changes in platelet surface glycosylation induced by treating platelets with various physiological and non-physiological agonists [43, 45]. The surfaces of platelets, like surfaces of all cells, are covered with carbohydrates. Most of them originate from cell surface glycoproteins [87]. Surprisingly little is known about the platelet carbohydrate coat, and even less is known about how it changes upon platelet activation:

- Structures of the carbohydrates linked to several of the surface glycoproteins have been elucidated [71, 72, 132, 133].
- Carbohydrate moieties on the GPVI complex are thought to be involved in its interactions with collagen [74].
- The chemical nature of the platelet carbohydrate coat changes as platelets age [140]. These changes serve as signals for platelet clearance by the liver macrophages and hepatocytes, [53, 111, 124] by mechanisms similar to those involved in clearance of aged erythrocytes and glycosylated plasma proteins [2].
- Carbohydrate-binding proteins selectins [1, 20] are present on platelet, leukocyte, and endothelial cell surfaces. They mediate adhesion and rolling of leukocytes, and CD62P – P-selectin—is involved in the platelet-leukocyute and platelet-endothelial cell interactions. This topic has already been

addressed in the very beginning of this article, when platelet role in maintaining vascular integrity was discussed [67, 139]. Selectins belong to the same family of the carbohydrate binding proteins (C-type lectins [1]) as the hepatic asialoglycoprotein receptor (ASGPR), already mentioned in the context of platelet clearance [41, 112].

- Galectins, [19] members of another lectin family (β-Galactose-binding proteins), interact with platelet surface carbohydrates and act as platelet agonists *in vitro*, but their *in vivo* role is not clear [113].
- Platelets release glycosyltransferases and regulate their activity as a part of the activation process, [140] but the role or the mechanisms of this regulation in the context of platelet activation are not understood.
- Differences in the composition of the platelet carbohydrate coat between platelets from healthy individuals and those from uremic or diabetic patients have been reported [81, 82].
- While progress is being made systematically analysing the glycome of, e.g., the immune system [50] and vascular endothelium, [120] a literature search using keywords "glycome" and "platelets" returns six publications, two of which are meeting abstracts. Similarly, among the entries found on the website of the Consortium for Functional Genomics (http://www.functionalglycomics.org/static/index.shtml), only one involves platelets [56]. In this context, a very recent review of the role surface carbohydrates play in platelet-endothelial cell interactions should also be mentioned [26].

Recently, we used fluorescently labelled plant lectins [1] to investigate platelet surface glycosylation changes as a result of agonist treatment. Using a panel of nine lectins and seven different agonists, we detected changes in platelet surface glycosylation upon stimulation and showed that these changes were agonist-specific (see also the illustration in Fig. 4) [45]. Some of them could be correlated with the expression of the granule-specific glycoproteins (CD62P for the α-granules and CD63 for the dense granules), while other changes did not correlate with secretion events. Our results allowed us to conclude that differences in the individual signalling pathways between different agonists were responsible for the differences in lectin binding to platelets activated with these agonists. While many questions remain to be answered, our study does suggest monitoring platelet surface glycosylation changes as a promising approach to studying platelet activation in the laboratory and to developing clinical diagnostic assays. It certainly offers much more in terms of diversity than existing antibody or soluble markers.

5. Development of assays for studying non-haemostatic platelet functions

While the development of new labels for studying platelet activation is a necessary step for unravelling their physiology and providing quantitative data for systems-biology approaches to model their signalling networks, all of the above studies share the same fundamental flaw: they focus on the range of agonists that stimulate procoagulant platelet functions. In order to understand the diversity of platelet functions, *in vitro* model systems need to be developed that transcend the reliance on the traditional set of platelet agonists. Possible search directions include co-cultures with endothelial cells and mesenchymal stem cells; interactions with leukocytes; use of inflammatory mediators as agonists or functional modulators; interactions with implants—because in this case, platelets are subjected to activating conditions that differ significantly from the natural ones they encounter in the body, and this may offer a unique insight into their physiology. Such a conceptual switch from the "coagulation" frame of mind is likely to lead to breakthroughs in the understanding of platelet regulation that will translate into their use in personalized, regenerative therapies. Indeed, such approaches are being explored [25].

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