Platelet functional responses and signaling: the molecular relationship. Part 2: Receptors.

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Abstract

Small, non-nuclear cells, platelets, are primarily designed to form aggregates when blood vessels are damaged, stopping bleeding. To perform this function, platelets can implement several functional responses induced by various agonists and coordinated by a complex network of intracellular signaling triggered by a dozen of different receptors. This review, the second in a series, describes the known intracellular signaling pathways induced by platelet receptors in response to canonical and rare agonists. Particular focus will be on interaction points and "synergy" of platelet activation pathways and intermediate or "secondary" activation mediators that transmit a signal to functional manifestations.

Keywords: hemostasis; platelet activation; platelet intracellular signaling; thrombosis; thrombus formation

Introduction

Platelets are blood cells that play a key role in the process of stopping bleeding [1], as well as in the formation of a hemostatic plug in trauma [2], maintaining the integrity of blood vessels [3–5] and modulating immune responses [6–8]. The protein composition of platelets is predetermined, although minor de novo protein synthesis is still present [9]. However, the main functional responses of the platelet (Figure 1) are independent of protein synthesis [10].

The first response to a meeting with an activator is a change in shape. Initially, discoid cells significantly increase their surface area due to the unraveling of the membrane folds inside in the form of an open canalicular system [11]. The shape change occurs in several stages [12]: microtubule depolymerization [13,14], rearrangement of the actin cytoskeleton [15] and the sequential formation of the filopodia and lamellipodia [12]. Platelets can spread on the

surface, and this process is majorly governed by the actin cytoskeleton [12,16].

The key functional response of the platelet, probably, is the transition to a proaggregant state, in which platelet integrins $\alpha\text{IIb}\beta 3$ [17] pass into a state with high affinity for fibrinogen [18] and provide platelet "adhesion" through the " $\alpha\text{IIb}\beta 3$ - fibrinogen - $\alpha\text{IIb}\beta 3$ bridges" [19]. Thus, the path from receptors to the activation of integrins is called "inside-out", while the enhancement of platelet activation, in which integrins already act as receptors, is called "outside-in" [20].

Another important functional response of the platelet is granule release [10]. Platelets contain three types of granules: α - and δ -granules and lysosomes, and it is believed that the release of alpha-granules occurs at weaker stimulation. In contrast, the release of dense granules is a sign of strong platelet activation [21]. α -granules contain various proteins, glycoproteins, and chemokines, while dense (δ) granules contain primarily low-molecular substances and ions [22]. All substances secreted by platelets play an essential role in the secondary activation of platelets and the regulation of the lumen of the vessel and the immune response [10].

Under certain conditions, for example, upon stimulation of platelets with the combination of thrombin and collagen, mitochondrial-dependent necrosis of a subpopulation of platelets occurs, which at the same time lose the capability to aggregate, exhibit a negatively charged phospholipid phosphatidylserine, which is one of the landing sites for tenase and prothrombinase complexes, which significantly accelerates the work of the blood plasma coagulation cascade [23,24]. This population is called "procoagulant". It is assumed that many procoagulant platelets in the thrombus nucleus promote the acceleration of fibrin polymerization, which strengthens the thrombus. [25,26]. Transitions of the platelets between different states with different

degrees of activation are shown in Figure 1.

A vast network of receptors and signaling pathways of platelets regulates implementing the listed functions where and when required [10]. How does it work? It turns out that the basic principles of information transfer are applicable to living cells. Namely, the system must have a “source”, “transmitter,” and “receiver” (Fig. 2). From the point of view of the platelet, the external signal is processed by receptors, which transmit signals to the inside of the cell to the transmitters – secondary messengers. This review will focus on analyzing how various platelet activators regulate the concentrations of secondary messengers, which, in turn, control the listed functional responses, which will be discussed in the third part of this series.

1. Secondary messengers of the platelets

The platelet receptor network can be roughly divided into several sub-networks, each controlled by its own set of secondary messengers, which may overlap in some cases. It can be argued that secondary messengers are at the center of intracellular signaling: they “collect” information from receptors and “transmit” it to functional systems. Therefore, in this review, we will look at how the concentration of the secondary messengers in the

platelets is regulated. In this case, one should not forget that platelets are non-nucleated cells, i.e., they have no pathways that induce protein synthesis. Below, we briefly list the primary, secondary platelet messengers.

Based on the previously published works [27–29], which aligns with our vision, the critical secondary messenger in the platelet is the cytosolic free calcium ions. Under resting conditions, its low levels are maintained by membrane ATPases PMCA and SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) [23,30], which “pump out” calcium from the cytosol to the extracellular space or to intracellular stores, respectively [23,29]. Calcium mobilization (i.e., release) can occur via varied membrane channels [23]. Usually, inositol-1,4,5-triphosphate (inositol-1,4,5-triphosphate, IP_3), which is generated by the phospholipase C on the plasma membrane, activates receptor channels to IP_3 (IP_3R) [31,32]. Based on the studies of calcium signaling in single cells, an increase in calcium concentration during platelet activation does not occur uniformly but in the form of individual spikes [29,33], which merge, possibly due to IP_3R clustering [34].

While the primary storage for calcium ions in platelets is the endoplasmic reticulum, calcium adhesions can also pump calcium into platelet

Figure 1. Different degrees of the platelet activation in hemostasis. Upon weak stimulation, platelets pass into a weakly activated state, in which there is no clustering of platelet integrins and no significant change in the shape of platelets. This weak activation is reversible, and it corresponds to the state of platelets in the outer layers (“coat”) of the thrombus. Upon stronger activation, platelet shape significantly changes. Platelets become irreversibly activated and aggregate. The secretion of platelet granules also occurs. At the maximum degree of activation, platelet mitochondria collapse, and platelets pass into a procoagulant state, exposing phosphatidylserine, which significantly accelerates blood plasma coagulation.

Figure 2. The basic scheme of signal transduction by a non-nucleated cell. From the extracellular environment comes some “signal” that activates the receptor. This leads to secondary messengers, which, passing through the “amplification”, induce intermediate and then functional responses. The system also contains regulators that play the role of positive or negative feedback loops.

mitochondria through a calcium uniporter. Calcium can also be pumped out of mitochondria through the NCLX pump [23]. However, strong activation, mitochondrial overload with calcium, a drop in mitochondrial potential, opening the mitochondrial pore, and mitochondrial collapse can occur. All this leads to necrosis and the transition of the platelets to the procoagulant state [23]. For example, it has been shown that if there are fewer mitochondria in platelets, then they become more prone to become procoagulant [35]. There are also hypotheses about the initial pre-determinism of a part of platelets to the transition to a procoagulant state, which may also be related to the number of their mitochondria [36]. Procoagulant platelets can be formed not only by the pathway of necrosis but also by the classical pathway of mitochondria-dependent apoptosis, induced, for example, by inhibitors of Bcl-proteins (e.g., ABT-737) [37].

IP₃ is also a secondary messenger, as it is formed due to phospholipase C activity combined with diacylglycerol (DAG) from a membrane phospholipid phosphoinositide-4,5-bisphosphate (PIP₂). Interestingly, the concentration of IP₃ in the platelet cytosol also depends on the enzyme IP₃-3 kinase (IP3K) [38]. IP3K catalyzes the phosphorylation of IP₃, thereby directly reducing the number of secondary messengers available to interact with IP₃R. According to [39], IP3K is activated by high concentrations of calcium-bound calmodulin, which are available only at high concentrations of cytosolic calcium (> 1 μM). It is assumed that upon platelet activation, IP₃ concentration reaches 1-2 μM (this concentration is enough to open IP₃R completely [23,28]), but it should be noted that experimental data on the absolute concentration of inositol-1,4,5-triphosphate in platelets are absent (despite the fact that this is the most active form of IP₃, other forms are also observed in intracellular signaling, primarily inositol-1,3,4-triphosphate, the concentration of which

can be much higher, and experimental separation of these substances is difficult) [40].

A secondary messenger comparable in importance to calcium is the membrane concentration of the phospholipid phosphoinositide-3,4,5-triphosphate (phosphoinositide-3,4,5-trisphosphate, PIP₃). Phosphorylated residues of inositide — phosphoinositides — account for 10–15% of the lipid composition of the inner layer of the plasma membrane of platelets [41]. Among membrane phosphoinositides, the most abundant is PIP₂, which is synthesized from phosphoinositide-4-phosphate by phosphatidylinositol-4-phosphate-5-kinase (PI4.5K) [41]. PIP₂ can independently act as a docking site for proteins associated with membranes: PIP₂ is a docking site for talin, a key protein required for the association of platelet integrins and the actin cytoskeleton [42,43]. Studies in mice deficient in specific isoforms of PIP5K indicate that several distinct pools of PIP₂ exist within the platelet membrane; PIP₂, produced by PIP5Ky, is important for membrane attachment to the underlying cytoskeleton, while PIP₂, synthesized by PIP5Kα and PIP5Kβ, is used as a substrate for secondary messengers [44]. PIP₂ is a substrate for the two key signaling enzymes, PLC and PI3K, which will be discussed below. Phosphoinositide-3-kinase (PI3K) phosphorylates various phosphoinositides at the 3rd carbon atom. PIP₃ is the most important of its products. [45]. PIP₃, like PIP₂, is recognized by specific PH-domains of proteins. It is also recognized by the PX and FYVE domains. Phosphoinositide signaling is associated with membrane docking and subsequent activation of proteins containing PH domains, such as PLCγ2, Btk/Tec, and serine / threonine kinase Akt [46]. Akt phosphorylation is the key effector of PI3K-dependent PIP₃ generation in platelets. It is noteworthy that Kindlin 3, a component of the platelet integrin activation complex that controls the avidity of integrins to fibrinogen, has an atypical FERM domain that also contains a PH domain [47].

PIP₃ clusters can be considered as the governing centers for signal propagation [42]. On the other hand, phosphoinositide signaling in platelets is regulated by lipid phosphatases (PTEN, SHIP1, SHIP2), which, by decreasing the PIP₃ level, suppresses PI3K-dependent platelet activation [17,48]. In particular, for mice, SHIP1 plays a major role in the downregulation of PIP₃ concentrations [49], however, its deficiency does not affect the “inside-out” activation of integrins [50].

The last secondary mediator of platelet signaling is the cytosolic concentration of cyclic nucleotides - cAMP and cGMP [51]. Cyclic nucleotides are generated by cyclases: adenylate cyclase (cAMP from ATP) and guanylate cyclase (cGMP from GTP) and are converted into monophosphoribonucleotides by low-specific phosphodiesterases (PDE) [52]. While PDE activity is mainly regulated by the availability of substrates, cyclase activity can be either increased or decreased by external signals. Thus, the concentration of cyclic nucleotides in platelets can change in both directions [52,53]. An increase in the amount of cAMP in the cell leads to the activation of cAMP-dependent protein kinase A (PKA) [54]. Activate PKA phosphorylates many regulatory proteins such as VASP (vasodilator-stimulated phosphoprotein). In addition, PKA indirectly regulates calcium signaling by phosphorylating IP3R and PMCA [51]. It is known that an increase in the level of cAMP suppresses all functional responses of platelets; however, the mechanisms of this suppression have been studied superficially. Perhaps phospho-proteomics of platelets in response to forskolin (an activator of AS) will give answers here [17].

2. G-protein coupled receptors (GPCRs)

Approximately half of the platelet receptors are serpentine receptors, otherwise called 7TM receptors or G-protein coupled receptors (GPCR) [55]. GPCRs are one of the largest protein families in humans. GPCRs recognize a wide variety of extracellular signals and control a variety of cellular and physiological responses [56]. GPCRs transmit signals into the cell by activating trimeric G-proteins. These proteins at rest exist as heterotrimers of α , β , and γ subunits, where the α subunit is associated with GDP. The G α GDP $\beta\gamma$ complex can associate with GPCR. When bound to an extracellular ligand, GPCR is activated and functions as a GEF (Guanine-nucleotide Exchange Factor) for G-proteins, catalyzing the exchange of α -subunit-associated GDP for GTP. This leads to the dissociation of the complex into activated GPCR, G α GTP, and $\beta\gamma$, which can independently transmit signals to various downstream effectors. Over time, GTP to G α is hydrolyzed back to GDP via the intrinsic GTPase activity of G α or via GAP (GTPase-activating protein). GAP-like activity is exhibited by direct targets of

G α GTP, for example, PLC β (phospholipase C β), or specialized proteins of the RGS family (Regulator of G-protein Signaling) [57]. G α GDP can bind $\beta\gamma$ and restore the original complex. It is believed that receptor activation leads to a significant increase in steady-state concentrations of free G α GTP and $\beta\gamma$ and a corresponding intracellular signal. After some time, activation decreases as a result of the disappearance of the ligand [58] or removal of the receptor from the cell surface (desensitization due to internalization) [59]. Under certain conditions, covalent unlinking of the receptor with G-proteins occurs. The homogeneity of G-protein activation by GPCR raises the problem of specificity in the GPCR signal, considered in the works of V.L. Katanaev et al. [56,60–62]. This problem is aggravated by heterogeneity in GPCR signaling: receptors and effectors usually do not distinguish between different $\beta\gamma$ subunits [63]. Moreover, receptors can activate G proteins containing different G α subunits [64]. There are multiple enzymes activated by G-proteins or simply localized by G-proteins at the membrane; we will consider only the most important ones for platelet activation.

Due to the importance of calcium signaling, we start with phospholipase C β (phospholipase C, PLC β), which hydrolyzes PIP₂ to inositol 3,4,5 triphosphate (IP₃) and diacylglycerol (DAG). PLC β activation consists in its localization at the plasma membrane close to its substrates [65,66]. It can be speculated that the longer the PLC stays near the membrane, the higher its activity. The key factor keeping PLC β near the membrane is type q G α GTP [23]. However, G $\beta\gamma$ is also able to retain PLC β (especially type 3 PLC β) near the membrane independently from G α , and together with the α -subunit, the amplification of attraction can be more than 6-10 times [57]. Moreover, activation by intracellular calcium concentration is also of essential importance for PLC [67,68]. According to [68], PLC has several Ca²⁺-binding sites, but only one of them is catalytic [69]. According to [70], the mutant type PLC, in which all calcium-binding sites are absent, except for the TIM catalytic cylinder, has kinetics similar to the wild type, which is described by the Hill equation with positive cooperativity. According to proteomics data [71], there are about 2000 molecules of PLC β 2 and PLC β 3 in a platelet, which can create a sharp increase in the IP₃ concentration in the cytosol with the rate of 200-400 s⁻¹. The second product of PLC operation is diacylglycerol (DAG) [46], which remains in the membrane and becomes an important docking site for many enzymes containing the C1 domain [72]. The most important DAG-activated enzymes are type C serine/threonine kinases (PKC), various isoforms of which are activated by binding to DAG and, sometimes, calcium, which leads to activation, degranulation, and changes in the shape of platelets [72]. Human platelets also express

several diacylglycerol kinases (DGKs) responsible for the phosphorylation of DAG. Inhibition of DGK is known to attenuate platelet calcium responses to activation, which highlights the importance of DAG for intracellular signaling in platelets[73].

The second enzyme activated by G proteins is called phosphoinositide 3 kinase γ (PI3K γ). The most characterized members of the PI3K family are PI3K class I, which are heterodimeric proteins: they consist of a catalytic subunit (p110 α , p110 β or p110 δ) linked to an SH2-containing regulatory subunit (five types, with p85 α being the most common). p110 β associates with the plasma membrane via the G $\beta\gamma$ subunits. PI3K class IB (PI3K γ) consists of the p110 γ catalytic subunit associated with the regulatory subunit (p84), which is also activated by binding to the G $\beta\gamma$ subunit [74]. The most important for platelet is PI3K activation from P2Y₁₂ [20,42], since its activation results in the release of the largest number of G $\beta\gamma$ subunits.

G12/13 - activates RhoA (GTPases Ras homolog gene family, member A) and Rho kinases. Activated Rho-kinase stimulates the phosphorylation of myosin light chains MLC (Myosin Light Chain) [75], thus participating in the platelet shape change and cytoskeleton rearrangement. Furthermore, RhoA is directly involved in granule secretion [18].

The last protein controlled by the platelet G-proteins, paradoxically, is adenylate cyclase (AC) [76,77]. The paradox here is related to the fact that AC controls the concentration of cAMP and, for many cells, for example, for myocytes, is the main enzyme in the regulation of energy metabolism. Since platelets live only a few days, the significance of glycogen stores and glycolysis/oxidative phosphorylation activity in platelets is questionable [78]. As has been mentioned earlier, the key role of AC in platelets is to control the concentration of cAMP. The dominating type of AC in platelets is AC3, a membrane enzyme that is activated upon binding to the Gs α GTP subunit and which is inhibited upon binding to the Gi or Gz α GTP subunit [79].

One of the interesting features of GPCR receptors is the possibility of their desensitization as a result of contact with an activator [80,81]. It is believed that desensitization is necessary for cells to regulate their responses to tonic signals, which can lead to pathological hyperactivation and death [80]. Upon activation of the GPCR receptor and dissociation of G α from G $\beta\gamma$, the GPCR itself can be phosphorylated by G-protein associated kinases (GRK). This leads to the landing on the GPCR of-arrestin and clathrin. PI3K also plays an important role in the desensitization process, as well as β -arrestin binds to phosphorylated GRK GPCRs, activate and produce PIP₃, which is necessary for recruiting the AP-2 adapter protein. AP-2, clathrin, and β -arrestin trigger endocytosis of the GPCR receptor [80]. Thus, the receptor is inaccessible for both the agonist

and the G-proteins. Desensitization of GPCR is reversible: upon internalization, dissociation of AP-2 and clathrin occurs, which decreases the affinity of β -arrestin for phosphorylated GPCR molecules [80]. This allows non-specific phosphatases (such as PP2) to dephosphorylate the GPCR. Dephosphorylated GPCRs return to the platelet plasma membrane and can again form complexes with G-proteins [80,81].

3. Platelet activation by thrombin

Thrombin is one of the key platelet activators and the key enzyme of the blood coagulation system. Thrombin activates platelets by binding to PAR (Protease-Activated Receptors). On the surface of platelets, there are two kinds of these GPCRs: PAR1 and PAR4 in humans and PAR3 and PAR4 in mice. Thrombin cuts off a small oligopeptide from the N-terminus of these receptors, which subsequently activates the corresponding receptor [82] and leads to instant platelet activation. For humans, the PAR1 receptor is considered to be the most important one since it has a higher affinity for thrombin than PAR4 (50-200 pM and 10-100 nM, respectively). PARs activate Gq- [23] and possibly G12/13 [83]. Gq signaling leads to an increase in the concentration of free calcium ions in the cell cytosol. At thrombin concentrations even of the order of 0.1 nM, the concentration of calcium ions in platelets can instantly increase tenfold. As we showed earlier [23], platelets need a pair of receptors with similar signaling pathways in order to expand the range of sensitivity to thrombin (together PAR1 and PAR4 are able to distinguish from 0.1 to 100 nM thrombin) and to increase the duration of the calcium response while maintaining a rapid initial response.

It is known that thrombin can cause the decrease of the concentration of cAMP in platelets via inhibition of adenylate cyclase (which catalyzes the conversion of ATP to cAMP) through the Gi protein associated with the thrombin receptor, or indirectly, via promoting the release of ADP, and an increase in the activity of phosphodiesterases (enzymes that catalyze the hydrolysis of cAMP in AMP). However, it is assumed that this effect is mediated by the P2Y₁₂ receptor activation by secreted ADP [21,42].

In addition to PAR receptors, thrombin can bind GPIb-V-IX. It is assumed that binding to GPIb enhances thrombin affinity to PAR receptors, dramatically accelerating their proteolysis, thereby accelerating PAR-dependent platelet activation [84].

Thrombin is one of the most potent platelet activators and leads to platelet aggregation, the release of ADP and ATP, synthesis of thromboxane A₂ (TxA₂), and, like collagen, can lead to the strongest degree of platelet activation - procoagulant activity. These signaling paths will be described later.

4. ADP-induced platelet activation

ADP is contained in dense platelet granules, which are secreted upon platelet activation [85]. Vascular endothelial cells, if damaged, also release the ADP they contain. When ADP is added to the system *in vitro*, platelet phospholipase A2 (PLA2) and cyclooxygenase 1 (COX1) become activated and produce TxA2 from arachidonic acid [86]. ADP also triggers calcium signaling, shape changes, protein phosphorylation, and platelet aggregation [87]. Platelet aggregation upon ADP stimulation increases in a dose-dependent manner [88]. Thus, in case of damage to the blood vessel wall, numerous platelets become activated, and ADP is released to the blood flow, activating other platelets.

There are two types of ADP receptors on the platelet surface, P2Y₁, and P2Y₁₂, both of which are GPCRs [89]. Resting platelets have about 160 P2Y₁ receptors and 745 P2Y₁₂ receptors on the surface [17]. When platelets become activated by a strong activator (thrombin or collagen), the number of these receptors on the platelet surface increases because these receptors are contained in its α -granules, secreted by the platelets upon activation [21].

The P2Y₁ receptor transmits a signal via the Gq protein, which leads to the same platelet signaling as thrombin, yet significantly less strong [81]. The binding of ADP to the P2Y₁₂ receptor leads to the activation of the Gi protein, which causes inhibition of adenylate cyclase and a decrease in the level of cAMP in the cell, as described above. In the absence or blockage of the P2Y₁ receptor, ADP is still able to inhibit the formation of cAMP, but its ability to induce calcium mobilization, change the shape of platelets, and their aggregation is greatly reduced. In P2Y₁^{-/-} mice, there is a slight increase in bleeding time and resistance to ADP-induced thromboembolism. On the other hand, these mice do not have a predisposition to spontaneous bleeding [18]. Platelets from P2Y₁₂^{-/-} mice are unable to aggregate normally in response to ADP administration. They retain the ability to change their shape and PLC activation, but this is due to the normal functioning of the P2Y₁ receptor. In such platelets, a decrease in the ability to inhibit the formation of cAMP in response to ADP is observed [18].

Considering ADP as a platelet activator, it should be noted that in plasma, ADP is hydrolyzed to AMP with a half-life of 10-15 minutes, which affects the number of platelets that it can activate. Hydrolysis of ADP in plasma occurs under the action of ADPase, which is produced by lymphocytes and endothelial cells [90]. Erythrocytes also participate in ADP hydrolysis [91]. This process is necessary for the prevention of the spontaneous activation of platelets in blood circulation.

5. Platelet activation by TxA2, serotonin, and adrenaline

TxA2 is also a weak platelet activator and is considered an even weaker activator than ADP. Upon addition of TxA2 (or its stable analog U46619), platelets *in vitro* exhibit all functional responses except for the procoagulant [87]. The TP receptor is the only receptor for TxA2 on the platelet surface. It is associated with Gq and G12/13 proteins, with signal transmission pathways being completely identical to those described above [18]. When a platelet is activated by a strong activator (thrombin or collagen), the number of these receptors on the platelet surface increases because these receptors are contained in its α -granules [92].

In TP^{-/-} mice, prolonged bleeding time was observed. Their platelets were unable to aggregate in response to TxA2 administration, and their aggregation time in response to collagen was increased. *In vitro*, in the presence of aspirin (inhibitor of TxA2 synthesis), platelet responses to ADP or collagen are impaired. The defect in response to thrombin looks like a shift in the concentration/aggregation curve, which indicates that TxA2 synthesis only supports platelet activation from thrombin but is not required for this process [18].

Platelet activation by TxA2 does not cause synthesis and release of TxA2 [93], but single platelet secretes about 10-8 nmol of ADP contained in dense granules, with a characteristic release time of about 5 seconds [94]. In the presence of platelets, TxA2 is hydrolyzed to thromboxane B2 (its half-life is about 30 seconds) [95], which severely limits its area of distribution and the number of platelets that it can activate.

Currently, the TxA2 signaling cascade in platelets is not completely understood. It is not clear why TxA2 synthesis is not initiated upon platelet activation by TxA2, although intracellular events, in this case, are completely identical to the case of platelet activation by ADP. It is possible that the short-lived TxA2 plays the role of a weak initiator of the platelet activation process, while the longer-lived ADP enhances this process and expands the platelet activation area.

Adrenaline and serotonin may also play a significant role in platelet activation [96]. Serotonin is contained in dense platelet granules and is secreted when activated. It has also been shown that platelets are one of the main stores of serotonin in the bloodstream of mammals [97]. Serotonin induces platelet activation in a Gq-dependent manner, triggering calcium responses via PLC β [96,98]. It has been shown that in patients with serotonin reuptake inhibitors (inhibitors of SERT pumps), which are used in depression treatment, platelet aggregation is impaired [97]. The adrenaline agonist on platelets is the adrenergic receptor, which induces similar signaling with the P2Y₁₂ receptor: β 2-receptor

inhibits the activity of adenylate cyclase via Gi and thus potentiates platelet activation [96].

6. Receptors that cause tyrosine-kinase signaling

The second important signaling branch in platelets is tyrosine kinase signaling. Tyrosine kinase signaling in platelets is built around a cascade of tyrosine kinases, which phosphorylate both each other and the surrounding effector proteins [99,100]. Each of these tyrosine kinases has a complex domain structure that determines the mechanisms of their activation and regulation [101,102]. Simultaneously, an equally important role is played by enzymes that reverse tyrosine phosphorylation - tyrosine phosphatases, which control both activation and inhibitory signals [102,103]. Despite the wide variety of tyrosine kinases, most of the regulatory domains responsible for their regulation are similar [101]. The most widespread regulatory domains are: calcium-binding domains (C2-domain, EF- "handles"); domains that recognize certain amino acid sequences (SH2, SH3, immunoglobulin-like domains, DED); membrane-binding domains (PH, SH4, C1); actin-binding domains (REM; FH2) [101,104].

The key kinases required for the initiation of tyrosine kinase signaling in platelets are the tyrosine kinases of the SFK family, as well as the kinases Syk and Btk. Tyrosine kinases of the SFK family, which include Src, Fyn, and Lyn in platelets [102], are graduated "switches": activation of their domains leads to an increase in their activity. These kinases consist of 4 main domains: a catalytic domain that conducts phosphorylation, as well as three regulatory domains SH2, SH3, SH4 [101,102]. The SH2 domain of SFK kinases allows them to bind to phosphorylated tyrosine residues on other proteins, for example, in phosphorylated YxxL sequences, which are called immune tyrosine-activated motifs (ITAM) [105]. The SH3 domain is required for SFK binding to polyproline regions (xPPxP) in proteins [106]: for example, due to SH3 domains, SFK kinases can associate with BCR and GPVI receptors, the cytoplasmic domains of which contain polyproline-enriched sequences [107]. Finally, the SH4 domain of SFK kinases is required for their localization in the juxta-membrane regions: SH4 is attached to palmitic or myristic acid residues, which are hydrophobic and, therefore, allow SFK to be "anchored" in the membrane [102]. SFK activation usually occurs according to the following scheme: initially, SFKs are in an autoinhibited state. Then CD148 phosphatase dephosphorylates them, which translates them into an active state [101,102]. Then, SFKs can SH3, and SH2 domains can attach to target proteins (for example, to receptors), which will transfer them to the $\frac{3}{4}$ active state. Thereafter, unfolded SFKs can become maximally active by autophosphorylation [101]. It is noteworthy that the

reaction opposite to the maximum activation reaction is carried out by the same phosphatase CD148 as the initial one [101].

Syk tyrosine kinases are OR switches - they can be activated by activating one of the two regulatory sites. The structure of Syk kinases is less complex than the structure of SFK kinases: Syk consists of three domains: one catalytic and two SH2 domains [108]. Syk is activated by simultaneous binding of two of their SH2 domains to phosphorylated tyrosine residues in YxxL sequences [101,108], or Syk can be activated by phosphorylation of their linker domain between the kinase and the first of the SH2 domains [108]. This reaction can be carried out by both representatives of SFK and active Syk [109].

Tyrosine kinases Btk and Tec [101] are "AND" type switches - for their activation, several conditions must be met at once [101]. Btk tyrosine kinases are composed of PH, SH3, SH2, and kinase domains [101]. During the production of PIP₃, Btk is localized on the plasma membrane, and their SH2 domain binds to phosphotyrosine [42,46], while SH3, as suggested by [101,102], may be required to attract other proteins containing polyproline [110]. All these events lead to the activation of Btk, which, in turn, lead to the activation of PLC γ 2, which are also localized in this region due to their SH2 domains and additional adapters vav and SLP-76 [46,99]. Currently, several pharmacological inhibitors of Btk kinases are used in clinics for the treatment of leukemia [111].

The activation of tyrosine kinases is the first step in initiating the assembly of signalosomes — large protein complexes — centers of intracellular signal propagation [112]. During tyrosine kinase signaling in platelets, the signalosome is based on the adapter protein LAT, which has a large number of phosphorylation sites [30]. Type I PI3K binds to phospho-LAT by the regulatory (p85) subunit, which has two SH2 domains. This leads to the unfolding and activation of the catalytic subunit p110, which, in turn, phosphorylates the membrane phosphoinositide PIP₂, producing PIP₃ [46]. PIP₃ is a substrate for the PH domain, which determines the docking on the plasma membrane of a number of components of the platelet tyrosine kinase signaling network, including Btk and PLC γ 2 [42,101].

It is important to note that not only kinases but also phosphatases play an important role in the activation of tyrosine kinase signaling. Thus, in addition to CD148, SFK can also be regulated by PTP1B and DUSP3 phosphatases, which are activated during the propagation of the activation signal by phosphorylation [103,113]. PTP1B is associated with the membrane of the endoplasmic reticulum and starts to participate when the platelet shape changes and intracellular organelles come to those in proximity to the plasma membrane [103]. On the other hand, negative regulators of activation, in particular, SHP-

1,2 phosphatase, also play an important role. SHP-1,2 possesses SH2 domains that can associate with phosphorylated tyrosines in immune tyrosine inhibitory motives (ITIMs). Activated SHPs perform ITAM dephosphorylation [114]. Phosphorylation of ITIM also leads to the activation of SHIP1,2, which carry out a reaction opposite to the reaction catalyzed by PI3K - dephosphorylate PIP_3 , thus inhibiting the activation of Btk [48].

7. Receptors that induce or inhibit tyrosine kinase signaling

There are 3 types of receptors on human platelets that trigger the activation of the tyrosine kinase cascade: the collagen GPVI receptor, the podoplanin receptor CLEC-2, and the immunoglobulin G receptor FcγRIIa [99,100]. Each of these receptors triggers a similar signaling cascade, but the specific features of their intracellular structure determine the characteristic times and the degree of strength of platelet activation when stimulated through them. Each of these receptors carries in its cytoplasmic domain one (CLEC-2) [115] or several (FcγRIIa) YxxL sequences [99] – ITAM and hemITAM, respectively. YxxL sequences can also be on receptor-associated proteins, for example, the cytoplasmic domain of GPVI is covalently linked to the FcγR chain carrying ITAM [116]. It is important to note that the tyrosine kinase signaling branch in human and mouse platelets is significantly different: for example, FcγRIIa is absent on mouse platelets [117]. Tyrosine kinase signaling in platelets can also be triggered by the receptor tyrosine kinases EphA4 and EphB1, which are activated by ephrin. It has been shown that their activation leads to adhesion and aggregation of platelets to fibrinogen. The key elements of the signaling cascade of these receptors are PI3K [118].

Besides activating tyrosine kinase-dependent receptors on the surface of platelets, inhibitory tyrosine kinase receptors are also present, which carry the ITIM and ITSM sequences in their cytoplasmic domains [114,119]. The most studied among them are the PECAM-1 (CD31) and G6b receptors [114]. It is believed that these receptors are necessary to limit platelet activation: for example, a platelet that interacted with a thrombus, but did not adhere to it, can return to a resting state largely due to the activity of PECAM-1 and G6b-B [114]. However, the mechanisms by which these receptors limit activation vary.

PECAM-1 is present not only on platelets but also on leukocytes and endothelial cells [120]. It has been shown that platelet PECAM-1 is involved in the interaction of platelets and endothelial cells, acting as an adhesion molecule [120]. In resting platelets, ITSM is hidden in its cytoplasmic domain; however, upon platelet activation, the serine residue in the near-membrane region of PECAM-1 is

phosphorylated [121]. This leads to the unfolding of the PECAM-1 cytoplasmic domain and the release of ITIM and ITSM, which can then be phosphorylated by SFK [122]. It has been demonstrated in vitro that ITIM and ITSM are phosphorylated upon stimulation of platelets by thrombin or collagen [122]. SHP-1 or SHP-2 phosphatases, which carry two SH-2 motifs, attach to the ITIM-ITSM motifs of PECAM-1. This leads to their activation: active SHP-1 and SHP-2 dephosphorylate ITAM motifs, Syk kinases, SFK kinases, and LAT adapters [123]. It has been shown that SHIP-1 and SHIP-2 can also bind to PECAM-1 [124]. However, there is no reliable data that they are actively involved in the propagation of the inhibitory signal.

The cytoplasmic domain G6b-B also consists of two ITAM-ITSM motifs. However, unlike PECAM-1, G6b-B is constantly available for phosphorylation by SFK kinases [125]. Thus, G6b-B is phosphorylated not only in activated cells but in resting cells as well, and the proportion of SHP-1 and SHP-2 phosphatases is maintained in a constantly active state [125]. There are also hypotheses according to which phosphorylation of G6b-B can be enhanced by binding to heparan sulfate. Thus, unlike PECAM-1, which is mainly an activation-limiting receptor, G6b-B is rather an activation threshold that does not allow platelets to become activated when signals are too weak [126].

8. GPVI – key platelet receptor for collagen

GPVI is a membrane glycoprotein that recognizes several glycine-proline-hydroxyproline (GPO) sequences characteristic of collagen [116]. The role of GPVI in platelet activation by fibrin [127], fibrinogen [128] and “outside-in” activation of fibrinogen-clustered integrins [100,129] has been reported. GPVI activation requires receptor clustering [116,130], the mechanism of which is not fully elucidated [131]. Lipid rafts, membrane regions enriched with cholesterol and sphingomyelin, in which diffusion is significantly slowed down, play an important role in the clustering of platelet GPVIs [132]. A large number of signaling molecules are associated with lipid rafts, among which are LAT and SFK kinases [102].

The cytoplasmic domain of GPVI is associated through salt bridges with the ITAM-containing FcγR chain, on which ITAM is present [99]. Through SH3 domains, SFK kinases are associated with GPVI, which are maintained in an active state by CD148 phosphatases [102]. Upon activation and clustering of GPVI SFKs phosphorylate ITAMs of the nearby GPVI-FcγR complexes [46,116]. Syk kinases bind to phosphorylated ITAMs, thus becoming active [101]. Due to the ability of Syk to autophosphorylation, positive feedback arises that accelerates the activation of Syk in the cytosol (Fig. 3). Active Syk phosphorylates LAT, to which vav1/3, SLP-76,

Figure 3. Scheme of tyrosine kinase signaling in platelets (based on the signal transduction network of CLEC-2 receptor). Upon binding of the receptor to the ligand (podoplanin, rhodocytin, or fucoidan), receptor clustering occurs. At the same time, it is believed that receptors are clustered in the region of lipid rafts (membrane microdomains enriched in cholesterol). GPVI and FcγRIIA also cluster after ligand binding. In resting platelets, small amounts of SFK are maintained in an active state by phosphatases CD148. Active SFKs can also support small numbers of active Syk. Upon clustering of receptors that trigger the activation of tyrosine kinases, either Syk (CLEC-2) or SFK (GPVI, FcγRIIA) phosphorylate their cytoplasmic domains, which leads to increased activation of Syk. Active Syk phosphorylates the adapter protein LAT, to which PI3K binds, becoming active. PI3K phosphorylates PIP₂, producing PIP₃, which acts as a docking site for Btk tyrosine kinase. When Btk lands on the membrane, it becomes active and phosphorylates PLCγ2, which also binds to phosphorylated LAT. Active PLCγ2 hydrolyzes PIP₂, producing a secondary activation messenger IP₃, which activates the IP3R receptor on the endoplasmic reticulum. The IP3R releases calcium ions from the intracellular platelet stores, which are returned there due to the activity of the SERCA pumps. Calcium ions can also inhibit IP3R. This leads to the initiation of calcium oscillations in the cytosol of platelets.

PLCγ2, and PI3K bind with their SH-2 domains, forming the signalosome [46]. PI3K activated in this process phosphorylates PIP₂, producing PIP₃ and thus attracting Btk [133]. Btk trapped in the signalosome are activated and trigger the activation of PLCγ2, which hydrolyzes PIP₂ to DAG and IP₃. IP₃ further triggers calcium signaling in platelets [115].

9. CLEC-2 – a receptor that is required for the prevention of blood-lymph mixing

Lymphatic endothelial cells express podoplanin, a glycoprotein capable of activating platelets through their CLEC-2 receptor, on their surface [115]. Moreover, CLEC-2, combined with GPVI, appears to be important in preventing inflammatory bleeding and maintaining vascular integrity [134].

The intracellular signaling cascade of the CLEC-2 receptor, with the exception of the initial stages of activation, is similar to the intracellular cascade of GPVI activation [109,116]. Unlike GPVI, the cytoplasmic domain of CLEC-2 contains only one amino acid sequence, YxxL, and also lacks a polyproline-rich region [109,135]. Thus, the activation of CLEC-2 is even more dependent on the clustering of receptors, as well as on the localization of CLEC-2 in the region rich in other signaling molecules [135]. Primary phosphorylation of CLEC-2 upon its activation is produced by Syk kinases, which

are maintained in an insignificant amount in an active state in resting platelets due to the activity of SFK [109]. Then, inactive Syk is attached to the phosphorylated and clustered CLEC-2, which makes them active and triggers a positive feedback loop (Fig. 3). The production of active Syk, similarly to GPVI, leads to the formation of the LAT signalosome and the activation of PLCγ2, which initiates calcium signaling in platelets.

10. Adhesion and immune receptors

One of the primary responses of platelets to the damage to the blood vessel walls is their adhesion in the damaged area. The key receptors for the platelet adhesion are glycoprotein Ib (GPIb) and α_{IIb}β₃-integrins. Glycoprotein Ib and α_{IIb}β₃-integrins do not directly activate tyrosine kinase signaling in platelets: neither GPIb nor α_{IIb}β₃-integrins have tyrosine residues, available for phosphorylation, that could serve as activation sites for initiating tyrosine kinase signaling [18,99]. However, indirectly, through the formation of complexes with other enzymes and with each other, both GPIb and α_{IIb}β₃-integrins can significantly contribute to platelet activation, initiating the activation of tyrosine kinases [129,136]. Other important integrins on the platelet surface area integrins α_vβ₃, α_vβ₁, and α₆β₁, which are necessary both for platelet interactions with fibronectin and

laminin, as well as for the formation of platelet aggregates with immune cells [137].

GPIb is a complex of three glycoproteins (GPIb-GPIX-GPV) that binds to the A2 domain of the flux-unfolded vWF [138,139]. This leads to the attraction of platelets to the activating surfaces - damaged endothelium or collagen of the subendothelial matrix - and initiates activation, adhesion, and aggregation [27]. GPIb has been shown to be a mechanosensitive receptor, but the sequence of intracellular events in platelets upon GPIb binding to the ligand is unclear. GPIb forms a complex with the adapter protein 14-3-3 ξ , which also possesses phospholipase activity [140], as well as filamin A [141]. These proteins bind GPIb to the actin cytoskeleton and can determine its mechanosensitivity. On the other hand, the relationship between GPIb and 14-3-3 ξ may be important for the activation of GPIb-bound PI3K kinase [142,143]. It is assumed that activation of GPIb leads to activation of PI3K, which, by producing PIP₃, contributes to the activation of Btk tyrosine kinase [46,143].

Integrin "outside-in" signaling is initiated upon integrin clustering [42,96,144]. Similar to GPIb, the detailed signaling sequence for this process is currently unknown. It has been shown that the initiation of "outside-in" activation requires the participation of the Fc γ R chains of the GPVI receptor, as well as the Fc γ RIIa receptor [129,144]. On the other hand, it was suggested that the cytoplasmic domain of α IIb β 3 integrin also contains several tyrosine residues that do not belong to any canonical motifs but can be phosphorylated [129]. Whether they affect, activation is currently unknown. We assume that the following chain of events is most likely: platelets receive a "mechanical" signal from GPIb by interacting with vWF, which attracts them to collagen. The platelets then receive a subsequent signal from the GPVI receptor when they come into contact with collagen [19,96]. This leads to the initiation of tyrosine kinase signaling. Activated GPIb also leads to the activation of associated PI3K, which enhances the activation of tyrosine kinases [143]. These signals jointly trigger the activation of integrins "inside-out" and then "outside-in", which occurs tyrosine kinase-dependent [20,99]. Thus, GPIb and α IIb β 3 induced signaling to become strong positive feedbacks for tyrosine kinase signaling in platelets.

It has been shown that in bacterial sepsis and the generation of an immune response, platelets can surround bacteria that have appeared in the bloodstream, which will lead to the death of bacteria [145]. Fc γ RIIa is the key immune receptor of the platelets [99,115]. The key function of this receptor is to recognize the constant amino acid sequence of IgG opsonizing antigens. Single IgGs are not capable of platelet activation via Fc γ RIIa [115,145]. Thus, like GPVI and CLEC-2, Fc γ RIIa requires clustering for

sufficient activation.

Among the receptors that induce platelet activation, a group of receptors that recognize pathogen-associated molecular patterns (PAMP) and molecular patterns associated with damage (DAMP) - Toll-like receptors (TLR) – stands out [146,147]. The main effector of TLR receptors in nuclear cells is the NF κ B transcription factor, which triggers gene transcription and de novo protein synthesis in response to activation, which is not applicable to platelets [148].

Activation of TLR receptors initiates the assembly of a signaling complex based on the adapter protein MyD88 — the middosome [149]. The middosome also consists of the TIRAP adapter, which leads to IKK β activation, as well as the NF κ B transcription factor [146,148]. On the other hand, the formation of the middosome complex also leads to the activation of soluble guanylate cyclase, which triggers the production of cGMP, as well as the activation of protein kinase G and further inhibitory signaling [148]. Finally, the middosome complex also includes the IRAK1, IRAK4, and TRAF6 adapters. PI3K is attached to TRAF-6 in the middosome complex, which leads to the activation of Akt and eNOS, which synthesizes NO, which also leads to the activation of guanylate cyclase, the production of cGMP, and the activation of PKG [150]. It has been shown that activation of IKK β leads to the activation of SNAP23, one of the key proteins required for platelet degranulation [151]. Thus, TLR-induced signaling can potentially lead to both platelet activation and platelet inhibition.

From the TLR family, the expression of TLR2 receptors on platelets has been unambiguously demonstrated both at the mRNA and at the protein level [152]. TLR-2 is a receptor that recognizes peptidoglycan sequences of gram-positive bacteria [152]. Stimulation by two typical periodontopathogens (*Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*) caused the formation of heteroaggregates of platelets and neutrophils [153,154], and this significantly enhanced the elimination of bacteria by neutrophils [153]. In this case, the formation of heteroaggregates was significantly reduced upon inhibition of TLR2 [153].

The platelet surface also contains a sufficient amount of the TLR4 receptor, which recognizes the components of the cell wall of gram-negative bacteria - lipopolysaccharides (LPS) [146]. However, incubation of LPS with platelets is more likely to have an inhibitory effect [51]. Like TLR2, TLR4 may be of great importance primarily in immune rather than thrombotic responses. For example, it has been shown that the stimulation of platelets by LPS can induce the synthesis of inflammatory mediators in them, including IL-1 β [9], sCD40L [155], RANTES [156]. It has also been shown that LPS can enhance the production of eicosanoids and markers of

oxidative stress [157].

TLR7 is expressed in platelets both at the protein and mRNA levels [152]. Recent data have demonstrated the potential role of platelet TLR7 in response to influenza virus [158], consisting in endocytosis of virions, formation of platelet-neutrophil heteroaggregates, and subsequent activation of neutrophils due to increased expression of CD62P and CD40L on platelets [158,159]. Functional TLR9 is also present on platelets, while TLR9 is localized in the near-membrane regions, being enclosed in vesicles [160]. It has been shown that stimulation by a number of thrombotic agonists leads to the movement of TLR9 from intracellular vesicles to the membrane using vesicle-associated membrane proteins VAMP7 and VAMP8. The expression of TLR9 on the platelet surface enhances the uptake and endocytosis of DNA, the TLR9 ligand [160]. It has also been shown that carboxy (alkylpyrrole) protein adducts (CAPs), lipid oxidation end products, are another ligand for platelet TLR9 [161], causing integrin activation and α granule secretion.

In addition to PAMP-induced signaling, platelets can also be a source of DAMP, which will be recognized by TLR receptors. Thus, endogenous mitochondrial DAMPs, mitochondrial DNA (mtDNA), and mitochondrial proteins are powerful immunostimulants [162]. The structure and proinflammatory effects of mtDNA are similar, if not identical, to bacterial DNA due to its proteobacterial origin. This similarity extends to the ability of mtDNA to stimulate TLR9 due to the presence of unmethylated CpG dinucleotides in mtDNA [163,164]. mtDNA can stimulate neutrophils and induce NETosis [165]. mtDNA can also induce endothelial cell permeability, thereby facilitating the transmission of a localized immunogenic response to distal organs [166].

HMGB1 is another well-studied DAMP that is passively released during both cellular stress and necrosis: HMGB1 paracrine signals “danger” to neighboring cells. It has been shown that HMGB1 binds to several members of the TLR family: HMGB1 can form a complex with CpG-DNA and bind to TLR9 and RAGE, increasing cytokine production in plasma dendritic cells [167]. When HMGB1 binds to nucleosomes, macrophages and dendritic cells are activated via TLR2 [168].

Finally, platelets also contain receptors for the components of the complement system: C3aR, C5aR. It has been shown that upon their activation, the secretion of platelet granules and the exposure of P-selectin occur, which also attracts immune cells. Also, during the secretion of granules, the C1qR receptor is exposed. It has been shown that in patients with the coronary syndrome, the expression of these receptors on the platelet surface is increased [169].

11. Inhibition of platelet activation

The platelet is maintained in an inactivated state by high concentrations of cyclic nucleotides. An increase in cAMP concentration stimulates the prostacyclin receptor, IP, associated with Gs proteins [170]. In addition to prostacyclin, there are also receptors for prostaglandin E2 (PGE2) on the platelet surface: EP3, which causes a decrease in the concentration of cAMP in a Gi-dependent manner, and EP4, on the contrary, increases the concentration of cAMP in platelets in a Gs-dependent manner [171]. The increase in the concentration of cGMP occurs under the action of nitric oxide (NO) synthesized by healthy endothelium [53,172]. Also, NO can be synthesized by platelet NO synthases NOS2 and NOS3 [173]. In platelets, NO stimulates the soluble guanylate cyclase sGC, which increases the synthesis of cGMP from GTP. An increase in the level of activity of soluble GC (sGC) leads to a decrease in the level of intracellular calcium [51,174]. Also, NO is able to catalyze the phosphorylation of TxA2 receptors, thereby preventing the activation of TxA2 platelets [174]. In addition, it has recently been shown that NO also affects the activation of integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ [174]. Although NO has an inhibitory effect on platelet aggregation at high concentrations, it rather stimulates granule secretion by acting through the cGMP pathway at low concentrations (biphasic effect) in vivo [174,175].

Conclusions

Platelet functioning is inextricably associated with the need to simultaneously receive and interpret a large number of external signals. Moreover, in some cases, these signals may contradict each other. Thanks to a complex and accurate network of receptors that trigger GPCR or tyrosine kinase signaling, platelets can “cope with such pressure” and adequately respond to arising breaches of the integrity of blood vessels. Moreover, signaling pathways in platelets can synergistically enhance each other (tyrosine kinase and GPCR) or, conversely, suppress (cAMP/cGMP signaling). Interestingly, platelets also contain voltage-gated receptors, such as the Kv1.3 and APMPA channels [176]. For such a unique cell as a platelet, such a set of receptors becomes fundamentally important since platelets can have a minimal impact on their protein composition during the life cycle.

It is noteworthy that synergistic enhancement of platelet activation pathways can occur both within the same type of signaling and for different types. So, on the one hand, ADP induces calcium signaling and activation of platelet integrins $\alpha_{IIb}\beta_3$ in a Gq-dependent manner through the P2Y₁ receptor. On the other hand, ADP can also reduce the concentration of cAMP in a Gi-dependent manner through the P2Y₁₂ receptor,

which greatly enhances platelet activation. On the other hand, the tyrosine kinase signaling branch and GPCR signaling can amplify each other: both of these signaling branches induce the activation of PI3K, which triggers the phosphoinositide signaling branch. Moreover, activation of phosphoinositide signaling during outside-in signaling via platelet integrins $\alpha_{IIb}\beta_3$ also becomes an important element of synergistic enhancement of platelet activation.

The interplay between the intracellular signaling pathways in platelets also allows them to be activated momentarily: platelets often become the first line of the body's response to pathology. Moreover, platelets are also important participants in immune processes, such as the body's response to a bacterial or viral infection, or to modulate the responses of components of cellular immunity. Secondary messengers that drive all of these platelet responses and provide interactions between activation signals and platelet functional responses will be the subject of the next part of our review series.

Author Contributions

A.A.M. drew the figures, wrote the text, and edited the paper; M.A.P. supervised the project, wrote the text and edited the paper. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest

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