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## **Platelets: From Formation to Function**

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#### Abstract

Platelets are small, anucleate cells that travel as resting discoid fragments in the circulation. Their average circulating life span is 8–9 days, and their formation is an elegant and finely orchestrated series of cellular processes known as megakaryocytopoiesis and thrombopoiesis. This involves the commitment of haematopoietic stem cells, proliferation, terminal differentiation of megakaryocytic progenitors and maturation of megakaryocytes to produce functional platelets. This complex process occurs in specialised endosteal and vascular niches in the bone marrow where megakaryocytes form proplatelet projections, releasing platelets into the circulation. Upon contact with an injured blood vessel, they prevent blood loss through processes of adhesion, activation and aggregation. Platelets play a central role in cardiovascular disease (CVD), both in the development of atherosclerosis and as the cellular mediator in the development of thrombosis. Platelets have diverse roles not limited to thrombosis/haemostasis, also being involved in many vascular inflammatory conditions. Depending on the physiological context, platelet functions may be protective or contribute to adverse thrombotic and inflammatory outcomes. In this chapter, we will discuss platelets in context of their formation and function. Because of their multifaceted role in maintaining physiological homeostasis, current and development of platelet function testing platforms will be discussed.

**Keywords:** platelets, megakaryocytes, megakaryocytopoiesis and thrombopoiesis, thrombosis and haemostasis, cardiovascular disease, platelet function testing

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### 1. Introduction

Efforts in coping with the socio-economic and health burden of CVD require further understanding of its aetiology and the risk factors behind it in order to develop cost-effective preventive strategies (primordial, primary, secondary and tertiary) to prevent and manage it. CVD risk factors can be classed as modifiable or non-modifiable. Modifiable risk factors included smoking, diabetes, unhealthy diet, cholesterol, physical inactivity (sedentary lifestyle and low cardiorespiratory fitness), overweight and obesity. Risk factors for CVD track from childhood into adulthood and are strong predictors of subclinical disease in early adulthood [1]. Up to 80% of CVD may be prevented if modifiable risk factors are evaded [2].

The main functions of blood are to supply oxygen and nutrients to tissues and cells, removal of waste, and regulation of pH and body temperature [3]. An average adult has approximately five litres of blood, accounting for about 7% of their body weight. Blood is composed of approximately 55% plasma; a pale yellow fluid mainly consisting of water, proteins, sugars and fat particles, and 45% blood cells. Blood cells include erythrocytes, leukocytes and platelets. Each of these cells are derived from a haematopoietic stem cell (HSC), which reside in the bone marrow and sit at the peak of a developmental hierarchy, with unique ability to self-renew and give rise to cells of all of the blood lineages [4]. In adults, nearly a trillion new blood cells are produced daily to sustain steady state in circulation [5]. In the classic model of haematopoiesis (the production of blood cells), an important bifurcation occurs between the lymphoid and myeloid branches, which then further divide into a number of progenitor cells (**Figure 1**). While the main function of red blood cells (RBCs) and white blood cells (WBCs) is oxygen transport and defence, respectively, this chapter will focus on platelets, the final product of one of the myeloid



Exposome Effect of Lifestyle and Nutrition on Megakaryocytopoiesis and Thrombopoiesis

**Figure 1.** Pictorial representation of megakaryocytopoiesis and thrombopoiesis, and the potential for exposome and epigenome modulation of platelet phenotype and function during this dynamic cellular differentiation process.

cell lines. In addition, the principle hypothesis and paradigm discussed will be that the modifiable lifestyle factors of physical activity and inactivity can impact on the processes of both *megakaryocytopoiesis and thrombopoiesis, via* epigenetic mechanisms. We propose that physical activity/ inactivity can modulate and program platelet phenotype and therefore function. Through recent studies, including our own (manuscripts in preparation), it is becoming increasingly evident that lifestyle factors such as physical (in)-activity and high BMI do impact on platelet function. Thus, various research studies have collectively demonstrated that platelets are indeed reflective of physiological and lifestyle changes, making them sensitive biomarkers of human health. Platelets represent a tangible link to physiological and pathological changes within the body. Future investigations will undoubtedly contribute to a greater mechanistic understanding of the relationship between cardiovascular health, lifestyle factors and platelet biology.

## 2. Platelets

#### 2.1. Platelet production

Gulio Bizzozero first described platelets as 'spherules piastrine' (little plates) as small cell fragments that clumped together at an injured blood vessel site. He also showed that these blood elements did not have a nucleus [6]. Circulating anucleate platelets are now described as dynamic specialised cells, formed in an elaborate style from their precursor cell, the mega-karyocyte. Normal platelet counts ranges between 150 and  $450 \times 10^3$  per microliter of blood, constituting the second most abundant cell type in blood after red blood cells. The average adult produces  $10^{11}$  platelets per day to preserve this count. Platelets travel as resting (quiescent) discoid fragments in the circulation, while an elaborate internal cytoskeleton allows shape changes to occur upon contact with an injured blood vessel.

The size of a mature platelet is approximately 2–4  $\mu$ m, making them the smallest cells in circulation, while their average thickness is 0.5  $\mu$ m [7] and their volume about 7  $\mu$ m<sup>3</sup>. Their small size facilitates in their role as 'guardians of the vasculature', as under laminar flow environments, platelets are pushed to the periphery by larger white and red blood cells. Consequently, they remain in close proximity to the blood vessel wall where they can quickly respond to any vascular damage [8]. This enables platelets to perform their main physiological function to prevent blood loss in primary haemostasis by the formation of a 'platelet plug' [9].

Platelets are formed and released into the bloodstream from megakaryocytes (MKs), which reside in the bone marrow [10, 11]. Their production is arguably the most elegant and distinct developmental process in eukaryotes [12]. While accounting for only 0.01% of nucleated bone marrow cells, MKs are also the largest cells, measuring between 50 and 100  $\mu$ m [13]. Both MK and platelet production, termed megakaryocytopoiesis and thrombopoiesis, are regulated by multiple cytokines, with thrombopoietin (TPO), a hormone produced by the liver and kidneys, being the key regulator. In response to TPO, HSCs differentiate into MKs by differential expression of various transcription factors. This maturation is characterised by a growth in MK size and DNA ploidy levels (endomitosis), enabling the accumulation of RNA, protein and organelles in the MK for packaging into platelets. MKs then migrate to the sinusoidal

blood vessels in the vascular niche where numerous long processes called pro-platelets are formed. MKs can extend as many as 20 pro-platelets which branch repeatedly over time. Platelets form at the tips of pro-platelets, receiving organelles, genetic material and granule contents that are transported from the MK cell body. The final point of platelet production occurs in circulation whereby anucleate fragments of pro-platelets bud into pre-platelets [14] and barbell-shaped platelets [15] that are subsequently converted into single platelets in a microtubule-driven process [16] aided by the shear forces within the bloodstream [11]. Platelets then have an average lifespan of 8–10 days, after which they are cleared via phagocytic cells such as macrophages in the spleen. Apoptosis (programmed cell death) is also well recognised in the anucleate platelet [17]. The constant number of platelets in circulation is a consequence of a homeostatic balance between their production and destruction/clearance.

#### 2.2. Platelet structure

#### 2.2.1. Internal

Platelets are unique in their structural composition and, while anucleate, contain a large variety of cellular organelles, granules and mitochondria. Granules are generally secretory vesicles that release their contents either to the platelet surface or to extracellular fluid by endocytosis. Over 300 proteins from platelet granules have been identified in the platelet releasate following activation [18, 19]. Three types of platelet granules have been identified: α-granules, dense granules, and lysosomal granules all of which derive their cargo from MKs.  $\alpha$ -granules are the largest and most numerous (50–80 per platelet) encompassing roughly 10% of the platelet volume [20]. They harbour a vast assortment of proteins important for primary haemostasis including integrins (aIIb<sub>β3</sub>) immunoglobulin family receptors (e.g. GPVI, PECAM), leucine-rich repeat family receptors (e.g., GPIb-IX-V complex), tetraspanins (e.g., CD9) and other adhesive proteins such as von Willebrand Factor (vWF), fibrinogen, and coagulation factors (Factors V, XI) that participate in secondary haemostasis. While it was previously assumed that platelet  $\alpha$ -granules were homogenous populations, [21] suggested that platelets have distinct subpopulations of alpha granules which differentially release their cargo in a context-dependent manner. Dense granules are smaller in size and number (3-8 per platelet) storing high concentrations of non-protein molecules that potentiate platelet activation such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), calcium, histamine, polyphosphate and serotonin [22, 23]. Lysosomal granules are sparse and harbour enzymes such as acid hydrolases and proteases). They function in the digestion of cytosolic components. Secretion of the lysosomal content has key extracellular functions including receptor cleavage, fibrinolysis and degradation of extracellular matrix (ECM) [24]. A recent report has described a possible new type of secretory granule termed a T-granule, after their tubular morphology [11]. These novel electron-dense granules have been proposed to function in toll-like receptor (TLR) organisation and signalling. Platelet granule deficiencies or defects such as the Grey Platelet Syndrome (α-granule deficiency) or Hermansky-Pudlak Syndrome (dense granule deficiency) can cause mild to severe bleeding disorders [25].

Platelets contain functional mitochondria, which despite being few in number, have higher rates of ATP turnover than resting mammalian muscle, suggesting they are very metabolically

active [26]. The traditional role of mitochondria in the platelet is to the supply of energy in the form of ATP for primary platelet functions. However, novel functions for mitochondria continue to emerge. Dual activation of platelets with collagen and thrombin results in a sub-type of platelets known as collagen and thrombin activated (COAT) platelets. COAT platelets display striking alterations in function and structure to typical "activated platelets" by exhibiting a myriad of features such as phosphatidylserine exposure due to cytoskeletal reorganisation, high microparticle release, and increased levels of fibrinogen on the platelet surface [27]. Mitochondrial membrane potential ( $\Delta \Psi_m$ ) is reduced in (COAT) platelets and decreases in parallel with elevated mitochondrial ROS levels that are necessary for facilitating platelet PS exposure upon activation [28]. Mitochondria are involved in the process of platelet apoptosis [29] and can be released from platelets as potential inflammatory mediators [30].

#### 2.2.2. Surface receptors

Platelets express a wide variety of receptors on their membrane, which are fundamental to platelet function and downstream signalling [9]. Major receptors include integrins, leucinerich repeat receptors (Glycoprotein GPIb/IX/V, Toll-like receptors), C-type lectin receptors (P-Selectin, CLEC-2), tyrosine kinase receptors (Ephrins and Eph kinases), proteins belonging to the immunoglobulin superfamily (GPVI, FcγRIIA) and other receptors shared with vascular cells (TNF receptor type, CD63, CD36, PSGL-1).

Integrins are type I transmembrane cell adhesion receptors [31] consisting of a short intracellular and larger extracellular domain. All integrins contain an  $\alpha$  subunit and a  $\beta$  subunit, capable of bi-directional signalling. During signal transduction, they transmit information concerning the chemical and mechanical status of the ECM to the cell [32, 33]. Platelets express five integrin receptors—each with affinity for specific ligands; αIIbβ3 (fibrinogen),  $\alpha 2\beta 1$ (collagen),  $\alpha 5\beta 1$ (fibronectin),  $\alpha V\beta 3$  (vitronectin) and  $\alpha 6\beta 1$  (laminin), all of which share related signal transduction processes [34]. On the cytoplasmic face of the plasma membrane, integrins regulate cytoskeletal dynamics and signalling complexes. On the extracellular side, integrins bind with high affinity to either ECM ligands or counter receptors on adjacent cell surfaces [35].  $\alpha$ IIb $\beta$ 3 is the most abundant platelet integrin, with copy numbers of ~50,000 per platelet, and is present in both alpha granules and the platelet surface. Platelets express a number of G-protein coupled receptors (GPCRs) which constitute a large family of receptors that can identify molecules outside the cell and initiate signal transduction pathways and ultimately cell function. The main GPCRs present on platelets include thrombin receptors called protease-activated receptors (PARs) (PAR1 and PAR4), ADP receptors (P2Y<sub>1</sub>, P2Y<sub>12</sub>), of which approximately 150 P2Y<sub>1</sub> receptors are present on the platelet [36], thromboxane receptors (TP $\alpha$  and TP $\beta$ ) and glycoprotein receptors [37].

#### 2.3. Platelet function

#### 2.3.1. Platelet function in primary haemostasis

Haemostasis can be subdivided into primary haemostasis, secondary haemostasis and fibrinolysis (**Figure 2**) [38]. Platelets prevent blood loss in primary haemostasis, the physiological process which halts bleeding at an injured blood vessel, while maintaining normal blood flow elsewhere in circulation, by the formation of a 'platelet plug' [11]. Secondary haemostasis refers to the deposition of insoluble fibrin that is generated by the coagulation cascade.



**Figure 2.** (A) Diagram showing key platelet membrane receptors involved in platelet function in haemostasis and thrombosis. (B) Distinct phases of platelet function in primary haemostasis: Initial platelet tethering and adhesion at a site of vascular damage, firm adhesion and shape change, activation, granule secretion and further recruitment of platelets leading to platelet aggregation. (C) Secondary haemostasis: Formation, deposition and cross-linking of insoluble fibrin, generated by the coagulation cascade, to stabilise the primary platelet plug. (D) Image of platelet aggregates (fluorescently-labelled, green) on collagen fibrils following *in vitro* blood flow at arterial shear (1800 s<sup>-1</sup>) using a parallel-plate blood perfusion chamber (*unpublished data—Gerardene Meade-Murphy*).

Finally, fibrinolysis results in the breakdown of blood clots during wound healing involving the interplay of a number of enzymes [38]. A healthy endothelium provides a non-adhesive surface for platelets. However, in areas of vascular injury, the sub-endothelium is exposed and platelets may adhere quickly to different extracellular matrix components, and then form a platelet plug. This process is achieved through three distinct processes—platelet adhesion, platelet activation and secretion, and platelet aggregation [39].

#### 2.3.2. Platelet adhesion

Platelet adhesion entails a collaborative effort of various platelet receptors, fundamentally leading to platelet activation and aggregation. ECM elements that platelets adhere to include proteins such as collagen, vWF, fibronectin, laminin and fibrinogen among others [40, 41]. Among these subendothelial substrates, the thrombogenic fibrillar collagens type I and III are the most powerful intermediaries of platelet adhesion due to their robust activating potential and affinity for vWF [42]. Following vascular damage, initial platelet 'tethering' is mediated by the interaction between the A1 domain of vWF deposited in the subendothelial matrix of the damaged vessel wall, and the GPIb $\alpha$  in the platelet receptor GPIb-IX-V. This interaction is particularly important at high shear rates supporting platelet translocation (i.e. decelerating platelets and keeping them in close contact with the endothelium) over the subendothelium, but not stable adhesion [43]. This interaction allows engagement of other platelet receptors. vWF/GPIb-IX interface also induces platelet activation signalling events, resulting in integrin activation [44].

Following platelet tethering, platelet collagen receptors, GPVI and  $\alpha 2\beta 1$  interact with exposed collagen and promote platelet adhesion and activation. GPVI is non-covalently coupled to the Fc Receptor chain (FcR $\gamma$ ) [45, 46] and has been acknowledged as the major signalling receptor for collagen. FcR $\gamma$  has an immunoreceptor tyrosine-based activation motif (ITAM) on its cytoplasmic sphere. After collagen binding to GPVI, the ITAM motif on the GPVI/FcR $\gamma$  complex is phosphory-lated, resulting in activation of the Syk kinase pathways that phosphorylate downstream targets, ultimately resulting in increased cytosolic Ca<sup>2+</sup> and subsequent platelet shape change, granule secretion and integrin activation. GPVI has a low affinity for collagen, rendering it unable to mediate stable adhesion alone. The  $\alpha 2\beta 1$  integrin then maintains stable adhesion to collagen.  $\alpha 2\beta 1$  stimulates downstream steps indirectly by reinforcing GPVI-collagen interactions [41, 47] and by direct signal-ling leading to activation of  $\alpha IIb\beta 3$ . Platelet Ca<sup>2+</sup> signalling is markedly dissimilar between GPVI and  $\alpha 2\beta 1$  suggesting that the alliance regarding GPVI and  $\alpha 2\beta 1$  supports optimal platelet adhesion.

The final step of platelet adhesion occurs via binding of platelets to other ECM components such as fibronectin, laminin and immobilised vWF. Platelets bind to fibronectin via the  $\alpha5\beta1$  receptor and  $\alpha$ IIb $\beta3$ , whilst adhesion to laminin is mediated by their  $\alpha6\beta1$  receptor. Stable binding of platelets elicits activation pathways involving tyrosine kinases and signal transduction GPCR receptor signalling, cumulatively resulting in elevated cytosolic Ca<sup>2+</sup> levels, cytoskeletal reorganisation and integrin activation.

#### 2.3.3. Platelet activation and secretion

Once platelet adhesion has occurred at the site of vessel wall damage, platelet activation needs to be maintained for haemostasis to continue. Essential for the amplification of platelet

activation is the production and release of soluble agonists at the site of damage [26], which act in an autocrine and paracrine manner to amplify platelet activation and recruit further circulating platelets. These agonists consist of  $TxA_{2'}$  ADP, epinephrine and thrombin. ADP is secreted from platelet dense granules and binds to its relevant receptors, P2Y<sub>12</sub> and P2Y<sub>1</sub> on the platelet surface [48]. ADP is also released from red blood cells at the site of vascular damage [37]. Binding of ADP initiates a full complement of activation events such as elevation of intracellular platelet Ca<sup>2+</sup>, TxA<sub>2</sub> synthesis, protein phosphorylation, shape change, granule release, and most importantly, activation of  $\alpha$ IIb $\beta$ 3 [49]. P2Y<sub>12</sub> is also the target of a class of antiplatelet drugs called thienopyridines (ticlopidine, clopidogrel, prasugrel), widely used in the prevention of vascular events in patients with CVD.

 $TxA_2$  is a potent platelet agonist synthesised from arachidonic acid through the COX pathway and  $TxA_2$  synthase enzymes. It subsequently binds to  $TP\alpha$  and  $TP\beta$  receptors that differ in their cytoplasmic tails, causing vasoconstriction, shape change, protein phosphorylation, secretion and platelet aggregation [50, 51]. Indeed, high levels of  $TxA_2$  have been implicated in CVD, whilst inhibition of  $TxA_2$  synthesis through aspirin-mediated COX inhibition is a major anti-platelet target.

The agonist thrombin rapidly accumulates at sites of vascular damage and has major functions in promoting and stabilising thrombus formation. Platelets release factors that support the activation of prothrombin, which after a complex series of sequential events in the coagulation cascade, results in the generation of thrombin [52]. The increase in cytosolic Ca<sup>2+</sup> after platelet activation results in platelet phosphatidylserine (PS) exposure on the activated platelet membrane providing a procoagulant surface for thrombin to interact with its PAR1 and PAR4 (G protein-coupled) receptors.

Uniquely, thrombin activates its PAR-receptors by cleaving an N-terminal part at a consensus site. Cleavage exposes a new binding site that acts as a ligand to activate the receptor. Thrombin is the most powerful platelet activator, initiating an entire complement of platelet responses (shape change, granule secretion,  $TxA_2$  synthesis, aggregation etc.) [53]. Thrombin can activate platelets at extremely low concentrations and within seconds, increases cytosolic level of Ca<sup>2+</sup>, eliciting downstream signalling events. Unlike ADP,  $TxA_2$  or thrombin, the catecholamine epinephrine is a weak agonist unable to cause shape change alone. However, it works collectively with the other agonists increasing their potential to activate. Epinephrine's mode of action is to inhibit cAMP formation by the platelet  $\alpha_2A$ -adrenergic receptor [54].

#### 2.3.4. Platelet aggregation

The ultimate step in primary haemostasis is platelet aggregation, caused by crosslinking of  $\alpha$ IIb $\beta$ 3 on adjacent platelets by fibrinogen (**Figure 2**). While platelet aggregation is a complex process involving different receptors ( $\alpha$ IIb $\beta$ 3 and GPIb $\alpha$ ) and ligands (fibrinogen, fibronectin, and vWF), the main process involves the integrin  $\alpha$ IIb $\beta$ 3. On resting platelets, integrin  $\alpha$ IIb $\beta$ 3 has a low affinity for its ligands fibrinogen and vWF, which dramatically increases upon platelet activation. Binding of the main agonists to their respective receptors induce intracellular signals that disrupt the complex between the cytoplasmic tails of  $\alpha$ IIb $\beta$ 3. This ultimately leads to a conformational change in its extracellular globular head domains from a

low affinity resting state to a high affinity activated state in order to bind extracellular ligands such as fibrinogen and vWF. Irreversible activation of  $\alpha$ IIb $\beta$ 3 is a prerequisite for the development of irreversible platelet aggregates. Due to the symmetrical nature of fibrinogen, platelets can be 'bridged' and platelet aggregates are formed [40]. While  $\alpha$ IIb $\beta$ 3 is the major player in platelet aggregation, couplings between other platelet receptors and their ligands could be incorporated in aggregation. Some of these comprise CD40 ligand [55] interaction with  $\alpha$ IIb $\beta$ 3, the vWF-GPIb complex [56], and an involvement of fibronectin in stabilising platelet aggregation [57, 58]. Cadherin-6 was recently acknowledged as a new counter-receptor for  $\alpha$ IIb $\beta$ 3, involved in platelet aggregation [59].

Bi-directional 'inside out' and 'outside in' signals are transferred by both integrin subunits, mediating receptor conformation and platelet function [44]. After platelet stimulation with ADP, the signal from the ADP receptors is conducted to the intracellular domain of the cytoplasmic tail of  $\alpha$ IIb $\beta$ 3 and subsequently transmitted through a series of events to the extracellular domain (inside-out) causing a conformational change in the extracellular domain that binds to its ligand. Inside-out signalling necessitates binding of talin and kindlins to the cytoplasmic domain of  $\beta$ 3. After ligand binding, a signal is sent to the cell (outside-in) to control platelet function such as filopodia and lamellipodia extension to dull platelet spreading [34, 44]. Furthermore,  $\alpha$ IIb $\beta$ 3 outside-in signals can also act as a break to curb excessive platelet activation by activated SHIP-1 [60].

#### 2.3.5. Bio rheological factors in platelet aggregation

A significant factor influencing platelet aggregation is the distinct shear environment experienced within the vascular system. Platelets are subjected to fluctuating haemodynamic conditions *in vivo* such as shear stress and shear rate. The latter refers to the rate of increase of blood flow velocity, whereas the former denotes the force per unit area on the vessel wall [61]. Shear rates experienced by platelets range from slow flow in veins (shear rate  $10 \text{ s}^{-1}$  to  $500 \text{ s}^{-1}$ ) to small arteries (approx.  $2000 \text{ s}^{-1}$ ) to diseased or pathological arteries, where extremely high shear rates (up to 40,000) have been described [61, 62]. Increasing shear rate activates platelets itself. At low shear rates (< $1000 \text{ s}^{-1}$ ), platelet aggregation is primarily facilitated by  $\alpha$ IIb $\beta$ 3-fibrinogen interactions. At shear rates typically over  $5000 \text{ s}^{-1}$  (but between 1000 and 10,000), a two-step sequential process occurs. The first depends on the adhesive properties of GP1ba and  $\alpha$ IIb $\beta$ 3 and is facilitated by the formation of reversible platelet aggregates. The second relies on the generation of platelet agonists and involves the irreversible activation of  $\alpha$ IIb $\beta$ 3 to form stable aggregates [63]. Accordingly, both fibrinogen and vWF, and receptors GPIba and  $\alpha$ IIb $\beta$ 3, have distinctive but complementary roles in platelet aggregation subject to the haemodynamic environment.

#### 2.3.6. Signal transduction during platelet function

The role of platelets in haemostasis is reliant on the equilibrium between activatory and inhibitory signals [64]. Inhibitory signals from the vasculature prevent platelet activation in healthy vessels. Activatory signals present at an injured blood vessel initiate platelet activation, and are managed by endogenous negative signalling regulators. Negative regulators

and pathways include the ITIM containing receptors that are postulated to reduce activation of PLC, PI3K and integrin  $\alpha$ IIb $\beta$ 3 [64]. The Wnt- $\beta$ -catenin pathway has recently gained attention as negative regulator of platelet function [65, 66]. Wnt3a is one of these glycoproteins, which is secreted from activated platelets. It has been suggested to activate the canonical Wnt- $\beta$ -catenin pathway as constituents of this pathway have been identified in platelets. The regulation of small GTPases such as Rap1, Rac1, RhoA and Cdc42 has been suggested as players in this inhibition of platelet function [65]. These pathways are less well characterised than the classical activation pathways [67]. The primary platelet inhibiting signals produced by healthy ECs are nitric oxide and prostacyclin. Both NO and prostacyclin relax blood vessels and prevent platelet activation [68]. NO is synthesised from several cells including platelets, ECs and RBCs and plays vital roles in maintaining platelets in a resting state [69–71]. Prostacyclin (PGI<sub>2</sub>) is a physiological anti-aggregating agent produced constitutively by ECs as a result of arachidonic acid metabolism by cyclooxygenase (COX) enzymes.

Following endothelial damage, endogenous inhibitory signals are overcome and platelets react rapidly to limit blood loss. The activating stimuli, such as collagen, vWF, ADP, TxA<sub>2</sub> and thrombin, which induce platelet adhesion, activation and aggregation ultimately regulate a central set of signalling mediators that support activation. Three principle mediator families of platelet activation are phospholipase C (PLC), protein kinase C (PKC) and phosphatidylinositide-3-kinase (PI3K), and underlie two crucial events in platelet activation—secretion of amplifying mediators and activation of integrin αIIbβ3.

#### 2.4. Platelet function beyond haemostasis

Platelets are recognised as pivotal players in numerous other processes ranging from inflammation and atherosclerosis, fighting microbial infection and tumour growth and metastasis. Platelets are equipped to influence inflammation, the innate immune response and infection, by acting as sentinels in pathogen detection. They express a collection of pattern recognition receptors called toll-like receptors (TLRs) that identify molecular motifs called pathogen associated molecular patterns (PAMPs) and initiate immune responses [72]. Platelets express functional TLR 1–9, whilst TLR2 stimulation in platelets by bacteria through the activation of the PI3K signalling pathway induces a pro-inflammatory response [20]. Platelet TLR9 activation has been associated with thrombosis and oxidative stress, and is found within the T-granules in platelets [11].

Platelets interact with leukocytes, monocytes and granulocytes through different receptorligand interactions (P-Selectin and PSGL-1 interaction) enabling intercellular communication [73]. The capacity of platelets to store and release copious inflammatory cytokines and chemokines is intimately associated to their role in inflammation. Platelets release microparticles linked to inflammatory pathways and which are associated with inflammatory diseases such as rheumatoid arthritis [74]. Platelets play key roles in infection and immune response to bacterial and viral infections. They are the first cell type to arrive at areas of vascular infection [75]. Thrombocytopenia (low platelet count) is a well-established manifestation of sepsis and studies suggests platelets play a functional role in the pathogenesis of sepsis and multi-organ failure [76]. Platelets have significant roles in the pathogenesis of metastasis [77]. Tumour cells can aggregate platelets *in vitro* and it has been proposed that platelet adhesion to metastatic cells can act as a 'cloak' around circulating tumour cells therefore acting as a shield for immune clearance. This phenomenon of platelet cloaking has resulted in pro-survival, pro-angiogenic and epithelial mesenchymal transition (EMT) in cancer cells [78]. Platelets also release growth factors such as VEGF and PDGF that can expedite tumour growth [79]. Platelets are deeply implicated in wound healing and bone health, and indeed the use of platelet rich plasma (PRP) therapy (i.e. rich in growth factors and bioactive substances) is effective in osteoarthritis [10] and in muscle damage such as rotator cuff tendinopathy [80].

#### 2.5. Assessing function: platelet function tests (PFT)

The different functions of platelets may be reliably detected with a wide spectrum of tests (**Table 1**). These can be utilised to identify inherited or acquired platelet dysfunction, monitor antiplatelet therapy, manage various aspects of platelet banking and transfusion, and to aid in the understanding of platelet physiology in basic research. PFTs are centred around principles of platelet function such as platelet adhesion and aggregation, platelet function under shear conditions, and measurement of the platelet releasate [81]. Platelet function testing began with the evaluation of the bleeding time (the time taken for platelets to occlude an *in vivo* wound), using the Duke procedure [82], before the development of light transmission aggregometry (LTA) revolutionised the study of platelet function. Considered the historical gold standard, LTA is a relatively easy technique that involves stirring a suspension of platelet rich plasma in a cuvette in the presence of a platelet agonist (such as ADP or collagen). The cuvette is placed between a light source and photocell. Agonist addition causes *in vitro* platelet aggregation and changes in light absorbance, which is detected by the photocell [83].

Investigation of platelet function in the environmental *milieu* of whole blood under conditions that take into account most of the physiological parameters that influence platelet adhesion and aggregation (red blood cells, white blood cells, plasma) is important. The PFA-100, The Impact-R Cone and Plate analyser and the global thrombosis test are examples of such assays. The PFA-100 assesses platelet aggregation under high shear where platelets are activated in whole blood by an amalgamation of high shear stress (5000–6000 s<sup>-1</sup>) and agonists (e.g. collagen and ADP), resulting in closure of an aperture [84]. The Impact-R Cone and Plate analyser is a point-of-care (POC) device which measures global platelet function by testing platelet adhesion and aggregation in whole blood, under arterial shear conditions [85, 86]. In this assay, platelet adhesion is dependent on plasma proteins vWF, fibrinogen and RBCs and WBCs. The addition of platelet agonists such as arachidonic acid (AA) and ADP in the system has enabled the evaluation of dual anti-platelet therapy [87–89]. The system is effective in the assessment of platelet function disorders in adults, [90] children [91] and new-borns [92].

#### 2.6. Platelet indices

Platelet indices are useful as inexpensive non-invasive biomarkers for assessing platelet activation [93]. Platelet indices are straightforwardly measured by semi-automated counters in complete blood counts (CBC) and usually include four factors; platelet count (PLT), mean

Method	Sample	Principle
Platelet aggregation assays		
Light transmission aggregometry (LTA)	Citrated PRP	Measurement of light transmission in response to agonist-induced platelet activation
Impedance Aggregation	Citrated WB	Monitors changes in electrical impedance in relation to agonist-induced platelet aggregation. Whole blood specimen diluted 1:1 with physiologic saline, prior to testing
VerifyNow	Citrated WB	Turbidimetric optical detection of platelet aggregation in whole blood in response to agonists/inhibitors
Plateletworks	Citrated WB	Platelet counting pre- and post-activation
Assays measuring release reactions		
Lumi-aggregometry	Citrated WB	Combination of LTA/WBA with nucleotide release
Soluble platelet release markers (Txb2, PF4, BTF, scD40L)	Urine, serum, citrated plasma	Typically measured by ligand binding ELISA immunoassays
Shear-based assays		
PFA-100/200	Citrated WB	High-shear platelet adhesion and aggregation during formation of a platelet plug
Impact R cone and plate analyser	Citrated WB	Shear-induced platelet adhesion-aggregation upon specific surface
Global thrombosis test	Native WB	High-shear platelet plug formation—measurement of time cessation of WB flow
Platelet activation based assays		
VASP phosphorylation	Citrated WB	Flow cytometry or ELISA measurement of VASP phosphorylation
Flow cytometry	Citrated WB, PRP, washed platelets	Measurement of platelet glycoproteins, and activation markers; platelet leukocyte aggregates, platelet microvesicles

Typical platelet function tests range from assessment of their primary haemostatic function including measurement of granule secretion using lumi-aggregometry to whole blood shear based assays, which measure platelet function under flow conditions. Platelet function can be tested in washed platelets, whole blood or platelet rich plasma.

Table 1. Platelet function tests.

platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), and depending on the analyser, platelet large cell ratio (PLCR). PLT is a universal indicator of haemostasis in a clinical setting and is utilised as a sensitive biomarker for a range of diseases. High PLT, even within the physiological range of 150–450  $\mu$ l, is associated with a greater risk of thrombosis and CVD suggesting that enhanced PLT encourage platelet hyperactivity and a pro-inflammatory state [94]. However, the consequence of high platelet numbers that are still within physiologic ranges remains unclear [95].

The indices MPV, PDW and PLCR are quantitative measures of the variability in platelet size. MPV reflects the average platelet size while PDW reflects the volume variability in platelet size

[94, 96]. The volume of circulating platelets is heterogeneous with subsequent functional differences. Some authors suggest that larger platelets are metabolically more active than smaller platelets, that they have faster rates of aggregation and release higher quantities of pro-thrombotic elements such as TxA<sub>2</sub> and ADP [97]. MPV and PDW levels can be altered in several diseases including T2DM [98], CVD and atherosclerosis [93] (Berger et al., 2010), and in this regard they have been suggested as markers of subclinical platelet activation. PLCR and PCT may serve as sensitive biomarkers of platelet health [99]. PLCR indicates the percentage of large platelets present in blood [93]. PLCR is significantly higher in subjects with dyslipidaemia compared to healthy subjects [100] and higher in children with T2DM compared to healthy children. Moreover, Rechcinski et al., have hypothesised that PLCR has the potential to be a prognostic biomarker [101]. Importantly, thrombogenicity of large platelets may put individuals at higher risk of acute cardiovascular events. PCT is the volume of blood occupied by platelets as a percentage, similar to the erythrocyte measurement of haematocrit (HCT). PCT reflects total platelet mass and is calculated as PLT × MPV/10<sup>7</sup>, providing comprehensive information about platelet activity. PCT has been proposed as a novel predictor of cardiovascular risk and higher PCT is associated with the risk of re-infarction and long-term mortality in CVD patients [102]. However, the clinical significance, reference values and efficacy of some of these parameters are still under exploration.

In peripheral blood, there is ample interplay between RBCs, WBCs and platelets [103] and altered levels of blood cells and their morphology have been associated with CVD [104]. Platelet adhesion and aggregate size, is influenced by platelet indices, RBC and WBC [86]. RBCs encourage platelets towards the vessel wall [105], which can affect platelet adhesion and aggregation. In this context, it is important to investigate the associations between the various indices of each blood cell to interpret the multicellular contribution to both thrombogenesis and CVD risk.

## 3. Conclusion

Novel techniques continue to emerge and develop the knowledge surrounding the platelet function regulation. The modern "omics" revolution enables simultaneous quantification of hundreds of molecules (e.g. protein or mRNA) from a single sample and their signatures may be reflective of platelet function changes. The amalgamation of transcriptomic and proteomic data and subsequent bioinformatic analysis will lead to a more complete characterisation of platelet function in response to environmental stimuli [106, 107]. Epigenetics and its ancillary elements, including platelet secreted microvesicles (MVs), and microRNA (miRNA), and regulation of the platelet mitochondrial genome are new avenues of investigation and testing in platelet research [108–110].

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## **Conflict** of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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