Nutraceuticals and Human Blood Platelet Function
Applications in Cardiovascular Health

Asim K Duttaroy

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Applications in Cardiovascular Health

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Nor weep nor doubt that still the spirit is whole,
And life and death but shadows of the soul.
– Bhagavad Gita

This book is dedicated to my elder brother, the late Subir K. Duttaroy (01.05.1947–10.05.2004), who encouraged and supported me through the entire postgraduate process, and never got to read this book.

I am forever indebted to my other elder brother, Mr. Asish K. Duttaroy, who gave me unrelenting support during my studies in India. Both of them routinely went beyond their duties to help me and to instil great confidence in my studies.

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Human Blood Platelets and Their Role in the Development of Cardiovascular Disease

Abbreviations Used in This Chapter

Cardiovascular disease, CVD; Glycoprotein IIb/IIIa, GPIIb/IIIa; von Willebrand factor, vWF; Tissue factor, TF; plasminogen activator inhibitor-1, PAI-1; cyclooxygenase, COX; TREM-like transcript-1, TLT-1; P-Selectin, CD62P; arachidonic acid, 20:4n-6, ARA; CD40 ligand, CD40L; phospholipase C; PLC, Phosphatidylinositol-4,5-bisphosphate, PIP2; Inositol-1,4,5-trisphosphate, IP3; Purinergic receptor P2Y12, P2Y12

1.1 Introduction

Human blood platelets are non-nucleated cells, produced in bone marrow from megakaryocytes [1]. Although very dynamic, blood platelets (around 2μm in diameter) usually prefer to remain in an inactive state in circulation, and get activated only when a blood vessel is damaged [1, 2]. The human body produces and removes $10^{11}$ platelets daily to maintain a normal steady state platelet count. Platelet production must be regulated to avoid spontaneous bleeding or arterial occlusion and organ damage.

The primary physiological role of platelets is to sense the damaged vessel endothelium and rapidly accumulate at the damaged site of the vessel, where they initiate blood coagulation process to stop the bleeding (Figure 1.1).
Circulating inactive platelets are biconvex but, upon activation, they become irregular and sticky, extending pseudopods and adhering to neighboring structures or aggregating with one another. The rapid interactions between activated platelets, their secreted components, or thrombin and endothelium at sites of damaged vessels, ensure the intravascular growth of the stable haemostatic plug [2].

Two different pathways mediate vascular homeostasis and thrombosis depending on vascular damage or vessel structure [1]. One is the intrinsic pathway mediated by collagen, while the other is the extrinsic pathway mediated by tissue factor (TF)-factor-VII complex. During normal hemostasis, damage to the endothelium may occur, and collagen from the sub-endothelial space is exposed.

Platelets, through their glycoproteins (GP) GPVI and GPIb/V/IX, interact with collagen and von Willebrand factor (vWF). Collagen exposure leads to platelet adhesion and formation of a platelet monolayer on the damaged surface of the vessel. Platelets form a three-dimensional structure by aggregating through their activated GPIIb/IIIa complexes, the fibrinogen receptors. Activated platelets aggregate with other circulating platelets by secreting platelet aggregatory/activating agents,
Human Blood Platelets and Their Roles

Figure 1.2 shows the expression of platelet membrane surface GPIIb/IIIa complexes induced by different aggregating agents through TxA₂ formation from ARA, liberated from membrane phospholipids. The tissue damage or plaque rupture leads to the release of TF from smooth muscle, adventitial cells, and pericytes. TF, with the help of activated factor VII(VIIa), mediates the conversion of pro-thrombin to thrombin, fibrin generation, and thus initiates the clotting cascade [1]. Activated platelets also accelerate the action of prothrombinase complex to produce thrombin from prothrombin.

Apart from hemostasis, platelets are involved in several processes in the cardiovascular system, such as atherosclerosis process, immune system, inflammation, and cardiac events [3–5]. Thus, human blood platelets play many pivotal roles in the pathophysiology of different diseases, from CVDs to tumor metastasis [6].

Figure 1.2 Expression of fibrinogen receptors. Fibrinogen receptors (GPIIb/IIIa complex) on the platelet surface represents the final common pathway, whereby platelet stimulation by various agonists leads to fibrinogen binding, platelet aggregation, and thrombus formation. Aspirin can inhibit collagen, adrenaline and, to some extent, ADP-induced expression of fibrinogen expression, but not the thrombin-induced expression of GPIIb/IIIa complex on platelet surface.
Upon activation, platelets secrete more than 300 components from their intracellular stores. Platelet dense granule components, such as ADP and polyphosphates, and Ca\(^{2+}\), contribute to blood coagulation and platelet aggregation. α-Granules secrete multiple cytokines, mitogens, and other components that contribute to the CVD processes. Anucleated platelets have stable mRNA transcripts with a long life, and use a variety of mechanisms to translate these mRNAs into proteins [7].

There are two important key regulators of translation processes such as ELF‐4e and ELF‐2a that are used by platelets. Platelets synthesize several proteins, such as integrins, αIIbβ3, TF, plasminogen activator inhibitor-1 (PAI-1), cyclooxygenase (COX), Factor XI, protein C inhibitor CCL5/ RANTES, and IL-1B [7]. mRNA translation is also regulated by miRNA in human platelets. In fact, the possibility of using miRNAs as biomarkers of atherosclerosis and cardiac episodes has been suggested. In platelets constitutively are synthesized proteins such as actin, PDGF, glycoproteins GPIIb/IIIa, and P-selectin [7].

Platelet hyperactivity, as occurs in obesity, smoking, sedentary life styles, and diabetes, insulin resistance is associated with secretion of different components, along with the shedding of membrane particles that play important roles in the development of CVD risk, especially in the development of atherosclerosis, blood flow, inflammation, and hypertension (Figure 1.3).

Continued research has revealed that platelet micro-particles have numerous functions. In addition to atherosclerosis, they are involved in thrombus and foam cells formation, and inflammation. Platelet membrane proteins GPIbα, GPV, GPVI, amyloid βA4, TLT-1 (TREM-like transcript-1), P-selectin (CD40L), amyloid-like protein 2 and semaphorin 4D are the most abundantly shed platelet proteins.

In this chapter, current understanding of human blood platelets and their roles in the development of CVD is discussed.

### 1.2 Human Blood Platelets: Structure and Function

Platelets have granular cytoplasm with no nucleus, and their diameter averages 2.5 μm, with a subpopulation of larger diameter 4–5 μm. Individual platelets, however, vary in terms of
Figure 1.3 The multiple roles of platelets in different diseases.
volume, density and reactivity towards agonists. The normal blood platelet count is in the range of 150–400 × 10^9/L. Under normal conditions, platelets circulate in the bloodstream for 8–10 days. Under conditions of hemostatic requirements, platelets move from the spleen to the peripheral blood circulation (70% of total platelets).

The normal peripheral blood platelet count is 150–400,000/μL [8]. This count represents only two-thirds of available platelets, because the spleen retains the rest of the platelets. Megakaryocytes develop in the bone marrow from hematopoietic stem cells [9]. The megakaryocyte undergoes endomitosis as chromosomes duplicate, and a polyploid nucleus is synthesized.

Within a five-day maturation period, DNA amplification occurs while mitochondria, endoplasmic reticulum, Golgi apparatus, membranous systems, and secretory granules develop [9]. Megakaryocytes mature into pro-platelets, and subsequently fragment into platelets [10]. Organelles and granules are synthesized in the megakaryocytes and then move towards the outer periphery using pseudopodia-like extensions. As the megakaryocytes enter the circulation, the pseudopodia-like extensions fragment, as a result of shear flow changes [10]. Fragmentation of megakaryocyte produces pseudopodia-like extensions, with around 10–20 anuclear, dumbbell-shaped pro-platelets (diameter of ≈ 2–3 µm). The pro-platelet buds fragment again to form platelets.

More than 120 proteins have been detected in platelets, including high concentrations of vWF, platelet factor 4, Factor V, Factor XIII, and plasminogen activator inhibitor-1 (PAI-1) [11]. These proteins are synthesized in the megakaryocytes, but are released when platelets become activated. In contrast, coagulation proteins, such as fibrinogen, high molecular weight kininogen, α2-antiplasmin, and α2-macroglobulin, are taken up from plasma by the blood platelets.

Platelets contain two unique membrane systems – the dense tubular system and the open canalicular systems [10]. The dense tubular system is a closed membrane system that is the primary storage reservoir of intracellular Ca^{2+}. The open canalicular system is a continuous channel, connected to the outer membrane surface, which allows the secretory granules contents to exit
from the platelet during activation [12]. As the megakaryocyte reaches maturity, it enters the sinusoidal blood vessels of the bone marrow and migrates towards circulatory vessels [10]. A circumferential bundle of microtubules maintains the microtubules, actin microfilaments, and intermediate microfilaments. These components regulate platelet shape change, extension of pseudopods, and secretion of granule contents.

Platelets contain two types of secretory organelles – alpha and dense granules – which, together, store over 300 different proteins and other small molecules [13] (Table 1.1).

The alpha granules are the largest in size, at ≈ 200–500 nm, and are the most abundant at 50–80 per platelet cell. The granules contain proteins that participate in coagulation, aggregation and inhibition of coagulation. As the platelet becomes activated, alpha granule membranes fuse, and their contents exit to the nearby microenvironment, where they participate in platelet adhesion and aggregation and coagulation. The dense tubular system sequesters Ca²⁺, and contains proteins that activate platelets. These are phospholipase A₂, COX-1, and TxA₂ synthetase responsible for liberation and metabolism of membrane phospholipid arachidonic acid, 20:4n-6 (ARA) to produce TxA₂.

A platelet has more than 50 different types of surface membrane receptors. The predominant platelet membrane receptors are purinergic receptor (P2Y12), fibrinogen receptor (GPIIb/IIIa), vWF receptor (GPIb/Factor V-Factor IX), and collagen

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Table 1.1 Platelet granules contents released on activation.
receptors, GPIa/IIa, and GPVI. The dense granules are formed later than α-granules during megakaryocyte differentiation. Small molecules are endocytosed, and are stored in the dense granules.

Compared with alpha granules, the dense granules are less abundant, numbering 3–8 per platelet. Dense granules contain ions and signaling molecules such as ADP, ATP, serotonin, histamine, Ca$^{2+}$, Mg$^{2+}$, pyrophosphate, and polyphosphate. The dense granules are referred to as being electron-rich, because they store ≈ 70% of the total platelet content of ions such as Ca$^{2+}$, Mg$^{2+}$, and polyphosphates [14]. In addition, transmembrane proteins, such as CD63, GP1b, and α2β3, are stored in the dense granules, and translocate to the membrane during platelet activation. Platelets contain lysosomes that participate in macromolecule degradation, mediated by acid hydrolases that can degrade proteins, carbohydrates and lipids.

The platelet membrane phospholipids are asymmetrically arranged. The neutral phosphatidylcholine and sphingomyelin predominate in the plasma membrane layer, while the anionic phospholipids, phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidylserine (PS) predominate in the inner cytoplasmic layer. These phospholipids, especially PI, support platelet activation by supplying ARA that is converted to different eicosanoids such as prostaglandins, lipoxins, and TxA$_2$ during platelet activation [15, 16].

PS flips to the outer surface upon platelet activation, and on which coagulation factor complexes such as factors VIII-IX and factors X-V can assemble. The plasma membrane phospholipids support platelet activation internally, and plasma coagulation externally. Platelet plasma membrane lipid rafts are involved in signaling and intracellular trafficking. These membrane proteins include CD36, CD63, CD9, G protein-coupled receptor (GPCR), GPIIb/IIIa, and GLUT-3. Human platelets also express thrombin receptors, protease-activated receptors-1 and 4 (PAR1 and PAR4), and activation of either is sufficient enough to trigger platelet activation secretion, and aggregation.

Thrombin, generated by blood coagulation pathways (extrinsic and intrinsic pathways), is the most potent platelet aggregating agent, and also activates endothelial cells and other important responses in vascular biology. Thrombin and platelets play a
central role in CVD and other pathological processes. The thrombin surface receptors of platelets also trigger the release of granules which play a role in multiple functions – namely, coagulation, inflammation, atherosclerosis, anti-microbial host defense, angiogenesis, wound repair, and tumorigenesis. Among these surface receptors, GPCR has been reported to play a crucial role in ADP secretion from dense granules. Asymmetrically arranged phospholipids (phosphatidylserine and phosphatidylinositol) present in the inner layer of the plasma membrane maintain the stability of platelet membrane surface during the inactive state.

Platelet aggregation response can be analyzed using various assays and measures of platelet activation. Platelet aggregation remains the gold standard, but other testing methods offer advantages for specific applications, such as detecting overall platelet hyper-reactivity in the presence of antiplatelet therapy, or detecting inhibition of the ADP receptor P2Y. Platelet behavior is dependent on metabolic potential and, therefore, the secretory granules, mitochondria, and glycoprotein receptors.

Light transmittance aggregometry, initially described by Born, can be used to measure platelet activity. This method was developed on the basis that plasma enriched with platelets (as a result of agonist-induced platelet aggregation) has altered light transmittance. Light transmittance aggregometry is potentially a good method for comparing the in vitro effects of different pharmacological agents (such as aspirin and clopidogrel). However, this method is limited in the ability to predict in vivo platelet activity in the pathological state, probably because a single agonist used in the test cannot reflect the complexity of pathophysiological signaling.

The PFA-100 (Platelet Function Assay https://en.wikipedia.org/wiki/PFA-100 - cite_note-PFA-1) is a platelet function analyzer. The membrane of the cartridges are coated with collagen and ADP, or collagen and epinephrine, inducing a platelet plug to form, which closes the aperture. The PFA test result is dependent on platelet function, plasma vWF level, platelet number. The normal value range is 84–160 seconds. The CT above 160 seconds suggests possible PLT hemostatic dysfunction in vivo. Standard testing protocols for platelet aggregation are needed to achieve consistency among studies.
1.3 Platelet Activation Pathways

The hemostatic roles of platelets are essential for the maintenance of the integrity of the vascular network. Platelet adhesion, aggregation, and secretion are the processes involved in platelet activation, and these processes often occur simultaneously. Damaged endothelial exposes the sub-endothelial collagen that binds and activates blood platelets. Upon stimulation of platelets, granules release their contents into the extracellular environment, contributing to further platelet activation and thrombus formation [18]. Several intracellular signaling pathways involving collagen, thrombin, TxA₂ and ADP are involved.

The initial docking of platelets at sites of vascular injury is mediated by GPIb/V/IX, a structurally unique receptor complex expressed in megakaryocytes and platelets[19]. vWF is the major ligand for one component of this complex, GPIb, and the absence of vWF causes defects in primary hemostasis and coagulation. Besides GPIb, several collagen receptors with a tethering function are found on the platelet surface – notably GPVI and GPIa, members of the immunoglobulin superfamily [19].

After the initial adhesion of platelets to the damaged vessels, the repair process requires a rapid response to autocrine and paracrine mediators, including ADP, thrombin, epinephrine, and TxA₂ [20]. These mediators amplify and sustain the initial platelet aggregation response. They recruit additional circulating platelets from the flowing blood, to form a growing hemostatic plug. Most agonists that activate platelets operate through G-protein-coupled receptors [20]. The final pathway for all agonists is the activation of the platelet integrins GPIIb/IIIa (αIIbβ3), the fibrinogen receptor, the main receptor for platelet adhesion and aggregation (Figure 1.4).

Usually, platelet activation begins with the activation of one of the phospholipase C (PLC) isoforms expressed in platelets. PLC cleaves the phosphatidylinositol-4,5-bisphosphate (PIP₂) to produce inositol-1,4,5-trisphosphate (IP₃), the second messenger that raises the cytosolic Ca²⁺ concentration [21]. The raised level of Ca²⁺ activates integrin and, thus, activation of platelets starts.

Different isoforms of PLC are activated by different agonists. Collagen activates PLCγ2 using a mechanism that depends on
scaffold molecules and protein tyrosine kinases, whereas thrombin, ADP and TxA2 activate PLCβ using G_q as an intermediary. The rise in the cytosolic Ca^{2+} concentration that is triggered by most platelet agonists is essential for platelet activation. In resting platelets, the cytosolic free Ca^{2+} concentration is maintained at approximately 0.1 μM, by limiting Ca^{2+} influx and pumping Ca^{2+} out of the cytosol, either out across the plasma membrane or into the dense tubular system. In activated platelets, the Ca^{2+} concentration rises tenfold to >1 μM, as Ca^{2+} pours back into the cytosol from two sources. The first is IP_3-mediated release of Ca^{3+} from the platelet dense tubular system. The second is Ca^{2+} influx across the platelet plasma membrane, an event triggered when depletion of the dense tubular system Ca^{2+} pool produces a conformational change in Stored Ca^{2+} Depletion-induced Oligomerization of Stromal Interaction Molecule 1 (STIM1), a protein located in the dense tubular system membrane.

Ultimately, it is the binding of fibrinogen or another bivalent ligand to α_{IIb}β_3 that enables platelets to stick to each other.
Proteins that can substitute for fibrinogen include fibrin, vWF and fibronectin. Average expression levels of $\alpha_{IIb}\beta_3$ range from approximately 50,000 per cell on a resting platelet, to 80,000 on an activated platelet. Fibrinogen plays an important role in maintaining the stability of a thrombus, by bridging GPIIb/IIIa integrins between platelets. Quiescent platelets contain the pre-mRNA of the molecule termed TF, the primary initiator of the coagulation cascade that leads to the conversion of prothrombin to thrombin and fibrinogen to fibrin. Mutations in $\alpha_{IIb}\beta_3$ that suppress its expression or function produce a bleeding disorder (Glanzmann’s thrombasthenia), because platelets are unable to form stable aggregates.

**1.4 Platelets and Vessel Wall Interactions**

The vascular endothelium controls platelet reactivity via different mechanisms, such as ARA-PGI₂ (prostacyclin) pathway, the L-arginine-nitric oxide (NO) pathway, and the endothelial ecto-adenosine diphosphatase (ecto-ADPase) pathway. Endothelial cells metabolize ARA into PGI₂ with COX-1 or COX-2, with the involvement of PGI₂ synthase. COX-2 appears to be important in prostacyclin synthesis by endothelium, on the basis of the effects of selective COX-2 inhibitors on the excretion of PGI₂ metabolites. PGI₂ inhibits platelet function by elevating intracellular cyclic AMP levels via G protein-linked receptor PGI₂ [22]. PGE₁ and PGI₂ share the same receptor on platelet membrane [22].

NO produced by the L-arginine-NO pathway can stimulate the production of cyclic GMP in platelets, and regulates cyclic GMP-dependent protein kinases, causing a secondary decrease in intracellular Ca²⁺ flux. This lowering of intracellular Ca²⁺ levels suppresses the conformational change in GPIIb/IIIa that is required for binding of the integrins to fibrinogen, thereby decreasing the number and affinity of fibrinogen binding sites on the platelet’s surface. Ecto-ADPase, an integral component of the endothelial-cell surface, limits the plasma level of ADP, ATP. The activity of this enzyme abrogates the critical recruitment phase of platelet reactivity, as the availability of nucleotides in the near environment is reduced.
1.5 Roles of Platelets in Atherosclerosis and Inflammatory Processes

The important role of platelets in atherosclerosis development in humans has emerged. Several platelet-derived chemokines and growth factors are detectable in atherosclerotic plaques. Moreover, platelet activation and shedding of membrane particles is associated with increased wall thickness of the carotid artery. Persistent platelet hyperactivity, as reflected by enhanced excretion of thromboxane metabolites and other secretory components, is associated with CVD risk factors.

Different pathways are responsible for contribution of platelets to atherogenesis, reduced blood flow, and hypertension, such as shedding of membrane particles, cytokines, and growth factors activating blood vessels. vWF mediates the recruitment of platelets at the site of vascular injury, and is also a determinant of atherosclerotic plaque development. COX-1-dependent thromboxane synthesis has been demonstrated to accelerate atherogenesis in animal models, suggesting that platelet activation increases the rate of plaque formation [22, 24].

P-selectin (CD62P) of platelets also stimulates monocytes and macrophages to release chemokines, and promotes the formation of platelet-monocyte aggregates. A significant association has been reported between platelet hyperactivity and carotid artery wall thickness in diabetes and in hypertension [25].

The correlation between platelet reactivity and the extension of coronary atherosclerosis was observed in a study of more than 300 patients. Patients with more extensive coronary atherosclerosis have hyperactivity of platelets. Platelets adhere to the endothelium of carotid arteries in apolipoprotein E (apoE)−/− mice before atherosclerotic lesions are visible. vWF, when secreted in large amounts by endothelial cells in response to inflammatory stimuli, can recruit platelets to the site; the interaction between GPIb and vWF allows platelets to roll on endothelial cells.

The acceleration of atherogenesis by COX-1-dependent thromboxane in LDL receptor−/− mice suggests that platelet activation increases the rate of plaque formation. The inhibition of the synthesis of platelet thromboxane, as well as the antagonism or deletion of the thromboxane receptor, delays
atherogenesis in murine models. Activated platelets can also influence the progression of plaque formation by releasing adhesive ligands, such as P-selectin expressed on the platelet membrane and mediate platelet-endothelium interactions. P-selectin can stimulate monocytes and macrophages to produce chemoattractants or growth factors. Moreover, engagement by P-selectin of the P-selectin glycoprotein ligand 1 on the monocye surface initiates the formation of platelet-monocyte aggregates and outside-in signaling, which induces the transcription of COX-2. Prolonged adhesion-dependent signaling promotes the expression of interleukin-1β. This cytokine enhances the stability of COX-2 mRNA, thereby promoting synthesis of the enzyme. All these factors render platelets to contribute to atherosclerosis.

Platelets are increasingly recognized to be involved in the fields of wound healing, immune, and inflammatory process. At sites of injuries or infections, platelets are the first cells to be recruited to the vascular endothelium. There, platelets interact with various cell types, including monocytes, neutrophils, and endothelial cells and, thereby, regulate cellular adhesion and extravasation. Platelets are known to induce to pro- and anti-inflammatory phenotypes, depending on the underlying pathology, site of inflammation, and experimental model employed [26].

Thrombin-activated platelets bind monocytes, inducing the production of pro-inflammatory cytokines. Depending on the experimental setting, membrane micro-particles released from platelets can either enhance the pro-inflammatory effects of macrophages, or inhibit pro-inflammatory cytokine/chemokine secretion. Platelet granules contain a wide variety of signaling factors, such as chemokines, serotonin, histamine, nucleotides, and proteases involved in inflammatory processes. In addition, platelet eicosanoids, such as TxA2 or PGE2, can modulate inflammatory responses. Activated platelets enhance IL-10 secretion and reduce TNFα secretion by monocytes, in order to counteract exaggerated pro-inflammatory immune responses.

Activated platelets alter the chemotactic and adhesive properties of endothelial cells by releasing these inflammatory molecules. These platelet-induced alterations of endothelial-cell function can modulate the chemotaxis, adhesion, and