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A guide to molecular and functional investigations of platelets to bridge basic and clinical sciences

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Platelets have been shown to be associated with pathophysiological processes beyond thrombosis, demonstrating critical additional roles in homeostatic processes, such as immune regulation and vascular remodeling. Platelets themselves can have multiple functional states and can communicate with and regulate other cells, including immune cells and vascular smooth muscle cells, to serve such diverse functions. Although traditional platelet functional assays are informative and reliable, they are limited in their ability to unravel platelet phenotypic heterogeneity and interactions. Developments in methods such as electron microscopy, flow cytometry, mass spectrometry and 'omics' studies have led to new insights. In this Review, we focus on advances in platelet biology and function, with an emphasis on current and promising methodologies. We also discuss technical and biological challenges in platelet investigations. Using coronavirus disease 2019 (COVID-19) as an example, we further describe the translational relevance of these approaches and the possible 'bench-to-bedside' utility in patient diagnosis and care.

Many studies in the past decade have elaborated the emerging functions of platelets beyond hemostasis and thrombosis. Molecular and functional studies have shown diverse roles of platelets in vascular integrity and remodeling, immunoregulation and tissue regeneration (Fig. 1). Although the function of platelets in the development and progression of cardiovascular diseases is well established, platelets were also demonstrated to be critical players in the pathophysiology of cancer, inflammatory diseases and infections such as that with severe acute respiratory syndrome coronavirus (SARS-CoV)-2, giving rise to COVID-19. Investigations of platelets and their heterogeneous states, platelet releasates and other target cells in various diseases have provided important mechanistic insights for diagnosis, prognosis and therapeutics. In this Review, we discuss the pathophysiological role of platelets and the latest developments in basic and clinical platelet methodologies. We also provide new insights into the development of guidelines for platelet investigation in which knowledge of underlying mechanisms might be important for diagnosis.

Platelets are heterogeneous first responders to vascular stress

Platelets are anucleate, small (2–4 µm), short-lived (7–10 d) circulating cells in the blood, shed from megakaryocytes[1]. Approximately, 10^{11} platelets are produced (and consumed) daily, maintaining a platelet count of between 150 and 450×10^9 platelets per l in the bloodstream of a healthy individual. Platelets have an essential function in thrombosis. Platelet releasates can initiate and propagate thrombosis following mechanical stress or injury, endothelial damage and collagen exposure. Circulating von Willebrand factor (VWF) binding to platelet glycoprotein (GP)Ib (GPIb–IX–V receptor complex) and platelet GPVI–collagen interactions contribute to initial thrombus formation at the damaged site. After initial adhesion to the damaged vessel wall and platelet activation, platelet-derived thromboxane A2 (TxA2) and release of ADP from δ-granules mediates recruitment of additional platelets. Platelet fibrinogen receptor αIIbβ3 primarily contributes to the formation and stabilization of the thrombus. The activation of coagulation factors and other cells, including monocytes, vascular smooth muscle cells (VSMCs), macrophages and the inflamed endothelium further facilitates thrombus development.

In addition to secreted thrombosis mediators including ADP, fibrinogen and VWF, platelets also contain other factors such as cytokines and chemokines (interleukin (IL)-1β, RANTES, MCP-1 and others), which allows them to respond to changes in the local environment and influence other cells (for example, leukocytes). The ability of platelets to influence other cells arises not only from granules (α-granules, dense granules and lysosomes) but also possibly via microvesicles, exosomes, apoptotic bodies and uptake of whole platelets. The procoagulant microvesicles (phosphatidylserine$^+$CD41$^+$CD42b$^+$) released by activated platelets in circulation bearing receptors for collagen and VWF contribute to hemostasis and thrombosis. Platelet-released microvesicles have also been proposed to promote thrombosis in clinical settings such as heparin-induced thrombocytopenia.

Platelets contain a heterogeneous content of coding and noncoding RNA species, machinery for processing RNA transcripts and the capacity to synthesize new proteins in response to stress stimuli. Indeed, the gradual loss of RNA content in ageing platelets might be a determinant of platelet lifespan[2]. MicroRNA (miRNA) species have been detected in human platelets, and platelet reactivity has been associated with the abundance of selective miRNA species, such as miR-223 and miR-126 (refs. [3,4]). Activated platelets can also

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release miRNA species and probably contribute to the increased levels of miRNA species in plasma[3–6]. Several platelet-derived miRNA species predominantly present in extracellular vesicles have been linked to cardiovascular diseases including coronary artery disease (CAD) and thrombotic events. Furthermore, select miRNA species have been shown to have considerable clinical utility in predicting risk and mortality and have been proposed as biomarkers for the assessment of the efficacy of an antiplatelet regimen[7–9].

Of note, it is also becoming apparent that circulating platelets can exist in different states, and their heterogeneity in size, age and responsiveness might be reflected at rest, upon activation or stimulation by agonists and as observed within the hemostatic plug. Newly synthesized, younger platelets are larger in size, with a higher mean platelet volume, also reflecting their greater prothrombotic potential. Elevated platelet size is associated with increased platelet aggregation and higher risk of cardiovascular and peripheral arterial disease[10], and mean platelet volume might be an important prognostic marker[10,11]. Immature platelets might be more resistant to inhibition with aspirin and clopidogrel, a P_2Y_{12} receptor antagonist[12]. In addition to differences in function based on age, platelets can be hyperactive[13], procoagulant[14] or apoptotic[13,15], all potentiating a thrombotic tendency. Our studies have shown that diabetes-induced oxidative stress (hyperglycemia, inflammation, lipids) leads to platelet dysfunction and apoptosis[13]. Paradoxically, platelet apoptosis can induce thrombosis and can result in a higher proportion of younger, more reactive platelets. Apoptotic bodies shed by dying platelets can promote hyperactivity of the remaining platelets[16] and lead to increased thrombosis risk, such as that seen in heparin-induced thrombocytopenia[17]. These and other such studies have highlighted the need to understand and identify the different states of platelets and their contributions to disease processes.

Intercellular interactions of platelets. Platelets execute many of their emerging pathophysiological functions (Fig. 1) by interacting with other cells directly or through platelet releasates (for example, growth factors, hemostatic factors, miRNA), reflecting further functional diversity. With endothelial damage, for example, VSMCs are exposed to circulating platelets. Platelets not only release growth factors such as platelet-derived growth factor (PDGF) that stimulate VSMC dedifferentiation to promote wound healing, but platelets themselves can also be taken up by VSMCs[2,7]. Platelet inhibition reduces endothelial dysfunction and limits the progression of atherosclerosis and atherothrombotic events[18]. Major antiplatelet therapies including aspirin and clopidogrel can improve endothelial function in patients with CAD and patients undergoing percutaneous coronary intervention. Platelet–endothelial interactions have also been observed in COVID-19 (refs. [19–21]). Activated platelets can also interact with monocytes to form platelet–monocyte aggregates, which participate in a broad spectrum of cardiovascular and other inflammatory diseases including atherosclerosis, venous thromboembolism and myocardial infarction[22–24]. The levels of circulating platelet–leukocyte aggregates are elevated in CAD and peripheral artery disease, and studies have shown evidence for platelet–neutrophil interactions at the sites of atherosclerotic plaque rupture and atherothrombosis[25]. Table 1 summarizes the interactions of platelets with other cells including endothelial cells, VSMCs, red blood cells (RBCs), pathogens and tumor cells that can occur during normal physiology as well as in disease pathology and the major techniques (to be described) that have been successfully used to study such interactions.

Over the past decade, advances in platelet studies have demonstrated platelet heterogeneity in functional states, content and interaction with other cells. Detecting these different states, contents and interactions will lead to recognition of important platelet roles in disease pathogenesis. However, studying platelets can be difficult and requires meticulous approaches (Box 1). Research laboratory techniques might prove clinically useful when complemented with clinical investigative methodology. We will outline some of these promising laboratory research approaches followed by currently used clinical approaches to provide a broad spectrum of what is currently available to investigate the latest developments in platelet heterogeneity and platelet–cellular interactions.

Current platelet techniques in the laboratory

Major progress has been made in the development of methods, both conventional and emerging, to study platelets in both the laboratory research setting and the clinic. Although classical platelet tests such as light transmission aggregometry (LTA) are still used globally to understand platelet responses, the emerging technologies reviewed below provide an expanded set of tools for a more in-depth evaluation of platelet biology and function. Although advantages and limitations are associated with each method (Table 2), the range of available techniques allows the researcher to choose the assay method best suited for the specific research goal. Owing to increasing evidence showing the critical roles of platelets in the prognosis of not only cardiovascular diseases but also in diseases with a previously unknown involvement of platelets, these technologies are ushering in an exciting new era for platelet studies and many might ultimately be used for clinical studies.

Aggregometry to study mechanisms. Platelet aggregometry is considered the gold standard test for platelet functional analysis. Aggregometry measurements take only minutes after sample processing and can be performed on whole blood (impedance-based), platelet-rich plasma (PRP) or washed platelet samples (LTA) with the use of specialized equipment (platelet aggregometer, reviewed by Hvas and Favaloro)[26]. Multiple-electrode aggregometry uses multiple electrodes and is high throughput. Although multiple-electrode aggregometry can be performed in whole blood, even in samples with low platelet counts, it remains less sensitive than traditional LTA[27]. In basic research, platelet aggregometry is commonly conducted to assess the effects of deleted or overexpressed genes in animal models or for testing antiplatelet activities of various therapeutic agents. Mouse platelet aggregation is often performed in pooled blood samples because of the scarcity of blood volume that can be drawn from live animals. To allow for individual mouse measurements, our group has optimized whole-blood impedance aggregometry, eliminating the need for euthanasia or sample pooling[28].

Flow cytometry. Flow cytometry is a diverse and reliable assay for studying multiple aspects of platelet function in both humans and mice. Flow cytometry allows for quantitation and detection of interactions between activated platelets and other blood cells including leukocytes. Interestingly, platelet aggregation can also be studied with the use of a flow cytometry assay. Flow cytometry assays offer the advantage of being highly sensitive and can be performed with platelet counts as low as 40,000 platelets per mm^3 or lower. Numerous platelet-specific markers have been incorporated into flow cytometry assays to study a variety of platelet functions. Platelet-activated glycoprotein αIIbβ3 antibody (PAC-1) is a specific monoclonal antibody that recognizes an epitope present on the activated form of human αIIbβ3 and can be used to differentiate resting platelets from activated platelets[29]. The presence of CD62P (P-selectin) and receptor TREML1 on the platelet surface, usually found in α-granules of platelets, is a marker of activation-coupled degranulation. Measurement of intraplatelet calcium levels with the use of calcium-binding fluorescent dyes can also be used to study platelet-activation kinetics and activation states. Although a Fluo-3-based assay has been shown to measure activation-associated intraplatelet calcium kinetics in whole-blood platelets, Fluo-4 and Fluo-5 have been used to study platelet-activation states and

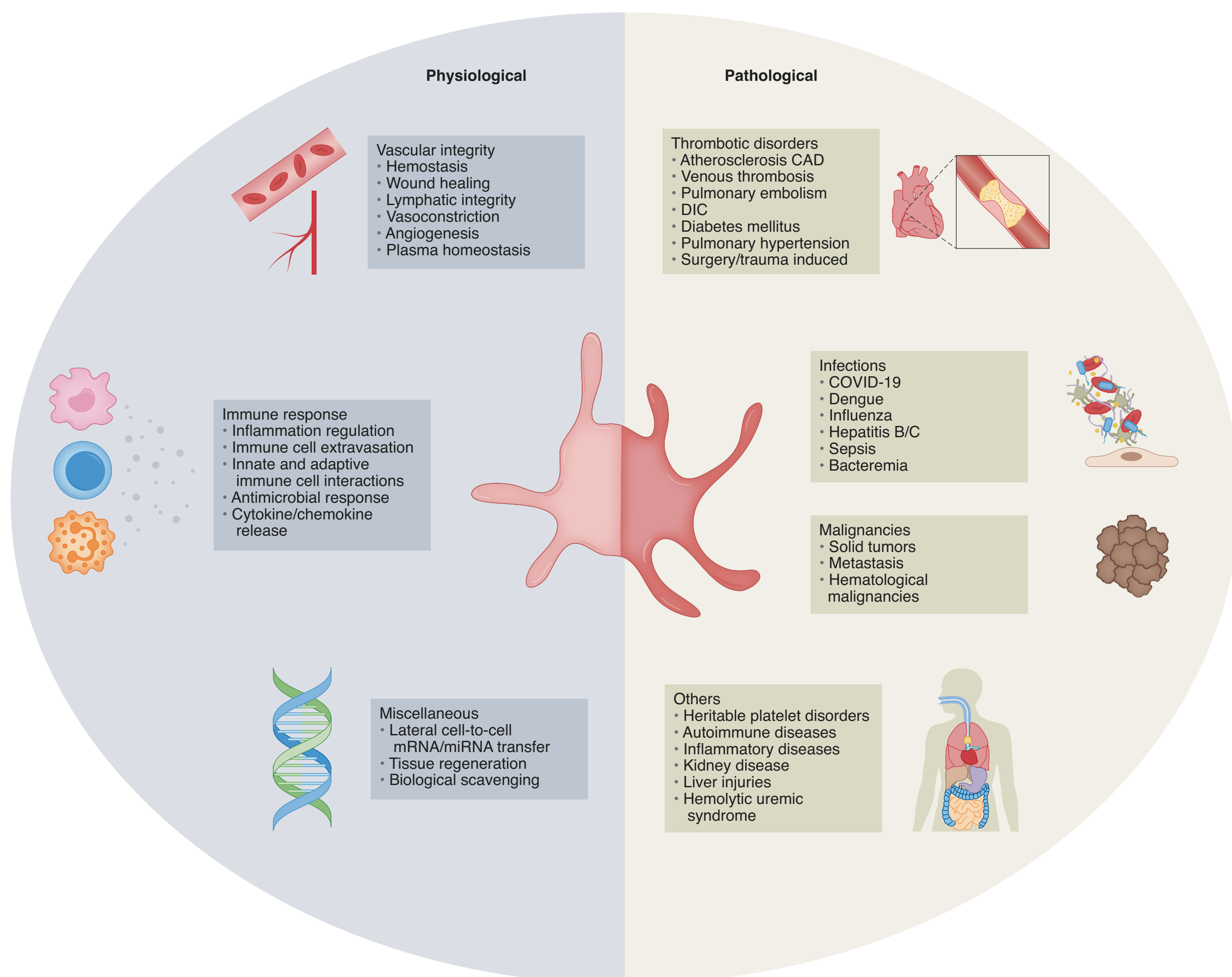

Fig. 1 | Schematic representation of physiological and pathological roles of platelets. Although classically considered as mediators of hemostasis and thrombosis, platelets have been demonstrated to play a wide range of other physiological and pathological roles. Their physiological functions include the maintenance of vascular integrity through interaction with the vascular wall and contributions to wound healing and angiogenesis[14,166]. Platelets also maintain plasma content homeostasis by storing and releasing many essential plasma factors such as growth factors (VEGF, TGF-β, etc.) and vascular tone regulators such as serotonin[166]. Platelets are intricately linked to physiological inflammatory responses through cytokine storage and release and by interacting with diverse circulating immune cells[167]. Many of these homeostatic functions are mediated through transfer to other cells (for example, mRNA or miRNA) or acceptance from other cells. Platelets are well studied for their pathological roles in thrombotic disorders[168], such as enhanced vascular thrombosis in diabetes mellitus and many other disease processes[14]. Platelets have also been linked to the pathogenesis of disease states such as infections (sepsis and viral infections including COVID-19 (refs. [19–21])) and malignancies[56] in addition to inflammatory disorders, autoimmune disorders, liver disease and kidney disease[57,58]. This functional diversity in both physiological and pathological contexts is unusual for a small, short-lived cell with no nucleus. Complementing basic investigations with clinical methodology can provide critical mechanistic insights into these diverse functions. DIC, differential interference contrast.

distinguish procoagulant platelets from activated but non-coagulant platelets[16]. Other ratiometric dyes such as Fura-2 have also been used to quantitate absolute calcium concentration changes during agonist-induced platelet activation[30]. Platelet mitochondrial depolarization, which is an important parameter for mitochondrial function, apoptosis[31] and externalization of phosphatidylserine, another property of an apoptotic, procoagulant platelet, can be also detected by flow cytometry[32]. Measurement of intraplatelet reactive oxygen species by flow cytometry with the use of specific cell-permeable dyes has been used in the study of platelet aging and oxidative stress[28].

Performing flow cytometry with the use of thiazole orange (a fluorescent dye that binds nucleic acids) can be used to distinguish the reticulated platelet subpopulation and is especially useful in the estimation of platelet production and megakaryopoiesis in thrombocytopenic disorders[33,34]. Automated blood analyzers (Sysmex XE-/XN-series) can also measure the immature platelet fraction in whole blood and are used often in clinical settings[34].

As platelet function inherently depends on the dynamic interplay between activating and inhibitory signaling, multiple methods have now been developed to study phosphoprotein patterns by flow cytometry, offering large advantages over more conventional approaches including immunoblotting and mass spectrometry that rely on lysis and extraction[35]. A smart application of the flow cytometry assay to platelet function analysis has been developed to identify heterogeneity in platelet responsiveness to agonists in healthy donors. Coined as a 'Phenomic' study by the authors, Dunster and coworkers used multiple agonists at varying concentrations to

Table 1 | Summary of platelet interactions with other cell types and study methods used

Cell type	Selected mechanisms of platelet interactions	Primary methods	Reference
Endothelial cells	Platelet (activated) P-selectin with endothelial PSGL-1 Platelet (resting) PSGL-1 with endothelial P-selectin Platelet αIIbβ3 with endothelial αvβ3 and ICAM-1 via fibrinogen engagement Platelet CD154 with endothelial CD40L Horizontal transfer of platelet miRNA	Intravital microscopy Flow chamber and microfluidic assays Flow cytometry Confocal microscopy	142,143
Neutrophils	Platelet P-selectin with neutrophil PSGL-1 Platelet GPIbα with neutrophil MAC-1 Formation of platelet–neutrophil aggregates	Flow cytometry Confocal microscopy Intravital microscopy ELISA	143,144
Monocytes	Platelet P-selectin with monocyte PSGL-1 Platelet interaction with monocyte ICAM-1 via fibrinogen Horizontal transfer of *TLR2* mRNA Horizontal transfer of platelet miRNA Platelet interaction with monocyte causing myeloid activation	Flow cytometry Confocal microscopy Single-cell RNA sequencing. Immunohistochemistry	132,143
Lymphocytes	Platelet P-selectin with lymphocyte PSGL-1 Platelet CD154 with lymphocyte CD40 Interaction with platelet P-selectin, CD40L and αIIbβ3 Release of PF4	Flow cytometry Confocal microscopy	145,146
Erythrocytes	Formation of aggregates with platelets via adhesive molecules Direct interaction with platelets via FasL/FasR Platelets interact with RBC microparticles	Flow cytometry Confocal microscopy Electron microscopy Microfluidics	147
VSMCs	Platelet PF4 stimulation of VSMC Platelet PDGF stimulation of VSMC Horizontal transfer of platelet miRNA causing VSMC differentiation	Cytokine and cell-proliferation assays Luciferase reporter assay Immunohistochemistry Electron microscopy Transgenic murine models	2,148
Hematopoietic stem cells	Horizontal transfer of platelet miR-1915-39 promotes megakaryocyte differentiation and platelet production	Cell flow cytometry Confocal microscopy and TEM	149
Kupffer cells	Platelet interaction with immune cell via hyaluronan–CD44 binding	Immunoblot analysis Flow cytometry RNA sequencing Confocal microscopy	150
Cancer cells	Platelet CLEC2 with tumor cell podoplanin Platelet TGF-β with tumor cell podoplanin Platelet TLR4 with tumor cell HMGB1	Flow cytometry Platelet-adhesion and -aggregation assays ELISA	151,152
Infectious pathogens (for example, bacteria and viruses)	Platelet GPIb, αIIbβ3 and FcγRIIa receptors adhere to and aggregate bacteria Platelet TLR4 and P-selectin bind to bacterial LPS Platelet TLR2 binds to DNA viruses SARS-CoV-2 binding to human *ACE2*-transgenic platelets? Platelet β-defensin induces NET formation PF4 induces bacterial phagocytosis Platelet IL-1β increases endothelial permeability in dengue infection Platelet degranulation and activation promotes pulmonary inflammation in H1N1 infection Enhanced αIIbβ3 activation, arachidonic acid metabolism and microparticle release in platelets during H1N1 infection Platelet deposition in lung tissues with SARS-CoV-2 infection	Confocal microscopy Flow cytometry In vitro platelet-adhesion and -aggregation assays TEM Histopathological analysis	127,130,153,154

Abbreviations: CD40L, CD40 ligand; CLEC2, C-type lectin-like receptor-2; HMGB1, high-mobility group box 1 protein; ICAM-1, intercellular adhesion molecule 1; LPS, lipopolysaccharide; NET, neutrophil extracellular trap; PF4, platelet factor 4; PSGL-1, P-selectin glycoprotein ligand 1; MAC-1, macrophage 1 antigen; TEM, transmission electron microscopy; TLR, Toll-like receptor; VSMC, vascular smooth muscle cell.

generate a platelet-response profile of each individual on the basis of surface marker expression[36]. Although this technique requires a more careful and elaborate assay design compared with that of conventional flow cytometry, this analysis could be potentially helpful in studying platelet function variability in a cohort with better reproducibility.

Proteomics. Platelets contain a vast pool of proteins (estimated to be more than 3,000) that includes membrane proteins, cytoskeletal proteins, signaling proteins, enzymes, coagulation factors and transcription factors. The discovery of this rich and diverse repertoire of platelet proteins has been largely achieved by platelet proteomic analysis. Platelet two-dimensional electrophoresis–mass

spectrometry (2DE–MS) proteomics to study thrombin-induced activation has led to the identification of specific proteins released by platelets[37]. In addition, platelet proteomic-analysis studies have identified numerous proteins that mediate hypoxia-induced platelet hyper-reactivity[38].

Proteomic advances have made it possible to study platelet subproteomes on the basis of post-translational modifications, and phospho-proteome analyses have revealed phosphorylation patterns in resting and activated platelets[39,40]. With the use of immobilized metal affinity chromatography (IMAC) and titanium dioxide coupled with nano-liquid chromatography (LC)–MS and isobaric tags for relative and absolute quantification (iTRAQ) (modified proteomic technique)[41], specific nodes in platelet-activation signaling have been identified. Studies of the glycosylated proteome showed a major role for *N*-linked glycosylation of adhesive proteins following binding to collagen[42]. Platelet protein ubiquitination is another important post-translational modification involved in protein degradation, signaling and protein–protein interactions and has been analyzed by studying the platelet ubiquitinome[43].

Mass cytometry time of flight (mass CyTOF) is a fairly new methodology that combines features of flow cytometry and mass spectrometry, improving upon the limitations of traditional flow cytometry through the use of antibodies covalently tagged by heavy-metal isotypes rather than fluorophores[44]. Despite the antibody dependence, the greatest advantage of mass cytometry is the large number of simultaneous parameters that can be analyzed, which allows for comprehensive characterization of cellular proteins and pathways. Mass cytometry has been used to identify previously unappreciated platelet subpopulations[45,46] and is emerging as a powerful single-cell proteomic technique.

Lipidomics. Diverse lipids and fatty acids including phospholipids, sphingolipids, steroids, eicosanoids (prostaglandins), phosphatidylinositides, diglycerides, triglycerides and cholesteryl esters are present in varying proportions in platelets and can regulate major aspects of platelet function including aggregation, morphological changes, coagulation and granule release[47]. Advances in mass spectrometry analysis of lipids, 'lipidomics', using high-resolution rapid scanning instruments have provided new insights into understanding the role of the platelet lipidome in the regulation of hemostasis and its contribution to the progression of thrombosis and atherosclerosis. Currently, lipidomics is commonly performed with the use of either 'shotgun' lipidomics, a high-throughput approach that detects the most abundant species, or LC–MS, a targeted and highly sensitive approach. A comprehensive analysis of the platelet lipidome has revealed that the platelet lipidome is altered during platelet activation and probably has a role in platelet procoagulant function[48,49]. Furthermore, studies on the composition of stored platelets[50] and extracellular vesicles have confirmed the presence of cholesterol and other lipids in platelets. High cholesterol levels as observed in hypocholesterolemia correlate with the elevated amounts of cholesterol found in platelet membranes and greater thrombosis risk[51]. Altered lipidomic profiles have been observed in patients with CAD[52] and myocardial infarction. Therefore, platelet lipidomic profiles could be potentially used as disease signatures (such as plaque progression in atherosclerosis), for risk stratification of patients and to determine treatment efficacy.

Genomics and transcriptomics. Although platelets lack nuclei, genomic sequencing (exon or whole genome) and/or genome-wide association studies (GWAS) can be informative by studying genetic mutations or single-nucleotide polymorphisms associated with platelet phenotypic traits or bleeding disorders. A whole-genome-sequencing study identified regulatory noncoding genomic loci affecting platelet function[53]. One single-nucleotide polymorphism in the *GNB3* gene associated with enhanced platelet function was identified in African American families with heritable premature CAD[54]. Platelet transcriptomics with the use of microarray and serial analysis of gene expression (SAGE) strategies have revealed platelets to represent around 15–25% of the human genes present in gene libraries[37,55]. All these genomic analyses are especially important for heritable platelet disorders and in cases of family history of cardiovascular risk. RNA sequencing and platelet RNA profiling of tumor-educated circulating platelets from over 220 patients with various types of cancers have revealed a unique platelet transcriptome profile that can accurately identify primary tumor sites[56]. Unique platelet transcriptomes that do not necessarily correlate with platelet hyperactivation signatures have also been characterized in myocardial infarction, lupus, kidney disease, obesity and sepsis[57,58]. Transcriptome analyses have highlighted the diverse roles of platelets beyond thrombotic activation and provide an important strategy for future investigations.

Despite advances over the past few years in high-throughput and high-sensitivity techniques, a challenge in platelet 'omics' studies is to attain a high level of purity of platelet samples. The possible presence of small numbers of leukocytes or RBCs in the isolated platelet population, even after centrifugation-based washing steps,

Box 1 | Pre-analytic challenges in platelet investigations

Platelets are highly sensitive to changes in temperature, pH, shear and/or mechanical stress and additive reagents after removal from their natural microenvironment in the circulation. Artificial materials and chemicals can cause spurious platelet activation; therefore, blood (most commonly venous) should be carefully drawn into polypropylene plastic or silicone-coated tubes with sodium citrate buffer as the recommended anticoagulant for many platelet function tests. Platelet preparation for basic or clinical investigations requires whole blood, platelet-rich plasma (PRP) or washed platelets. Although PRP isolation from whole blood is a single centrifugation step, the preparation of washed platelets requires sequential centrifugation steps. The anticoagulant preferred for washed platelets is an acid-citrate-dextrose buffer (pH 6.5), which not only chelates calcium ions but also maintains lower pH to prevent platelet activation during isolation. The avoidance of possible platelet activation or aggregation during centrifugation of PRP and subsequent washing without exerting excessive inhibition is a critical factor to be considered. Prostaglandin (PG)I2, PGE1 and/or apyrase can be added to PRP as appropriate before centrifugation to prevent such activation. Further details of various steps including buffer and reagent compositions have been described previously[169]. According to the recommendations of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis[170], patient variables such as exercise, smoking, caffeine and medications should be taken into account when interpreting platelet aggregometry results. Additional variables that can affect the results of platelet function testing include platelet count ($<150\times10^9$ platelets per l or $>600\times10^9$ platelets per l)[170] and hematocrit[171]; all clinical tools ideally require platelet counts above 100×10^9 platelets per l. Patients being investigated often have a bleeding phenotype with platelet counts below 30×10^9 platelets per l, leading to difficulties in interpretation. The high volumes of blood required can pose technical difficulties, especially in pediatric populations in which blood specimens are limited. Additionally, evaluating platelet function in neonates is particularly challenging due to substantial differences between platelets from neonates and adults. Given these challenges, along with the limited number of parameters evaluated by these methods, a substantial proportion of patients with suspected platelet bleeding and/or clotting disorders remain without a clear diagnosis even after comprehensive clinical laboratory testing[163].

makes it mandatory to estimate the purity of an isolated platelet population. An average of one to five leukocytes per 10 million platelets (<0.00005% contamination) has been suggested as the acceptable limit. Flow cytometry assays make it possible to gate platelet populations with the use of CD41- or CD42b-specific antibodies. PCR or western blotting are other reliable methods, using a CD45-targeted primer or antibody, respectively, to detect leukocyte contamination. The low yields of platelet RNA and the potential for leukocyte contamination makes RNA analysis challenging. However, this challenge can be overcome by adding RNA-amplification steps into the standard protocols. Leukocyte depletion with magnetic bead-based antibodies is preferred for platelet RNA studies, although carefully conducted centrifugation steps along with PCR-based purity checks might bypass the need for leukocyte depletion. Platelet RNA analysis can also be performed by PCR-based arrays with the use of standard primers for the candidate genes or using commercially available high-throughput arrays[28].

Platelet imaging. Many imaging tools using fluorescence or phase contrast have advanced our understanding of platelet biology. A range of specialized microscopy techniques (Table 2) has allowed the visualization of platelet activation and receptor profiles, the formation of platelets from megakaryocytes and even the cytoskeletal rearrangements for the formation of thrombi[59]. Microscopy methods include wide-field microscopy, encompassing phase-contrast, total internal reflection fluorescence, reflectance interference-contrast and differential interference-contrast microscopy[59–61]. A platelet-adhesion assay tests the ability of platelets to adhere to and spread on different assay surfaces and can be visualized in static mode or under flow with the use of perfusion chambers and immunofluorescence microscopy[62]. Similarly, time-lapse phase-contrast microscopy with high magnification has been used to visualize and quantitate platelet migration after activation in the context of innate immune responses to bacterial challenges[63]. The development of in vivo imaging techniques such as intravital microscopy allows for dynamic analysis of interactions between platelets and vascular cells in real time[64].

In the last decade, the emergence of super-resolution microscopy and other nanoscopy techniques such as structured illumination microscopy and single-molecular-localization microscopy have allowed visualization at the nanometer resolution while overcoming the limitations of light micrsocopy[65]. Given that biological function is preserved during sample preparation for these techniques, these approaches have provided useful insights into cytoskeletal rearrangement, actin and tubulin fibers in platelets and receptor-colocalization studies[66–68]. However, these methods have been largely limited to in vitro and ex vivo studies because they are invasive and technically challenging to perform.

The photolithography technique coupled with immunofluorescence microscopy has been applied to quantitate the mechanical traction force generated by the platelet cytoskeleton during adhesion and spreading[69]. Customized atomic force microscopy has also been used to study the mechanics of platelet contraction at the single-cell level during clotting, which examines single platelets spread between two fibrinogen-coated surfaces[70]. Although less physiologic than in vivo imaging, microfluidic device imaging represents another approach to study platelet interactions with vascular cells and leukocytes in real time[71]. In conjunction with other technologies (for example, flow cytometry), these assays can provide useful information on the role of platelets in inflammation and immunity[72].

The development of high-throughput imaging and deep learning has taken platelet imaging to the next level. Intelligent image-activated cell sorting (iLACS) technology, which was initially developed for nucleated cells[73,74], has been applied successfully to platelets in the form of 'intelligent platelet morphometry'. This technique combines optofluidic time-stretch microscopy with a convolutional neural network in a deep learning algorithm to image and quantify morphological features of platelet aggregates, activated by different types of agonists in a high-throughput (>1,000 cells per second) approach[75]. This technology might be useful for monitoring antiplatelet therapy or identifying patients with increased thrombotic risk, such as those with cardiovascular disease or diabetes or patients hospitalized with COVID-19. Other single-cell microfluidic assays using real-time deformability flow cytometry and light-emitting diodes for mechanical phenotyping might offer additional insights into platelet physiology; however, they have yet to be validated for studying platelets[76].

Electron microscopy. Electron microscopy has been traditionally used for the study of platelet morphology and ultrastructure, both as a basic research method and a diagnostic tool. Transmission electron microscopy (TEM) studies have provided detailed insights into the molecular mechanisms of platelet granule release, platelet microparticle formation and release and platelet mitochondrial dysfunction[77–81] (Table 2). Moreover, TEM has also been a critical tool in discerning the differences in overall platelet morphology and cytoskeletal organization in platelet function defects[82,83]. Immunogold labeling, with the use of antibodies loaded with nanometer-size colloidal gold particles, allows the study of localized proteins and macromolecules within platelets. In combination with TEM, immunogold labeling has been used to determine the subcellular localization of many platelet proteins in α-granules, the open canalicular system and mitochondria as well as in extracellular vesicles[84–86].

In the clinical setting, TEM is commonly recommended as part of the diagnostic work-up for storage pool disorders and other structural abnormalities[87], including genetic disorders such as Hermansky–Pudlak syndrome and Chediak–Higashi syndrome[82]. In particular, whole-mount TEM has been shown to have clinical utility in evaluation of dense granule deficiency in patients with suspected platelet function disorders[88]. Given that whole-mount TEM has a superior sensitivity for dense granule deficiency than for platelet aggregometry and ADP-release assays, this technique is considered the gold standard for diagnosis of storage pool disorders. However, this method is fairly expensive, requires specialized equipment and is therefore only available in specialized centers.

In contrast to TEM, which is two-dimensional, conventional scanning electron microscopy (SEM) provides high-resolution imaging of three-dimensional (3D) surface topology. SEM is particularly useful in characterizing the composition and structure of thrombi and morphologic changes in platelets following activation[81]. Although SEM provides valuable information about clot and fibrin networks[89,90], typical analysis is manual and tedious. Newer automated platforms are now being developed to increase accuracy and reproducibility in imaging clots[91]. Several studies have used SEM to facilitate understanding of thrombus formation, platelet adhesion and platelet properties under flow and mechanical stress[81,92–94]. SEM has also been used to analyze the structure and composition of thrombi in myocardial infarction and ischemic stroke and to characterize the distinct heterogeneity of arterial and venous thrombi[77,95].

Cryogenic electron microscopy (cryo-EM) allows TEM of hydrated biological samples, preserving the native biological organization of cellular organelles and macromolecules[81,96]. Cryo-EM has been used to characterize the platelet open canalicular system and its interaction with the dense tubular system and α-granules along with platelet spreading and cytoskeletal changes at nanometer to subnanometer resolution. Serial block face SEM and focused ion beam SEM incorporate serial sectioning with SEM, allowing the acquisition of serial images with nanometer-size z-plane spacing (~20–30 nm for serial block face SEM and ~5 nm for focused ion beam SEM)[81,96]. These techniques have been used to obtain 3D structures of whole platelets with detailed characterization of platelet size, volume, activation events and organelles including

Table 2 | Summary of major basic research techniques used for platelet studies

Technique		Platelet characteristic/event	Advantages	Limitations	Reference
Imaging	Confocal microscopy	Temporal thrombus studies Platelet–fibrin interactions Platelet interactions with other cells Platelet spreading Morphology/activation/apoptosis Platelet deposition in tissues Protein localization	Protein markers High-resolution imaging Localized quantitation Cytoskeletal studies 3D reconstruction Single-platelet-level study	Time sensitive Cost of reagents Limited fluorescence channels Requires washed platelets	155
	Intravital imaging	Real-time in vivo thrombogenesis Platelet–vessel wall interaction dynamics under flow Platelet dynamics in live animal before/after	Real-time visualization in physiological environment Rapid No variations in sample processing	Limited resolution Requires specialized equipment and training Not at single-platelet level Anatomical challenges Expensive	156,157
	Conventional/bight field/wide field	Thrombus formation Effects of shear stress Large platelet aggregates	Accessibility Less time consuming No specialized training Low cost	Low resolution Lack of protein markers Limited by wavelength Only size-based identification	155
	Microfluidic devices	Platelet adhesion and hemostasis Biophysical forces	Whole-blood, real-time thrombus formation Thrombus dynamics	Not validated in the clinical setting	120,125
	iLACS	Single-platelet morphology and activation Platelet–platelet or platelet–immune cell interaction	High-throughput imaging Direct whole-blood application Benefits of both flow cytometry and imaging Rapid	Requires specialized equipment and training Expensive	135
Flow cytometry		Platelet activation, aggregation/thrombotic risk markers Apoptotic cell quantitation Platelet degranulation markers Diagnosis of platelet function disorders and thrombocytopenia Reticulated platelets Detection of platelet interaction with other cells Microparticle release Purity check	Minimal sample volume Millions of platelets analyzed within seconds with high accuracy Selection of marker-based platelet population Greater control during data acquisition Simultaneous determination of resting as well as activated state, apoptosis of platelets Can be done on fixed or unfixed samples Multiple quantitation options at cell and protein level Multiplexing possible	Interlaboratory standardization needed Availability of fluorescent reagents/antibodies Fairly expensive technique Best done on same-day sample Limited use for intraplatelet events	33,35,158
	Mass cytometry (CyTOF)	Platelet heterogeneity across resting and stimulated samples Identification of platelet subpopulations	Improves upon the limitations of traditional flow cytometry Analysis of large number of simultaneous parameters Low/zero background binding activity; look for exceedingly rare events	New method and not well established for studying platelets Expensive Requires operator expertise to run and interpret results	45,46

Continued

Table 2 | Summary of major basic research techniques used for platelet studies (continued)

Technique		Platelet characteristic/event	Advantages	Limitations	Reference
Electron microscopy	TEM	Ultrastructural evaluation of platelets (granule numbers, platelet defects) Protein localization at nm scale Organelle dynamics Viral/bacterial internalization imaging	Diagnostic utility Detailed information at nm scale Highest possible magnification/resolution Modifications reveal multiple ultrastructural details	Expensive specialized equipment Highly trained professional needed Time consuming (processing takes days) Fluorescent labeling cannot be done	81,82,96
	SEM	Super-high-resolution surface view Shape change dynamics Platelet–pathogen interactions Activation stage imaging	Best possible surface details Magnification/resolution	Expensive specialized equipment Highly trained professional needed Time consuming (processing takes days)	81,86
	CLEM/3D cryo-EM	Platelet secretion; platelet production from megakaryocytes Structural dynamics at ultrastructural level Organellar distribution Viral particle/microparticle studies	Super-high-resolution 3D visualization Intraplatelet structural details	Samples need to be mounted on a grid; precise solvent requirements Specialized trained technician required Time consuming Expensive	96
Proteomics	LC–MS, iTRAQ Label-free MS	New protein identification Global molecular insights Pathway analysis Post-translational modification detection Protein interactions Genotype–phenotype bridging	Global profiling Subfractionation analyses Reliability Variations available for targeted chemical modification analysis	Biased against low-abundance proteins Reproducibility, standardization and sample purity Time consuming, expensive	35,159
Lipidomics	'Shotgun' lipidomics LC–MS/MS	Platelet lipid content Lipid species in platelet membrane and extracellular vesicles Lipid uptake by platelets	High throughput and sensitive Targeted and quantitative identification Global profiling	New and in nascent stages for platelet studies Need for development of computational and bioinformatic tools for analysis of large datasets Laborious and time consuming for accurate identification	48,52,160
Transcriptomics	RNA-seq microarrays	Coding transcriptome analysis Global noncoding RNA analysis Global molecular insights Pathway analysis Expression identification	Global profiling Subfractionation analyses Reliability	Stringent platelet purity required Additional RNA amplification required Complex data analysis Time consuming, expensive	56
Genomics	DNA microarray SAGE GWAS	Diagnosis of heritable platelet disorders Mutation detection Identification of genes associated with platelet function Regulatory mechanism studies for platelet production	Platelet isolation/processing not required Meta-analyses from genomic databases Individual- or population-level data analysis	Highly limited conditional variation studies Time consuming, expensive Requires analysis of family members	55
Gene amplification	RT–qPCR	Gene expression of individual genes Splicing isoform identification miRNA validation Platelet purity assessment Genotyping	Validation method Gene product size-based identification Fairly cheap Highly reproducible No specialized training Rapid and simpler data analysis	Small-scale application Highly pure platelet sample needed	28,161

Continued

Table 2 | Summary of major basic research techniques used for platelet studies (continued)

Technique		Platelet characteristic/event	Advantages	Limitations	Reference
Immunoblotting	Western blot IP	Protein expression of individual genes Post-translational modification detection Protein cleavage/isoform identification Protein–protein interactions Signaling pathway studies	Validation method Protein size and expression detection Semi-quantitative Routine laboratory instrumentation and protocol	Small-scale application Availability of specific antibody Time consuming Greater manual effort Troubleshooting steps Requires relatively higher volume of washed platelets	28,32

Abbreviations: IP, immunoprecipitation; LC–MS/MS, LC with tandem mass spectrometry; RT–qPCR, quantitative PCR with reverse transcription; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

mitochondria, Golgi and granule secretion events. Correlative light and electron microscopy (CLEM) is a new technique that combines the advantages of both fluorescence imaging and electron microscopy, with fewer limitations than conventional electron microscopy (Table 2). CLEM has been used to characterize in detail the spatial regulation of platelet activation during hemostasis[94].

Although electron microscopy imaging has its challenges owing to the technical expertise and expensive instrumentation required, it remains the gold standard for studying platelet morphology and ultrastructure, both for understanding platelet biology and identifying various platelet abnormalities and storage pool deficiencies.

Non-imaging immunoassays. Although high-throughput and increasingly sensitive proteomic techniques have their unique advantages, their use is limited for routine analysis of platelet biology (Table 2). Immunodetection methods use specific antibodies and allow easy validation and a relatively inexpensive platform to identify proteins and detect quantitative differences. Enzyme-linked immunosorbent assays (ELISA) have been regularly used for the measurement of soluble platelet-activation markers including soluble P-selectin and platelet factor 4 as well as the platelet secretome (from α-granules and dense granules) and the platelet sheddome (GPIbα, GPVI and other membrane proteins)[97–99]. Changes in the levels of plasma platelet-activation markers have been associated with thromboembolic events and form a valuable tool for acute event prognosis and timely individualized adjustment of the treatment regimen to prevent thrombosis. However, despite quantitative results, ELISA is limited to analyzing the plasma compartment and does not provide isoform-specific information. By contrast, western immunoblotting has long been used as a classical tool to distinguish specific protein expression in platelets and for the investigation of both intracellular signaling pathways and surface markers. Although semi-quantitative, western immunoblotting is considered one of the most common and reliable techniques to also validate results from more extensive proteomic studies. The use of fluorescently labeled secondary antibodies has substantially improved the quantitative accuracy of platelet proteomes, thereby forming a rapid and reproducible routine platform for quantitative proteomic studies of platelets.

Several of the above-described techniques have been used to study interactions of platelets with other cells including endothelial cells, VSMCs, RBCs, pathogens and tumor cells that occur both in normal and disease states (Table 1). Therefore, basic research techniques might provide important insights with clinical relevance. We will next discuss the development and current status of the major clinical assays used for platelet function tests.

Current methodology for clinical evaluation

Traditional platelet testing and platelet aggregometry in clinical medicine. Traditional clinical laboratory testing for platelet disorders generally relies on platelet counts and platelet function testing. The bleeding time test, introduced in the early 20th century, was the first functional test to evaluate primary hemostasis and platelet disorders but is no longer used in clinical practice owing to its low sensitivity, poor reproducibility, scarring potential and absence of correlation with clinical outcome. The ability to test platelet function in the clinical laboratory has substantially improved with the introduction of platelet LTA, which rapidly became a widely used and informative methodology for diagnosing and managing platelet disorders[26]. Subsequent variations of aggregation-based platelet function tests have been developed and include whole-blood impedance platelet aggregometry (multiple-electrode aggregometry with the use of a Multiplate Analyzer)[100,101] and VerifyNow[102,103] (Table 3).

The development of multiple-electrode aggregometry that uses electrical impedance offers platelet function analysis in whole blood. Multiple-electrode aggregometry has been demonstrated to be useful for the diagnosis of heparin-induced thrombocytopenia[104,105] and as a point-of-care test for pediatric patient samples[106] in which sample volumes are very low. Other studies, however, found multiple-electrode aggregometry to be less sensitive for detection of abnormal platelet function and to have lower prognostic value than LTA in patients[27,107]. Nevertheless, multiple-electrode aggregometry offers several advantages over LTA, such as a lower sample volume requirement, and it is faster to perform on a per-sample basis.

Although platelet aggregation responses have historically been one of the most common ways of evaluating platelet disorders, these methods are limited by their sensitivity, labor intensiveness and number of parameters evaluated. However, certain factors must be considered while interpreting the aggregation assay data in which platelet function is biologically distinct such as in the case of neonates. Studies have shown that neonatal platelets are hypofunctional with agonist stimulation by aggregation-based methods. However, full-term neonates typically maintain normal physiologic hemostasis[108] despite elevated hematocrit and increased VWF levels, which likely counterbalance intrinsic platelet hyporeactivity. A small but considerable proportion of patients with suspected platelet or other bleeding disorders remain without a diagnosis even after comprehensive clinical laboratory testing.

Thromboelastography. In vitro assays that simulate physiologic whole-blood hemostasis can evaluate relative contributions of platelets, fibrinogen and thrombin generation. The critical feature of such methods for platelets is that their function is assessed under more physiological conditions, with contributions from the other blood and plasma components that drive hemostasis. Thromboelastography (TEG) and rotational thromboelastometry are similar viscoelastic technologies that use whole blood to analyze dynamic changes over time, including clot formation, strength and lysis[109]. These methods are primarily used for the assessment of global hemostasis (that is, coagulopathy versus hemorrhage)

Table 3 | Summary of common techniques used for platelet clinical studies

Technique	Principle	Clinical utility	Advantages	Limitations	Reference
Bleeding time	Assessment of platelet function and hemostasis by making a superficial wound and measuring time to bleeding cessation	Screening test for bleeding disorders	In vivo physiologic test	Invasive, poor reproducibility Low sensitivity and specificity Obsolete in developed countries	162
LTA	Evaluates platelet aggregation response following stimulation with agonists by measuring light transmission	Evaluation of bleeding and platelet function disorders; also used to monitor antiplatelet therapies	Different platelet agonists and concentrations for pathway investigation	Requires large volumes Processing required to isolate PRP Requires expertise to perform and interpret results Sensitive to platelet count and antiplatelet therapies	163
Multiple-electrode aggregometry (impedance platelet aggregometry)	Variation of traditional platelet aggregometry that measures changes in electrical impedance	Evaluation of bleeding and platelet function disorders; also used to monitor antiplatelet therapies	Similar to LTA but can use whole blood Multiple channels and semi-automated	Results affected by platelet counts and hematocrit	100,101,104
Lumiaggregometry	Platelet aggregometry combined with luminescence	Useful in evaluation of platelet granule release and storage disorders	Combined assessment of platelet aggregation and granule release	Semi-quantitative assay Less commonly used	164
VerifyNow system	Disposable cartridges containing platelet agonists and fibrinogen-coated beads	Assessment of efficacy of antiplatelet therapies (aspirin, P_2Y_{12} and αIIbβ3)	Simple point-of-care test Fully automated with high reproducibility	Expensive with limited number of parameters analyzed	102,103
Plateletworks	Measuring changes in platelet counts before and after agonist stimulation	Primarily used for surgery and cardiology to monitor antiplatelet therapies	Point-of care test that uses minimal blood volumes and provides rapid information on platelet function	Indirect assay that requires accurate platelet counts; limited data in literature Less commonly used	165
TEG and ROTEM	Rotational force transmitted to an electromechanical or optical detection system, which allows for quantitative measurement of clot dynamics	Assessment of global hemostasis and supports targeted therapy, blood produce usage in patients during trauma or major surgery; can be used to monitory antiplatelet therapies (that is, TEG platelet mapping)	Whole-blood point-of-care test that measures dynamic properties of clot formation Provides rapid information in real time	Low sensitivity to many aspects of platelet function and not recommended for diagnosing platelet function disorders Not standardized for thrombocytopenic samples	109
PFA (PFA-100 and PFA-200)	High-shear platelet adhesion and aggregation that measures the closure time of a membrane aperture coated with agonists	Rapid screening of platelet function disorders and monitoring antiplatelet therapies	Simple and rapid whole-blood test Minimal volume of blood Stimulates physiologic flow and high-shear environments	Affected by platelet count and hematocrit Moderately sensitive for platelet dysfunction Performance across a range of channel geometries	118
Microfluidic devices	Miniaturized multichannel flow devices; platelet adhesion and aggregate stability under variable shear rates	Mostly limited to research settings with few devices that have been tested clinically	Whole-blood, real-time thrombus formation Performance across a range of channel geometries	Few devices validated with appropriate standardization in the clinical setting	112,120,123,125

LTA, light transmission aggregometry; PRP, platelet-rich plasma; ROTEM, rotational thromboelastometry; TEG, Thromboelastography.

and to support targeted transfusion therapy in patients during trauma or major surgery (for example, cardiopulmonary bypass and liver transplantation) (Table 3). Together, modifications to the standard TEG system and the addition of platelet agonists (for example, ADP, arachidonic acid and thrombin analogs) provide a new assay, known as TEG platelet mapping, that allows for a more specific assessment of platelet function[110]. This technique has been shown to have utility in monitoring antiplatelet therapies[111]. However, this approach has a fairly low sensitivity for platelet function and is therefore not recommended for diagnosing platelet functional disorders. The TEG6s Platelet Mapping assay, which uses disposable multichannel microfluidic cartridges, has been shown to have clinical utility in evaluating platelet function[112–114]. This method was shown to have less interindividual variability and greater ease of use than other viscoelastic assays. Evaluating hemostasis in patients with COVID-19 demonstrated the usefulness of TEG6s Platelet Mapping in monitoring antiplatelet and anticoagulant therapy[115].

Table 4 | A concise guide to research approaches used to assess platelet properties

Platelet state	Immature/young	Resting	Activated	Apoptotic	Defective	Cell conjugates
Technique	Flow cytometry (reticulated platelets) Hematology analyzer (Sysmex IPF, Abbott retPLT)	Flow cytometry Western blotting Imaging (SEM, TEM) Phase-contrast/ confocal microscopy Proteomics RNA sequencing	Flow cytometry Western blotting Aggregometry Imaging (SEM, TEM) ELISA PFA-100 Proteomics RNA sequencing Mass cytometry Intravital imaging	Flow cytometry Western blotting Spectroscopy Confocal microscopy	Flow cytometry Lumiaggregometry ROTEM platelet PFA-100 Imaging (TEM) Genomic sequencing Immunofluorescence	Flow cytometry Confocal microscopy Single-cell morphometry Mass cytometry Real-time microfluidic assays Intravital imaging
Markers/dyes	Thiazole orange polymethine (XE-series), oxazine (XN-series) CD4K530	Lowered activation markers (CD62p, TLT-1, CD69) Platelet morphology	Activation markers (CD62p, TLT-1, CD69) Surface and ultrastructural morphology PMV release Calcium levels (Fura-2, calcein-AM)	Annexin V Caspase substrates/ antibodies Mitochondrial depolarization (TMRE)	Platelet morphology Surface proteins Granule content (α, dense) Granule staining (mepacrine) ATP release	Cell-specific markers (CD45, CD14b, CD11b, PSGL-1, E-selectin, CD68)
Basic research application	Platelet turnover/ lifespan Platelet production from megakaryocytes Developmental thrombopoiesis	Platelet biology Intraplatelet signaling Cytoskeletal studies Organelle studies Species differences Global proteome/ transcriptome profiling Platelet population heterogeneity Plasma protein homeostasis	Platelet biology Hemostasis Activation pathways Organelle studies Molecular profiling Platelet population heterogeneity Biomechanics and bioenergetics PMV studies	Platelet lifespan Platelet death pathways (apoptosis, necroptosis, pyroptosis) Mitochondrial studies	Hemostatic function and pathway Platelet biology and morphology	Platelet interaction with other cell types Role beyond hemostasis (innate and adaptive immunity) Vessel wall remodeling Lymphatic integrity
Clinical relevance	Thrombocytopenia ITP Thrombocytosis Hereditary platelet diseases Chemotherapy/stem cell transplantation Risk assessment in cardiovascular diseases Response to therapy On/off target effects of other drugs	Normal platelet physiology assessment and monitoring for antiplatelet therapy Screening of new antiplatelet compounds Platelet transfusions Tissue regeneration	Thrombo-embolism Cardiovascular risk Inflammatory disease (acute lung injury, arthritis) Wound healing Response to therapy On/off target effects of other drugs	Thrombocytopenia (heparin induced, drug induced, ITP) Stem cell tranplantation Bone marrow defects Infections Response to therapy On/off target effects of other drugs	Bleeding disorders Inherited platelet disorders (Bernard–Soulier, gray platelet, Wiskott–Aldrich syndrome) Storage pool disease Platelet release disorders	Thrombosis Ischemic stroke Bacterial/viral infection Tumors Hepatitis/liver injury Chronic kidney disease

Abbreviations: ITP, immune thrombocytopenic purpura; PMV, platelet microvesicles; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TLT-1, TREM-like transcript 1; TMRE, tetramethylrhodamine ethyl ester.

Clinical microfluidics and shear-induced platelet responses. A major limitation in current platelet function testing is the difficulty of replicating biorheological conditions in vitro. Vascular thrombus formation is a dynamic process, dependent on shear forces generated from physiologic blood flow and blood viscosity, which drive platelet adhesion, activation and aggregation[116]. Several platelet function tests (Table 3) have been developed to study shear-induced platelet responses to better simulate the physiologic processes that occur in the vessel. The simplest and most widely used test is the platelet function analyzer (PFA; PFA-100 and PFA-200), which uses citrated whole blood and measures the closure time of a membrane aperture coated with agonists[117,118]. The PFA assesses platelet adhesion and aggregation under flow and high-shear conditions that simulate primary hemostasis. Although the PFA is moderately sensitive for platelet dysfunction, it has low specificity and is primarily used as a rapid clinical tool for screening for von Willebrand disease and monitoring the efficacy of antiplatelet therapies[119]. Given that the PFA assay is sensitive to low platelet count and low

hematocrit, results need to be adjusted for thrombocytopenia and anemia, and its clinical utility might be limited in these settings.

Although these technologies are useful for studying various aspects of thrombus formation and platelet interactions under uniform shear stress, they are unable to sufficiently simulate the dynamic flow in vessels in which bifurcation or stenosis is common. The development of microfluidic devices has allowed for more complex designs of networks that better model the vasculature[120,121]. The Total Thrombus-Formation Analysis System uses whole blood under physiological shear conditions with disposable microchips coated with activators such as collagen and tissue factor[122] and has demonstrated promise in detecting platelet function and coagulation disorders. The method has also shown potential in monitoring antiplatelet therapies in patients with CAD and predicting periprocedural bleeding in patients undergoing percutaneous coronary intervention[123,124]. More recently, a microfluidic device has been developed that measures platelet contractile forces under shear gradients[125]; this technique might be useful for detecting platelet inhibition, guiding antiplatelet therapy and predicting bleeding risk and transfusion needs in patients with trauma. Although these newer technologies might eventually be useful complements to established laboratory evaluation of platelet function, most have not been rigorously tested in a clinical setting; a need exists for standardization and validation before routine clinical use[126].

The use of rigorously optimized methods for platelet preparation and isolation and platelet functional testing is of primary importance for achieving reproducible and clinically relevant results in all platelet-investigative studies. Integrating the basic research methodologies with clinical utility assays allows both platelet biologists and clinicians to comprehensively advance the understanding of the pathophysiological role of platelets in cardiovascular and other diseases. Moreover, the combined use of these various approaches also provides a means to study the effects of antiplatelet agents and their mechanisms of action.

Investigating platelets in COVID-19

COVID-19 is a prime example in which basic investigations, beyond standard platelet counts and aggregation assays, have complemented clinical investigations to improve diagnostic and therapeutic strategies. Platelets can contribute to the overall procoagulant and inflammatory states during viral infection, potentially leading to thrombosis, vascular complications and high mortality[127]. COVID-19 is associated with thrombocytopenia, thrombosis and hemorrhage in the setting of underlying endotheliopathy, coagulopathy and inflammation, with each serving as biomarkers for disease severity and poor prognosis[19,128]. Several mechanisms have been investigated and proposed for the development of thrombocytopenia, including platelet activation and/or consumption and subsequent clearance by the reticuloendothelial system, including platelet consumption in response to increased endothelial damage, platelet auto-antibody formation and subsequent platelet clearance, bone marrow or megakaryocyte suppression owing to inflammation or direct viral infection[19,129,130]. Studies have used the above-described techniques to elucidate possible functions for platelets in the thrombo-inflammatory activation responses of COVID-19. A combination of flow cytometry, confocal microscopy, TEM and RNA sequencing demonstrated increased formation of platelet–neutrophil and platelet–monocyte aggregates, increased platelet activation and altered expression of over 3,000 genes in patients hospitalized with COVID-19 (ref. [131]). Platelet–monocyte aggregates were also identified in another study of patients critically ill with COVID-19 with the use of flow cytometry assays[132]. In a separate study, Althaus and colleagues used flow cytometry assays and western blotting to investigate COVID-19-associated platelet apoptosis mediated by circulating immune complexes in patient sera[133]. Mass cytometry was also used to characterize a unique platelet hyperactivation phenotype of platelet surface proteins in COVID-19 (ref. [134]). A remarkable application of the latest imaging technology for platelets in COVID-19 used large-scale single-cell platelet imaging by intelligent platelet morphometry[135]; the investigators analyzed whole-blood samples to identify platelet aggregates in nearly 90% of all patients with COVID-19. TEM demonstrated uptake of SARS-CoV-2 through attachment to microparticles, independent of angiotensin-converting enzyme 2 (ACE2) (ref. [136]). Moreover, transcriptomic profiling revealed the presence of both platelet apoptosis and necroptosis[136]. The application of these diverse basic sciences' platelet methodologies highlights the potential utility of these emerging assays for clinical use. Increasing evidence of COVID-19 thrombocytopathy associated with platelet–leukocyte interactions, various states of activation or hyperactivation, apoptosis and necroptosis has facilitated and encouraged the use of antiplatelet agents (for example, low-dose aspirin in addition to anticoagulation) to combat thrombosis in COVID-19 (refs. [137,138]). The results of these studies have been promising with improved morbidity and mortality. Many other drugs targeting thrombosis are also being tested[19]. Techniques such as TEG, especially TEG6s, have proven useful in investigating the levels of thrombogenicity markers[139] and also evaluating the pharmacodynamic response to anticoagulants and aspirin in patients with COVID-19 (ref. [115]). Also related to patients with COVID-19, platelet function tests have proven useful in cases of vaccine-induced thrombotic thrombocytopenia, a rare but life-threatening condition, in vaccinated individuals. The use of both conventional and emerging assays in patients with vaccine-induced thrombotic thrombocytopenia is another example of the importance of these assays in the clinic[140,141]. Thus, using both conventional assays and cutting-edge techniques to study platelets in normal physiology and in disease and the incorporation of these assays into clinical decision making has proven to be beneficial and warranted.

Conclusion

On the basis of major advances in basic science investigations, platelets can no longer be thought of as a homogeneous population but rather as a heterogeneous interactive population with distinct subgroups that can protect against or contribute to disease processes. The classical and advanced methods and studies described in this review have enhanced our understanding of the heterogeneity of platelets, the functions of distinct platelet subsets and the mechanistic complexities of the roles of platelets in diseases (Table 4). The frontier will be to continue to characterize platelets at a single-cell, single-disease and single-person level. The study of individual-level variabilities in platelet function and response efficiently and accurately by emerging assays can be the key step towards enhanced clinical safety of antiplatelet drugs. Some of these developing techniques might also be adapted for high-throughput screening in the clinic and especially for patients with trauma for quick decision making as well as to identify new therapeutics targeting unique populations of platelets. These techniques can also potentially be used to monitor therapeutic efficacy. These technologies and discoveries might ideally lead towards the development of more precise and personalized diagnostics and point-of-care devices that will increase our ability to understand disease etiology and bleeding risk, integrating information on each patient's history, presentation and risk factors. This knowledge will allow for customized, targeted therapies for treatment and prevention of cardiovascular diseases and other diseases.

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