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STATE OF THE ART

Heterogeneity of platelets and their responses

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Abstract

There has been increasing recognition of heterogeneity in blood platelets and their responses, particularly in recent years, where next-generation technologies and advanced bioinformatic tools that interrogate "big data" have enabled large-scale studies of RNA and protein expression across a growing list of disease states. However, pioneering platelet biologists and clinicians were already hypothesizing upon and investigating heterogeneity in platelet (and megakaryocyte) activity and platelet metabolism and aggregation over half a century ago. Building on their foundational hypotheses, in particular Professor Marian A. Packham's pioneering work and a State of the Art lecture in her memoriam at the 2023 International Society on Thrombosis and Haemostasis Congress by Anandi Krishnan, this review outlines the key features that contribute to the heterogeneity of platelets between and within individuals. Starting with important epidemiologic factors, we move stepwise through successively smaller scales down to heterogeneity revealed by single-cell technologies in health and disease. We hope that this overview will urge future scientific and clinical studies to recognize and account for heterogeneity of platelets and aim to apply methods that capture that heterogeneity. Finally, we summarize other exciting new data presented on this topic at the 2023 International Society on Thrombosis and Haemostasis Congress.

KEYWORDS
blood platelets, genomic medicine, hematology, heterogeneity, multiomics

Essentials

- Historically, researchers have identified heterogeneity in blood platelet form and function.
- Next-generation technologies have led to recognition of platelet heterogeneity at a molecular level.
- Here, we survey multiscale evidence for platelet heterogeneity in health and disease.
- We also summarize novel data on this topic from the 2023 International Society on Thrombosis and Haemostasis Congress.

Sally Thomas and Sarah Kelliher contributed equally to different components of this review.

1 | INTRODUCTION

In any standard clinical diagnostic hematology laboratory, automated whole blood counters distinguish and count platelets based on electrical impedance, determined by their size. Counted in this automated manner, platelets appear nearly identical across the ranges of age, biological sex, and disease states of the individuals whose complete blood count is enumerated. Likewise, to a biomedical scientist or hematologist examining a blood film (smear) under a light microscope, platelets look fairly similar within and between individuals. Here, we advance what we believe is an often overlooked concept of platelet heterogeneity: that beneath the superficial similarities, there are differences in platelet biology and, therefore, platelet function responses. This concept was first introduced in the 1960s when subpopulations of platelets were defined by distinctive metabolic and aggregation profiles [1,2]. Blazing through the last 50 years to contemporary technologies, we now have large "omics" data sets in healthy donors and patient cohorts that point strongly to significant platelet heterogeneity at the level of RNA [3–5], protein [6,7], and phenotype [8–11].

Heterogeneity of platelets and their responses may be viewed broadly on 3 levels (Figure 1): 1) heterogeneity at the population or system level, where constitutional factors such as race, age, and sex contribute to differences between healthy individuals; 2) cellular within-individual heterogeneity, on the other end of the spectrum, where we recognize that the platelet population in any one individual constitutes varying subpopulations, with each likely having distinct functional properties, and 3) between-individual heterogeneity, which arises due to genetic factors, disease state, or other acquired influences.

At this year's International Society on Thrombosis and Haemostasis (ISTH) meeting in Montreal, there were a number of studies presented that related to platelet heterogeneity. In this State of the Art review, we discuss the historical and contemporary concepts of platelet heterogeneity as a setting for key work presented at ISTH 2023 and point to future directions in this field. In essence, we echo the words of pioneering biologist Simon Karpatkin from over 50 years ago urging us to study platelet heterogeneity: "these data make it necessary to interpret all future, as well as past, platelet metabolic or function studies with caution" [2].

2 | HISTORICAL AND CONTEMPORARY PERSPECTIVES AND TECHNOLOGIES

The earliest mention of platelet heterogeneity we found was from Professor Simon Karpatkin and colleagues in 2 publications titled "Heterogeneity of Human Platelets I" [1] and "Heterogeneity of Human Platelets II" [2]. The authors established that blood from human donors contained platelets of varying sizes. When they compared large and small platelets, they found differences in metabolic activity and aggregation. Notably, they conclude that the larger platelets that aggregate more readily are younger platelets, an observation that remains relevant in contemporary platelet research [12].

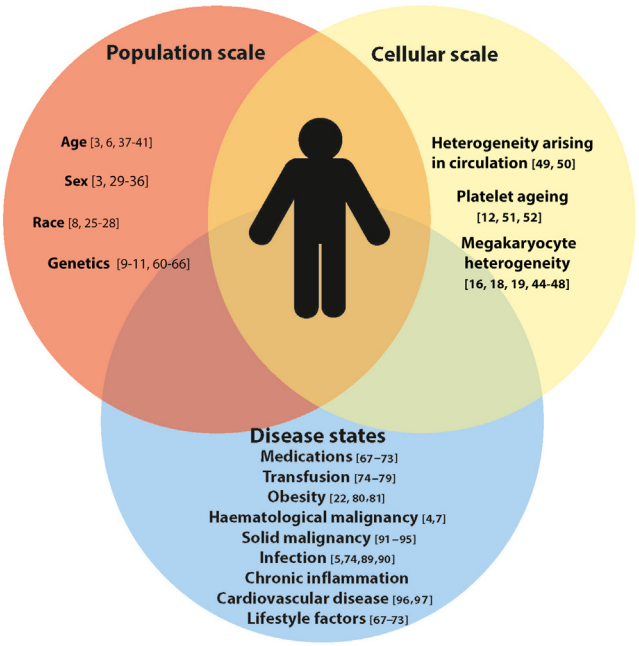

FIGURE 1 Heterogeneity of platelets and their responses may be viewed broadly on 3 levels: 1) population or systems, 2) cellular/within-individual, and 3) between-individual.

These and other early studies [13] (including that of Professor Packham) introduced key ideas on the causes of platelet heterogeneity, importantly introducing the concept that platelet heterogeneity arises not only due to influences acting on platelets in circulation [14] but also from factors [15] impacting the megakaryocytic population [16]. Several recent advances in technology build on these early ideas. Single-cell technologies have opened a unique window into understanding intraindividual heterogeneity among hematopoietic stem cells [17] and megakaryocytes [18,19]. Transcriptomics [20], proteomics [21], and novel lipidomics [22] have enabled multiple pathways to be examined simultaneously in large and varied cohorts of individuals.

3 | PLATELET HETEROGENEITY AT THE POPULATION LEVEL: CONSTITUTIONAL FACTORS

Fundamental to applying omics approaches to compare groups is the assumption of stability in healthy platelet profiles within an individual across time. This was established nearly 20 years ago [23] in healthy individuals, and their platelet aggregation phenotype remained consistent over time, with data covering periods up to 3 years. More recently, consistency of platelet function over time was shown using flow cytometry [24], and similar stability and reproducibility have been demonstrated for the platelet transcriptome [5]. In a healthy cohort who underwent repeated platelet transcriptome analysis, almost all individuals demonstrated an extremely close "within-individual" correlation when their RNA expression signature at

baseline was compared with their profile 4 months later. This was preserved over longer periods, with samples at 4 years correlating as close to baseline as those at 2 weeks [5].

These reference studies also help pinpoint potential constitutional factors that contribute to platelet heterogeneity in the population. In the aggregation study, women were overrepresented in a group with hyperreactive aggregation [23]. In the transcriptome study, between-individual correlations were closer when samples were grouped by race, age, and sex, and genes previously associated with race or sex were among the transcripts showing high interindividual variation [5].

3.1 | Race and ethnicity

Differences in outcomes in cardiovascular disease according to race seen in epidemiologic studies [25] have triggered investigation into the possible contribution of differences in platelet aggregation. Activation of the thrombin receptor protease-activated receptor 4 (PAR4) has been observed to cause greater aggregation in platelets from Black individuals, with differential expression in transcripts correlated with *PAR4* activation [8]. Differential responses to platelet antagonists have also been observed *in vitro* [26].

It is well documented that racially and ethnically minoritized populations are underrepresented in clinical trials [27]. Drug regulators are beginning to mandate that clinical trial design ensures recruitment is representative [28].

3.2 | Biological Sex

Biological sex-related differences in platelets might be expected based on evolutionary selection imposed by the hemostatic challenge of childbirth [29], from observed differences over the menstrual cycle [30,31], or rates of arterial thrombotic events between males and females [32,33]. More recently, a large transcriptomic study identified 54 messenger RNA (mRNA) transcripts differentially expressed according to sex [3]. Acknowledging the contribution of sex to platelet heterogeneity is critical in basic [34] and clinical studies [35,36].

3.3 | Age

Even among adults, there is evidence from transcriptome data of platelet heterogeneity by age: there is differential expression of genes by subject age in pathways including signal transduction and granule secretion and in mitochondrial mRNAs [3]. Functional studies, conversely, have shown no difference in platelet responses in aggregometry in older adults [37].

Platelet heterogeneity and age are particularly notable in the context of neonatology, highlighted in the setting of platelet transfusions. Where donor platelets (from adult donors) are transfused to neonates, permissive transfusion thresholds are associated with harm [38]. Although the explanations for this are unclear, comparison of neonatal and adult platelets shows that their transcriptomes are broadly similar [39,40], whereas there are differences in proteomes [6,41].

4 | PLATELET HETEROGENEITY AT THE CELLULAR LEVEL: WITHIN-INDIVIDUAL FACTORS

As historic platelet researchers characterized platelet heterogeneity by size and density, we are increasingly recognizing the importance of platelet subpopulations within an individual [42]. Platelets are generated by megakaryocytes, released into the circulation, and (if not consumed in hemostasis or removed by immune responses) live for 7-10 days before being cleared in the liver [43]. In any one individual, the platelet pool is therefore a heterogeneous mix of platelets with different megakaryocyte origins, different influences from the circulation, and different ages since release.

4.1 | Megakaryocyte heterogeneity

Heterogeneity among megakaryocytes has long been suspected to contribute to platelet heterogeneity [16], and it is an area that has seen significant advances in our understanding in recent years [44]. The recognition that megakaryocytes are found outside the bone marrow, including in the liver, spleen, and lung [45], has generated interest. It might be expected that platelets generated by extramedullary megakaryocytes might have different properties from those of platelets made in the bone marrow. However, recent work in mice suggests that lung megakaryocytes make only a small contribution to platelet synthesis [46].

Single-cell RNA sequencing enables the transcriptomes of individual cells to be examined to identify subgroups of cells and infer differentiation trajectories [47]. Applying this to megakaryocytes, which are not abundant and are large and fragile, has only recently been achieved. This has revealed a diversity of subpopulations of bone marrow megakaryocytes with distinct profiles [18,19], only some of which generate platelets. Given that megakaryocytes actively and selectively transport RNA into platelets [48], it will be fascinating to unravel whether this translates into producing distinct subpopulations of platelets.

4.2 | Platelet properties altered by the circulation

Platelets are altered by influences they encounter in the circulation. Regarding the platelet transcriptome, platelets exchange RNA with cells in the vasculature [49]. The transcriptome is potentially altered further by receptor-triggered alternative splicing taking place in circulating platelets [50].

4.3 | Platelet age

The properties of platelets change as they age over their lifespan of 7 to 10 days in the circulation. Individuals therefore contain a heterogeneous population of platelets with different properties, determined by how long it has been since those platelets were released into the circulation [12]. Our knowledge of the properties of younger and older platelets has been improved by the development of new approaches to reliably age platelets [51]. Proteomic studies have revealed alterations in pathways suggestive of reduced aggregation potential as platelets age [52].

4.4 | Laboratory isolation methods

Methods of platelet isolation may themselves introduce heterogeneity in platelet characteristics. Platelets derived from platelet-rich plasma have shown higher levels of P-selectin and annexin V as compared with those shown by platelets isolated from a buffy coat or using apheresis [53]. In recent work using a novel platelet phenomics assay [54], platelets from healthy individuals were stratified into 6 distinct platelet phenotypes based on their reactivity and shown to be consistent between different cohorts and across time. Storage of platelets has also been reported to introduce subpopulations, including a predominance of degranulating platelets after cryopreservation [55] and populations arising over 7 days of storage from stress-induced platelet activation and cell death [56], with potential functional consequences [57]. These efforts prompt us to better recognize the importance of optimum methodology in platelet isolation [58] and the significance of the subpopulations (eg, procoagulant, aggregatory, resting, and apoptotic) to specific platelet functions [59].

5 | PLATELET HETEROGENEITY AT THE INDIVIDUAL LEVEL: INHERITED, ACQUIRED, AND DISEASE FACTORS

There are extensive and growing data on the effects of genetic factors, disease states, and other acquired influences on platelets. However, for the most part, these analyses are performed on bulk-isolated platelets. Therefore, we do not know to what extent these changes reflect a blanket effect on all platelet generation or whether they reflect a change in the relative size and contribution of subtypes of platelets to the pool.

5.1 | Genetic factors

We recognize that while many external and/or somatic factors influence platelets, not all interindividual platelet heterogeneity is acquired. It has been established that many of the platelet parameters that vary between individuals, including platelet count and platelet function, are largely determined by inherited genetic factors [9–11,60–66].

5.2 | Medications and lifestyle factors

Platelet subpopulations may be responsible for differences observed in treatment response. We know that activated platelets are required to stimulate the innate immune system [67], while platelet subpopulations have been shown to respond differentially to antiplatelet therapy [68,69]. Effects on platelet function are also seen in medications prescribed for other indications, including antidepressants [70] and antiretrovirals [71]. Likewise, lifestyle factors such as diet, smoking, and alcohol affect platelet function, but at what scale they become relevant is an ongoing study [72,73].

5.3 | Transfusions

Platelet transfusions are frequently part of the care of patients with hematologic malignancies, those undergoing cardiothoracic surgery, and those in intensive care [74]. Given that these are obtained from donors, platelet transfusion entails elements of platelet heterogeneity inherent in the population variables above. Furthermore, heterogeneity in units arises from different donor reactions to apheresis [75], changes occurring with storage over time [76], and the specific method of storage [77]. Progress is being made on *ex vivo* synthesis of platelets for transfusion [78,79], and their properties compared with those of donor platelets may need to be considered.

5.4 | Obesity

Obesity is associated with increased risk of cardiovascular events. Studies of the platelet proteome and phosphoproteome in obese individuals and matched controls have identified differentially activated signaling pathways that translate into altered *in vitro* aggregation phenotypes [80,81]. More recently, comprehensive study of platelet lipids—the "lipidome" —has revealed differences between obese and nonobese individuals [22].

5.5 | Clonal bone marrow disorders

Heterogeneity in platelet function in bone marrow disorders is of interest, especially in the context of potential megakaryocyte heterogeneity. Some of our own work [4,7,82–85] has been in this area, in myeloproliferative neoplasms (MPNs) and clonal hematopoietic stem and progenitor cell malignancies characterized by aberrant megakaryocyte proliferation and thrombocytosis [86,87]. Patients with MPNs, including essential thrombocythemia, polycythemia vera, and myelofibrosis, experience progressively more severe clinical features, including complications with thrombosis and hemostasis and an increased propensity for transformation to acute myeloid leukemia [88]. A genetically heterogeneous megakaryocyte population [17] is expected in these patients. Our recent work on the platelet transcriptome in patients with MPN compared with that in healthy

controls identified distinct signatures by MPN subtype, driver mutation, and treatment [4]. Building further from the transcriptome, we also saw differential platelet protein expression in proteomic profiles of patients with essential thrombocythemia and polycythemia vera [7].

5.6 | Infectious diseases

At a practical level, clinicians are familiar with platelets playing a role in infection in the context of using higher platelet transfusion thresholds in the presence of sepsis [74]. Transcriptomic studies reveal distinct transcriptomic signatures in infection [5,89], which is congruent with increasing recognition of the active role that platelets play in the response to infection [90]. Sepsis alters the transcriptional landscape of platelets [89].

5.7 | Solid tumors

The platelet transcriptome is altered in solid malignancy [91], creating reprogrammed "tumour-educated" platelets [92]. Transcriptional alterations are indicative of the presence of solid malignancy [93] and have been distilled to identify signatures specific to tumor type [94] and even occurrence of metastasis [95].

5.8 | Cardiovascular disorders

Heterogeneous platelet molecular phenotypes are associated with a myriad of diseases characterized by chronic inflammation and vascular dysfunction [96]. Some aspects of heterogeneity in cardiovascular phenotypes are attributed to more rapid platelet turnover, leading to a higher proportion of young platelets and, therefore, different transcriptomes and prothrombotic behavior [97].

6 | ISTH CONGRESS REPORT

As we demonstrate in our review thus far, platelet heterogeneity at an individual scale is becoming increasingly recognized, not only in terms of variability across states of health and disease but also with regard to heterogeneity in platelet function beyond thrombosis and hemostasis, including immune response and inflammation. Platelets, as peripherally accessible and functionally diverse cells, are being harnessed using novel approaches for diagnostic and therapeutic purposes. While we trace the study of platelet heterogeneity back over 70 years, novel data continue to emerge, expanding our understanding of the diverse phenotype and myriad of functions possessed by these anucleate cells. The theme of platelet heterogeneity was seen throughout a range of clinical and scientific abstracts presented at the 31st Congress of ISTH held in Montreal in 2023, from a population level to a person/disease-specific and cellular scale (Figure 2).

Jeltuhin et al. [98] showed that neonates have higher platelet contraction forces than adults, while Denorme et al. [99] crucially discovered a causal role of a common platelet PAR4 functional variant that may explain the racial disparity in stroke outcomes for Black individuals. Potential sex-specific changes in platelet reactivity and megakaryocytes were shared by Goldfinger et al. [100], who described increased platelet reactivity to thromboxane A2 with enhanced mitochondrial activity specific to male Argonaute 2 (AGO2) deleted mice; no difference was observed in female counterparts. Heterogeneity in platelet activity during pregnancy was characterized *in vitro* [101], and a novel role for platelet-derived growth factor β in mediating intrauterine growth restriction was uncovered *in vivo* [102].

In addition to platelet diversity in the general population, platelet heterogeneity can also be considered as broad and varied biological processes mediated by platelets beyond their recognized function as effectors of coagulation. There is increasing evidence recognizing the critical and heterogeneous contribution of platelets to immune function in states of health and disease [103]. Abstracts presented at the 31st Congress of ISTH further expanded our understanding of platelets as immunomodulators with targeted and variable responses during infectious processes. Platelets were shown to have a context-specific effect across numerous infectious processes [104]. Greenman et al. [105] demonstrated differences in megakaryocyte subsets of murine models of chronic schistosome infection. Using ploidy analysis, an increase in megakaryocytes with potential immune cell function with a reduction in thrombopoietic and niche-maintaining megakaryocytes was observed. This was associated with enhanced platelet clearance through infection-induced changes, including increased desialylation and phosphatidylserine expression. Ngo et al. [106] described a platelet protective effect in bacterial sepsis through platelet factor 4 (PF4)–enhanced bacterial internalization and PF4-mediated prevention of bacteria-induced endothelial dysfunction, neutrophil release of extracellular traps (NETosis), and coagulopathy. Furthermore, platelets have also been demonstrated to influence immune function in "sterile" conditions [107]. Bourne et al. [108] used *in vivo* stroke models to investigate neuroinflammation and observed platelet extravasation and interaction with activated, proinflammatory microglial cells. Additionally, resting platelets have also been shown to modulate immune function in the absence of pathogenic stimuli. Li et al. [109] reported that platelets maintain monocyte immune tolerance in healthy conditions by regulating monocyte immune-metabolic processes.

At a cellular level, platelets are dynamic cells. Platelets demonstrate heterogeneous responses to systemic changes and, in turn, influence their surrounding circulatory microenvironment. This can be illustrated through the study of thromboinflammation, an important pathogenic process describing the interdependent and synergistic relationship between the classic hemostatic and proinflammatory pathways [110]. Platelets are critical components and cellular mediators of thromboinflammation through exposure of procoagulant surface proteins and release of their granular contents upon activation [111]. Banerjee et al. [112] suggest that IFITM3 is a novel regulator of platelet secretion (through interaction with VAMP8 and secretion of

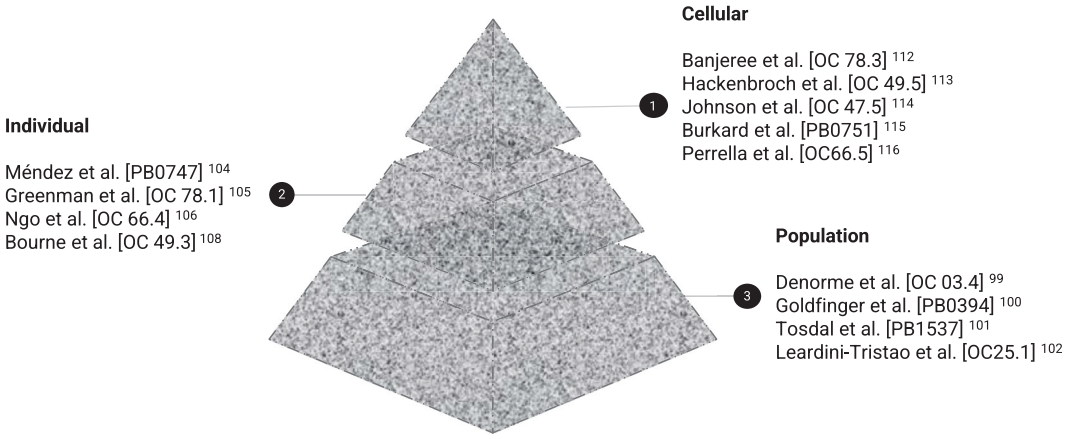

FIGURE 2 Novel data investigating platelet heterogeneity presented at the 2023 International Society on Thrombosis and Haemostasis Congress from a population, individual/disease-specific, or cellular levels.

PF4, P-selectin, and CD63) and platelet aggregation (platelet-leukocyte aggregates) and contributes to thrombotic events (under flow conditions) during systemic inflammation. Hackenbroch et al. [113] demonstrated a previously unknown mechanism of platelet-mediated thromboinflammation: platelet secretion of leukotriene B4 with direct recruitment of neutrophils to sites of endothelial damage. Similarly, neutrophil recruitment via platelet-derived integrin and tetraspanin-enriched tethers [114] and platelet GPVI–driven NETosis was demonstrated in lung models of acute inflammation [115]. In addition to platelets as drivers of thromboinflammation, platelets may, in turn, be influenced by their surrounding inflammatory milieu. Perrella et al. [116] provide evidence for NETosis-induced S100A8/A9 expression on platelets, driving phosphatidylserine exposure in a GPIbα-dependent manner.

The scientific community is employing novel technologies to harness and uncover platelet heterogeneity. Novel quantitative flow cytometry approaches have been developed to identify platelet subpopulations to better understand heterogeneity of platelet response and variability in patient outcomes in thrombotic conditions [117]. Badior et al. [118], Robertson et al. [119], and Leung et al. [120] described state-of-the-art technology using lipid nanobodies to transfect platelets with translationally active mRNA enabling expression of exogenous proteins. This provides a novel research technique to study protein synthesis in platelets and may represent the possibility of engineered platelets for therapeutic purposes in the future.

Platelet heterogeneity can be leveraged to develop pathobiology-specific treatment modalities. For example, differences in antigen expression between fetal and maternal platelets may give rise to the potentially catastrophic condition fetal and neonatal alloimmune thrombocytopenia. Geisen et al. [121] described progress toward prophylactic treatment with the use of a subcutaneous anti HPA-1a monoclonal antibody (RLYB212), which is intended to drive phagocytosis of fetal-derived HPA-1a antigen in maternal circulation to prevent alloimmunization. At the ISTH Congress, the authors showed elimination of HPA-1a platelets from circulation following treatment with (RLYB212). Moreover, Chen and Moskowitz [122] investigated a stabilized platelet-based drug delivery system with anticancer effects, while Malvestiti et al. [123] transfected platelets with therapeutic small interfering RNA and successfully reduced oncogenicity in a pancreatic ductal adenocarcinoma cell line. However, not all platelet heterogeneity introduced therapeutically is intentional. Artifactual platelet variability has been reported due to storage conditions of apheresis platelets prior to transfusion. Halliday et al. [124] have shown alterations in platelet glycan expression at 7 days of storage at 22 °C. The clinical implications of storage-related heterogeneity remain unknown. Collectively we envisage that with novel technologies and increased understanding of platelet heterogeneity, future studies hold significant potential for the development of personalized diagnostic and therapeutic strategies and enhancing healthcare and improving patient outcomes.

7 | FUTURE DIRECTIONS

Future directions in this still-understudied area of platelet heterogeneity abound. For instance, although recent single-cell omics studies explore the cellular landscape in various hematologic disorders [125–128], these have largely been limited to measuring single modalities at a time. Multiomic investigations of each of progenitor stem cells, megakaryocytes, and platelets from the same patient are currently lacking. Patient-specific cellular heterogeneity multiomics maps will help build a framework toward understanding therapy response and adapting treatment by subpopulation-specific strategies. Findings from such studies will also challenge our current approaches evaluating platelet and (megakaryocytic) functional heterogeneity solely by surface markers and instead suggest that it is closely linked to their cell state and gene expression signature. Future studies, particularly in hematologic cancers, will be motivated, therefore, to simultaneously capture cellular lineage potency/affiliation, potential genetic mutational status, and any molecular reprogramming in addition to the surface markers. We expect that advances in single-cell RNA sequencing methods will help overcome our current limitations in working with extremely low individual

platelet RNA content [129]. Patient-derived organoid models will be critically needed to obtain a more complete understanding of the range of platelet biological heterogeneity. Another noteworthy direction to pursue would be to unravel specific megakaryocyte/platelet mechanisms involved in the emergence of different marrow- or lung-derived megakaryocyte (and therefore platelet) subsets in response to inflammatory cues. Any future data on platelet heterogeneity will of course lay the groundwork for focused functional analyses of individual subpopulations of platelets. Collectively, a deeper understanding of platelet heterogeneity will provide significant new information on the composition, signaling pathways, and cellular communication in conditions of health and disease (initiation, evolution, and progression) and before and after treatment.

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AUTHOR CONTRIBUTIONS

A.K. presented the core content of this review as part of her invited State of the Art Marian A. Packham memorial lecture at the 2023 International Society on Thrombosis and Haemostasis Congress and subsequently invited S.T. and S.K. to contribute to the writing.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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