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Platelet "-omics" in health and cardiovascular disease

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HIGHLIGHTS

- "-omics" technologies have enabled characterization of the platelet proteome, transcriptome, metabolome and lipidome.
- Inter-lab differences in mass-spectrometry quantification or inability to analyze platelet subpopulations are limitations.
- Future breakthroughs will likely be enabled by novel "-omics" technologies capable of studying single cells.

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ABSTRACT

The importance of platelets for cardiovascular disease was established as early as the 19th century. Their therapeutic inhibition stands alongside the biggest achievements in medicine. Still, certain aspects of platelet pathophysiology remain unclear. This includes platelet resistance to antiplatelet therapy and the contribution of platelets to vascular remodelling and extends beyond cardiovascular disease to haematological disorders and cancer. To address these gaps in our knowledge, a better understanding of the underlying molecular processes is needed. This will be enabled by technologies that capture dysregulated molecular processes and can integrate them into a broader network of biological systems. The advent of -omics technologies, such as mass spectrometry proteomics, metabolomics and lipidomics; highly multiplexed affinity-based proteomics; microarray- or RNA-sequencing-(RNA-seq)-based transcriptomics, and most recently ribosome footprint-based translatomics, has enabled a more holistic understanding of platelet biology. Most of these methods have already been applied to platelets, and this review will summarise this information and discuss future developments in this area of research.

1. Introduction

In 1881, *in vivo* imaging experiments by Bizzozero [1] led to the discovery of platelets. Bizzozero correctly attributed haemostasis as important function to platelets. In 1906, histological studies as well as clinical observations led Wright [2] to conclude that platelets are shed from their megakaryocyte precursors in the bone marrow. Only recently was it shown that other organs such as the lungs may also be sites of substantial thrombopoiesis [3]. From megakaryocytes, platelets inherit their molecular content. Due to being anucleate, the discovery of platelet protein synthesis in 1966 by Warshaw et al. [4], confirmed by later studies [5], was rather surprising. With the advent of -omics technologies in the past decade, it became apparent that the platelet transcriptome and proteome only show a weak correlation [6], thereby questioning the physiological relevance of protein translation in platelets [7,8]. The large amounts of data amassed by proteomic, lipidomic, metabolomic and transcriptomic analyses of platelets have led to an extensive characterization of the molecular composition of platelets and now platelet -omics technologies are also being tested for their use in clinical practice (Fig. 1).

There are obstacles however, such as inter-laboratory differences in mass-spectrometry quantification or the inability to capture differences in specific platelet subpopulations that limit the advances of -omics technologies in clinical research. Nevertheless, platelet-based diagnostic and therapeutic innovations might have potential to improve morbidity and mortality from cardiovascular diseases, given that dysregulated or excessive activation of platelets underpins the most prevalent cardiovascular diseases such as stroke and myocardial infarction [9]. This review will cover platelet research in cardiovascular disease from an -omics perspective. It will discuss how the abilities and limitations of these technologies improved over time and provide an outlook for future applications (Fig. 2).

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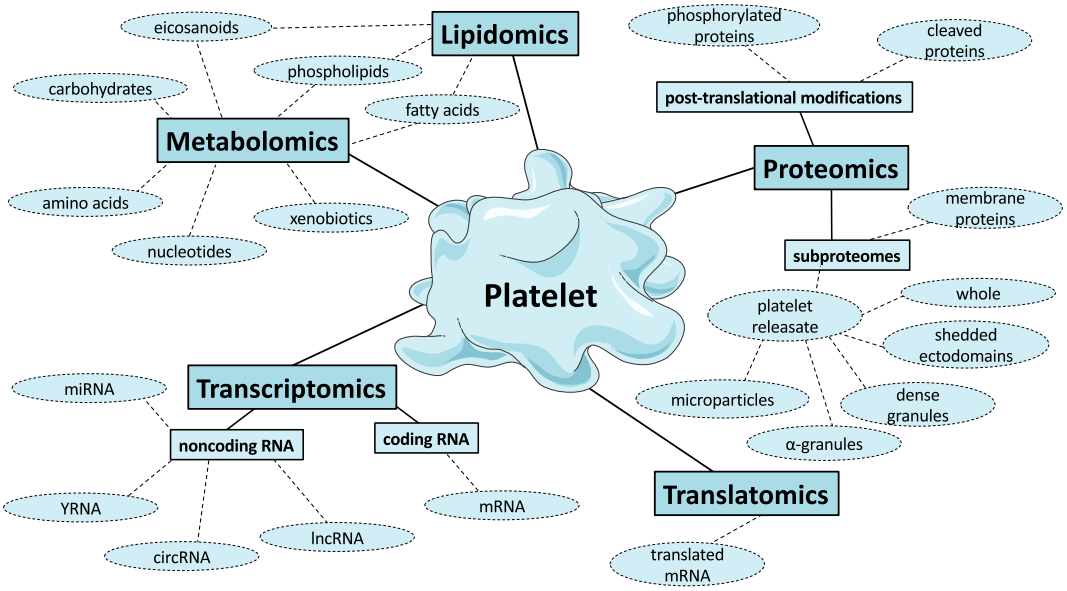

Fig. 1. Schematic overview on -omics studies in platelets.

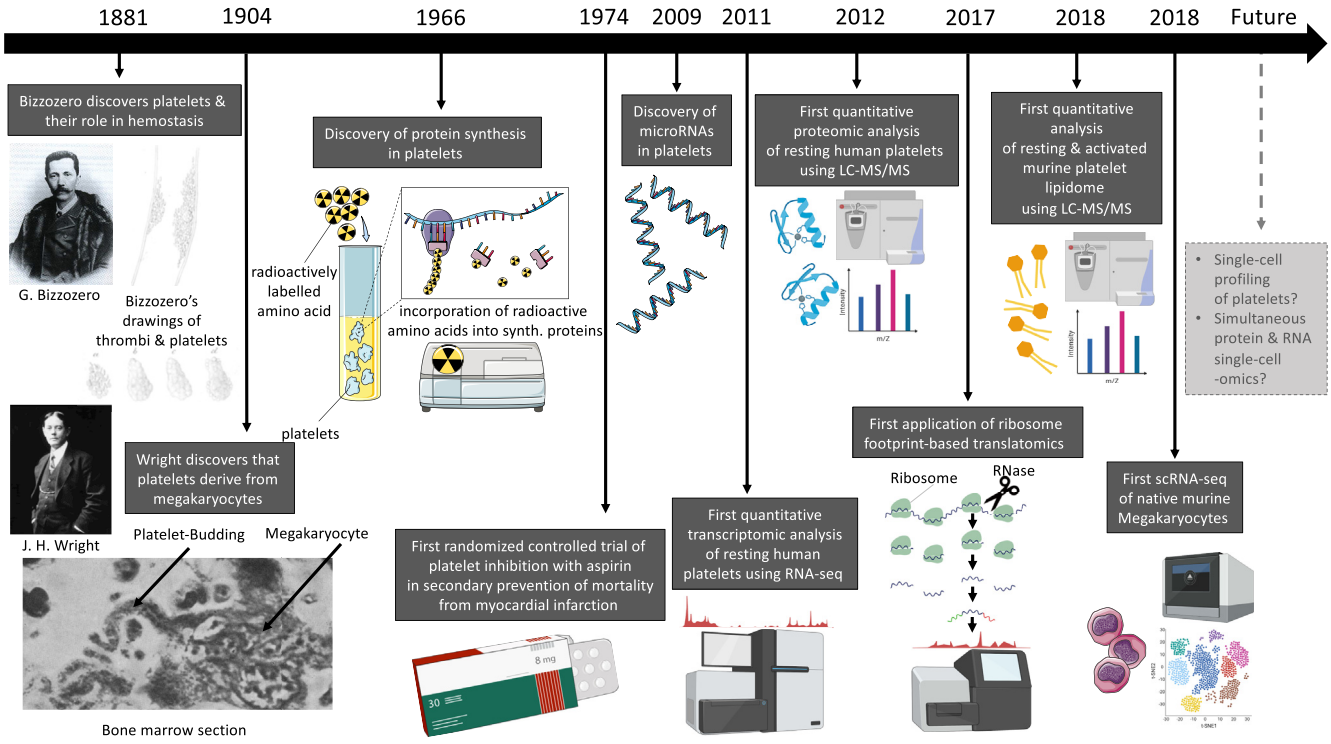

Fig. 2. Scientific and technological breakthroughs in platelet research.
1881: Bizzozero [1] discovers platelets and their role in hemostasis by *ex vivo* and *in vivo* imaging, reported as simple drawings because no cameras were available to him. 1904: Wright [2] discovers that platelets originate from megakaryocytes by a budding process in the bone marrow. 1966: through incubation of platelets with radio-labelled amino acids and subsequent Western blot and autoradiography, Warshaw et al. [4] conclude that platelets are capable of synthesizing proteins. 1974: first randomized controlled trial on platelet inhibition with aspirin shows striking reduction of mortality in patients with myocardial infarction [10]. 2009: platelets are found to be enriched in microRNAs and to contain microRNA processing proteins Dicer and Argonaute 2, thereby initializing research on the noncoding platelet transcriptome [11]. 2011: Rowley et al. [12] publish the first dataset of the complete coding transcriptome of human and mouse platelets. 2012: Burkhart et al. [6] publish the first quantitative proteomic dataset of human platelets. This proteomic dataset showed only a weak correlation with the transcriptomic dataset of Rowley et al. [12]. 2017: first publication of translatomic changes upon platelet activation [13]. 2018: first quantitative analysis of resting and activated mouse platelet lipidome [14]. 2018: first application of scRNA-seq in native murine megakaryocytes [15]. Potential future –omics breakthroughs: single-cell -omics of platelets, simultaneous protein and RNA analysis on single-cell level through DNA-barcoded antibodies.

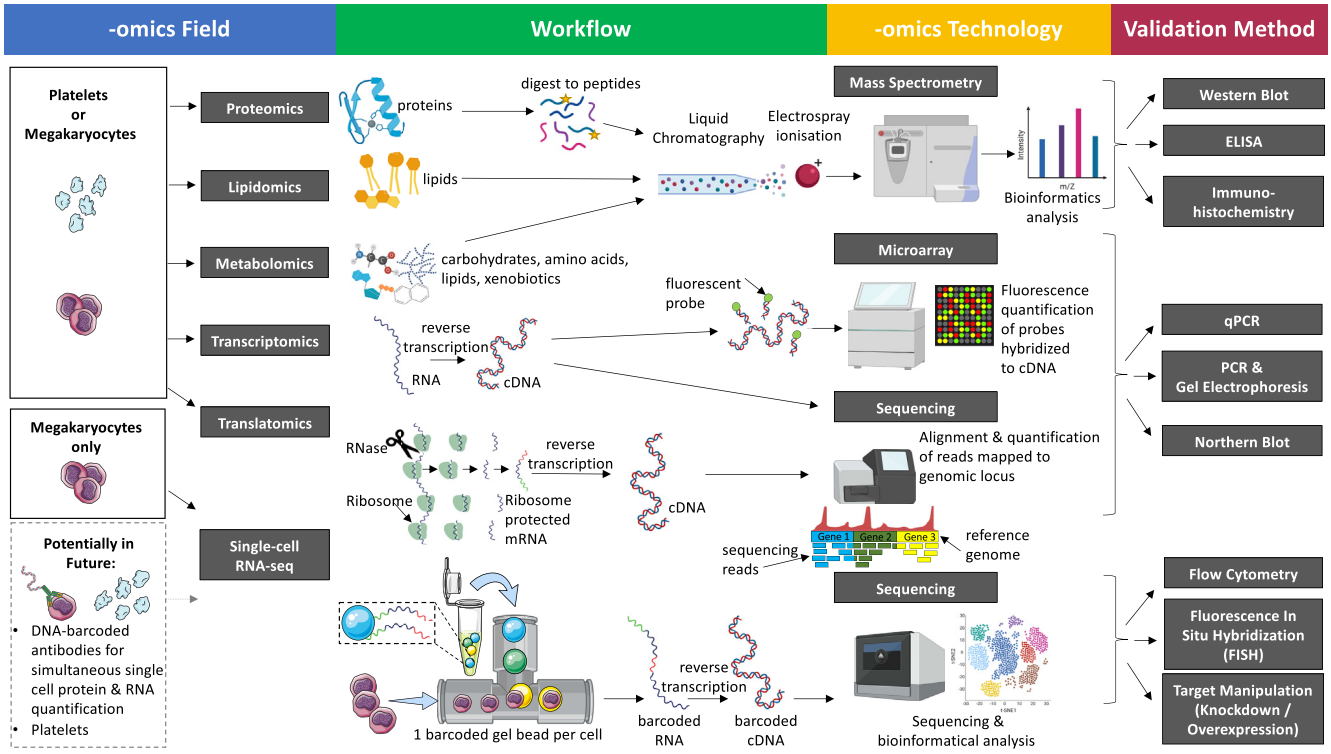

Fig. 3. Overview on methods used in –omics studies of platelets and megakaryocytes.

2. Platelet proteomics

2.1. The early beginnings of platelet proteomics

Proteomics refers to the analysis of all proteins contained in a sample. The analysis of the platelet proteome was pioneered in 1979 with two-dimensional gel electrophoresis and Coomassie staining. With these simple methods, abundant platelet proteins such as actin, myosin, tubulin, fibrinogen and proteins of the glycoprotein (GP) family were identified [16]. Gel-based techniques continued to be applied in early proteomics studies and provided a useful starting point for subsequent in-depth analysis of promising individual proteins. This included studies on the proteome of resting [17] and activated [18] platelets, as well as studies on specific subproteomes, such as membrane proteins [19], platelet releaseates as a whole [20], or microparticles [21], α-granules [22], dense granules [23] and shedded ectodomains of membrane proteins (the sheddome) [24] in particular. With the improvement of both mass spectrometry technology and subsequent data analysis, proteomics enabled also more in-depth quantitative analysis (Fig. 3). This technological advance provided unprecedented insights into molecular changes in platelets under different conditions. In 2012, Burkhart et al. [6] reported the first quantitative proteomic analysis of resting platelets isolated from healthy volunteers. Using 100 μg of protein per donor (~4 x 10^7 platelets, equivalent to a few ml of blood), this dataset provided an important reference for subsequent proteomic studies by other groups. Wijten et al. [25] for instance compared this dataset with their own proteomics data of activated platelets, thereby estimating protein release upon activation. Interestingly, the number of identified platelet proteins released upon activation in this study (124) was less than half of the number reported by Coppinger et al. [20] in the first ever published platelet releasate proteome. Reasons why proteomics studies may identify more proteins include the application of less stringent protein and peptide identification criteria, better sample prefractionation before mass spectrometry analysis, longer run times and more sensitive mass spectrometry instruments.

2.2. The platelet proteome – where does it originate from?

Platelets contain all components that are necessary for protein synthesis: precursor messenger RNA (pre-mRNA), a spliceosome to process this pre-mRNA to mature mRNA, endoplasmic reticulum, ribosomes and transfer RNA (tRNA) [26]. Signal-dependent splicing has been demonstrated for the pre-mRNAs of tissue factor in platelets from healthy volunteers [27] and of interleukin-1β in $CD34^+$ progenitor cell-derived proplatelets [26]. Functionality of the platelet protein synthetic machinery is also suggested by the presence of newly synthesized proteins inside platelets. Evidence for *de novo* protein synthesis, however, relies on experiments in which platelets were incubated with radio-labelled amino acids. Incorporation of these amino acids into growing peptide chains can then be assessed by combined gel electrophoresis and autoradiography. *De novo* protein synthesis can be blocked by inhibitors of protein synthesis, suggesting that the detected signal is specific to newly synthesized proteins and does not derive from non-specific interactions. Since the first report of platelet protein synthesis by Warshaw et al. [4] in 1966, this remains the only method with which platelet protein synthesis has been successfully assessed. It was applied to important platelet proteins such as cyclooxygenase-1 [28], eukaryotic translation initiation factor 4E and 2α [29], GP IIIa [30], interleukin-1β [31] and tissue factor [27,32]. One study found this translational activity to be mostly confined to reticulated (an indicator of young) platelets during the first 6 h of their generation [33]. Another study reported that platelet translation is controlled by RNA-DNA hybrids, which are formed through endogenous reverse transcriptase activity of the Long Interspersed Element-1 (LINE-1) [34]. It should be noted that radio-detection is an extremely sensitive approach that may detect very low protein amounts, the physiologic relevance of which remains unclear.

Doubts about the physiologic relevance of platelet protein synthesis were raised by indirect assessments, such as comparative analyses of the proteome and the transcriptome. Burkhart et al. [6] for instance compared their quantitative proteomics data to published RNA-seq data [12]. Here, only a weak correlation was observed between the platelet

transcriptome and proteome [7,8]. Further analysis of these datasets revealed that the correlation strength could be increased when mRNAs and proteins with low abundance were excluded from the analysis [7]. This might be attributable to the higher sensitivity of RNA-seq for mRNAs compared to mass spectrometry for proteins, which is also reflected in the fact that more proteins had a corresponding mRNA (87%), than mRNAs had a corresponding protein (30%). Taken together, it appears that protein "inheritance" from megakaryocytes and also protein uptake from other cells or plasma, facilitated by the platelets' open canalicular system [35], has a much greater effect on protein composition than endogenous translation.

Studies on platelet translation continue to date, taking advantage of new technologies. Ribosome footprint profiling was first reported in 2009 by Ingolia et al. [36] as a method to enable large scale analysis of translational events, thereby establishing the field of "translatomics". This method is based on RNA-seq of ribosome-protected fragments of mRNA. Alignment of these fragments to the genome allows the identification of translated rather than just transcribed mRNAs (but does not allow for detection of the synthesized protein itself). In 2017, this approach was applied to platelets by Mills et al. [13]. In this study, a comprehensive analysis of the translatomic changes upon thrombin-induced platelet activation was performed. Since agonist-induced activation occurred *ex vivo*, the detected changes must have taken place in platelets. The large-scale translatomic approach by Mills et al. [13] enabled, for the first time, a correlation of the platelet transcriptome with the platelet translatome, and the platelet translatome with the proteome (the latter being retrieved from public depositories). This time the correlation strength was moderate (Spearman $\rho = 0.39$ and $\rho = 0.37$, respectively).

In another study [37], it was recently shown that sepsis alters both the transcriptional and translational landscape of human and murine platelets. This included increased transcription, translation and activation of integrin α-IIb, a protein known to be important for platelet aggregation. Whether these transcriptional and translational changes have any relevant *in vivo* effects remains unclear. In summary, translatomic studies support the concept of limited platelet protein synthesis but have not yet demonstrated whether these processes are physiologically relevant.

2.3. Studying platelet function through integrative approaches

High abundance proteins such as cytoskeleton proteins (important for morphological changes upon activation and adaption to a high shear stress environment) as well as α-granular proteins such as platelet factor-4 (essential for prothrombotic effects) can be indicative of platelet function [6]. Signalling processes, however, might deliver more mechanistic insights. For this purpose, studies on post-translational modifications with enrichment workflows for phosphoproteins [38] and protein cleavage [39] have been conducted. Using a tyrosine phosphoproteomic analysis, Izquierdo et al. [40] have recently identified novel components of the C-type lectin-like receptor 2 signalling cascade in platelets, including adaptors, kinases/phosphatases and membrane proteins. Another recent study has characterized the interactome of the Trem-like transcript 1 protein, identifying several novel interactors and phosphorylation sites [41]. This protein has been shown to have a mechanistic role in bleeding triggered by inflammatory insults, but detailed knowledge on the exact signalling cascade had been limited [41].

Simultaneous metabolite, (phospho-)protein and lipid extraction (SIMPLEX) with subsequent mass-spectrometry-based analysis as reported by Coman et al. [42] demonstrated the feasibility of a multi-omics approach. Indeed, the same group used a similar integrative approach to quantify the global proteome, phosphoproteome and proteolytic cleavage to characterize altered platelet functions in Scott syndrome [43]. They revealed major differences in phosphopeptide levels and calpain-induced cleavage of cytoskeleton-linked and signalling proteins between agonist-treated platelets of patients with Scott syndrome (higher phosphorylation, lower cleavage) and control individuals (lower phosphorylation, higher cleavage).

As proteins are just one effector of biological functions, lipidomics and metabolomics have recently also been applied to platelets. In 2018, Peng et al. [14] have published the first comprehensive quantitative analysis of the resting and activated murine platelet lipidome, four years after publication of the first quantitative analysis of the murine platelet proteome [44]. They reported that platelets contain almost 400 lipid species with a concentration range of 7 orders of magnitude. Approximately 20% of the lipidome undergoes changes upon activation, involving mainly lipids containing arachidonic acid. Notably, hyperlipidaemic patients often present with a prothrombotic phenotype [45]. Although the precise mechanisms are still unclear, hyperlipidaemia increases platelet production and activation [46] and membrane cholesterol correlates with platelet activation [47], probably due to increased membrane fluidity. Hypercholesterolemia also appears to have a procoagulant effect that is mediated by platelets: one study has shown that the content of cholesterol in platelet membranes is positively correlated with platelet tissue factor activity [48].

Similarly, the platelet metabolome has attracted attention. Among platelet proteins, enzymes involved in glucose metabolism are highest in abundance [6], indicating the importance of glycolysis for platelet function. Metabolomics has also been applied to correlate platelet metabolites with mitochondrial energetic parameters [49,50]. The mitochondrial stress test for instance uses the cellular oxygen consumption in response to different mitochondrial inhibitors to create the bioenergetic profile of intact cells. Chacko et al. [49] have recently applied this method to intact platelets and combined it with untargeted metabolomics measurements of the same sample. Using xMWAS analysis, they could then integrate metabolomic and bioenergetic data and define the bioenergetic-metabolite interactome in platelets [49]. This represents a timely contribution since it has become apparent that platelets might serve as markers for mitochondrial dysfunction [51]. Platelet bioenergetics has been implicated as indicator of therapeutic efficacy/toxicity of anti-cancer drugs [52] or severity of diseases such as Alzheimer's [53] or sickle cell disease [54].

2.4. Targeted proteomics applications

Despite the importance of discovery approaches, advances in targeted mass spectrometry technologies allow the quantification of hundreds of preselected proteins with reference peptide standards, providing data on specific biological processes without the need for binders such as antibodies or aptamers. This could benefit laboratory measurements in clinical practice, as protein markers are currently routinely measured by enzyme-linked immunosorbent assays (ELISAs) and consumable costs are high. A recent study has provided a proof of principle for this approach, by using targeted proteomics to quantify platelet proteins related to platelet activation and functional disorders [55]. Introduction into clinical practice will require standardized workflows, as targeted mass spectrometry-based quantification of the same sample in six different laboratories yielded substantial differences in the reported protein concentrations [56].

2.5. Identification of platelet subtypes through proteomics

Circulating platelets differ in various characteristics, some of which are associated with increased risk for adverse cardiovascular outcomes. It is therefore of clinical relevance to further characterize these subtypes so that risk prediction as well as treatment decisions can be improved. To date, assessment of platelet heterogeneity in clinical settings relies on rather simple markers such as mean platelet volume (MPV) and reticulation. MPV as a measure of platelet size is a standard measurement in clinical blood tests and positively associated with risk for myocardial infarction [57,58]. This correlation was also seen in

patients on antiplatelet therapy, suggesting that this risk factor is unaffected by current treatment strategies [57]. Reticulation as a morphological indicator of high intraplatelet RNA-abundance can also be assessed in clinical settings and was found to be strongly correlated to MPV [59]. Similar to high MPV, a high reticulated platelet count was associated with adverse outcomes in patients with myocardial infarction [59,60]. Interestingly, reticulated platelets were also associated with a reduced response to different antiplatelet drugs [61]. A hyperreactive role of reticulated platelets is supported by the finding that their relative contribution to thrombi is much higher than that of non-reticulated platelets [62]. Both high MPV and reticulation are considered a feature of newly formed platelets, which increase as a response to acute thrombocytopenia [33].

Reticulated platelets are also increasingly formed in diabetes and in inflammation [63,64], both of which are conditions known to be associated with a reduced antiplatelet response [65–68]. The release of reticulated platelets under these conditions might differ from that of "normal" continuous thrombopoiesis. While the latter is controlled by thrombopoietin and predominantly involves a slow budding process of platelets from megakaryocyte membrane extensions, inflammatory processes may favour a rather rapid release of platelets through megakaryocyte rupture, which is under control of interleukin-1α and independent of thrombopoietin [69]. Other descriptions of platelet subpopulations rely on a differential response to collagen and thrombin (COAT platelets) [70]. In the membrane of these platelets, increased levels of α-granule proteins and phosphatidylserine can be found, which confer the ability to bind fibrinogen despite treatment with GP IIb/IIIa inhibitors [71].

Platelets are heterogenous and certain platelet subpopulations may increase the risk for adverse cardiovascular outcomes. However, no treatments and no diagnostic tests exist, which are able to prevent the formation of hyperreactive platelet subtypes or are able to inform treatment decisions, respectively. Currently used markers of platelet heterogeneity rely on simple morphologic features that are unable to specifically capture the molecular culprits responsible for adverse cardiovascular outcomes.

A novel approach to study platelet heterogeneity is the use of proteomics. A first study to characterize the proteomic profile of platelets of different ages was made by Thiele et al. in 2016 [72]. In this study, three consecutive platelet apheresis procedures were conducted on a single healthy male volunteer to lower his platelet count and provoke the increased production of new platelets. In line with the latter, MPV was increased following apheresis. Proteomic analysis of isolated platelets with two-dimensional difference gel electrophoresis (2D-DIGE) and in-solution mass spectrometry yielded similar results: both approaches identified around 1,000 proteins, of which around 50 (~5%) were differentially expressed over the platelet depletion time-course. Analysis of these differentially expressed proteins revealed that two protein clusters in the 2D-DIGE and four protein clusters in the in-solution digest followed similar kinetics. After this first study based on a single donor, another study used a density ultracentrifugation-based protocol to separate small from large platelets of healthy volunteers and analyse their differences in the proteome [73]. For this purpose, a novel density ultracentrifugation-based protocol was developed to separate platelets by size. Of the 894 proteins identified by mass spectrometry, 80 (~9%) were differentially expressed, when large and small platelet preparations were normalized to total protein. When absolute protein amounts of proteins involved in platelet activation such as GP Ia, Ib, IIIa, VI and the $P2Y_{12}$ receptor were analysed, large platelets showed 30–50% higher abundance levels. Large platelets furthermore showed higher reactivity for the majority of analysed reactivity indices. This included an elevated P-selectin expression as a measure of α-granule release following thrombin-receptor-activating peptide (TRAP) and collagen stimulation; increased α-IIb-β-III integrin activation following collagen stimulation (but decreased following TRAP and adenosine diphosphate); elevated membrane phosphatidylserine exposure; faster adhesion and spreading on a collagen surface; and faster aggregation upon light transmission aggregometry measurements following collagen and adenosine diphosphate stimulation (but no difference in maximal aggregation).

2.6. Platelet proteomics in acute myocardial infarction

There are few studies applying proteomics techniques to platelets in the setting of myocardial infarction. These studies primarily used 2D-DIGE before applying mass spectrometry for the identification of differentially expressed protein spots. An early study compared patients with no cardiovascular disease with those with angina or non-ST elevation myocardial infarction, and found 6 out of 400 resolved proteins to be differentially regulated [74]. In another study, 18 patients with a diagnosis of myocardial infarction, and 10 matched patients with stable coronary artery disease donated platelet samples immediately on arrival to hospital, and at 5 days and 6 months later [75]. The study identified increases in the GPVI and GPIIb/IIIa signalling pathways in patients with myocardial infarction. The protein SPARC was reduced in platelets at the time of non-ST elevation myocardial infarction (NSTEMI). A second study recruited 11 patients with a specific diagnosis of ST-elevation myocardial infarction (STEMI), compared them with 15 stable coronary artery disease controls, and again followed patients with STEMI at day 5 and 6 months [110]. Of approximately 2,400 identified proteins, the authors validated an increase in phosphorylated proto-oncogene tyrosine-protein kinase Src (pTyr418-phosphorylated Src), and its adaptor Crk-like protein by immunoblotting. In an independent cohort, the authors confirmed their previous findings of increased GPVI signalling in STEMI, demonstrating an increase in pTyr418 phosphorylated Src when platelets from patients with STEMI were treated with collagen-related peptide, a GPVI specific agonist.

2.7. Platelet proteomics in stroke

The first study on changes of the platelet proteome following stroke was published in 2008 [76]. Although this study recruited patients with any kind of arterial thrombosis that took place 1 week to 6 months before blood collection, 90% of patients (26 of 29) had an ischemic stroke. Using 2-D DIGE and subsequent mass spectrometry, the platelet proteome of these patients was compared with the platelet proteome of 24 healthy donors. Proteomic differences between both groups were predominantly related to cytoskeletal components. Regardless of the kind of arterial thrombosis or treatment, the platelet content of integrin-linked kinase, fructose bisphosphate aldolase and glyceraldehyde-3-phosphat dehydrogenase was decreased, whilst actin binding protein, coronin-1A, non-muscle myosin heavy chain, pyruvate kinase M2 isoenzyme and phosphoglycerate kinase were increased [76].

A more recent study compared the platelet proteome of samples from 65 patients, taken within 24 h of incidence of ischemic stroke, with samples from 42 healthy controls [77]. Based on label-free proteomics analysis, 83 platelet proteins were found to be differentially expressed between both groups. These included proteins important in inflammation, cellular movement, immune cell trafficking, intercellular communication, haematological system development and nucleic acid metabolism [77]. A caveat of these case-control studies is the potential confounding effect of medication on the platelet proteome.

2.8. Affinity-based proteomic platforms

Whilst mass spectrometry approaches offer the current gold standard for assessment of the proteome, other technologies such as the SomaScan and O-link proteomic platforms offer higher throughput but are restricted to a preselected range of proteins. The O-link proximity extension assays combine the specificity of antibody techniques with DNA amplification steps. Proximity extension assays rely on the binding

of two antibodies to a single protein, bringing their conjugated, complementary DNA strands into proximity and amplifying and detecting the resulting double-stranded DNA. Antibody-DNA pairs can be multiplexed, allowing currently the discovery of a total of 1,164 proteins in plasma [78]. As an antibody technique, O-link is dependent on the availability of good quality antibodies and the accessibility of the epitopes. Detection of the double-stranded DNA is performed by polymerase chain reaction meaning saturation effects can be expected if proteins are more abundant. Unlike mass spectrometry, however, low abundant proteins, including cytokines or chemokines can be better detected. To date, no studies of the platelet proteome have been undertaken using the O-link proteomics approach.

The SomaLogic aptamers use unique, protein-binding DNA oligonucleotides to increase the depth of proteomic analysis [79]. SomaScan panels covering over 1,000 and 5,000 proteins are available, offering scalable detection of proteins. In a recent study, a comparison of the measurements of the same 1,129 proteins across 3 genotyped cohorts reported that the expression levels of 268 proteins could be determined by single nucleotide polymorphisms [80]. Using these data, protein quantitative trait loci (pQTLs) were generated, to predict plasma protein levels based on an individual's genome. External validation through direct measurements of pQTL targets in patients with clinically determined indices of atherosclerosis, confirmed C-type Lectin Domain Family 1 Member B (CLC1B or CLEC-2), a platelet immuno-receptor [81], and platelet-derived growth factor receptor-β as biomarkers for the presence of stable atherosclerosis.

Both the SomaLogic and O-link platforms are optimised for use in plasma, meaning saturation effects can be expected when applied to tissues or cell lysates such as platelets. The aptamers face additional technical challenges as highlighted recently [82]. However, the presence of the platelet membrane receptor CLC1B as a differentially expressed biomarker for atherosclerosis risk in plasma upholds the idea that traditionally prepared plasma is composed, at least in part, from platelet releasate and debris. Future affinity-based approaches to platelet proteomics may use platelet-specific panels to investigate this phenomenon in large cohort plasma samples, linking the results with other measures of platelet function, or attempt to optimize the use of O-link or SomaScan aptamer panels for platelet lysates.

3. Platelet transcriptomics

Transcriptomics provides snapshots of the entire landscape of RNA transcripts in a given cell type or tissue at a given time and condition. Besides transcriptomic studies on coding mRNAs, the noncoding transcriptome (microRNAs, YRNAs, circular RNAs, long noncoding RNAs) is also gaining attention for its role as a marker of platelet function in various conditions [111,84]. Microarrays and RNA-seq are the two predominant methods for transcriptomic analyses. While microarrays require the design of predesigned probes and therefore rely on *a priori* knowledge of its targets, RNA-seq represents a less biased approach that enables the discovery of novel transcripts. Bias can still be introduced by differences in library preparations, the methodological approach as well as the subsequent data interpretation. As with any -omics technology, key findings require validation by independent techniques. In case of transcriptomics this is usually performed by conventional real-time polymerase chain reaction-based methods.

Associating the transcriptomic profile of platelets to different pathologic conditions has been shown for many diseases but establishing whether transcriptomic changes are causal or consequential remains challenging. Studies that address these unsolved questions hold promise in offering novel diagnostic and/or therapeutic opportunities. Platelets show characteristic expression patterns across a diverse range of disease classes such as inflammatory [85], haematological [86], oncologic [87], autoimmune [88], metabolic [89], nephrological [90] and cardiovascular conditions. The latter include distinct platelet transcriptomes for patients with stable and acute coronary heart disease [91–93], coronary artery bypass graft surgery [94], peripheral artery disease [95] and atrial fibrillation [96]. It remains to be seen whether these platelet comparisons were confounded by medication and co-morbidities that could provide an alternative explanation for alterations in the platelet transcriptome.

4. Single-cell -omics – the future of platelet profiling?

One limitation of current -omics technologies is that they are based on “bulk”-analysis of large numbers of cells or tissue homogenates, which only gives information on the “average” transcriptome (in transcriptomics) or proteome (in proteomics). Future breakthroughs in the identification of platelet subtypes are therefore likely to derive from -omics technologies that are able to characterize single cells. A landmark study in this regard was the sequencing of the whole coding transcriptome of a single cell in 2009 by Tang et al. [97]. Single-cell RNA-seq (scRNA-seq) has since then seen a rapid development in terms of throughput and applicability to various cells.

The coding transcriptome aside, single-cell analysis of small noncoding RNAs such as microRNAs, transfer RNAs and small nucleolar RNAs could be shown for the first time in 2016 [98], while simultaneous scRNA-seq of microRNAs and mRNAs was first reported in 2019 [99].

Another breakthrough was the recent development of simultaneous protein and RNA measurements in single cells. This approach was reported by two independent groups in 2017, using similar methodologies: both CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing [100]) and REAP-seq (RNA expression and protein sequencing [101]) use antibodies with conjugated DNA-barcodes for protein detection, instead of using “traditional” fluorophores. The same sequence containing the DNA-barcode is also equipped with a poly(dA) tail and a next generation sequencing read, enabling the simultaneous capture of mRNA and the subsequent sequencing analysis.

In platelets, the feasibility of single-cell applications remains to be shown. In theory, platelets meet several criteria for successful scRNA-seq. A sufficient RNA content for instance is critical, as defined by number and diversity of transcripts per cell. Given that platelets contain a diverse coding (9,500 mRNAs based on bulk RNA-seq [102]) as well as noncoding transcriptome [83] (~750 different microRNA species based on RNA-seq [102] and ~21,000 circular RNAs identified in circular-RNA-seq data of RNase R treated RNA [103]), single platelets should retain enough transcripts to distinguish different platelet subpopulations based on differences of one, or a combination of different platelet RNA classes. Cell size is another limiting factor for single-cell applications. In case of the most commonly used Chromium™ Single Cell Solution platform, this only applies to large cells however (> 30 μm), while there is no limitation for small cells (platelets are 2–3 μm in diameter). The initial barcode labelling and library preparation steps of single platelets can therefore be expected to be feasible. Successful distinction of cell populations also depends on sequencing depth and breadth. Cell types with low total RNA amounts , such as platelets, generally require lower sequencing depth. Sequencing breadth on the other hand can be ensured by the high throughput capacity of commercial platforms such as the most commonly used Chromium™ Single Cell Solution, which allows profiling of up to 10,000 cells per reaction – a number that can be readily obtained with a single blood donation.

The rapid development in profiling single cells will likely result in a clearer definition of cellular subtypes and make it easier to attribute pathophysiologic features to some of them. This is especially true for platelets, where successful identification of hyperreactive platelet subpopulations could improve prediction of adverse cardiovascular outcomes and inform treatment decisions accordingly. It is possible that future clinical applications of scRNA-seq-based platelet tests can be performed with a simple finger prick test, given the fact that human blood contains 150,000–450,000 platelets per microliter and currently

available single-cell applications such as the Chromium™ Single Cell Solution make use of only 10,000 cells per reaction.

Besides identifying different platelet subpopulations, single-cell applications might also make it possible to trace the source of these subpopulations. Identifying the source of hyperreactive platelet subtypes could result in therapeutic opportunities to prevent their formation. It has been speculated that many platelets are produced from extramedullary megakaryocytes in inflammatory conditions [69] or diabetes [63,64]. Simultaneous scRNA-seq of platelets and their megakaryocyte precursors from different anatomical sites (bone marrow, lungs) under different conditions (stable haematopoiesis, increased haematopoiesis following acute thrombocytopenia, inflammatory or hyperglycaemic conditions) could identify the source of each platelet subtype based on whole transcriptome correlations, e.g. platelet-bone marrow megakaryocyte clusters vs. platelet-lung megakaryocyte clusters. A shift in the relative contribution of these clusters may indicate the response at different sites of platelet production. This approach could also reveal to which extent platelets alter their transcriptome in the circulation and to which extent differences in the platelet transcriptome under disease conditions are due to processes that take place in megakaryocyte precursors.

Until recently, our understanding of the megakaryocyte transcriptome has relied on bulk analysis of *in vitro* differentiated $CD34^+$ progenitors, immortalised MEG-01 cells or induced pluripotent stem cells [104–106], while insufficient sample numbers of this rare cell type have impeded direct transcriptomic analysis of native megakaryocytes. Only by using scRNA-seq, Davizon-Castillo et al. made it recently possible to analyse the whole coding transcriptome of native, single megakaryocytes [15]. Here, the investigators could distinguish seven megakaryocyte clusters, of which three were particularly enriched in megakaryocyte transcripts such as von Willebrand factor, platelet factor 4, integrin α-IIb, integrin β-3, erythroid transcription factor and the thrombopoietin receptor; presumably representing the pro-platelet forming groups of cells. In another study, the same investigators showed that aging-associated platelet hyperreactivity in mice is associated with transcriptional reprogramming of inflammatory, mitochondrial and metabolic pathways in megakaryocyte precursors [107].

Single-cell technologies could also bring improvements to technical problems that are inherent to platelet research. Platelet studies are particularly prone to pre-analytical variation. Differences in the type of anticoagulant used in collection tubes, differences in sample processing time and differences in centrifugation speed can lead to differential platelet activation. Another problem common to all centrifugation-based platelet isolation protocols is that they are contaminated with leukocytes to varying degrees. The absolute RNA concentration of single leukocytes is around 1,000 times higher than that of platelets [108]. Bulk transcriptomic analysis of platelet preparations therefore inevitably leads to a substantially confounded transcriptomic profile of platelets. Current alternatives to centrifugation-based platelet preparations are protocols based on cell sorting. Although this approach reliably yields pure platelet preparations, the long protocol duration and the sorting process usually lead to substantial platelet activation. With single-cell profiling, any contaminating leukocytes can easily be identified as such in the resulting dataset. With platforms such as the Chromium™ Single Cell Solution, phenotypic manipulation of platelets could be kept to a minimum, since cells are immediately lysed upon bead capture (i.e. within the first few seconds of the single-cell protocol).

5. Conclusion

Advances in science are often enabled by progress in technologies. While in the 19th century Bizzozero [1] was able to use merely descriptive tools to obtain first insights into platelet function, molecular biology techniques enabled identification of the mechanisms of platelet aggregation, which could then be targeted by antiplatelet medication. The first applications of -omics technologies were similarly descriptive but are now increasingly used for mechanistic studies as well as for diagnostic purposes. Still new approaches are needed to investigate platelets. In this regard, the development of scRNA-seq can provide both coding and non-coding transcriptomic data of primary megakaryocytes within the bone marrow. Furthermore, this technology could enable transcriptomic studies of single platelets. This would avoid the problems of leukocyte contamination in isolated platelet preparations and enable the potential identification of platelet subtypes that might contribute to adverse cardiovascular outcomes. Insights gained from such studies could lead to better risk prediction to inform treatment decisions as well as novel therapeutic strategies to target hyperreactive platelet subtypes. Both of these diagnostic and therapeutic innovations might have the potential to reduce cardiovascular morbidity and mortality.

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Declaration of competing interest

M.M. has filed and licensed patent applications on microRNAs as platelet biomarkers. The other authors have nothing to disclose.

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