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Multi-omics approaches to study platelet mechanisms

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Abstract

Platelets are small anucleate cell fragments (2–4 μm in diameter) in the blood, which play an essential role in thrombosis and hemostasis. Genetic or acquired platelet dysfunctions are linked to bleeding, increased risk of thromboembolic events and cardiovascular diseases. Advanced proteomic approaches may pave the way to a better understanding of the roles of platelets in hemostasis, and pathophysiological processes such as inflammation, metastatic spread and thrombosis. Further insights into the molecular biology of platelets are crucial to aid drug development and identify diagnostic markers of platelet activation. Platelet activation is known to be an extremely rapid process and involves multiple post-translational mechanisms at sub second time scale, including proteolysis and phosphorylation. Multi-omics technologies and biochemical approaches can be exploited to precisely probe and define these posttranslational pathways. Notably, the absence of a nucleus in platelets significantly reduces the number of present proteins, simplifying mass spectrometry-based proteomics and metabolomics approaches.

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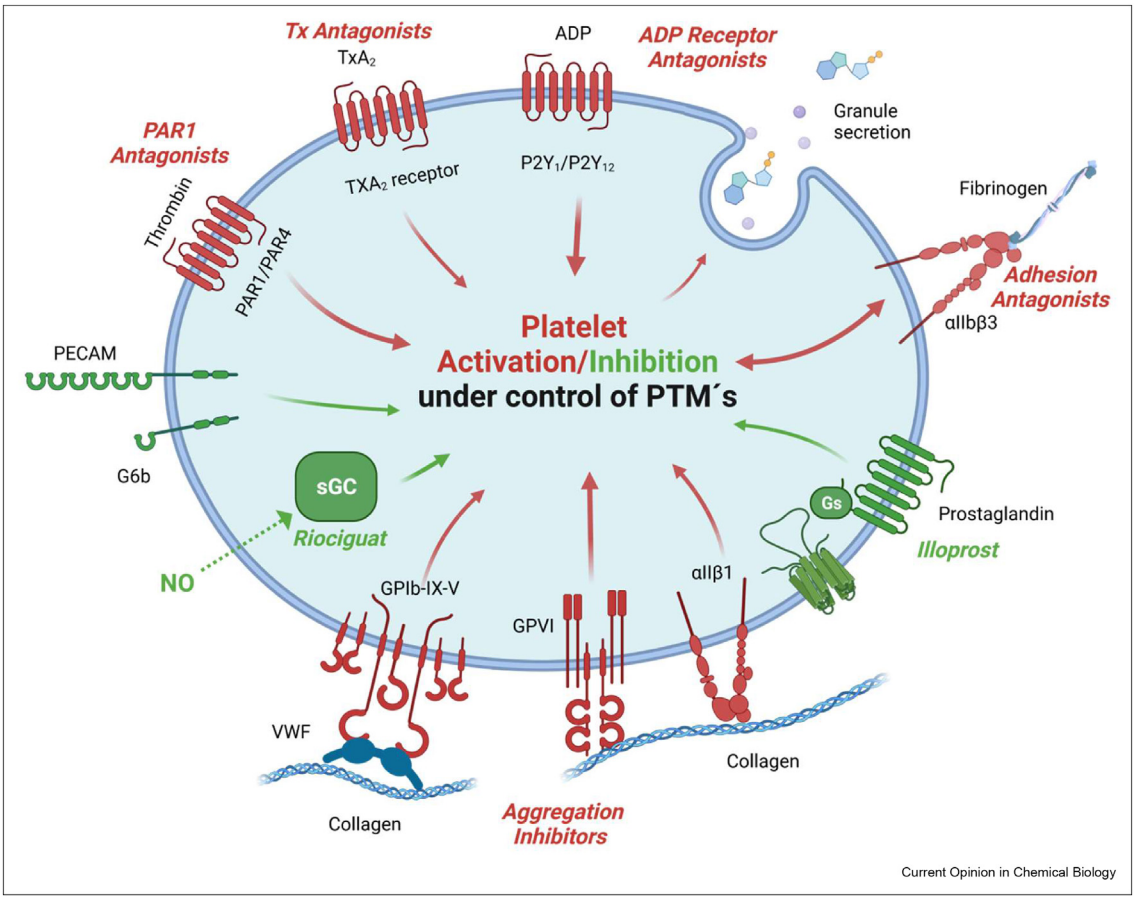
Multi-omics, Platelet, Mass spectrometry, Posttranslational modification, Chemical proteomics, Activity based probe

Introduction

Platelets are small anucleate cell fragments (2–4 μm in diameter) in the blood, which play an essential role in hemostasis and blood coagulation. Genetic or acquired platelet dysfunctions are linked to bleeding, increased risk of thromboembolic events and cardiovascular diseases [1]. Hence, platelets contribute to one of the major causes of death worldwide. Advanced proteomic approaches and techniques in related research fields can pave the way to a better understanding of the roles of platelets in hemostasis and pathophysiological processes, such as inflammation, metastatic spread, and thrombosis [2].

Further insights into the molecular biology of platelets are crucial to aid drug development and identify diagnostic markers of platelet activation. Platelet activation is known to be an extremely rapid process and involves multiple post-translational mechanisms at sub-second time scale, including proteolysis and phosphorylation (Figure 1) [3]. Multi-omics technologies and biochemical approaches can be exploited to precisely probe and define these post-translational pathways. Platelets are highly sensitive to changes in their natural microenvironment in the circulation during isolation and react on factors, such as temperature, pH, shear stress, etc. Thus, the detailed analysis of platelets' function is altered and subsequent validation of omics data is mandatory [20]. Notably, the absence of a nucleus in platelets significantly reduces the number of present proteins, simplifying mass spectrometry-based proteomics and

Figure 1



Overview: Platelet signaling under the control of PTM's. Platelet activation and inhibitory mechanisms are ruled by changes in PTM modifications, in particular (de)glycosylation and (de)phosphorylation. When adhering to a (damaged) vessel wall, flowing platelet become activated and change shape from discoidal to semi-round with filopodia. In response to agonists like collagen and thrombin, activated platelet with filopodia release ADP, thromboxane A_2 (TxA_2), which act as autocoids. In the cytosol, multiple protein kinases and phosphatases become activated and start phosphorylation and dephosphorylation events of their target proteins. In addition, depending on the platelet activation state, proteases become activated and cleave their protein substrates. Furthermore, changes in the platelets lipids and glycan composition are part of the platelet activation mechanisms.

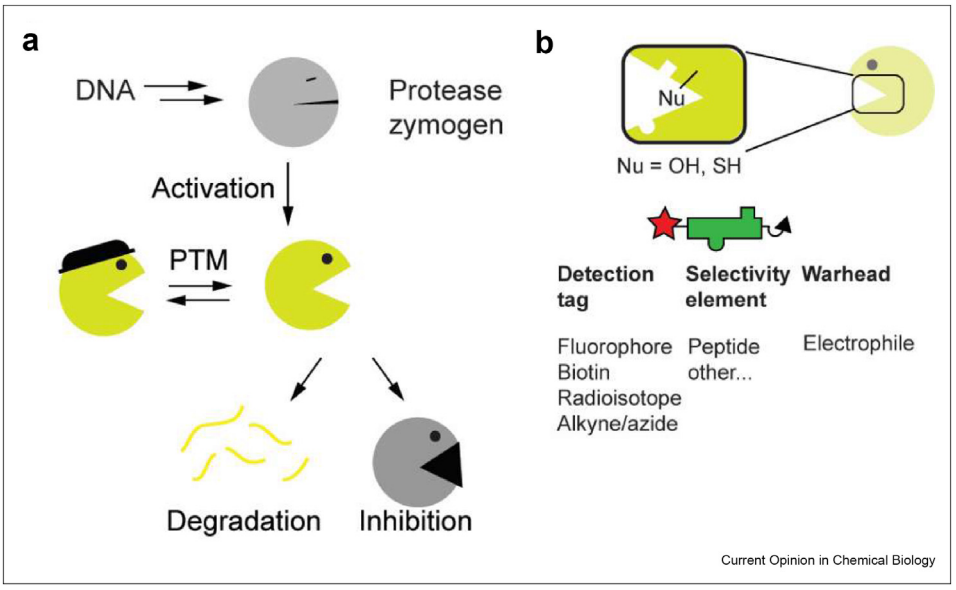
metabolomics approaches. Genomic sequencing, genome-wide association studies, and transcriptomics were used to investigate platelet phenotypic traits and remaining gene expression [16–19,51–53]. In this review, we will present an overview of established and new multi-omics strategies, which can be used to study platelet biology in health and disease.

Covalent chemical probes and chemical proteomics

Covalent chemical probes are small molecules that label proteins according to a certain trait, such as enzymatic activity or the binding to a certain drug or metabolite [8]. The multiple post-translational signaling mechanisms that activated platelets undergo can be investigated with tailor-made chemical probes and mass spectrometry. This is, in particular, the case for proteolytic processes — the cleavage of peptide bonds in proteins by proteases. Intra- or extracellular proteolysis is an irreversible process that needs tight regulation. The platelet proteases involved are activated by various mechanisms, including the transition of an inactive zymogen by (auto)proteolysis or conformation changes, the relief of auto-inhibition by other proteins, or increases in the cytosolic Ca^{2+} concentration (Figure 2a).

A well-known proteolytic signaling cascade in platelets involves the cleavage of the protease-activated receptors PAR-1 and PAR-4, which both are G protein-coupled receptors. They can be cleaved not only by thrombin but also by other proteases, such as matrix metalloproteinases (MMP) [46]. In platelets, this cleavage induces multiple downstream signaling events, including the activation of protein kinases and a rise of the cytosolic Ca^{2+} concentration [4]. As a result of this increased concentration, the cysteine protease calpain becomes activated [5,47] and acts on a variety of intracellular proteins, including those of the cytoskeleton. Enhanced

Figure 2



Active proteases and their detection. (**a**) Most proteases are present as inactive zymogens, which undergo activation by proteolysis or conformation changes. The active protease forms can be influenced by PTMs and (endogenous) inhibitors. Active proteases are usually subjected to inactivation or degradation. (**b**) Activity-based chemical protease probes can consist of (1) an electrophile that reacts with the active site nucleophile (serine, cysteine, or threonine proteases); (2) a selectivity element, often a peptide that reflects the substrate specificity of the target protease; and (3) a detection tag, which can be a fluorophore for microscopy, a biotin for enrichment, a radio-isotope for imaging, or a bio-orthogonal handle, such as an alkyne/azide [9].

activation can be induced by the release of MMP isoforms from platelets, which then cleave PAR-1 and PAR-4 [6]. Although platelets display only limited protein synthesis, the protein-degrading proteasome complex, involved in the degradation of proteins, also appears to play a role in platelet signaling and function [7].

Such probes have been useful in the functional elucidation of specific proteases [9,10]. Activity-based probes utilized for proteases undergo a mechanism-based [48] reaction with a nucleophile in the protease active site (Figure 2b). The result is the labeling of only the active forms, allowing the study of the dynamics of protease regulation.

To study other enzyme activities, such as kinases and phosphatases, the use of covalent chemical probes is combined with advanced MS-based proteomic approaches. Over the last decade, this approach has revealed invaluable insights into disease-related mechanisms and led to the identification of potential novel drug targets [11,12]. To date, only a few applications of chemical proteomics for platelet biology have been reported. *Wong and colleagues* investigated the platelet proteins binding ATP, cAMP, and cGMP by using immobilized analogs of these second messengers [13]. This work successfully demonstrated not only the enrichment of platelet sub-proteomes but also revealed differences in the nucleotide-binding proteins upon platelet activation [13].

Holly and colleagues applied a competitive activity-based protein profiling method for the discovery of a selective inhibitor for the serine hydrolase AADACL1 [14]. Using this inhibitor, the authors revealed the role of this serine hydrolase in platelet aggregation and whole-blood thrombus formation. The group of *Cravatt* developed a selective inhibitor of the platelet-activating factor acetyl hydrolases 1b2 and 1b3 (PAFAH1b2/3) by making the use of a chemical probe-enabled screening method [15]. This work revealed that tetrahydropyridine scaffolds can act as potential starting points for future medicinal chemistry studies on the function of the PAFAH1b isoforms in pathophysiological processes [15]. Both examples highlight the possibilities for chemical proteomics investigation on platelets for a better understanding of platelet biology.

Omics technologies as a readout for platelet chemical biology

Several omics technologies can be used to explore the mechanisms of platelet changes in health and disease. These include transcriptomics, global proteomics, phospho-proteomics, N-terminomics, glycoproteomics, and lipidomics.

Transcriptomics

As anucleate cell fragments, platelets lack DNA, but they do contain a complex transcriptome of mRNA, miRNA, long noncoding RNA, pre-mRNA, and circular RNA species [49]. These genetic transcripts are acquired from the megakaryocyte precursor cells during proplatelet shedding in a process called thrombopoiesis. There is also experimental evidence that mRNA forms can be acquired by unidentified cell−cell transfer mechanisms. In addition, platelets have the capability of processing pre-mRNA to generate mRNA that can potentially be translated into proteins. Despite the absence of DNA, the platelet transcriptome is not static. It varies in response to external stimuli, such as inflammatory signals, invading pathogens, and tumor metastasis, by using alternative start and stop sites, exon skipping, and intron retention [16].

To obtain better insights into the platelet transcriptome, the Blueprint consortium has recently generated one of the largest databases by genome-wide cataloging of RNAs in multiple blood cell types [17]. Regarding platelets and megakaryocytes, this work led to the quantification of 57,849 transcripts and opened new possibilities for elucidating the composition and biological function of platelets [18]. Based on this work, *Hung and colleagues* examined the distribution pattern of all expressed gene-linked transcripts and showed that about 20,000 of these were present at relevant levels in platelets and/or megakaryocytes. A classification of 5200 platelet-expressed proteins and 14,800 protein-coding transcripts revealed a more or less homogenous distribution pattern over 21 UniProt-based protein localization and function classes [19]. This remarkable achievement indicates that the platelet proteome is considerably larger than the 4000 proteins expected in 2012 [20].

Proteomics

The term proteome refers to all the proteins produced by a biological system at a certain time point under defined conditions [21]. Elucidation of the entire proteome of a biological system, such as platelets, is a challenging task due to the wide range of protein abundances. In addition, post-translational modifications (PMTs) increase the proteome complexity and complicate the evaluation of data. Fortunately, recent developments of mass spectrometry-based proteomics have made it possible to partially overcome the complexity problems by empowering the detection of more proteins with better sensitivity in less time [3].

The recent first and to date most comprehensive platelet proteome analysis confirmed the presence of almost 4000 proteins [20]. However, with the introduction of more sophisticated proteomics acquisition techniques, such as data-independent acquisition (DIA) in combination with parallel accumulation-serial fragmentation (PASEF), we expect that it will become feasible to achieve a higher coverage of the platelet proteome in the near future. This will provide a deeper insight into so far unidentified key protein players *(e.g.*, low abundance transcription and regulation factors) in platelet activation mechanisms and platelet-derived biomarkers for platelet-related and other disorders [22,23].

Phosphoproteomics

Among the fast PTM-mediated changes in activated platelets are those of protein phosphorylation. As phosphorylation of proteins is a reversible process by a complex interplay of protein kinases and phosphatases, it can control many biochemical and signaling pathways. In most proteins, only a sub-stoichiometric amount of serine, threonine, or tyrosine is phosphorylated, meaning that phosphor-proteomics analyses heavily rely on phosphopeptide enrichment techniques, mostly at the peptide level [24].

So far, the common technique for the enrichment of platelet phosphopeptides is that of metal oxide affinity chromatography (MOAC). Titanium dioxide beads are commonly used for enrichment and subsequent hydrophilic interaction liquid chromatography (HILIC [50]), followed by quantification using isobaric tags for relative and absolute quantification (iTRAQ) or tandem mass tags (TMT) [25]. For platelets, this strategy has allowed the quantification of almost 3000 individual phosphopeptides, which revealed unknown activation and inhibition mechanisms, for example, by comparing samples from healthy and diseased individuals [26−28].

Recently, *Swieringa and colleagues* studied the phosphorylation events in platelets from patients suffering from Albright hereditary osteodystrophy syndrome (AHO) [28]. This syndrome is linked to a maternally inherited mutation in the GNAS complex locus, encoding for the GTPase subunit Gsαa [29,30]. As a consequence, defects in the protein kinase A (PKA)-mediated protein phosphorylation pathway are expected in AHO syndromic platelets. Platelet analysis using either iTRAQ- or TMT-based phosphoproteomics revealed 2516 phosphorylation sites, of which 453 were regulated by the Gsα-PKA pathway. This study revealed new molecular links to this pathway.

Even though isotopic label approaches have been used for a medium-scale throughput of platelet phosphoproteomics analysis, it is to be expected that this throughput will further increase with the introduction of automated sample preparation and liquid handling systems [31]. We expect that automated peptide enrichment for label-free phospho-proteomics will become particularly interesting. In such platform, sample

number will no longer be a limiting factor, and up to 96 samples can be processed simultaneously. This will provide better statistical analysis and potentially more confident targets. A similar automated workflow has been already applied to monitor EGF-induced signaling in hippocampal neurons, revealing a comprehensive phosphoproteome [32].

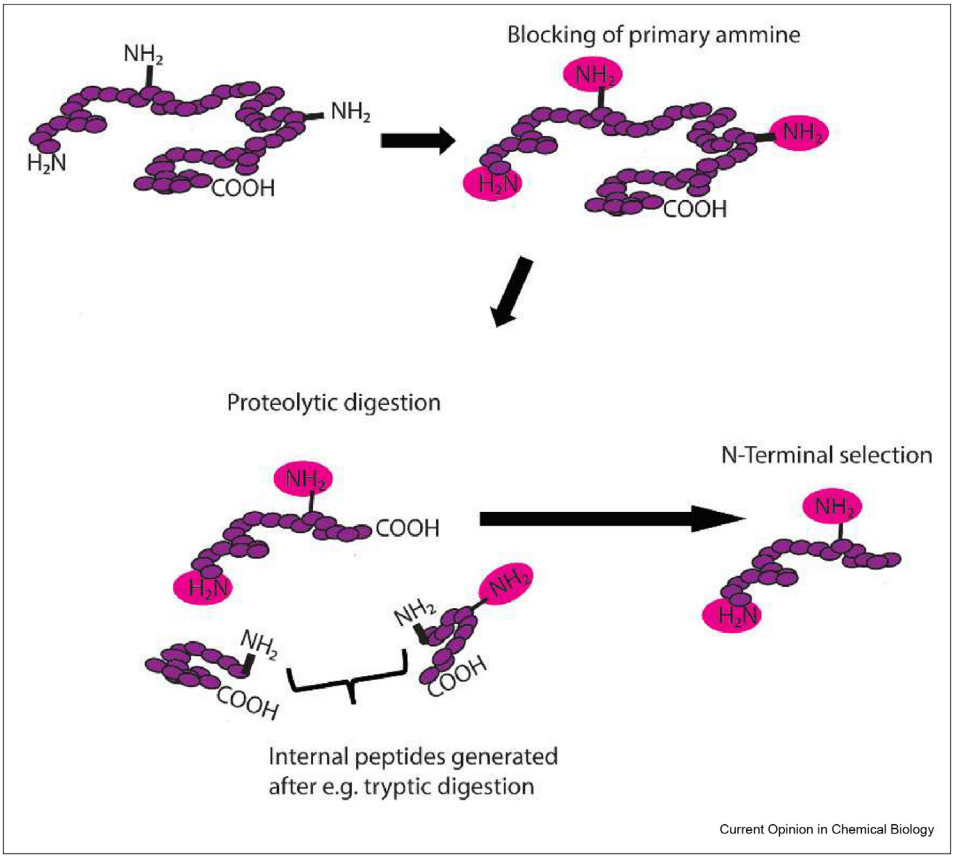
N-terminomics

Another PTM involved in platelet activation is the cleavage of proteins by proteolytic enzymes/proteases. Protease substrate identification by proteomics methods is collectively termed "terminomics," where most approaches focus on the enrichment of the neo-N-termini that are formed upon a cleavage event (N-terminomics).

Because of the sub-stoichiometric nature of proteolytic events and to ensure the separation of original and neo-N-termini of cellular proteins from those generated by tryptic digests, enrichment techniques are required. So far, various proteomics enrichment techniques are available [33,34], including terminal amine isotopic labeling (TAILS) [35], combined fractional diagonal chromatography (COFRADIC) [36], charge-based fractional diagonal chromatography (ChaFRADIC) [37], and the method of subtiligase-N-terminal biotinylation [38]. The enrichment is either done by positive selection (pulling out the desired N-termini), for example, by selective biotinylation before tryptic digestion or by negative selection (pulling out the undesired tryptic peptides), for example, by first blocking the desired primary amines by methods, such as demethylation, followed by the removal of undesired tryptic peptides (Figure 3). These methods can be used with multiplexing by dimethylation for a direct comparison of three samples [37] or by iTRAQ technology, allowing the analysis of up to eight samples. Both multiplexing quantification methods have been used for TAILS and ChaFRADIC.

Prudova and colleagues studied the role of proteolytic processing during the storage of platelet concentrates,

Figure 3



Specific isolation of neo-N-terminal peptides. Enrichment of neo-N-terminal peptides involves the blocking of primary amines for instance by the reactive amine-interacting stable isotope labels iTRAQ or TMT. In bottom-up proteomics approaches, this step is followed by proteolytic digestion, e.g. trypsin for the analysis of multiple (neo) N-terminal peptides. By N-terminal selection, undesired (internal) peptides are depleted, and the peptides of interest are analyzed by LC-MS/MS.

which are of therapeutic use, but have a limited shelf life. By using the TAILS method, the authors assessed N-terminal methionine excision, post-translational Nα acetylation, protein maturation, and proteolytic processing of platelet proteins. Interestingly, metalloproteinases were found to be primarily responsible for proteolytic processing during storage [39]. This study provided novel insights into the effects of extracytosolic proteases on protein integrity and platelet function during storage.

Glycoproteomics

The glycosylation of membrane proteins is an additional, important PTM relevant for the roles of platelets in hemostasis and vascular integrity. In particular, platelet glycoproteomic analyses are considered to be relevant for the elucidation of interactions of platelets with other blood and vascular cells. To date, glycoproteomic approaches are based on the capturing of glycopeptides from isolated platelets using Hydrazide Affinity Capture for N-glycosylation [40] or Concanavalin A Affinity purification for lectin-based glycopeptide enrichment [41]. These approaches are followed by a mandatory treatment before proteolytic digestion using PNGaseF to detach the glycan motifs from the peptides. This allows the identification of glycosylations via LC-MS/MS analysis.

Shah and colleagues reported an interesting example of glycoproteomics on platelets, by analyzing the platelet glycoproteome under stimulation of aspirin, which led to the identification of 799 glycosides [40]. The study provided new information on the relation between glycosylation and aspirin, a widely used drug in cardiovascular disorders, paving the way for investigating novel therapeutic approaches.

Lipidomics

Mass spectrometry-based approaches to identify and quantify lipid molecular species — free or in interacting with other molecules — are designated as lipidomics. Differences in (phospho-)lipid composition may not only influence platelet activation or inhibition but also discriminate between certain healthy and diseased individuals [42]. *Peng and colleagues* [43] assessed the platelet lipidome and identified 400 lipid species, including phospholipid and eicosanoid. The authors also compared the lipidomics network of resting and activated murine platelets, which was further validated in human platelets. It was revealed that less than 20% of the platelet lipidome is changed upon activation and that these changes mainly involve phospholipid molecular species containing arachidonic acid [43]. This study paves the way for further investigation of lipid-related processes in platelets. A recent lipidome analysis of murine platelets revealed an altered lipid profile in genetically modified mice with mildly elevated levels of plasma lipids and cholesterol linked to high-fat diet intake [44]. In this study, particularly free cholesterol and cholesteryl esters, lipid species were found to be elevated in platelets from mice experiencing mild hyperlipidemia.

A semi-multi-omics approach for studying platelet mechanisms

Multi-omics approaches aim to integrate distinct omics data sets to elucidate complex molecular mechanisms and understand the pattern of biomolecular changes linked to platelet activation or inhibition. *Solari and colleagues* reported an initial multi-omics iTRAQ-based combined proteomic approach to elucidate the molecular mechanisms underlying platelet procoagulant activity, which thus far was not well understood [27]. The authors determined differences in the global proteome, phosphoproteome, and N-terminome between control platelets and platelets from a Scott syndrome patient, a rare genetic bleeding disorder characterized by *ANO6* mutations and malfunction of the platelet procoagulant response mechanism [27]. In the combined proteome and phospho-proteome approach, *Solari and colleagues* were able to quantify 2278 unique proteins and 1566 phosphopeptides in the (stimulated) control and patient platelets [45]. The study provided novel insight into the Ca^{2+} dependency of platelets and in anoctamin-6 and calpain-related platelet activation mechanisms, when the platelets were stimulated with thrombin, convulxin, or Ca^{2+}–ionophore ionomycin [45]. The absence of anoctamin-6 in the patient's platelets was confirmed by western blot analysis. This work elucidated the role of calpain in the platelet procoagulant response by quantifying N-terminal peptides. In sum, this multi-omics study paved the way for further investigations of patient platelets in other disorders.

Conclusions and outlook

In this short review, we highlighted the advancements and possibilities of multi-omics approaches for the study of platelet biology and function. In the last few years, the emerging field of platelet proteomics has significantly contributed to our understanding of platelet biology and molecular signaling hubs. This is also demonstrated by an increasing number of publications, emphasizing the importance of mass spectrometry-based proteomics, lipidomics, and metabolomics approaches on platelets. We conclude that further integration of the multi-omics techniques will open up new horizons in elucidating novel molecular and biochemical pathways to explain the complex role of platelet in health and disease.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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