The vital role of platelets in hemostasis was recognized >130 years ago. Nowadays, it is established that platelets are involved not only in many more physiological but also in pathophysiological processes: they act as safeguards of vascular integrity and contribute to inflammation, tumor metastasis, liver regeneration and the genesis, and progression of cardiovascular diseases. Both, the absence of a nucleus (and consequently limited levels of protein synthesis) and the regulation of platelet activity at the level of post-translational modifications (PTM), render proteomics an invaluable tool for characterizing the fundamental processes that affect platelet homeostasis and thus determine the roles of platelets in health and disease. In this article we provide a critical overview on the achievements, the current possibilities, and the future perspectives of platelet proteomics to study patients experiencing cardiovascular, inflammatory, and bleeding disorders. (Circ Res. 2014;114:1204-1219.)

Key Words: bleeding ■ blood platelets ■ cardiovascular diseases ■ hemorrhage ■ proteome ■ proteomics

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.114.301598/-/DC1.
characterizing the fundamental processes that affect platelet homeostasis and thus determine the roles of platelets in health and disease. Moreover, platelets can easily be isolated from the human body as a relatively pure and homogeneous population of primary cells. The great variety in functions beyond hemostasis renders them—and hence platelet proteomics—interesting for different application fields, where the use of genomic approaches might be limited. In this article we provide a critical overview about the achievements, the current possibilities, and the future perspectives of platelet proteomics.

### Current State of Platelet Proteomics

The proteome can be described as the complete set of proteins expressed within a defined sample under distinct conditions. As basically all biological processes are regulated by proteins, the proteome—in contrast to the genome—is highly dynamic and can change its composition both qualitatively and quantitatively over time. Recent studies demonstrated that using modern proteomics technology, ≈10,000 proteins can be detected in human cell lines, covering a dynamic range of 7 orders of magnitude. More than 300 known PTM considerably narrow its application range: (1) Large, small, hydrophobic, and basic proteins cannot be resolved well; (2) the sensitivity and linear/dynamic range depend on the applied staining procedure; (3) before MS, each protein spot of interest has to be excised, washed, and subjected to in-gel proteolytic digestion, thus reducing the detection limit at the expense of costs/time per protein identification; (4) 2-DE quantification is based on spot intensities after staining, but in complex samples, spots usually contain more than a single protein, rendering quantification prone to errors; and (5) the availability of only few identifications can impede a thorough validation to estimate the share of false-positive protein identifications. However, 2-DE has several inherent limitations that considerably narrow its application range: (1) Large, small, hydrophobic, and basic proteins cannot be resolved well; (2) the sensitivity and linear/dynamic range depend on the applied staining procedure; (3) before MS, each protein spot of interest has to be excised, washed, and subjected to in-gel proteolytic digestion, thus reducing the detection limit at the expense of costs/time per protein identification; (4) 2-DE quantification is based on spot intensities after staining, but in complex samples, spots usually contain more than a single protein, rendering quantification prone to errors; and (5) the availability of only few identifications can impede a thorough validation to estimate the share of false-positive protein identifications. However, 2-DE can resolve protein isoforms, and in contrast to liquid chromatography (LC)-MS, the proteomic characterization of larger cohorts using 2-DE has been established for many years.

Most of the aforementioned limitations of 2-DE are overcome with the advent of advanced gel-free proteomics techniques. This in particular concerns 3 developments: (1) availability of improved mass spectrometers providing higher sensitivity, specificity (mass accuracy, resolution), and speed/throughput (as well as novel scanning techniques); (2) better high performance LC systems and materials providing an enhanced separation of highly complex samples; and (3) novel bioinformatics strategies enabling highly complex data interpretation at well-defined quality measures. Yet, these technical developments require robust protocols and extensive quality control to translate big data into reasonable results. Using state-of-the-art LC-MS bottom-up setups (Text Box 1), it is possible to identify and quantify thousands of proteins within hours at a given quality level, usually referred to as false discovery rate (FDR, Text Box 1), representing an estimated share of unwanted but still inevitable random identifications. One limitation of these bottom-up approaches is that information about protein isoforms can be lost because identification and quantification are conducted on the peptide level.

Owing to the continuous propagation of MS-based proteomics throughout life sciences, there has been a tremendous development of approaches to analyze proteins, their abundances, their interaction partners, their mature N and C termini, as well as their PTM patterns. Although several of these MS-based techniques have been used to deepen our understanding of human platelets (Figure 1), we are only beginning to exploit the range of possibilities, which is far beyond the mere identification of the most abundant proteins.

Depending on the study design, different types of information can be obtained from platelet proteome analyses (Figure 1). Proteomics can provide detailed insights into the (quantitative) protein composition of platelets, of their subcompartments (eg, the plasma membrane) or platelet-derived microparticles, examined at resting or activated conditions. In addition, proteomics can provide quantitative information about changes in protein and PTM levels, for instance on perturbation of the resting state (eg, agonist treatment) or when comparing platelets derived from different donors (genomic background or healthy versus disease). Accordingly, platelet proteome studies cannot only contribute to revealing functional mechanisms but also help to identify target proteins that associate with the genesis or progression of a pathological condition. Such studies thus promise to provide potential biomarkers for prevalent diseases. Yet, examples of proteomics-derived biomarkers, which have made it into application, are scarce. However, the steady and fast progress of proteomic technologies in the past few years has paved a way toward application in clinical research and holds a great potential for the future. One big challenge for platelet (biomarker) analysis still is preanalytical interference for instance because of impurity or preactivation as the levels of known platelet biomarkers are known to be influenced by platelet isolation protocols. To circumvent artificial results, there is an urgent need for robust and standardized protocols of platelet preparation and activation, as well as protein isolation.

### Quantitative Human Platelet Proteome

Owing to their lineage as anucleate fragments of megakaryocytes and their unique role, it is evident that platelets differ considerably from nucleate cells and cultured cell lines. Platelets can be isolated as a relatively homogenous population of primary (human) cells, and hence, corresponding data

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2-DE</td>
<td>2-dimensional gel electrophoresis</td>
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<tr>
<td>FDR</td>
<td>false discovery rate</td>
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<td>LC</td>
<td>liquid chromatography</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modifications</td>
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</table>

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**Text Box 1**

- Development of advanced gel-free proteomics technologies providing higher sensitivity, specificity (mass accuracy, resolution), and speed/throughput (as well as novel scanning techniques); (2) better high performance LC systems and materials providing an enhanced separation of highly complex samples; and (3) novel bioinformatics strategies enabling highly complex data interpretation at well-defined quality measures.
Based on the high degree of correlation (coefficient of determination \( R^2 \approx 0.9 \)) and, using spectral counting, we compared our MS data with copy numbers of 24 proteins found in the literature. Of \( <1\% \) and, using spectral counting, we compared our MS data with copy numbers of 24 proteins found in the literature. For a proper analysis, contamination of erythrocytes, circulating stem cells, and plasma have to be minimized and preferentially monitored, as these clearly bias the proteome analysis. Peptide sequences are usually identified by comparing the molecular masses of the peptide (parent) ion and corresponding fragment ions, as determined in MS and MS/MS scans, with the predicted peptide/fragment masses obtained from in silico digestion of a protein database. Nowdays, results are reported at defined false discovery rates, representing the share of random hits within large data sets. Therefore, for a selected protein database (eg, uniprot human or mouse), all sequences are usually reversed to generate a concatenated target/decay (forward/reverse) database. On searching MS data against such a database, the number of decoy hits represents the number of random hits in the target database, such that the share of false-positive hits can be assessed on the peptide spectrum match, the peptide, and the protein level. However, the validity of the underlying statistics depends on the availability of sufficient data points (peptide spectrum match, peptide, and protein identifications). Notably, the presence of genetic polymorphisms still poses a challenge for global proteomic workflows because they can alter peptide and fragment ion masses. Nevertheless, they can contribute to platelet dysfunction and, therefore, will represent an important field for future platelet proteomics studies.

For the past decade, MS has been the method of choice to identify and quantify post-translational modifications (PTM) in proteins and peptides, given that those induce a detectable mass shift and are either stable during sample preparation and MS detection, or, before analysis, can be chemically/enzymatically modified for this purpose. Notably, a direct detection of the intact PTM is preferable to indirect methods, which rely on induction of artificial mass shifts providing a more confident way of identification. In principle, MS can identify the presence of a PTM but also pinpoint its distinct localization within the peptide sequence. As common search algorithms for peptide identification are prone to errors if modifiable amino acids are in close proximity, the use of specific algorithms to assess the quality and confidence of PTM localizations is mandatory. Because the implementation of such algorithms is relatively new, it should be kept in mind that public PTM databases might contain a certain level of wrong annotations, especially in case of older data sets.

Despite the relevance of knowledge of PTM, their sheer versatility and usually low stoichiometry render the usage of specific enrichment strategies for removal of the bulk of nonmodified peptides inevitable. However, especially when PTM-directed antibodies are used for the analysis of ubiquitination or acetylation, sensitivity and consequently large sample amounts can be an issue, even requiring several milligrams per sample for a comprehensive analysis.

Text Box 1. Liquid Chromatography–Mass Spectrometry Analysis

Although also intact proteins and complexes can be analyzed by liquid chromatography–mass spectrometry (MS), the method of choice in MS-based proteomics is still so-called bottom-up, in which proteins are enzymatically cleaved into peptides (commonly with trypsin) before analysis, leading to multiplication of sample complexity from 1000s of proteins to 100 000s of peptides. Advantages are that, for peptides, (1) separation by chromatography, (2) ionization, and (3) fragmentation are more efficient, (4) their masses are less heterogeneously distributed, and (5) their identification through database searches is more straightforward. Complex peptide mixtures can be either directly analyzed by liquid chromatography–MS using long gradients or fractionated by hyphenation of different separation techniques beforehand to reduce sample complexity and increase depth and coverage of the proteome analysis. Peptide sequences are usually identified by comparing the molecular masses of the peptide (parent) ion and corresponding fragment ions, as determined in MS and MS/MS scans, with the predicted peptide/fragment masses obtained from in silico digestion of a protein database. Nowadays, results are reported at defined false discovery rates, representing the share of random hits within large data sets. Therefore, for a selected protein database (eg, uniprot human or mouse), all sequences are usually reversed to generate a concatenated target/decay (forward/reverse) database. On searching MS data against such a database, the number of decoy hits represents the number of random hits in the target database, such that the share of false-positive hits can be assessed on the peptide spectrum match, the peptide, and the protein level. However, the validity of the underlying statistics depends on the availability of sufficient data points (peptide spectrum match, peptide, and protein identifications). Notably, the presence of genetic polymorphisms still poses a challenge for global proteomic workflows because they can alter peptide and fragment ion masses. Nevertheless, they can contribute to platelet dysfunction and, therefore, will represent an important field for future platelet proteomics studies.

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may yield more relevant insights compared with cultured and endogenous cells with major differences in differentiation stages. Detailed knowledge of the protein composition of platelet proteome studies. For example, in disease or on drug intake, and for which the accessible blood volume might be limited, the reproducibility of the platelet preparation is as important as its purity. If the level of nonplatelet proteins remains constant but limited, contamination by other blood components can be tolerated. For high reproducibility and confidence of
the generated data, it is important to monitor the entire workflow including (1) patient selection, (2) blood drawing, (3) platelet isolation and purity, (4) lysis and proteolytic digestion, along with analytic steps such as (5) chemical labeling, (6) prefractionation of the peptide mixture, (7) LC-MS analysis, and (8) MS data interpretation.

At present, costs and effort to conduct LC-MS–based proteome-wide analyses from individual patients are relatively high. These require state-of-the-art equipment and either the usage of costly stable isotope labeling techniques which allow multiplexing of ≤10 samples to save time or the successive analysis of single samples by label-free quantification strategies to characterize larger cohorts (Text Box 1). In the past few years, 2-DE was preferably used for disease-driven platelet proteomics, but as mentioned above, this technique has limitations when compared with gel-free LC-MS–based proteomics (Table I in the online Data Supplement).

Because of the rapid progress in the development of MS instrumentation, costs and time per identified (and quantified) protein will reduce further, but it is questionable whether this will equally apply for stable isotope labeling reagents. Alternatively, so-called targeted LC-MS assays, such as multiple reaction monitoring and high-resolution targeted-MS/MS, allow fast, accurate, and sensitive quantification of predefined sets of proteins in complete cell lysates (Figure 2; Text Box 2). These techniques have already been used to monitor protein signatures in a variety of studies and might be suitable to monitor defined proteomic changes in platelets from larger patient cohorts. In this context, recently introduced SWATH-MS clearly has good prospects. Here, based on a priori information from spectral libraries, samples are analyzed in a data-independent manner, such that all ions present are fragmented in consecutive 25-Da windows. This allows for quantification of many more analytes than with conventional targeted methods with similar accuracy, however less sensitivity. One drawback might be the dependence on spectral libraries for precise data interpretation, which is not required in other data-independent strategies.

**Platelet Proteome and Transcriptome**

In support of the observations that essential components of the RNA translational machinery are present in platelets, microarray-based studies discovered that ≤32% of all
human genes are expressed in platelets at the messenger RNA level. Several studies focused on analysis of the platelet transcriptome and some of these compared protein and transcript levels, deducing a certain degree of correlation. A comprehensive comparison between our quantitative proteome data and published transcriptome data revealed only weak correlation. The messenger RNA found in platelets originates from megakaryocytes and is likely to be affected

**Figure 2. Typical liquid chromatography (LC)-mass spectrometry (MS) based proteomic workflows.** A. Sample preparation. Proteins are extracted and enzymatically cleaved into peptides (1–4). Modified peptides need to be specifically enriched in case of post-translational modification (PTM) analysis. B. LC-MS analysis. For discovery, qualitative or label-free quantitative (*) analysis of individual samples (5) or stable isotope labeling (SIL) of peptides (6) with subsequent multiplexing and analysis of pooled samples can be applied. Both strategies can be combined with fractionation to reduce sample complexity and to increase proteome coverage of the LC-MS analysis (7) and following data interpretation. Stable isotope labeling can be performed with nonisobaric and isobaric labels (Text Box 2). Whereas nonisobaric labeling requires quantification based on parent ions (MS1 level) and leads to duplication/triplication of sample complexity, isobaric labels are quantified on the level of fragment ions (MS/MS) and thus allow multiplexing of ≤10 samples without considerable increase in sample complexity. For validation and screening, specific approaches such as targeted MS/MS can be applied (8), allowing for a sensitive detection and quantification of specific peptides/proteins in complete cell lysates. Demonstrated is the detection of a peptide from sortilin (~700 copies per platelet) with high signal-to-noise, obtained by a 2-hour LC-MS analysis of 1 µg of complete platelet lysate, which consists of >5000 different proteins, covering ~5 orders of magnitude. TIC indicates total ion chromatogram; and UV, ultraviolet.

**Text Box 2. On the Use of Stable Isotopes**

Incorporation of heavy stable isotopes (¹³C, ¹⁵N, ¹⁸O, and ²H) is a widely used strategy for quantification in mass spectrometry (MS)-based proteomics. For the hypothetical peptide sequence PLATELETPROTEOMICS, the light (endogenous) and the heavy stable isotope-coded versions (e.g., containing ¹³C₆-labeled Arg) have the same chemical properties but differ in physical properties. They thus behave equally during sample preparation, liquid chromatography separation, and MS ionization. Yet, based on the 6-Da mass shift derived from the presence of 6 additional neutrons in the heavy version, both forms are distinguished by MS, and their signal intensities can be used to determine their relative ratio. Incorporation of stable isotopes is achieved by chemical and metabolic labeling, but use of the latter method is limited to cell culture and some model organisms, among those the SILAC (Stable isotope labeling by amino acids in cell culture) mouse. In case of isobaric chemical labeling techniques such as isobaric tag for relative and absolute quantitation (iTRAQ) and tandem mass tag (TMT), all differently labeled forms of a peptide have the same mass, as represented by a single signal on the MS1 level (Figure 2). Once the peptide is isolated and fragmented, reporter ions corresponding to relative amounts of the peptide within the labeled samples are released for each label and detected in a subsequent MS/MS scan. iTRAQ and TMT currently allow multiplexing of ≤ 8 and 10 samples, respectively.

Reductive dimethyl labeling is a comparatively cheap alternative, however, at the expense of several disadvantages when compared with the more costly iTRAQ and TMT technologies. (1) Currently multiplexing is limited to only 3 samples and, as for all nonisobaric labels, 2-plexing will double and 3-plexing will triple the complexity of the sample. (2) Incorporation of deuterium causes retention time shifts between light/medium/heavy forms of the peptide during reversed phase chromatography and thus can complicate quantification by liquid chromatography-MS. Hebert et al recently introduced a novel strategy for neutron-encoded labeling, which will increase the multiplexing capabilities of the aforementioned labeling strategies, but requires mass spectrometers with a resolution power of ≈500 000 and above, currently supported by only few instruments in conjunction with reasonable sensitivity and scanning speed.
Platelet Subproteomes

A variety of studies has focused on the characterization of platelet subproteomes. In contrast to the global analyses, subproteomes can provide insights into the subcellular localization or PTM patterns of proteins. Subproteome analyses can reveal (patho)physiologically important processes and, therefore, hold the potential for unraveling previously unknown mechanisms. Figure 3 gives a summary of published platelet (sub)proteome studies.

The platelet plasma membrane represents the interface for interaction with other blood components and transmits responses to external stimuli. Many drug targets are membrane proteins, and thus there is considerable interest in the plasma membrane subproteome. Dedicated studies used gel-based or gel-free approaches, with the latter yielding ≈650 membrane proteins, identifying even low abundant 7 transmembrane domain receptors, such as the P2Y<sub>1</sub> receptor for ADP (≈150 copies per platelet). These studies clearly extended our knowledge of plasma membrane proteins and receptors. Despite the increasing power of proteomic workflows, membrane proteins can easily escape detection and thus may be under-represented. Especially 2-DE is not well-suited for analysis of hydrophobic membrane proteins, which often are quantitatively lost. Even for gel-free strategies, the hydrophobic nature of membrane proteins renders sample preparation challenging. For instance, the absence of trypsin cleavage sites within transmembrane domains may require the use of alternative enzymes/strategies. In addition, frequently present glycosylation can hamper proteolytic digestion and also impede identification by MS. Despite these problems, ≈75% of the known platelet membrane receptors have been identified in the 2 to date most comprehensive LC-MS–based proteomic studies (Table 1).

Platelet-derived microparticles are common components of the blood, which are increasingly released on aging and during activation of platelets, increasing in number under pathological conditions. Several hundreds of proteins could be identified in platelet microparticles after gel electrophoresis and LC-MS analysis of 26 excised bands. A preliminary analysis showed that platelet microparticles can be separated in size classes with different protein composition; however, the applied data interpretation does not meet current standards in the field. Recently, microparticles induced by thrombin or shear were compared using 2-DE, resulting in 26 differential proteins.

Activation of platelets triggers α-granule secretion, leading to the redistribution of granule membrane proteins to the plasma membrane and the release of soluble proteins into the blood. Several studies analyzed the proteome of α-granules isolated from platelets. Wijten et al used a quantitative gel-free proteomics approach to compare the proteome of collagen- and thrombin-stimulated platelets with that of unstimulated platelets to identify proteins that are downregulated because of secretion. The authors describe a high correlation between their platelet proteome data and our previously published quantitative platelet proteome and could identify 124 released proteins. However, already in 2004, Coppinger et al identified >300 proteins after MS analysis of a platelet releasate depleted from microparticles and intact cells. One reason for this discrepancy might be contamination derived from unanticipated platelet lysis as Wijten et al analyzed changes in the proteome of intact platelets rather than in the releasate itself. Moreover, Coppinger et al used an elaborate data interpretation workflow, but no methods for estimating FDRs were available at that time. Nowadays, proteomics data are usually provided with a defined FDR (routinely 1%), which results in an improved validity, usually at the expense of reduced protein numbers.

Activity-based proteomics technologies use active site–directed chemical probes, which can specifically and irreversibly modify enzyme active sites (eg, for subsequent enrichment and direct analysis of enzyme activity, respectively). Activity-based protein profiling approaches have been used to analyze ATP-binding proteins in platelets and to enrich cyclic nucleotide–binding proteins (discussed in a recent review by Holly et al). In one study, proteins from resting and collagen receptor–stimulated platelets were incubated with cAMP/cGMP-coupled beads, and the pull-down eluates were analyzed by LC-MS/MS. Owing to the enrichment provided by specific probes, activity-based protein profiling

Figure 3. Summary of 47 platelet proteomics studies. A, Platelet proteomics has been used to characterize the global proteome, activity-based protein profiling (ABPP), and post-translational modifications (PTM). B, These studies are mainly (40%) based on 2-dimensional gel electrophoresis (2-DE), whereas gel-free methods (23%) are still under-represented. Combination refers to studies using 2-DE/DIGE in combination with other approaches. COFRADIC indicates combined fractional diagonal chromatography; and MudPIT, multidimensional protein identification technology.
Table 1. Platelet Membrane Receptors

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<th>Gene</th>
<th>Protein Name</th>
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From a set of 73 known platelet membrane receptors, \(\approx 75\%\) have been identified by the 2 most comprehensive global proteome analyses. Notably, this list might contain nonplatelet proteins (eg, as the insulin receptor could not be detected immunologically.) \(^{126}\)

can be highly sensitive and access proteins hidden from common proteome analyses. However, it should be kept in mind that they can also enrich nonplatelet contaminations.

**PTM and Signaling Proteomics in Platelets**

PTM have a strong effect on protein structure and, therefore, can modulate protein function, localization, interaction, and
stability. MS-based proteomics has the capability to systematically screen for and identify such modifications (Text Box 1). Among PTM, protein phosphorylation is the most abundant and best-studied, and it affects the regulation of many protein functions, such as enzymatic activities, folding, localization, and interactions with other proteins and other molecules, such as nucleic acids and lipids. Protein phosphorylation is tightly regulated by protein kinases and phosphatases, which allow fast and dynamic regulation of biochemical processes. Platelet activation and inhibition is regulated by phosphorylation- and dephosphorylation-dependant signaling cascades, leading to significant changes in phosphorylation patterns already after millisecond seconds to seconds of stimulation. Phosphoproteomics is the method of choice to investigate the complex underlying signaling networks in highly responsive cell types such as platelets. To date, only few platelet studies used dedicated phosphopeptide enrichment procedures (Text Box 1) in conjunction with LC-MS to identify phosphopeptides and phosphorylation sites. The first one published by us in 2008 identified >500 phosphorylation sites in resting human platelets. Another recent study used phosphotyrosine peptide immunoprecipitation in conjunction with quantitative MS to quantify changes in 28 of 214 identified phosphorylation sites in platelets stimulated via the glycoprotein VI collagen receptor. Among these was Tyr370 in oligophrenin-1, which may play a role in platelet filopodia formation.

In our own studies, we have mapped ≈6000 phosphorylation sites in platelets with high confidence (unpublished data), but merely qualitative information about the position of phosphorylated amino acids has only limited value. In contrast, state-of-the-art procedures use quantitative phosphoproteomics to monitor changes in phosphorylation levels of hundreds to thousands of peptides from relatively low sample amounts (∼0.1–1 mg of protein per condition), thus providing valuable insights into important biochemical processes. To date, mostly gel-based approaches in conjunction with phosphorysine immunoprecipitation have been used to study platelet signaling processes (eg, mediated by activation of thrombin receptors, collagen receptors, C-type lectin-like receptor 2, and integrin αIIbβ3 outside-in signaling). Because of the limitation in sensitivity, these approaches often did not provide MS-based identification of phosphorylation sites but identified new phosphoproteins and signaling pathways, hence expanding the knowledge about platelet regulation.

The newly found phosphoproteins comprised regulatory proteins such as myosin light-chain isoforms; Dok-2, whose phosphorylation is mediated by integrin αIIbβ3 outside-in signaling and is implicated in stabilization of thrombus formation; and RGS18, a GTPase-activating protein which turns off signaling by G-protein–coupled receptors. Gegenbauer et al describe RGS18 as an interface molecule between activating and inhibitory pathways. Whereas RGS18 Ser49 is phosphorylated during platelet activation, Ser216 is phosphorylated on platelet inhibition by protein kinase A (PKA) and protein kinase G. These 2 cyclic nucleotide-dependent protein kinases govern inhibitory pathways in platelets which, despite their important role, have been addressed by only few proteomic studies to date. Up to now, only 15 PKA and 11 protein kinase G substrates have been confirmed. Lately, a new PKA and protein kinase G substrate was identified, namely calcium and diacylglycerol-regulated guanine nucleotide exchange factor 1, the main activator for Rap1b in platelets. Quantitative MS analysis of 4 putative phosphorylation sites revealed Ser387 as the major PKA target, which on phosphorylation causes inhibition of Rap1b activity.

To gain an improved insight into the inhibitory cAMP/PKA pathway, we used quantitative MS and monitored time-resolved changes of phosphorylation patterns in human platelets treated with different concentrations of the stable prostacyclin analog, iloprost. Quantifying >2700 phosphorylation sites over 4 different time points, we could identify ≈300 unique proteins that are differentially phosphorylated on platelet inhibition. Our data indicate that platelet inhibition is not merely mediated by PKA but involves the interaction with other signaling pathways. For instance, 16 protein kinases and 7 protein phosphatases showed differential phosphorylation patterns on iloprost treatment, including increased phosphorylation of the inhibitory site Tyr530 of Src kinase.

Beside phosphorylation, glycosylation is among the most abundant PTM, especially for extracellular proteins, as such cell surface markers and secretory proteins. Glycosylation can either occur as a static modification or as a dynamic signaling process. Even with state-of-the-art instrumentation, the extreme heterogeneity and complexity of glycosylation patterns render their analysis challenging, such that many studies focus on the enrichment and identification of glycosylated residues rather than on characterizing the glycan structures. Yet, glycosylation patterns may dramatically influence processes such as platelet biogenesis and activation. To date, only few dedicated glycoproteomics studies have been conducted on human platelets, identifying several hundreds of N-glycosylation sites mainly on membrane proteins. However, the advent of more sensitive and robust protocols for the enrichment and quantitative analysis of glycopeptides is promising for the future.

Another PTM in platelets that was recently studied using proteomics is palmitoylation. Palmitoylation is the covalent but yet reversible attachment of palmitate to cysteine residues, thereby modulating protein–protein and protein–lipid interactions. Using a dedicated enrichment protocol in conjunction with MS, Dowal et al identified 215 palmitoylated proteins. However, the authors did not directly detect the intact PTM by MS, and additional work is required to confirm these findings.

What Can Quantitative Proteomics Tell Us About Platelet Biology?

Because the proteomics field is rapidly moving from discovery/inventory (listing the proteins and PTM in a cell) to understanding/function (knowing the numbers of proteins and their dynamics), it becomes relevant to consider what proteomics can teach us about the precise functions of cells in health and disease. Our first large-scale quantitative analysis of the human platelet proteome, covering ≈80% of the entire amount of proteins in platelets, has refined the insight into the physiological functions of platelets in several ways. Figure 4 provides an overview of the cumulative copy numbers of the...
Figure 4. Distribution among functional categories of the 500 most abundantly expressed proteins. The top-100 (A), -200 (B), and -500 (C) most abundant proteins in human platelets, such as determined by quantitative proteomics analysis. The top-100 proteins have an estimated >35k copies per platelet, the top-200 proteins >16k copies per platelet, and the top-500 >6.7k copies per platelet. ER indicates endoplasmic reticulum.

Within the top-100, top-200, and top-500 proteins present in an average human platelet. Among the 100 most abundant proteins, together encompassing 11606k copy numbers (each >35k copies per platelet), more than half of all copies can be attributed to the actin-myxosin and the microtubule cytoskeleton, respectively, most prominent being β-actin (795k copies) and α-tubulin (185k copies). This may be a consequence of the relatively high surface–volume ratio (and hence large membrane cytoskeleton content) of platelets in comparison with larger, nucleated cells. The high cytoskeleton content (1) ensures maintenance of the discoid structure of resting platelets in the circulation, (2) promotes the profound shape changes during pseudopod and lamellipod formation on platelet adhesion and activation, (3) mediates the conversion into rounded, microparticle-releasing structures during the platelet procoagulant response, and (4) enables a growing thrombus to withstand the high shear forces in the arterial circulation.

Within the top-100, several α-granular proteins (16%), that is, platelet factor-4 (CXCL4, 706k), β-thromboglobulin (CXCL7, 479k), and thrombospondin-1 (101k), have a surprisingly high abundance. These proteins are released on platelet activation and can deposit at the (damaged) vessel wall. The protein CXCL4 acts as a chemoattractant for leukocytes and vascular cells and has also been shown to coat bacteria, enabling their recognition by circulating antibodies and targeting destruction, which may account for its high level. Other highly expressed proteins in the α-granules are fibrinogen (89k) and the transglutaminase, coagulation factor XIa (83k), which points to a major role of platelets in fibrin clot formation.

The relatively high abundance of proteins that can be classified as cytoskeleton-linked signaling proteins (9%) is also of particular interest. Most prominent among these are the integrin α<sub>IIb</sub>β<sub>3</sub>-linked proteins, talin (116k), fermitin member URP2 (116k), filamin A (88k), and vinculin (81k). Linked to the actin cytoskeleton via GPIb-V-IX complexes (von Willebrand factor receptors) are the so-called 14-3-3 adapter or modifier proteins, of which 5 members seem to be highly expressed (sum 572k). The cytoskeleton also plays an important role in regulating α<sub>IIb</sub>β<sub>3</sub> and GPIb-V-IX as 2 key adhesive receptors, and data from proteomics have added to this recognition by revealing actin cytoskeleton rearrangements connected to these receptors. Moreover, both α<sub>IIb</sub>β<sub>3</sub> (64k–83k) and GPIb-V-IX (49k) are present in the top-100 platelet proteins, thus providing sufficient copy numbers to modulate actin cytoskeleton-dependent platelet shape. Interestingly, the only other highly expressed membrane protein is the chloride channel CLIC1 (44k), the function of which is still poorly understood, but may include modulation of ADP receptor signaling.

Among the most profusely expressed cytosolic proteins are small GTP-binding proteins and their regulators, in particular 2 variants of Rap1b (299k) and RapA (125k), which are known to regulate integrin activation in a cytoskeleton-dependent way. Other abundant small GTP-binding proteins are 2 ADP-ribosylation factors (80k) and Rab27b (36k), of which at least the latter controls platelet dense granule secretion. Directly or indirectly linked to actin-dependent integrin signaling are also the adaptor protein, VASP (45k), and the integrin-linked kinase (60k). The same holds for the Ca<sup>2+</sup>-dependent protease, calpain-1 (51k), which cleaves many cytoskeletal components and controls integrin closure in procoagulant platelets.

Another remarkable fact is that 2 of the highly expressed signaling proteins play a role in PKA-dependent inhibition of platelets, that is, the adenylyl cyclase–associated protein CAP1 (42k) and VASP, which is the major substrate of this cAMP-dependent protein kinase. Although earlier data with mice indicated that the prostacyclin receptor (a key cAMP-elevating receptor on platelets) is not required for normal hemostasis, the abundance of these proteins supports a quantitatively important role of cAMP signaling in the control of platelet functions (see also above). Interestingly, the majority of top-100 proteins controlling metabolic processes are confined to glucose metabolism (glyceraldehyde 3-phosphate dehydrogenase, fructose bisphosphate aldolase, αVT-enzyme, pyruvate kinase, lactate dehydrogenase, triosephosphate isomerase, and phosphoglycerate kinase; each 36k–106k). Hence, glycolysis seems to be most essential for the metabolic control of platelet functionality.

When comparing the categories of top-100 proteins with those of the top-200 (13929k copies, >16k per protein) and top-500 (16943k copies, >6.7k per protein), several changes are noteworthy (Figure 4). The top-200 and top-500 sets show a small decrease in the relative abundance of microtubule and actin–myosin cytoskeletal proteins, as well as α-granular proteins, thus indicating that the most prominent of these proteins are expressed at high copy numbers. The same holds for the category of cytoskeletal-linked signaling proteins. In
contrast, there is an increase in abundance of small GTPases and their regulators (5%–8%), with major contributions of the (regulators of) Cdc42, Rab, Rac, and Rho isoforms that organize intracellular membrane interactions and platelet shape change. Furthermore, the top-500 comprises a marked increase in copy numbers of signaling and adaptor proteins (6%–12%). These include significant numbers of the most poorly defined adaptor proteins; several proteins of the ubiquitin–proteasome system (sum >80k); well-known (Src, Btk, Lyn) and less known tyrosine protein kinases; various serine/threonine protein kinases (protein kinase Cα, PKA, ROCK, MAPK isoforms); protein phosphatases (G6b, PP1-3 isoforms); Ca²⁺-binding proteins; classical GTP-binding proteins Grq and Gqα; and components of the thromboxane synthase complex. Prominent membrane receptor proteins in the top-500 are several HLA-class histocompatibility antigens, the collagen receptor GPVI, the tetraspanin CD9, and the thrombospondin receptor CD36. Jointly, the majority of these proteins can be seen as part of the hard-core signaling network, controlling platelet activation to adhesiveness, secretion, and aggregation. However, the top-500, furthermore, shows a shift from proteins involved in glucose metabolism to nonglucose metabolism (2%–5%). The latter category contains, for example, antioxidant enzymes and enzymes involved in the conversion of glutathione or nucleotides. Prominent proteins in the endoplasmic reticulum (2% of top-500) are protein disulfide isomerases (sum 34k) and isoforms of the SERCA-type Ca²⁺-pumps regulation calcium homeostasis (sum 25k). In mitochondria (3%), these include ATP synthase (28k) along with other proteins involved in ATP synthesis.

Remarkably, the top-500 list contains hardly any proteins that control megakaryocyte development and proplatelet formation on the one hand or platelet apoptosis on the other hand. Together, it can be concluded that this list, obtained by quantitative proteomics, gives improved insight into the massive expression patterns and might represent target proteins for follow-up investigations.

### Platelet Phenotyping and Variation in Protein Composition in Health and Disease

In principle, quantitative proteomics provides a powerful, high-throughput technology to reveal multiple changes in platelet structures and functions, both in health (genetic variation, platelet formation, life span) and disease (thrombocytopenia, cardiovascular disease, diabetes mellitus, cancer). However, only little is known to date about which types of changes in protein expression levels predict for abnormal platelet functions in defined genetic or clinical settings. Our earlier work indicated that, for platelets from healthy subjects, the intersubject variance was small for both low and high copy numbers, with 85% of the quantified proteins showing (almost) no variation between healthy donors. From this, one can hypothesize that only a subset of platelet proteins determines variation in platelet functions. Clearly, comparison of the platelet proteome of many more individuals is needed to establish this.

As described above, quantitative analysis of the most abundant (structural) proteins can provide general information on cellular functions. However, for proteins that are more scarcely expressed, the link to function is less obvious because often the extent and type of PTM may be even more important for the protein activity than the expression number. An example is provided by the platelet protein kinase Cαβ isoforms (8k–10k) that regulate almost every platelet response. These protein kinases not only require multiple phosphorylation steps to become catalytically active, but their activity is also modulated by additional phosphorylation events on platelet activation. Hence, for full understanding of the role of the majority of cellular proteins, quantitative information about phosphorylation processes and other PTM is needed. Based on the literature, >2500 phosphorylation sites and >1000 other PTM have been identified in platelets. However, this information has not been coupled to the quantitative proteome. To date, there is limited evidence that the platelet protein composition may vary with age and in patients with cardiovascular or other diseases. In addition, there are still groups of proteins that are difficult to access using proteomics but are nevertheless important for platelet function. Accordingly, current global proteomics procedures might result in underestimation of heavily modified and membrane proteins with high hydrophobicity; splice variants, isoforms of related proteins, and proteins expressed at low copy numbers (<0.5k). More dedicated approaches using enrichment procedures or targeted MS analyses described above need to be used in these cases.

### Current and Future Use of Proteomics to Study Platelet Function Disorders

It is to be expected that many functional disorders of platelets are attributable to alterations in protein expression levels and PTM patterns, which might be reflected already in the composition of the top-100/200/500 proteins derived from the quantitative proteome. Despite the tremendous efforts made in the past 130 years of platelet research, even for well-established diseases such as Glanzmann thrombasthenia, we know little more than the single protein defect. However, further secondary changes on the protein and PTM level can be expected. This is in accordance with our own preliminary quantitative proteome data on platelets from healthy donors treated with aspirin or serotonin uptake inhibitors, as well as platelets from thrombathenic patients. Already from a limited number of analyses, unanticipated proteins show differential expression patterns and might represent target proteins for follow-up investigations.

The most thoroughly characterized platelet disorders are those associated with an impairment in platelet function or too few or too many platelets (ie, thrombocytopenia and thrombocytosis, respectively). An increase in platelet count above the normal range (150–400×10⁹/mL) is known as essential thrombocythaemia. This is a rare chronic disorder that is associated with the overproduction of platelets in the bone marrow. Approximately 50% of patients have a V617F mutation in the tyrosine kinase Jak2, which gives rise to a low level of constitutive signaling and potentiation of the response to the thrombopoietin receptor, c-Mpl. The phenotype is influenced by other modifier genes and is associated with small
changes in expression of surface glycoprotein receptors in either direction. The diagnosis of essential thrombocythaemia is achieved by ruling out other causes of an increase in platelet count and a genetic test for V617F. The causative mutation in V617F-negative essential thrombocythaemia patients is not known, and proteomics may have a role in their investigation. This potential, however, needs to take into account the heterogeneity in protein levels between patients and the potential influence of platelet-lowering drugs such as anagrelide.

Thrombocytopenia can be inherited or acquired, with the most common form of the conditions being through immune thrombocytopenia. In this acquired, antibody-mediated disorder, the use of proteomics is needed to understand why some but not all patients experience severe bleeding episodes, despite having similar platelet counts. Proteomics may also help in the diagnosis of immune thrombocytopenia. The demonstration of a platelet proteome that is different to that of healthy controls may provide evidence of a contributing genetic cause of the reduced platelet count.

Individuals with inherited thrombocytopenia are in comparison relatively rare, numbering in the order of 1:100000 of the population, with many of these having a mild reduction in platelet count of <40% of the normal range. Many individuals with a mild reduction in platelet have no clinical history of excessive bleeding and are diagnosed through routine blood tests (e.g., in association with pregnancy). However, other individuals with a similar, mild reduction in platelet count have a significant history of bleeding, indicative of impairment of platelet function. In ~50% of cases, the causative gene is known, ranging from transcription factors (e.g., Runx1 and GATA-1) to proteins that play a critical role in platelet formation (e.g., GPIbα, MYH9, and WASp). Proteomics has the potential to find causative genes (proteins) in individuals, in whom the genetic defect has not yet been identified, either through the identification of the gene or as a guide to next-generation sequencing studies.

The most heterogeneous and challenging group of patients for investigation are those with a clinical history of bleeding consistent with an impairment in platelet activation but where the cause is not known. Such patients are relatively rare, in the order of 1:10000 in the population. In approximately half of these, a defect in platelet function can be demonstrated using the gold standard platelet function assay of Born aggregometry. Many of these patients present early in life with bleeding symptoms, suggesting that the defect is inherited, but a significant number only present later on when subject to a challenge such as surgery, tooth removal, or menstruation. In patients from nonconsanguineous relationships, the major challenge is the lack of penetrance between family members, reflecting the multifactorial nature of the bleeding diathesis. In other words, the affected individual has almost certainly inherited >1 mutation, and it is the combination of mutations that gives rise to bleeding. Although next-generation sequencing approaches are being increasingly used to identify candidate mutations, the demonstration of cause and effect will be dependent on platelet functional studies and techniques, such as quantitative proteomics, to demonstrate significant changes in protein expression or function. Even then, it will be challenging to establish the degree to which bleeding is dependent on the gene mutations and the corresponding changes in platelet function.

The challenge in linking candidate mutations in platelets and proteins and changes in protein expression is even higher in disorders involving platelet activation including arterial thrombosis. Platelets are now recognized for their important roles in inflammation, cancer metastasis, innate defense, organ development, and repair, as well as in an increasing number of cardiovascular diseases, including deep vein thrombosis, atherosclerosis, tissue infarctions, and ischemia–reperfusion injury. The molecular basis of the role of platelets in the majority of these pathologies is still under debate, thereby making it challenging to use proteomics to establish the molecular mechanisms through which platelets contribute to disease processes.

### Future Directions of Platelet Proteomics

In the past few years, it has become increasingly clear that platelet proteomics can provide novel insights into basic research questions and thus improve our understanding about the fundamental processes that regulate platelets and can also contribute to the diagnosis of platelet disorders, as summarized in Table 2. The protein composition and the phosphorylation patterns of platelets will be useful to understand certain disease states and therapeutic interventions. In particular, quantitative phosphoproteomic studies will pave the way for a refined understanding of platelet properties, beyond the classical biochemical studies which focused on the description of linear pathways such as stimulus→receptor→second messenger→phosphoprotein→effect on cell function. Although these approaches undeniably have made and will make important discoveries, the past few years clearly showed...
that signaling is much more complicated, branched, and non-linear than originally anticipated, including a considerable level of cross talk, in platelets for instance between inhibitory and activatory pathways. In this context, the extensive cross talk between different PTM and the existence of a PTM code that defines the functions of proteins, protein complexes, and signaling pathways have gained more and more attention.178–180 The vast amount of data that comes from quantitative proteomics studies, however, will require a thorough evaluation using systems biology approaches not only for obtaining a systematic interpretation of signaling networks and nodes but also for the simulation of experiments and conditions that cannot be realized in vivo or in vitro.181–186 This will lead to the description of (phospho)protein clusters that are characteristic for the various levels of inhibitory and activation pathways or that represent molecular signatures for those pathways, which are known to be affected in disease. We expect that recent strategies to analyze protein complexes using quantitative MS will further improve our understanding of such molecular clusters.187–191 In addition, quantitative MS-based profiling methods for cell organelles can provide information about the spatial distribution of proteins192,193 and reveal dynamic changes in this distribution. Given the role of circulating platelets as sentinels of vascular integrity, proteomic analyses and PTM signatures could lead to new approaches for diagnosis and treatment of platelet disorders.

A successful contribution of proteomics for improving clinical diagnosis and treatment will rely on interdisciplinary collaborations between chemists, biochemists, and clinicians. On its own, proteomics can pinpoint target proteins, but it is important to consider that conclusions can only be clear and strong at validated combinations of (1) study design, (2) platelet preparation, (3) proteomic sample preparation, (4) LC-MS analysis, and (5) MS data interpretation. Still, even for extensively quality-controlled studies, a small level of uncertainty will remain for MS-derived identifications. Fortunately, the level of uncertainty, as expressed in the FDR, can be quantitatively assessed. For PTM peptides, the probability for correct site localization of the modification is nowadays routinely estimated. This provides an advantage over the use of site-specific antibodies, which are often not immune to recognize adjacent modification (eg, phosphorylation) sites.

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Disclosures

None.

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What Can Proteomics Tell Us About Platelets?
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