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Platelet Proteomes, Pathways, and Phenotypes as Informants of Vascular Wellness and Disease

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ABSTRACT: Platelets rapidly undergo responsive transitions in form and function to repair vascular endothelium and mediate hemostasis. In contrast, heterogeneous platelet subpopulations with a range of primed or refractory phenotypes gradually arise in chronic inflammatory and other conditions in a manner that may indicate or support disease. Qualitatively distinguishable platelet phenotypes are increasingly associated with a variety of physiological and pathological circumstances; however, the origins and significance of platelet phenotypic variation remain unclear and conceptually vague. As changes in platelet function in disease exhibit many similarities to platelets following the activation of platelet agonist receptors, the intracellular responses of platelets common to hemostasis and inflammation may provide insights to the molecular basis of platelet phenotype. Here, we review concepts around how protein-level relations—from platelet receptors through intracellular signaling events—may help to define platelet phenotypes in inflammation, immune responses, aging, and other conditions. We further discuss how representing systems-wide platelet proteomics data profiles as circuit-like networks of causally related intracellular events, or, pathway maps, may inform molecular definitions of platelet phenotype. In addition to offering insights into platelets as druggable targets, maps of causally arranged intracellular relations underlying platelet function can also advance precision and interceptive medicine efforts by leveraging platelets as accessible, dynamic, endogenous, circulating biomarkers of vascular wellness and disease.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: hemostasis ■ inflammation ■ immunity ■ proteomics ■ vascular endothelium

PLATELETS AS SENTINELS OF ALTERED VASCULAR FUNCTION

Upon encountering molecular or biophysical cues of aberrations in vascular flow, form or function, platelets promptly initiate a set of responses at the endothelium to limit vessel leakage.[1,2] In the context of primary hemostasis, rapid platelet reactions—including platelet adhesion to endothelium, shape change, secretion, and aggregation—are physiologically critical to prevent bleeding. However, the essential utility of platelets as swift guardians of vascular integrity often comes with an ultimate price for their lifelong loyalty—most notoriously as drivers of atherothrombosis and sudden death following atherosclerotic plaque rupture.[3] Accordingly, mechanisms of platelet activation in hemostasis and their dysregulation in thrombus formation have been, and continue to be of high relevance to translational efforts, where there has been important, although incomplete success in targeting platelets to limit morbidity and mortality in cardiovascular disease.[4–6]

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In contrast to rapid shape change and other responses characteristic of platelets in hemostasis and thrombosis, platelets can also undergo more subtle and extended transitions in phenotype that are increasingly associated with chronic disease.[1,7,8] As a generalized concept, phenotype refers to the observable, distinguishable, or measurable type of phenomenon exhibited by a biological entity resulting from the interaction of its genotype and environment.[9,10] Over the past few decades, the notion

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Nonstandard Abbreviations and Acronyms

BTK	Bruton's tyrosine kinase
CD40L	CD40 ligand
CLEC-2	C-type lectin domain family 2
COVID-19	coronavirus disease 2019
CRP-XL	crosslinked collagen-related peptide
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C chemokine receptor
FCGR2A	low-affinity immunoglobulin gamma Fc region receptor II-a
GPIb	glycoprotein Ib
GPVI	glycoprotein VI
IGF-1	insulin-like growth factor 1
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor κB
oxLDL	oxidized low-density lipoprotein
P2Y12	P2Y purinoceptor 12
PAR	protease-activated receptor
PI3K	phosphoinositide 3-kinase
PLCγ2	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase γ 2
Syk	spleen tyrosine kinase
TP	thromboxane A2 prostanoid receptor
TLR	Toll-like receptor
VASP	vasodilator-stimulated phosphoprotein
VWF	von Willebrand factor

Highlights

- Platelets serve essential roles in hemostasis to prevent bleeding, but also contribute to inflammatory responses and disease progression.
- Specific, potentially maladaptive, platelet subpopulations are associated with inflammatory diseases, but molecular mechanisms of platelet heterogeneity are not clear.
- Rapid as well as progressive changes in platelet reactivity come about through receptor activation and signaling.
- Factors associated with inflammation and disease may prime platelet reactivities through specific signaling pathways.
- Pathway maps of signaling relations in proteomics data sets may provide a means to define platelet phenotype.

of platelet phenotype has emerged to describe single-cell properties of platelets or platelet subpopulations that deviate from normal, quiescent platelets in circulation, and that may be indicative or causative agents of disease. Indeed, in vitro laboratory studies as well as in vivo observations from the clinic have begun to catalog heterogeneous subpopulations of platelets described as procoagulant, angry, coated, exhausted, or sticky—in vascular diseases,[11] metabolic syndrome,[12] trauma,[13] shock,[13] neurodegenerative disorders, circadian cycles,[14] chronic mental stress,[15] gut dysbiosis,[16,17] mechanical intervention, infection, lupus,[18] dermatitis,[19] cancer,[20] aging,[21] and other states marked by systemic inflammation.[22–24]

DETERMINING AND DEFINING PLATELET PHENOTYPE

Thousands of biochemical, cell biological, and translational studies have built a wealth of knowledge around molecular processes that rapidly bring about platelet activation states in contexts of hemostasis and thrombosis. However, it remains unknown how, many now classic, platelet activation mechanisms may also contribute to changes in platelet phenotype in a manner supporting or resultant of chronic disease. Such knowledge gaps in understanding platelet phenotypes in health and disease remain, in part, due to conceptual hurdles. For instance, it is unclear whether or how platelet phenotypes may be specified at a molecular level, where platelet cell surface markers and flow cytometry tools offer a promising means to define some platelet subpopulations.[25,26] Standardized methods to prepare samples of platelets with specific phenotypes from whole blood are also lacking—where there has been recent progress in enriching platelet subpopulations based on size as well as RNA content (ie, acridine orange staining) before functional or wider omics characterization.[27,28]

Unbiased omics approaches have long offered a promising means to profile and define cell phenotypes as well as biomarkers and targets in platelets in the context of disease.[29–33] Ongoing studies of the platelet transcriptome,[34] metabolome,[35–37] lipidome,[38] and proteome highlight variations in the levels of some specific platelet mRNAs, metabolites and proteins over different individuals, disease conditions, and activation states; however, how these alterations mechanistically relate to platelet function and human physiology still remains unclear.[39] As platelet hemostatic, inflammatory and other activation programs are largely coordinated by intracellular enzymatic and signaling processes—in particular, global changes in reversible protein phosphorylation as mediated by protein kinases and phosphatases[40–42]—measuring and comparing dynamic, biochemical events that globally control platelet function may provide a relevant means to define and understand platelet phenotypic variations, where phosphoproteomic profiling tools can provide an especially powerful and promising approach.

Here, we briefly highlight and discuss emerging concepts on how profiling intracellular processes in platelets may inform and help to define platelet phenotypes

at a molecular level—particularly in terms of the composition, organization and modification of the platelet proteome, or platelet proteotype.[43,44] Ultimately, mapping omics data sets on to causative, molecular-level relations underlying platelet phenotype and function could facilitate discussion and hypothesis generation while advancing efforts to better understand platelets as therapeutic targets as well as endogenous, responsive biomarkers of health and disease.

PLATELET LIGAND-RECEPTOR RESPONSES PRECEDE PHENOTYPIC CHANGES

Molecular-level changes in platelet proteome and cellular architecture can cause deviations in platelet size, morphology, reactivity, and other properties to upregulate or downregulate platelet function in a variety of circumstances. For instance, congenital platelet disorders[45] such as Glanzmann's thrombasthenia[46] and Bernard–Soulier syndrome[47] provide examples of how gene and protein alterations at the level of platelet receptors can affect platelet phenotype as well as organismal physiology and health. In sharp contrast to genotype-driven changes in platelet phenotypes, platelets undergo rapid (within seconds) morphological transitions in response to physiological agonists and cell interactions to mediate vessel wall repair.[1] For example, VWF (von Willebrand factor), collagen, and ADP sequentially engage specific platelet receptors to orchestrate platelet adhesion, secretion, and aggregation at damaged endothelium (Figure 1, top).[2,41,48] Similar, although more gradual and less detailed responses initiated by platelet receptor interactions at dysfunctional endothelium are also increasingly associated with platelet heterogeneity and the progression of vascular inflammation in a range of chronic diseases (Figure 1, bottom).[49–51] Upregulation of platelet receptor expression and activity—as well as loss of functional receptors on the platelet cell surface through cleavage and shedding—are also hallmarks of some chronic inflammatory states that may contribute to disease progression.[52,53] For instance, surface expression of the platelet collagen receptor GPVI (glycoprotein VI) can increase in obesity in a manner potentially upregulating proinflammatory and prothrombotic platelet GPVI responses, including GPVI receptor shedding.[54,55]

Following from their potential maladaptive contributions to thrombosis and inflammation, platelet agonist-receptor responses have provided several unique molecular targets against platelet activities in disease.[4] Aspirin is well known to target platelet cyclooxygenases to prevent arachidonic acid oxidation, thromboxane A2 generation, and feedback activation of platelet TP (thromboxane prostanoid receptor). Purinergic receptor antagonists (ie, clopidogrel, ticagrelor) are also well-established antiplatelet agents that prevent secondary activation of platelets by ADP. Studies with other platelet inhibitors directed against thrombin/PARs (protease-activated receptor),[56–58] integrin $\alpha_{IIb}\beta_3$,[59] and more recently GPVI,[60,61] also provide examples of how platelet ligand-receptor responses can support platelet heterogeneity in thrombotic diseases in a mechanistic manner. Roles for these and other platelet ligand-receptor systems in the progression of chronic diseases are less clear than in acute thrombosis; however, precedence grows to target specific platelet receptors in vascular inflammation, where blockade of platelet GPIb (glycoprotein Ib) and GPVI can limit atherosclerosis in vivo in mouse models.[51,62–64] Modulation of human platelet receptor responses associated with inflammation is less straightforward,[65] and, likely far away in effective clinical

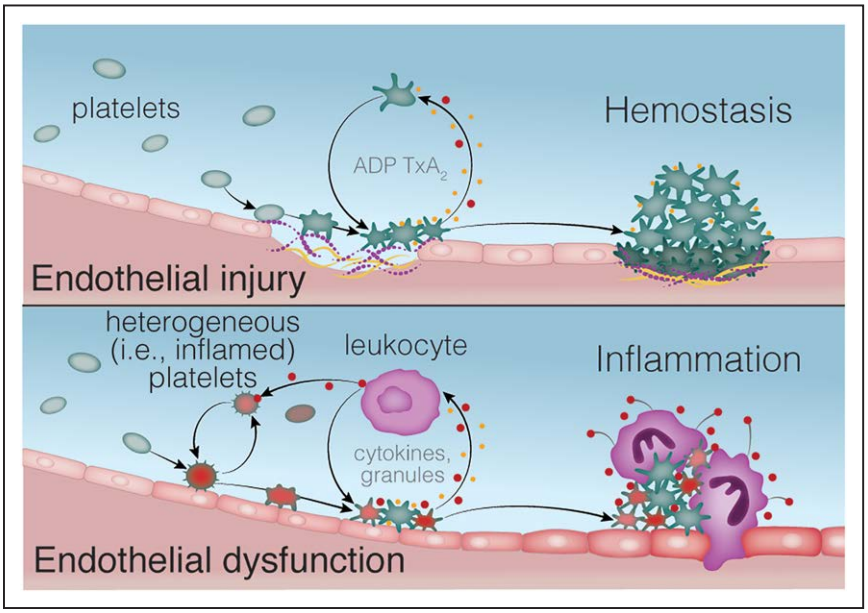

Figure 1. Platelet phenotype and function in endothelial injury (hemostasis) and dysfunction (inflammation).
Upper, Upon encountering cues of endothelial injury (ie, VWF [von Willebrand factor], subendothelial collagen), circulating platelets rapidly respond through adhesion, secretion (ie, ADP, granules), thromboxane generation (TXA2), and aggregation to mediate hemostasis and prevent vessel leakage. **Lower**, Platelets can also more transiently interact with dysfunctional endothelium, where factors associated with chronic conditions may give rise to more heterogeneous inflamed platelet subpopulations (shaded in red) to progress vascular inflammation, further fueling platelet secretion (ie, cytokines, granules), platelet-leukocyte aggregate formation, and other responses driving disease.

practice; however, recent findings from trials such as CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) and COLCOT (Colchicine Cardiovascular Outcomes Trial) point to the possibility of targeting platelet- and other cell-associated inflammatory processes as drivers of chronic diseases.[8,62,66,67]

INTRACELLULAR SIGNALING MODULATES PLATELET PHENOTYPE

Downstream of ligand-receptor interactions at the extracellular surface of the platelet plasma membrane, a combinatorial sequence of signals through kinases, phosphatases, GTPases, phospholipases, phosphodiesterases, Ca^{2+} ions, lipids and potentially thousands of other distinct intracellular effectors ultimately decide the phenotypic and functional fate of platelets in physiological context (Figure 2). Relative to hemostatic platelet agonist-receptor systems (ie, collagen/GPVI, ADP/P2Y12 [P2Y purinoceptor 12]), it is less clear how inflammatory stimuli (ie, interleukins [IL], cytokines) evoke platelet signaling responses, and consequently, any changes in platelet proteotype, phenotype, and function (Figure 2). While platelet reactivity can be primed or dulled in a manner involving a range of platelet receptors and intracellular signaling pathways, it remains unclear how extended interactions of platelets with circulating inflammatory factors and diseased cells may adjust signaling responses to tune platelet function in health and disease. In addition to direct platelet interactions, many other underlying physiological factors likely influence phenotypic differences in platelets. For instance, perturbations to megakaryocytes may result in the production of maladaptive platelets,[68,69] which, in turn, can reprogram bone marrow to further alter hematopoiesis and platelet phenotype in disease.[70] Nonetheless, regardless of the origin of platelet heterogeneity, the molecular basis of platelet phenotype remains be specified, where signaling mechanisms offer a number of insights for hypothesis building.

Platelets have long been recognized to participate in immune and inflammatory responses[71]; however, specific platelet receptor-driven signaling mechanisms associated with inflammation did not begin to emerge until the late 1990s—when the immune receptor FCGR2A (Fc gamma RIIA) was found to drive platelet responses through PI3K (phosphoinositide 3-kinase) and PLCγ2 (1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2).[72] Soon thereafter, other platelet cell surface molecules, including CD40 and CD40L (CD40 ligand), were found to transduce specific signaling events in platelets in a manner associated with pathologies such as inflammatory bowel disease and type 2 diabetes.[73–75] Though the early 2000s, an expansion of studies around platelet MAPK (mitogen activated protein kinase), NF-κB (nuclear factor κB), and especially PI3K/Akt[76] pathways linked platelet intracellular signaling to phenotype and disease in a manner also related to hemostasis and thrombosis.[77] For instance, a number proinflammatory molecules and receptors have been noted to prime platelet activities through PI3K/Akt signaling, including CXCL16 (C-X-C motif chemokine ligand 16)/CXCR6 (C-X-C chemokine receptor 6),[78] CXCR7,[79] IGF-1 (insulin-like growth factor 1), and thrombopoietin.[80,81] ILs, such as IL-6, can alter platelet phenotype in a manner that may involve platelet Jak/STAT (Janus kinase/signal transducer and activator of transcription) signaling downstream of gp130.[22,82–84] Other receptors associated with innate and adaptive immunity expressed by platelets—including TNF-α (tumor necrosis factor) receptors,[37] TLRs (Toll-like receptors),[53,85] complement receptors (C3R, C5R),[86] and lectins (CLEC-2 [C-type lectin domain family 2],[87] dendritic cell-specific ICAM-3-grabbing nonintegrin[88])—may also interact with a diverse range of ligands to modulate platelet intracellular signaling systems as well as platelet function.

The intersection of platelet hemostatic and immune receptor signaling systems in physiology and disease is particularly noteworthy for platelet ITAM (immunoreceptor tyrosine-based activation motif)-coupled receptors, including GPVI/FcγR and CLEC-2.[89] Roles for GPVI, CLEC-2, and their downstream signaling responses in hemostasis, thrombosis, and development have been extensively reviewed.[89,90] In addition to collagen, many inflammation-associated molecules have been reported to engage GPVI to prime or activate platelets in disease, including fibrin,[91] β-amyloid,[92] cathelicidins,[93] and EMMPRIN (extracellular matrix metalloproteinase inducer).[89,94] Similarly, a systematic upregulation of GPVI signaling associates with increased reactivity of platelets from patients with myocardial infarction[95] as well as obese subjects[55]; however, disease and ligand specific GPVI responses remain to be resolved.[96] Other ongoing studies suggest a disease-associated crosstalk between GPVI and other platelet receptors, including the scavenger receptor CD36.[97,98] Following interaction with glycated or oxidized lipids (ie, oxLDL [oxidized low-density lipoprotein]), CD36 can upregulate Jnk, ERK5 [extracellular signal-regulated kinase 5], reactive oxygen species, and other signaling systems to support prothrombotic and procoagulant platelet phenotypes (phosphatidylserine exposure), particularly in dyslipidemia.[97–100] Cell physiological, metabolic, and other factors (ie, hypoxia, reactive oxygen species generation) associated with vascular disease progression can also bring about maladaptive platelet phenotypes more prone to activation through similar ERK5 pathways that are also upregulated in platelets from patients with ST-segment–elevation myocardial infarction.[97,101] These examples highlight the complex similarities and disparities between hemostatic and

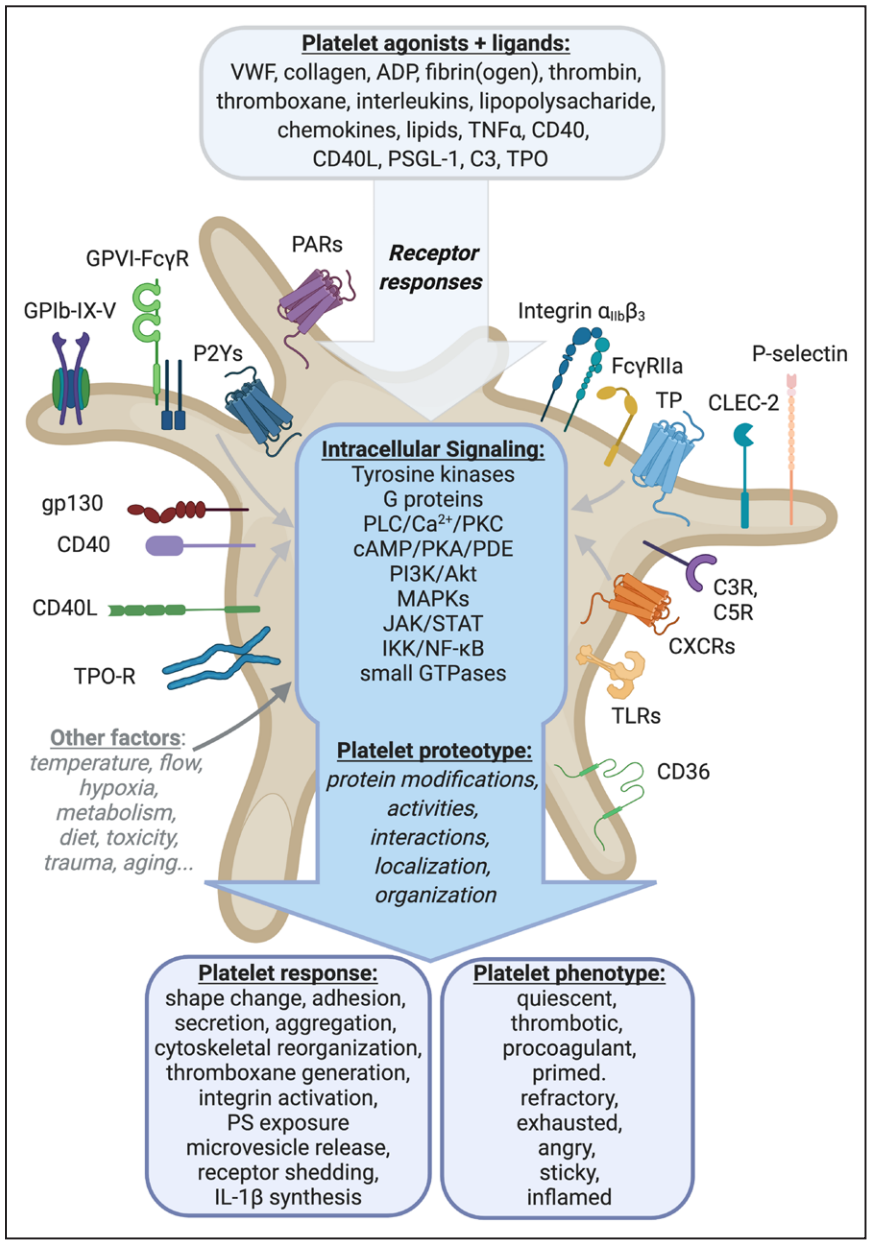

Figure 2. Platelet ligands, receptors, and intracellular signaling pathways determine platelet function and phenotype.
A variety of hemostatic (ie, VWF [von Willebrand factor], collagen) and inflammatory (ie, IL-6 [interleukin 6], lipopolysaccharide) agonists and other ligands (ie, thrombopoietin [TPO]) engage a number of different receptors expressed on platelets to result in different platelet responses and platelet phenotypes. Following ligand binding and initial receptor responses, diverging signaling pathways globally reorganize platelet intracellular protein modifications and relations (platelet proteotype) to solicit specific platelet responses in physiological context (ie, shape change, secretion). Specific signaling events downstream of platelet receptors are mediated by tyrosine kinases, PLC (phospholipases), PKC (protein kinase C), adenylyl cyclase, PKA (protein kinase A), PDE (phosphodiesterases), PI3K (phosphoinositide 3-kinase), Akt, MAPKs (mitogen activated protein kinases), Jak/STAT, IKK/NF-κB, and, potentially thousands of other distinct effectors. How these signals and their systemic intracellular consequences globally determine platelet phenotype and function remains to be elucidated. CLEC-2 indicates C-type lectin domain family 2; GPVI, glycoprotein VI; IKK, inhibitor of nuclear factor kappa-B kinase; Jak, Janus kinase; NF-κB, nuclear factor κB; PAR, protease-activated receptor; PSGL-1, P-selectin glycoprotein ligand-1; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; and TP, thromboxane A2 prostanoid receptor.

inflammatory signaling interactions in platelets, many of which could offer insights into chronic vascular conditions and platelet phenotype.

Many other specific intracellular signaling proteins regulating platelet phenotype and function have been revealed through clinical pharmacology—where therapeutically druggable kinases such as Syk (spleen tyrosine kinase), BTK (Bruton's tyrosine kinase), and PI3K have emerged as potential targets in platelets against thrombotic as well as inflammatory conditions.[4–6,102] Given their key roles in activation and proliferation programs of other inflammatory cells and hematologic cancers, these and other kinases represent important therapeutic targets for rheumatoid arthritis, immune thrombocytopenia, and lymphoma. However, many kinase inhibitor drugs have been associated with bleeding and other hemostatic abnormalities due to undesirable side effects on platelet function. It is now becoming more apparent that some kinase inhibitor therapies may serve as effective antiplatelet and antithrombotic agents in some specific contexts. Indeed, inhibitors targeting BTK (ie, ibrutinib, acalabrutinib) may already show promise against several platelet-related conditions, such as atherogenesis,[103] heparin-induced thrombocytopenia,[104] multiple sclerosis,[105] and coronavirus disease (COVID-19).[106,107] In addition to protein and lipid kinases, other platelet enzymes offer readily druggable targets in pathology, including the lipid deacetylase AADACL1 (arylacetamide deacetylase like 1).[108] Signaling through Ral GTPases may also specifically support secretory platelet phenotypes in inflammatory diseases in a targetable manner.[109]

PLATELET PHENOTYPES AND PLATELET SIGNALING EVENTS AS BIOMARKERS

Beyond providing therapeutic targets, heterogeneous subpopulations of platelets with specific molecular properties and phenotypes may offer a means to define, predict and diagnose platelet-associated conditions—especially vasculopathies that are progressed by inflammatory, procoagulant, and other platelet responses. Platelets can already provide physiologically relevant information as biomarkers in vascular diseases, as well as cancer, multiple sclerosis, Alzheimer disease, Parkinson disease,[110] and COVID-19.[111] A number of platelet indices associated with these and other conditions already have direct or potential prognostic and diagnostic utility, including platelet count,[112] plateletcrit,[113,114] mean platelet volume,[115] platelet mitochondria function,[37,116–118] platelet RNA content,[110,119] and platelet receptor shedding,[120,121]—as well as levels of circulating platelet-monocyte aggregates[122] and platelet-derived microvesicles.[123]

As cellular scale properties of platelets can indicate specific pathologies, the molecular processes that bring about cell biological changes in platelets in chronic conditions may likewise provide insights to, or, biomarkers of specific platelet states in disease. To this end, readily measurable signaling responses in platelets—in particular, site-specific protein phosphorylation events—may reflect specialized platelet phenotypes that come about following the activation of different signaling systems in disease states. For instance, a recent study from the Heemskerk team finds that platelet phosphoproteomic profiles can reveal global changes in platelet kinase activities and inform diagnosis of pseudohypoparathyroidism and Albright hereditary osteodystrophy syndrome.[124]

Given their roles as dynamic messengers of cellular function, phosphoproteins offer an encouraging source of biomarkers for elusive, chronic conditions. However, few biomarkers based on site-specific protein phosphorylation have yet made their way to clinical practice.[125–127] Site-specific protein phosphorylation biomarkers of promising utility include Tau (pT_{181}, pT_{217}) in cerebrospinal fluid, which can follow Alzheimer disease progression[128,129]; similarly, phosphorylation of Rab10 in peripheral blood cells may serve as a biomarker for Parkinson disease.[130] Intriguingly, some platelet phosphoproteins also already offer potential as biomarkers, such as VASP (vasodilator-stimulated phosphoprotein) S_{239} phosphorylation, as readily measured by flow cytometry or ELISA.[131] Following its discovery in unbiased autoradiography studies of prostaglandin treated platelets in the 1980s,[132] phospho-VASP has gone on to offer a means to predict high on-treatment platelet reactivity and resistance to antiplatelet therapies.[133,134] In addition to phospho-VASP, many thousands of other site-specific protein modifications now undergoing mechanistic characterization may similarly inform specific aspects of platelet phenotypes in disease (ie, platelet acetyl-CoA carboxylase S_{79} phosphorylation by AMPK [5' adenosine monophosphate-activated protein kinase] in coronary artery disease patients).[135] Moreover, other dynamic protein modifications (ie, lysine acetylation, methylation)[136,137] with less defined roles in platelet function and disease also have the potential to provide additional molecular information regarding platelet phenotype.

CAUSAL RELATIONS IN PROTEOMICS DATA INFORM PLATELET PHENOTYPE

Over the past 2 decades, proteomics studies of platelets have resulted in groundbreaking mechanistic and translational discoveries,[138] where mass spectrometry-driven phosphoproteomics approaches have identified many specific protein phosphorylation sites in platelets important to hemostasis; however, such studies have been limited in detailing complete signaling pathways and networks underlying platelet function.[139–142] In recent years, mass spectrometry instrumentation, reagents, and computation tools have synergistically improved the ability to perform quantitative studies beyond that of earlier work with foundational although less complete results. Phosphoproteomics workflows that take advantage of Orbitrap mass analyzers, triple stage tandem mass spectrometry, and synchronous precursor selection tools to quantify between (at present, up to 16) different tandem mass tag labeled samples are especially powerful.[143–145] With tandem mass tag–synchronous precursor selection–triple stage tandem mass spectrometry tools (ie, TMT-SPS-MS3), our team recently measured global changes in site-specific protein phosphorylation in resting versus CRP-XL (crosslinked collagen-related peptide) stimulated platelets to profile platelet GPVI signaling systems at unprecedented depth and resolution.[146] We found that >5000 different site-specific protein phosphorylation modifications are readily measurable in platelets, supporting the use of next generation mass spectrometry tools for quantitative deep proteomic comparisons and profiling (Figure 3). In addition to more extensive profiles of other hemostatic (ie, ADP, thrombin) and inflammatory (ie, IL-6) agonist responses, future studies of platelets from specific populations will further deepen phosphoproteomics insights of platelets in disease, where quantitative studies of the platelet phosphoproteome in Scott's syndrome[147] pseudohypoparathyroidism[124] and obesity[54] have already set precedence.

Despite potential for hypothesis testing and discovery, proteomics experiments and data sets often suffer from multiple complexities.[148] Informatics tools can help to organize omics data sets as interactomes of general relations (ie, STRING [search tool for recurring instances

of neighbouring genes])[149] or as an enrichment of specific pathway members (KEGG [Kyoto Encyclopedia of Genes and Genomes], Reactome), but lack mechanistic depth.[150,151] Recognizing a need to better interrogate accumulating high-throughput omics data with prior knowledge, Demir et al developed BioPAX as a computational ontology to aggregate publicly available biochemical data through a common platform, Pathway Commons.[152–155] As a comprehensive, integrated database of databases, Pathway Commons allows for programmatic queries to computationally model biochemical regulatory systems from >2 million indexed biological interactions. A recently developed Pathway Commons application, CausalPath,[156,157] processes quantitative data from multiscale (phospho) proteomics experiments and then matches the data to logic maps of curated protein phosphorylation events in Pathway Commons to produce causal, step-wise explanations about how the data came to be. With CausalPath, we found that 290 significant phosphorylation events measured in CRP-XL stimulated platelets mapped through >300 well-established and more novel causal signaling relations in GPVI activation programs.[146] Importantly, CausalPath also noted >1000 significantly regulated phosphorylation events that were not directly explainable by pathways in curated literature, highlighting areas for novel insights, and exploration. Ongoing studies from our group and others now aim to determine how high-resolution phosphoproteomes as well as interactomes and secretomes[158–160] not only quantitatively differ in specific platelet responses and disease states—but also how causal relations support and systematically explain measurable events in data sets. Rather than representing omics data sets between platelet resting versus activated states or healthy versus diseased individuals as quantitative heatmaps or hairball interactomes—in the very near future, pathway maps (ie, phosphorylation of protein A at site Y causes phosphorylation of protein B at site Y, etc) could provide circuit-like signatures of signaling events in platelets to specify platelet phenotypes and subpopulations in a manner more useful for mechanistic as well as diagnostic and therapeutic efforts.

CONCLUSIONS AND FUTURE PERSPECTIVES

A continued expansion and integration of platelet functional and omics studies will undoubtedly advance the understanding of how molecular mechanisms systematically underlie platelet phenotype in physiology and

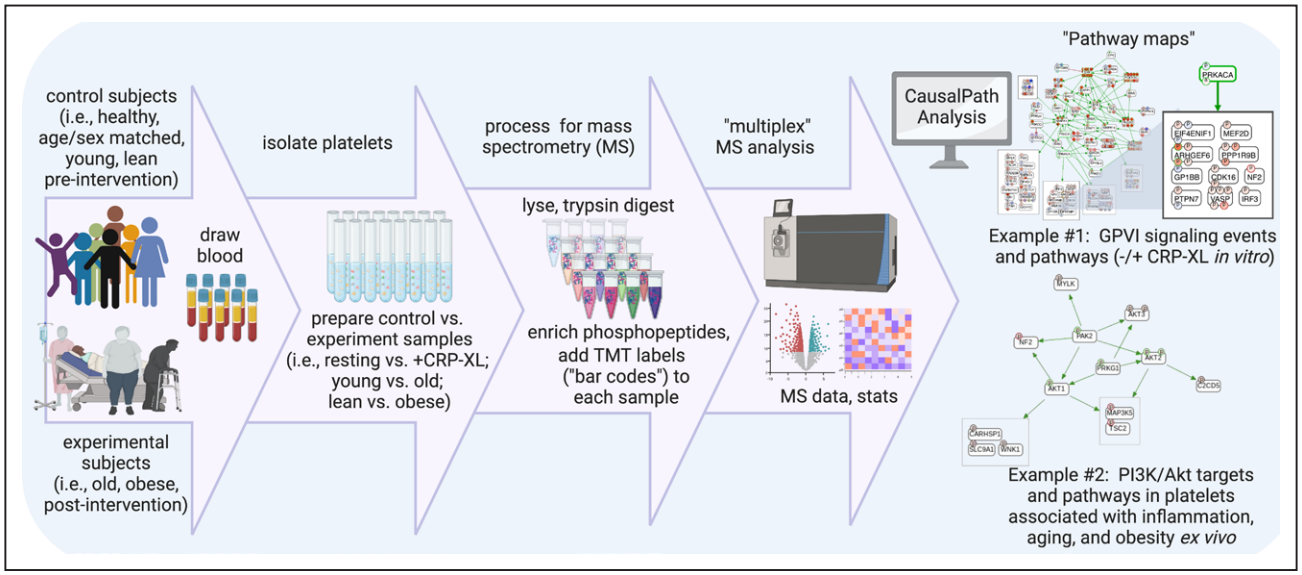

Figure 3. From platelet phenotype to (phospho) proteome and pathway map.
Example workflow highlighting steps from blood collection and platelet preparation, through quantitative mass spectrometry (MS) and sample analysis and pathway map generation. First, blood is drawn from a set of control (ie, healthy) and experimental subjects to prepare platelets. After isolation from whole blood, purified platelets may be kept in resting state, or stimulated with specific agonists (ie, –/+CRP-XL [crosslinked collagen-related peptide] to activate GPVI [glycoprotein VI]). Following sample lysis and tryptic digestion to prepare peptides, phosphopeptides are enriched with reagents such as TiO_2. Next, each sample of enriched phosphopeptides is labeled with a specific tandem mass tag (TMT) label that serves as a bar code to inform the mass spectrometer which sample each peptide corresponds to. Finally, all TMT-labeled samples are mixed together for multiplexed, quantitative mass spectrometry analysis. Quantitative data and statistics of thousands of phosphopeptide reporter ion intensity measurements between samples are analyzed with CausalPath to note causal relations in each data set (phosphorylation of protein A at site X causes phosphorylation of protein B at site Y…). Example No. 1 shows a subset of causal relations associated with platelet GPVI activation in vitro, where specific changes downstream of PKA (PRKACA) activity are highlighted.[146] Example No. 2 illustrates some PI3K (phosphoinositide 3-kinase)/Akt pathways that may be associated with disease when platelets are analyzed ex vivo from obese, elderly or other subjects with upregulated vascular inflammation relative to healthy control subjects.

disease.[161] But exactly what picture of platelets will emerge in the coming decades from multi-dimensional omics profiling, machine learning and other big data methodologies is still not clear. With time, several unbiased omics and targeted high-resolution platforms will likely be able to work together in parallel to simultaneously collect and analyze phenotypic information from platelets to, perhaps, customize disease detection and intervention beyond what is currently imaginable. For instance, deep, molecular insights into platelet subpopulations may ultimately advance efforts to target maladaptive megakaryocyte and platelet phenotypes in vivo with CRISPR (clustered regularly interspaced short palindromic repeats) tools; or, to engineer designer platelets for in vitro production and universal transfusion or other applications.[162–164] More immediately, by following pathway relations underlying platelet phenotypes in a personalized manner, platelet function may be tunable with lifestyle, nutrition, or pharmacology—where findings from off target effects of kinase directed cancer therapies already suggest alternative strategies to modulate platelet signaling networks and responses in disease.[165]

A number of high-throughput cell phenotyping tools are already in place to inform clinical research,[166] including reverse-phase protein arrays,[167] which can assay hundreds of protein phosphorylation markers in platelet-rich plasma to follow the efficacy of kinase inhibitors in patients with cancer.[168–170] Combinations of other common (ie, Western blot, ELISA) and more advanced flow cytometry,[171–174] microscopy,[175] and mass spectrometry[156,176] tools also now provide a means to deeply profile platelet phenotypes in a manner that is likely to expand clinical investigation. Precision medicine programs such as SMMART (serial measurements of molecular and architectural responses to therapy),[177] Network medicine,[178,179] P4 medicine,[180,181] and LifeTime medicine[182] also offer frameworks to help better connect large scale omics profiles and networks of networks to individual physiology and disease populations—but also acknowledge the limitations of reductionist approaches in medicine and recognize the need for new paradigms in understanding mechanisms of health and disease.[181,183]

Historically, pathway models have helped to reveal emergent properties of cellular systems from large scale experimental and data collection efforts. As a classic example, studies of glucose metabolism over the 20th century fueled an expansive era of enzymology[184]—identifying several individual, enzymatic steps in carbon metabolism, and ultimately, resulting in an emergence of organized biochemical pathways as a means to model cells by their metabolic flux rather than a sum collection of enzyme and metabolite components.[185] Similarly, cutting-edge, large-scale multi-omics endeavors will continue to break down cellular disease phenotypes into an increasing number of smaller and smaller puzzle pieces to inform precision medicine strategies. As we approach a postomics era, increasing effort will be required to determine how to best bring these pieces together to reveal a coherent picture of physiology and disease, and, ultimately place pictures together to tell a coherent story. To this end, multiple concepts from omics, statistics, computational biology, and systems science will need to coalesce in a meaningful and practical manner—where platelets themselves can serve as important puzzle components and also provide physiologically relevant examples of how to piece together proteotype, phenotype, and cellular function while informing vascular wellbeing.

ARTICLE INFORMATION

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