

## e-Blood

# The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways

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**Antiplatelet treatment is of fundamental importance in combatting functions/dysfunction of platelets in the pathogenesis of cardiovascular and inflammatory diseases. Dysfunction of anucleate platelets is likely to be completely attributable to alterations in posttranslational modifications and protein expression. We therefore examined the proteome of platelets highly purified from fresh blood donations, using elaborate protocols to ensure negligible contamination by leukocytes, erythrocytes, and plasma. Using**

**quantitative mass spectrometry, we created the first comprehensive and quantitative human platelet proteome, comprising almost 4000 unique proteins, estimated copy numbers for ~ 3700 of those, and assessed intersubject (4 donors) as well as intrasubject (3 different blood samples from 1 donor) variations of the proteome. For the first time, our data allow for a systematic and weighted appraisal of protein networks and pathways in human platelets, and indicate the feasibility of differential and comprehensive**

**proteome analyses from small blood donations. Because 85% of the platelet proteome shows no variation between healthy donors, this study represents the starting point for disease-oriented platelet proteomics. In the near future, comprehensive and quantitative comparisons between normal and well-defined dysfunctional platelets, or between platelets obtained from donors at various stages of chronic cardiovascular and inflammatory diseases will be feasible. (Blood. 2012;120(15):e73-e82)**

## Introduction

Platelets play a crucial role in hemostasis and in the pathologic genesis and progression of cardiovascular and inflammatory diseases. As those are the major cause of death in industrialized countries, antiplatelet treatment is recognized as a pharmacologic target of considerable importance. Patients suffering from cardiovascular or cerebrovascular disease benefit from antiplatelet treatment through reduced prevalence of recurrent—mostly fatal—events, and improved safety in case of angiographic intervention.<sup>1</sup> In recent years, however, evidence has been accumulating that a significant subset of patients does not respond adequately to certain drugs.<sup>2-4</sup> This insufficient responsiveness to antiplatelet treatment imposes an increased risk for severe cardiovascular or other adverse secondary events<sup>5</sup> and might be a direct consequence of differences in protein expression patterns and/or posttranslational modification (PTM) states of individual humans. More recently, it has been increasingly recognized that platelets also have major inflammatory functions and affect innate and adaptive immunity and inflammatory diseases.<sup>6</sup> The anucleate nature of platelets renders proteomics a valuable tool to help and better understand physiologic as well as pathophysiologic processes related to hemostasis and thrombosis. In various proteomic studies, we and others identified ~ 2000 proteins,<sup>7-13</sup> and more than 1000 PTM in human platelets.<sup>12,14,15</sup>

However, from qualitative data, conclusions can be inferred only to a limited extent. Deeper understanding of the individual role and contribution of signaling components to the initiation and propagation of platelet activation demands quantitative data for the protein components involved as it allows a weighted appraisal of the relevance of diverse signaling pathways in platelet activation and inhibition, which is mandatory for reconstruction and modeling of pathways. In principle, quantitative global as well as PTM-centric proteomics of reproducibly prepared platelets might allow for the assessment of differences between platelets from healthy individuals or from individual patients or patient cohorts. This appears of interest because platelets are not only a major component of hemostasis but serve as a sensitive monitor of vascular integrity especially in chronic diseases.

To assess the potential of quantitative proteomics to address these issues of utmost relevance, we conducted a systematic study determining the general biologic variance of human platelets, freshly isolated from 4 healthy donors. We identified more than 2500 phosphorylation sites, almost 4000 unique proteins and estimated copy numbers per platelet for ~ 3700 of those. In addition, we relatively quantified ~ 1900 proteins between 4 different donors (intersubject variation) and ~ 1500 proteins between 3 different blood

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**Table 1. Reference set of 24 platelet proteins for which determined copy numbers were found in the literature**

Uniprot accession	Protein name	CN/literature	Estimated CN	%	References
Actin	ACTB + ACT + ACTA	2 000 000	2 186 000	9	Watson and Harrison <sup>25</sup>
P02675	FIBB: fibrinogen beta chain	100 000	88 900	-11	Fischer et al <sup>26</sup>
P50552	VASP: vasodilator-stimulated phosphoprotein	78 900	44 600	-43	Eigenthaler et al <sup>27</sup>
P08514	ITA2B: integrin alpha-IIb	70 000	83 300	19	Fischer et al <sup>26</sup>
P05106	ITB3: integrin beta-3	70 000	64 200	-8	Fischer et al <sup>26</sup>
P21926	CD9: CD9 antigen	49 000	8000	-84	Protty et al <sup>28</sup>
P40197	GPV: platelet glycoprotein V	25 000	30 200	21	Lopéz and Berndt <sup>29</sup>
P07359	GP1BA: platelet glycoprotein Ib alpha chain	25 000	18 900	-24	Berndt et al <sup>30</sup>
P13224	GP1BB: platelet glycoprotein Ib beta chain	25 000	49 000	96	Berndt et al <sup>30</sup>
P14770	GPIX: platelet glycoprotein IX	25 000	32 400	30	Lopéz and Berndt <sup>29</sup>
P06396	GELS: gelsolin	20 000	52 900	165	Hartwig <sup>31</sup>
P16671	CD36: platelet glycoprotein 4	12 000	16 700	39	van Schravendijk et al <sup>32</sup>
P16284	PECA1: platelet endothelial cell adhesion molecule	10 000	9400	-6	Varga-Szabo et al <sup>33</sup>
P16109	LYAM3: P-selectin	10 000	8900	-11	Shattil et al <sup>34</sup>
P05771	KPCB: protein kinase C beta type	9800	9700	-1	Eigenthaler et al <sup>27</sup>
P48509	CD151: CD151 antigen	6800	3000	-56	Protty et al <sup>28</sup>
Q9HCN6	GPVI: platelet glycoprotein VI	3700	9600	159	Best et al <sup>35</sup>
P17301	ITA2: integrin alpha-2	3000	4600	53	Cosemans et al <sup>36</sup>
P13598	ICAM2: intercellular adhesion molecule 2	3000	3000	0	Diacovo et al <sup>37</sup>
P08962	CD63: CD63 antigen	2940	2200	-25	Protty et al <sup>28</sup>
O75954	TSN9: tetraspanin-9	2800	2200	-21	Protty et al <sup>28</sup>
P04156	PRIO: major prion protein	2000	910	-55	Holada et al <sup>38</sup>
P12318	FCG2A: low-affinity immunoglobulin gamma Fc region receptor II-a	1500	1000	-33	Kelton <sup>39</sup>
P07942	LAMB1: laminin subunit beta	700	830	19	Geberhiwot et al <sup>40</sup>

CN indicates copy number; and %, deviation.

donations of the same volunteer (intrasubject variation), covering a dynamic range of 4 orders of magnitude. Our results clearly demonstrate on a global level that in both cases ~ 85% of the platelet proteome show no variation.

We calculate a total of ~ 20 million protein molecules per platelet, corresponding to 1.5 mg of protein/10<sup>9</sup> platelets which fits well to common laboratory values (1.8 ± 0.2 mg). Based on this and the protein coverage we achieve for human platelet-related pathways taken from the manually curated Reactome website ([www.reactome.org](http://www.reactome.org)), we estimate a coverage of 80%-85% of the entire platelet proteome, concluding that human platelets comprise approximately 5000 rather than the afore-estimated 2000-3000 proteins.<sup>16</sup> Taken altogether, this study significantly improves our knowledge of quantitative platelet composition and biologic variance, and demonstrates that quantitative proteomics is a powerful ready-to-use tool for conducting disease-related studies of high clinical relevance,<sup>17-19</sup> starting already with small blood donations.

## Methods

### Platelet isolation and purification

Human platelets were prepared as reported,<sup>20</sup> with small modifications. The additional centrifugation step after dilution with buffer was introduced to remove leukocytes and improves sample purity substantially. Blood was obtained from healthy volunteers according to our institutional guidelines and the Declaration of Helsinki. Our studies with human platelets were approved and reconfirmed (September 24, 2008) by the local ethics committee of the University of Würzburg (studies 67/92 and 114/04).

Blood was collected in ACD solution (12mM citric acid, 15mM sodium citrate, 25mM D-glucose), apyrase, and EGTA were added to final concentrations of 0.01 U/mL and 3mM, respectively. Platelet-rich plasma (PRP) was obtained by 5-minute centrifugation at 330g. To reduce

leukocyte contamination, PRP was diluted 1:1 with PBS and centrifuged at 240g for 10 minutes. Subsequently, the supernatant was centrifuged for 10 minutes at 430g and pelleted platelets were washed once in CGS buffer (120mM sodium chloride, 12.9mM trisodium citrate, 30mM D-glucose, pH 6.5). A small aliquot was resuspended in HEPES buffer (150mM sodium chloride, 5mM potassium chloride, 1mM magnesium chloride, 10mM D-glucose, 10mM HEPES, pH 7.4) to a final concentration of 3 × 10<sup>8</sup> platelets/mL for quantifying leukocyte contamination using a leukocount kit (BD Biosciences).

### Sample processing for quantitative mass spectrometry

Experimental details for sample preparation/processing are given in supplemental Methods (see the Supplemental Materials link at the top of the article). To assess reproducibility of the entire workflow, proteolytic digests were controlled before LC-MS analysis.<sup>21</sup>

To assess the intersubject variance of the platelet proteome, samples from 4 donors were independently processed and individually labeled with iTRAQ on the peptide level. Thus, isobaric tags of different stable isotope compositions are attached to all primary amines (free peptide N-termini and Lys residues) which allows pooling of samples and consequently relative quantification based on specific reporter ion signal intensities in corresponding MS/MS spectra.<sup>22</sup>

Experimental details for iTRAQ labeling, peptide fractionation, TiO<sub>2</sub> enrichment, mass spectrometry, spectrum processing, and database searching are given in the supplemental Methods. Search engine results were postprocessed using PeptideShaker software (<http://peptide-shaker.googlecode.com>) and peptide to spectrum matches (PSM) were exported to the PRIDE repository (<http://www.ebi.ac.uk/pride/>; accession numbers 22201-22203, 22206).

### Protein quantification

Spectrum counting for estimation of protein copy numbers was accomplished using the normalized spectral abundance factor (NSAF)<sup>23</sup> chosen for its robustness.<sup>24</sup> NSAF relates the number of identified mass spectra to the length of a protein as a measure of its abundance. To estimate copy numbers of the platelet proteome, NSAF were correlated with a set of

24 reference proteins for which copy numbers were found in the literature (Table 1). Relative quantification between donors was conducted using the iTRAQ methodology. Details are given in the supplemental Methods.

### Comparison of quantitative proteomics and transcriptomics data

We compared our quantitative proteome data with RNAseq data from Rowley et al<sup>41</sup> and with SAGE data from Dittrich et al<sup>42</sup> as well as quantitative proteome data from HeLa<sup>43</sup> and U2OS<sup>44</sup> cells. Details are given in supplemental Methods.

## Results and discussion

### Sample purity

In contrast to cell-culture models, for proteome analyses of primary tissues and blood components such as platelets, sample preparation and purity have to be considered thoroughly. In platelet concentrates, changes in activity and degradation can alter proteome patterns, so we decided to analyze platelets directly isolated from fresh blood donations, based on an optimized protocol guaranteeing high purity.<sup>20</sup> As determined by the leukocount kit, all platelet samples contained less than 1 leukocyte per 10<sup>6</sup> platelets and less than 1 erythrocyte per 10<sup>4</sup> platelets.

### The platelet proteome

In this study, we initially identified more than 3700 proteins in human platelets with a false discover rate (FDR) of < 1% using a multipronged approach (Figure 1). We conducted a 2-step enrichment of phosphopeptides with low and high selectivity to identify additional proteins which were not accessible to the global approach—most likely because of low abundance and/or the presence of only few unique peptides within the respective sequences. This led to the identification of 1355 and 1052 phosphorylation sites of high and lower confidence (regarding the unambiguous localization of the phosphoamino acid) from 263 additional proteins at < 1% FDR (supplemental Table 2).

Because global proteomics studies are generally biased against membrane proteins,<sup>45</sup> we added ~ 200 further proteins which were only confidently identified in our previous platelet membrane proteome study,<sup>11</sup> generating a combined dataset comprising, in total, ~ 4200 proteins (supplemental Table 3).

### Estimation of protein copy numbers

Protein abundance estimation was conducted based on NSAF. Since nonunique peptides, shared between different proteins still pose a major challenge to proteomics, these might lead to deviations of estimated copy numbers for isoforms/homologs. Thus, ambiguous peptide sets derived exclusively from different protein isoforms or homologous proteins were considered as protein groups in our final proteome dataset; ambiguous peptide sets derived from nonrelated proteins were omitted. Based on the correlation to 24 reference proteins (Table 1), copy numbers for 3718 identified protein/protein groups were estimated, ranging from 2.2 · 10<sup>6</sup> (actin) to less than 500 copies per platelet for low abundant proteins. In general, our data correlate surprisingly well with known literature values yielding a Pearson coefficient of  $R^2 = 0.90$ . Notably, these copy numbers are estimates which shall provide researchers a general overview of platelet composition—although in good accordance with the current knowledge, in individual cases, copy number estimates can significantly differ from real copy numbers. This can be attributed to general limitations of large-scale spectral counting-based quantification of cells, which comprise sample preparation (eg, tryptic digestion),

mass spectrometric detection/identification, and data interpretation. (1) Only unique peptides can be unambiguously assigned to a single protein rendering protein isoforms problematic; (2) hydrophobic proteins can be depleted during sample preparation; (3) proteins with multiple transmembrane domains (TMD) generate comparatively few tryptic peptides accessible for the analysis and may be underestimated; and (4) proteins which are extensively modified (eg, glycosylated) are underrepresented because digestion might, and identification will be, hampered.

In the case of CD9, the reported 49 000 copies in the literature differ extremely from the estimated 8000 copies in our study. This discrepancy might be ascribed to the generation of only 2 fully tryptic peptides < 20 amino acids (and 3 additional ones > 25 amino acids), because of the presence of 4 TMD within only 228 amino acids.

From the obtained copy numbers and the corresponding protein molecular weights, we calculate an approximate protein concentration of 1.5 mg per 10<sup>9</sup> platelets which is in close agreement with typical laboratory reference values (1.8 ± 0.2 mg). The final list of identified proteins and copy numbers is given in supplemental Table 3.

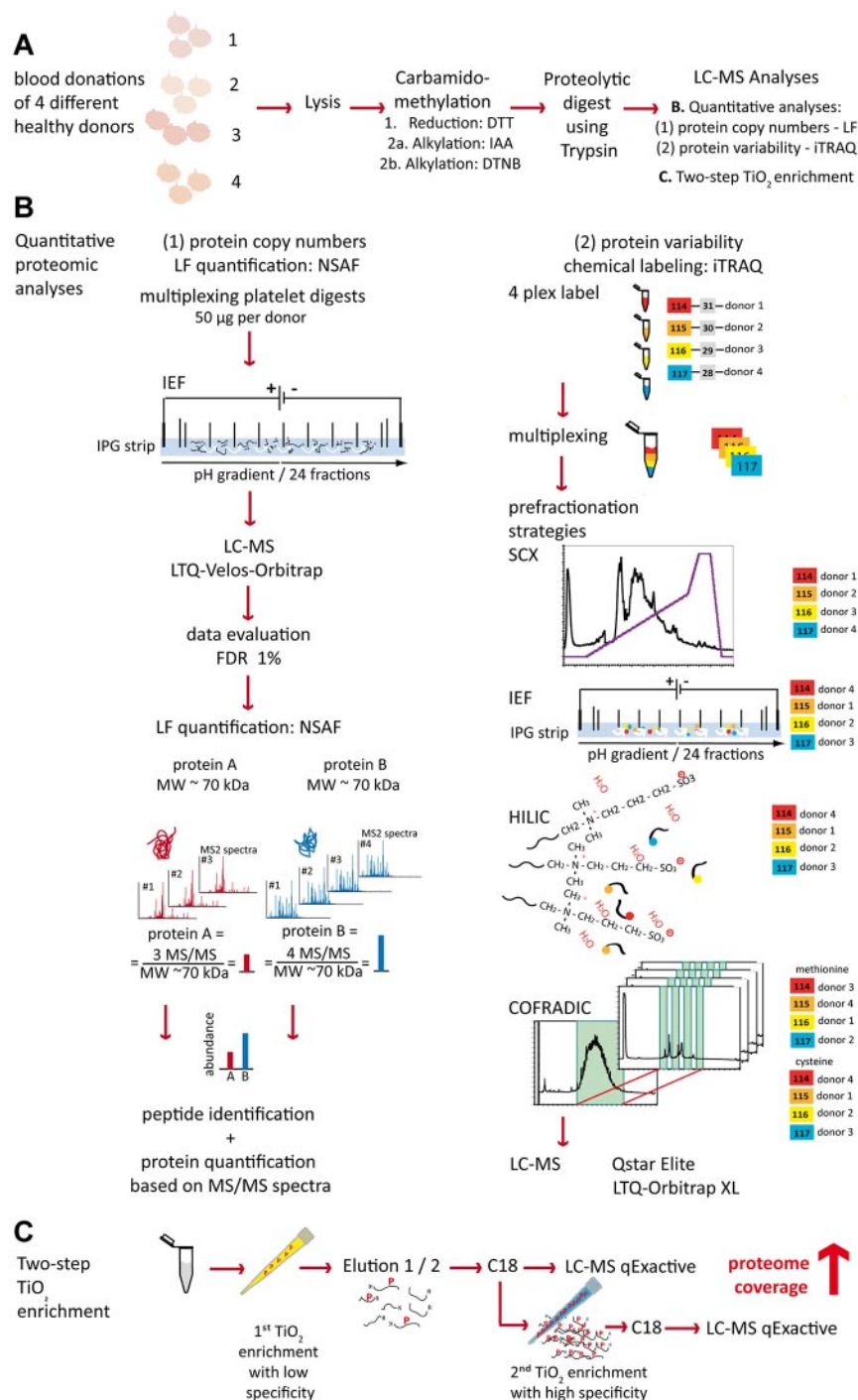
### Comparison to transcriptome data

To compare our proteome to transcriptome and SAGE RNA data, we generated a merged dataset comprising 9505 entries, 8070 of which referring to individual proteins (3598 from proteomics, 6202 from RNAseq, and 2737 from SAGE). The overlap between all 3 datasets is considerably low with 8%, whereas 12%, 38%, and 9% of proteins were exclusively found in proteome, RNAseq, and SAGE data, respectively. From the 20th percentile of the proteins quantified by RNAseq, 71% are shared with proteome data, whereas from the 20th percentile of the MS-derived proteins, 85% are shared with RNAseq data. However, for the most frequent proteins/transcripts, no correlation of RPKM and protein copy number can be observed ( $R^2 < 0.1$ ).

Purity of all datasets was evaluated based on 68 established CD markers (Table 2) selected from UniProt (<http://www.uniprot.org/docs/cdlist>), HPRD (<http://www.hprd.org/>), and the BD Biosciences CD marker handbook, of which 28 were shown to be expressed in platelets. Indeed, all of these except for CD23, and also 2 established nonplatelet proteins, CD81 and CD97, were identified in our proteome. CD37, present in ~ 110 000 copies per leukocyte,<sup>46</sup> is also present in platelets (own FACS data). It was also identified by Dowal et al in their recent analysis of platelet palmitoylation,<sup>13</sup> and is estimated to ~ 790 copies per platelet based on our MS data. In comparison, RNAseq data contain 15 nonplatelet CD marker transcripts, whereas in the SAGE data 5 nonplatelet markers were found, yet at significantly higher rates than in the RNAseq data.

### Protein stoichiometry

Platelet interaction with subendothelial layer matrix proteins and prothrombotic plasma components is mediated by heterooligomers of glycoproteins and particularly integrins of specific stoichiometry. The integrin heterodimer  $\alpha 2\beta 3$  is the most relevant integrin for platelet aggregation, acting as fibrinogen receptor. The 2 subunits are found almost equally expressed in the proteome (83 000 and 64 200 copies) and also in the transcriptome (1357 and 1223 RPKM in RNAseq). The subunits of the von Willebrand factor receptor GPIX/Ib $\alpha$ /Ib $\beta$ /V are found in stoichiometric ratios of 1.00:0.58:1.51:0.93 in the proteome, while GPIb $\alpha$  is missing in the transcriptome data and GPV is only detected in trace amounts. Collagen interacts with platelets



**Figure 1. Overview of the analytical strategy.** (A) Platelets were isolated from fresh blood donations and lysed, and proteins were carbamidomethylated and digested using trypsin. (B) Quantitative analysis was conducted in a 2-pronged way. (1) For absolute quantification of protein copy numbers, equal amounts of digest from the 4 donors were combined and peptides separated by in-solution isoelectric focusing. Obtained fractions were analyzed by LC-MS/MS on an LTQ Orbitrap Velos and copy numbers were calculated based on the NSAF method. (2) For quantifying the biologic variance of the human platelet proteome, 100  $\mu\text{g}$  of each sample were labeled with iTRAQ 114, 115, 116, and 117, respectively. Samples were multiplexed and fractionated using different techniques, namely SCX, IEF, HILIC, and COFRADIC. Obtained fractions were analyzed by LC-MS on Orbitrap XL and Qstar Elite mass spectrometers. (C) To increase the coverage of the human platelet proteome, a 2-step  $\text{TiO}_2$  enrichment, first with low and then with high specificity for phosphopeptides was conducted and samples analyzed by LC-MS/MS on a q-Exactive mass spectrometer.

via the integrin  $\alpha 2\beta 1$ , GPIV, or GPVI/Fc receptor  $\gamma$  complex. Glycoprotein GPIV is the most abundant of these followed by GPVI/FcRg (1.00:0.85 stoichiometry), and  $\alpha 2\beta 1$  (2:1 stoichiometry).

In quantitative proteomics, the I $\alpha$  (P10644), I $\beta$  (P31321), II $\alpha$  (P13861), and II $\beta$  (P31323) regulatory and  $\alpha$  (P17612) and  $\beta$  (P22694) catalytic subunit isoforms of PKA were found. The regulatory to catalytic subunit stoichiometry as derived from proteomics data with 1:0.82 is close to the expected 1:1 ratio and with ~ 9600 copies of catalytic subunit twice as much as published.<sup>27</sup> In contrast, RNAseq data are clearly deviating, not reflecting the expected stoichiometry.

## Pathway analysis

We analyzed the coverage of known platelet pathways for G-protein, integrin, calcium, and cyclic nucleotide signaling. Because of the central role of G-protein signaling in platelet regulation, we analyzed proteome, RNAseq, and SAGE data with respect to the presence of G-protein-coupled receptors (GPCR). Whereas the platelet transcriptome contains 20 GPCR (19 in RNAseq and 4 in SAGE), our initial proteome dataset comprised only 4: ADA2A, PAR4, P2Y12, and V1AR, with an additional 7 GPCR present in our previous membrane study (included in the final dataset supplemental Table 3). This discrepancy between the global and the

**Table 2. Overview of CD expression**

CD	Cell	Platelet	RNA	SAGE	MS copy numbers
CD19	B cell	-	+	-	-
CD20	B cell	-	+	+	-
CD146	Endothelial cell	-	-	-	-
CD35	Erythrocyte	-	-	-	-
CD44	Erythrocyte	-	+	-	-
CD66b	Granulocyte	-	-	-	-
CD49a	Monocyte	-	-	-	-
CD117	Stem cell	-	-	-	-
CD243	Stem cell	-	-	-	-
CD33	Stem cell	-	-	-	-
CD34	Stem cell	-	-	-	-
CD90	Stem cell	-	-	-	-
CD133	Stem cell	-	-	-	-
CD135	Stem cell	-	-	-	-
CD4	T cell	-	-	-	-
CD8a	T cell	-	+	-	-
CD8b	T cell	-	-	-	-
CD81	T cell	-	+	+	1000
CD97	Granulocyte	-	+	+	580
CD37	B cell	+	+	-	790
CD93	Neutrophils	+	+	-	750
CD92	Platelet	+	+	+	3300
CD41	Platelet	+	+	+	83 300
CD61	Platelet	+	+	+	64 200
CD62P	Platelet	+	+	-	8900
CD9	Platelet	+	+	+	8000
CD23	Platelet	+	-	-	-
CD31	Platelet	+	+	-	9400
CD36	Platelet	+	+	+	16 700
CD42a	Platelet	+	+	+	32 400
CD42b	Platelet	+	-	-	18 900
CD42c	Platelet	+	+	+	49 000
CD42d	Platelet	+	+	-	30 200
CD49b	Platelet	+	+	-	4600
CD49f	Platelet	+	+	-	11 500
CD51	Platelet	+	-	-	1400
CD84	Platelet	+	+	-	2900
CD109	Platelet	+	+	-	1700
CD110	Platelet	+	+	+	1600
CD147	Platelet	+	+	-	2900
CD151	Platelet	+	+	+	3000
CD226	Platelet	+	+	-	2800
CD107a	Platelet (activated)	+	+	-	3000
CD107b	Platelet (activated)	+	+	+	2000
CD63	Platelet (activated)	+	-	+	2200
CD111	Stem cell	+	-	-	570
CD112	Stem cell	+	+	-	750
CD11c	Dendritic cell	o	+	-	-
CD123	Dendritic cell	o	-	-	-
CD62E	Endothelial cell	o	-	+	-
CD236	Epithelial cell	o	+	-	-
CD235a	Erythrocyte	o	-	-	-
CD238	Erythrocyte	o	-	-	-
CD247	Lymphocyte	o	+	-	-
CD62L	Lymphocyte	o	+	-	-
CD131	Megakaryocyte	o	+	-	-
CD203c	Megakaryocyte	o	-	-	-
CD14	Monocyte	o	+	-	-
CD56	NK cell	o	-	-	-
CD228	Stem cell	o	-	-	-
CD280	Stem cell	o	-	+	-
CD338	Stem cell	o	-	-	-
CD277	Stem cell	o	+	-	670
CD318	Stem cell	o	-	-	-
CD227	Stem cell	o	-	-	-
CD3d	T cell	o	+	-	-
CD3e	T cell	o	+	-	-
CD3g	T cell	o	-	-	-

Platelet refers to the literature and our own data. Estimated copy numbers per platelet are given. SAGE indicates presence in SAGE data; MS, presence in MS data; and o, not tested.

membrane-dedicated study can be mainly attributed to the combination of (1) low abundance, (2) strong hydrophobicity (7 TMD), and (3) sample complexity, impeding generation as well as detection of proteotypic peptides in case of global studies. Amisten et al evaluated the gene expression profile of platelet GPCR<sup>47</sup> with PAR1 (292nd highest abundance in our platelet membrane proteome) and P2Y12 (296th) receptors being the prevalent GPCR followed by SUCR1 (not present), P2Y1 (482nd), and LPA (530th) receptors, in agreement with our previous results<sup>11</sup> as well as RNAseq data. For G-protein  $\alpha$  subunits, the inhibitory G $\alpha$  subunits (G $\alpha$ I-3, G $\alpha$ Z) account for 61%, followed by G $\alpha$ Q with 24%, G $\alpha$ I2 with 10%, and G $\alpha$ S with 5% of all subunit copies. The corresponding G $\beta$  and G $\gamma$  subunits are almost equal (34 400 vs 42 300 copies) yet less expressed than G $\alpha$  subunits. In contrast, in RNAseq data, G $\alpha$ S is found almost exclusively (82%) and in SAGE data G $\alpha$ I3 and G $\alpha$ I2 together account for 72% of all G $\alpha$  subunit transcripts.

A steep increase of intracellular calcium constitutes the initial and essential signal for platelet activation.<sup>48</sup> Two major mechanisms contribute to the fast increase: calcium entry through the nonselective P2X1 ion channel and mobilization of calcium from intracellular stores.<sup>49</sup> P2X1 is the only ligand-gated calcium channel identified in the proteome and the transcriptome and found at a moderate copy number (1400). Calcium mobilization occurs through IP3 receptors (inositol 1,4,5-trisphosphate receptor type 1, 2, and 3 with 2400, 1700, 750 copies; IRAG with 3500 copies). IP3 is formed from phosphatidylinositolbisphosphate by phospholipase C which is expressed in several subtypes and isoforms (PLCB2, PLCB3, PLCB4, PLCG2: 2500, 1700, 1000, 2000 copies). The secondary calcium influx can be achieved by 2 major pathways which have been proposed for platelets: the calcium sensor/calcium release activated channel (STIM1/CRAC) and transient receptor potential channels (TRPC).<sup>50,51</sup> Apparently, both pathways are present in human platelets (STIM1: 7400, CRACM1: 1700, TRPC6: 1100 copies). However, proposed interaction partners of STIM1 such as TRPC1 and IPLA2<sup>52</sup> or other TRPC were detected neither in the platelet proteome nor the transcriptome.

For the resting state of platelets, maintaining low intracellular calcium levels is fundamental. While plasma membrane calcium transporters are only present in low copy numbers (CaATPases PMCA4: 640, and ATPase 2C1: 2200, Na<sup>+</sup>/Ca<sup>2+</sup> Exchanger SLC8A3: 580), mitochondrial calcium transporter (MCU, MICU1, 5900, 1400) and particularly ER/SR CaATPases (SERCA2 and 3 with 9000 and 16 300), which also maintain the filling state of Ca stores, are found in exceedingly high quantity. These numbers underline the outstanding importance of intracellular calcium stores for platelet calcium regulation.<sup>53</sup>

The major endogenous platelet inhibitory pathways are based on cyclic nucleotide regulation. cAMP is formed by adenylyl cyclases which are anchored in the cell membrane by two 6-TMD each. In our proteome data, only adenylyl cyclase 6 (ADCY6) is present, while in our membrane proteome and in the RNAseq data, AC3 and AC5 are present as well. As expected from previous studies from us and others, only soluble guanylyl cyclase could be detected in platelets: in all datasets, solely GC  $\alpha$ 3 (Q02108) and  $\beta$ 1 (Q02153) subunits are present, with quantitative proteomics indicating a 1:1 stoichiometry (3500 and 3700 copies). Cyclic nucleotides are degraded by 3',5' cyclic phosphodiesterases; in our current understanding, platelets predominantly express 3 phosphodiesterases: PDE2A, PDE3A, and PDE5A, with PDE5A being the most relevant. Deducing PDE stoichiometries from different data sources results in similar ratios: (1) 1.00:0.17:0.04 for PDE5:PDE3:

PDE2 from Western blot,<sup>54</sup> (2) 1.00:0.07:0.04 from RNAseq, and (3) 1.00:0.12:(n/a) from quantitative proteomics, with PDE2 only detected after phosphopeptide enrichment, most likely because of low abundance. Combining quantitative proteomics and Western blot data < 300 copies per platelet can be estimated. The major effectors of cyclic nucleotides are the cyclic nucleotide-dependent protein kinases PKA and PKG. Of the PKG subtypes, only PKGI is consistently found in all datasets, yet proteome data indicate a 6-fold lower expression (3500 copies) than literature data.<sup>27</sup>

Generally, the comparison with transcriptome data clearly indicates that platelet transcripts do not reliably reflect protein expression. The lack of correlation with copy numbers, even for highly expressed proteins, and the rather unlikely frequency distribution of transcriptome data suggest that in platelets, the occurrence of proteins is not interrelated to the presence of transcripts.

### Global analysis of the platelet proteome and comparison to quantitative proteome data from human cell lines

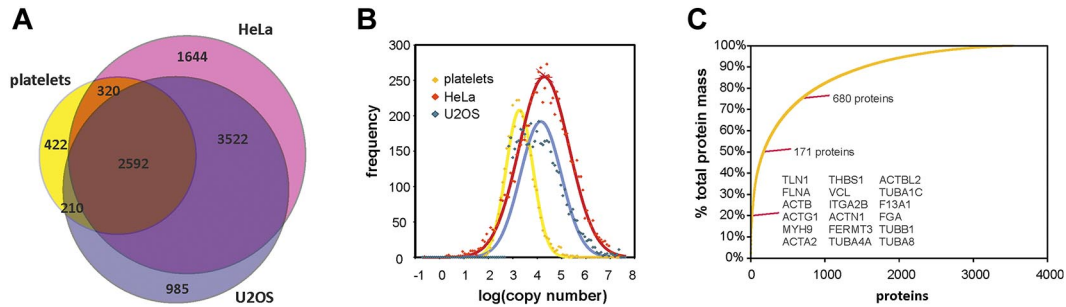
To further assess the general quality/coverage of the present dataset on a global scale and thus evaluate whether it is in accordance with current knowledge, we analyzed our ~ 4200 platelet proteins with regard to enriched GO terms and coverage of platelet-related pathways in the Reactome database. Although validity and integrity of the underlying protein-interaction and function databases are a matter of debate, the obtained information can nevertheless help to appraise the quality and reasonableness of proteomic data and furthermore allows for comparing large-scale proteomic data.

Compared with the human proteome (Uniprot human, ~ 18 000 entries), our platelet proteome is highly enriched in GO terms which can be related to platelet function, such as "endomembrane system," "actin cytoskeleton," "platelet  $\alpha$  granule," "platelet dense granule," "intracellular signal transduction," or "filopodium."

Among the 10 most significantly enriched pathways were "platelet activation," "hemostasis," "response to elevated platelet cytosolic Ca<sup>2+</sup>," "membrane trafficking," and "platelet degranulation." Surprisingly, this analysis also suggested an important role for ubiquitination in platelet regulation.

As all the aforementioned data confirm a highly pure and comprehensive analysis of the platelet proteome, we analyzed several platelet-specific human pathways in the Reactome database more in detail, namely "GPVI signaling" (stable identifier React\_1695.3), "GP1b-IX-V signaling" (React\_23847.2), "platelet homeostasis" (React\_23876.2) and "thrombin signaling through PAR" (React\_21384.2; all summarized in supplemental Table 4). However, here one of the limitations of global proteome analyses (that, often, isoform resolution and differentiation of highly homolog proteins is not possible because of the absence of sufficient unique peptide sequences) has to be considered. Hence, some small G proteins or PP2A and PDE subunits are missing; we therefore considered these as protein groups as usually several but not all of these members/subunits were unambiguously detected (in analogy to our final protein list). Thus, we achieve 97% (32 of 33 proteins) coverage of the Reactome "GPVI signaling" pathway, 90% (9 of 10) coverage of "GP1b-IX-V signaling," 90% (18 of 20) coverage of "platelet homeostasis," and 89% (8 of 9) coverage of "thrombin signaling through PAR." Concluding, we estimate a coverage of 80%-85% of the complete platelet proteome, even when taking into account missing isoforms.

To reveal discrepancy or congruency of protein expression and copy numbers between proteomes of human platelets and human



**Figure 2. Comparison of the platelet proteome to proteomes of human HeLa and U2OS cells, encompassing a total of 9696 proteins.** (A) All 3 proteomes show a remarkable overlap, which is virtually independent from copy numbers. (B) The distribution of copy numbers is similar for the 3 proteomes. However, because of the smaller cell volume in platelets the mean of the frequency distribution is shifted to lower copy numbers which is in accordance with Lundberg et al who reported a correlation between cell size and protein expression levels.<sup>55</sup> (C) Contribution of proteins to the total protein mass in the platelet proteome: only 18 proteins account for 20%, 171 proteins for 50%, and 680 proteins for 75% of the total protein mass in human platelets.

cell lines, we aligned our data to quantitative proteome data from HeLa<sup>43</sup> and U2OS<sup>44</sup> cells. The combined datasets encompass a total of 9696 individual proteins (disregarding splice variants). The overlap is rather complete with only 4% of the proteins exclusively found in platelets, 69% present at least in 2 sets and 27% in all sets (Figure 2). Thirty-two percent of the platelet proteome overlaps with both sets. The overlap of the highly expressed proteins (95th percentile) of each dataset (platelets, HeLa, U2OS) is clearly higher. Fifty-eight percent of the 671 proteins in this set are present in all 3 cells and 88% in at least 2 cells. A subset of 28 proteins (4%) is exclusively present in platelets and is mainly composed of  $\gamma$ -actin, glycoproteins, and platelet and coagulation factors (supplemental Table 5A).

According to Engelhard et al,<sup>56</sup> the coverage of the total protein mass for the HeLa proteome is  $\sim 51\%$ , compared with 80% for the platelet proteome, whereas for U2OS data an appropriate analysis was not possible because of the cutoff thresholds in the data. The frequency distribution of logarithmized copy numbers indicates almost identical maximum and full width at half maximum values for HeLa ( $\mu = 4.4$ ,  $\sigma = 1.03$ ) and U2OS ( $\mu = 4.2$ ,  $\sigma = 0.85$ ). In contrast, the maximum of the frequency distribution of the platelet proteome is shifted to lower copy numbers and is narrowed ( $\mu = 3.16$ ,  $\sigma = 0.55$ ) reflecting the lower protein expression levels and a limited protein inventory (Figure 2). Protein copy numbers of the different cells do neither correlate for the complete proteome nor subsets based on the stratified data. The ratio of copy numbers for the platelet proteome in relation to the HeLa proteome is mainly between 0.02 and 0.1, in relation to the U2OS proteome between 0.05 and 0.5 (supplemental Table 5B). The distribution of protein sizes in the 3 datasets is almost identical to the total human proteome as provided by Swissprot, demonstrating the absence of any bias for protein size in mass spectrometry analysis.

In summary, the qualitative comparison of the human platelet proteome to HeLa and U2OS proteomes indicates considerable similarities which are virtually independent from copy numbers, while the distribution of copy numbers is similar for the 3 cell types. However, for platelets the smaller cell volume shifts the mean of the frequency distribution to lower copy numbers, whereas highly expressed proteins exclusively found in the platelet proteome are typical platelet proteins.

#### Potential contamination with other blood components

Because estimated copy numbers and stoichiometries in this study substantially reflect and extend present knowledge, we addressed the important issue of contamination with other blood components. In particular, the sponge-like structure of platelets<sup>57</sup> leads to an

extensive perfusion of the open canalicular system with plasma components. Consequently, a complete removal of plasma components is almost unattainable and it remains questionable to which extent the respective plasma proteins indeed are an actual and vital part of the platelet proteome. We therefore compared our dataset with the so-far most comprehensive plasma proteome study providing concentration estimates for a large set of plasma proteins.<sup>58</sup> Indeed, 149 of the 150 most abundant plasma components are also present in our dataset (supplemental Table 3), with albumin estimated to  $\sim 53\,000$  copies per platelet. The established concentration of albumin in plasma (50 mg/mL) corresponds to the occurrence of  $\sim 450\,000$  copies per fL. Considering the mean platelet volume of  $9.7 \pm 0.5$  fL, we conclude that our samples contained approximately 1% by volume (0.1 fL) plasma per platelet. From the occurrence of the second most abundant plasma protein transthyretin (0.77 mg/mL corresponding to 29 000 copies per fL) at 4500 copies per platelet, almost the same share, namely 1.5% by volume (0.15 fL) plasma per platelet can be deduced.

Considering that a significant amount of hemoglobin can be ascribed to marginal hemolysis during platelet preparation a relevant contamination by erythrocytes can almost be ruled out. The absence of one of the most abundant erythrocyte proteins, the band 3 anion transporter (1 000 000 copies per erythrocyte),<sup>59</sup> underlines this assumption. However, even if the detected hemoglobin (280 000 000 copies per erythrocyte) resulted from erythrocytes, the estimated 61 000 copies would account for less than 1 erythrocyte per 5000 platelets.

#### Platelet proteome variation

For the 1900 proteins relatively quantified between 4 different donors and covering a dynamic range of 4 orders of magnitude, we calculated SD over the obtained ratios, with the resulting median SD over all quantified proteins amounting to 0.14. To account for (1) technical variations during sample processing and (2) MS analysis, we classified all proteins with SD 2 times higher than the median SD as potentially differential. Although this is significantly lower than the usually expected biologic variance between humans ( $\gg 2\times$ ), 85% of the quantified proteins are below this cutoff and therefore show no/almost no variation between the analyzed donors.

Whereas individual deviations increase with lower copy numbers, there is no clear tendency toward variation for low abundant proteins; one-fourth of all proteins and one-fourth of the proteins with  $SD > 0.28$  have less than 1000 copies per platelet.

To assess the impact of a single outlier ratio (ie, 1 donor), for those proteins with  $SD > 0.28$ , we determined  $SD^{3\text{patients}}$  values

when omitting the most deviating ratio and set the  $SD^{3\text{patients}}$  cutoff to 0.14 ( $1 \times$  median  $SD$ ). Then, still 171 proteins (9%) show potential variation.

The group of differential proteins comprises potential/known contaminants such as hemoglobin, MHC molecules, and apolipoproteins, most likely because of slight differences during platelet isolation. In principle, for individual proteins, a differing degree of PTM as well as single amino acid exchanges might simulate a variation in protein abundance, caused by the reduced recovery of respective peptide sequences.

In contrast, proteins central to platelet function have low  $SD$  which predominantly reflect the technical error of iTRAQ-based protein quantification ( $\sim 20\%$ ): GPIb $\alpha$  0.05; GPIb $\beta$  0.11; GPIV 0.16; GPVI 0.24; GPIX 0.10; PAR4 0.25; platelet basic protein 0.09; platelet factor 4 0.13; STIM1 0.07; P2X1 0.17; VASP 0.11; LASP 0.10; PKA  $\sim 0.20$ ; PKG 0.10; actin  $\sim 0.10$ .

In principle, these results agree with Winkler et al who compared platelets from healthy donors using the difference in gel electrophoresis (DIGE) strategy and determined a coefficient of variation (CV) of 18% based on the relative quantification of 500 reproducibly found spots.<sup>60</sup> However, 2-dimensional gel spots can easily contain up to 10 or more proteins, rendering accurate and global quantification on the protein level challenging. In contrast, in this study, 1900 proteins were relatively quantified by MS (CV of 13%) yielding a substantial increase of quantitative information.

In addition, we assessed the intradonor variation by quantitatively analyzing platelet samples purified from 3 different blood donations of the same donor. We independently processed 2 technical replicates per sample and analyzed all 6 samples without prefractionation by LC-MS. Since omitting the prefractionation reduced the number of identified spectra per protein, the obtained NSAF values were used only for relative comparison. From the 1505 proteins which were identified in all samples with high confidence (CV 12.2%),  $\sim 85\%$  showed no variation (CV  $> 24.4\%$ ). Furthermore, technical variances determined using the 2 replicates per sample were 12.7%, 14.8%, and 13.8%, respectively (supplemental Table 6). Only 7 proteins were considered as regulated in both intrasubject and intersubject comparisons, namely DDX3X, SAMM50, UQCRH, GLTP, EIF5A, BCL2L1, and ACAA1, of which 3 are of mitochondrial origin.

These results confirm that platelet formation as membrane-encapsulated fragments of megakaryocytes is a highly reproducible and regulated process.

## Conclusion

We conducted a systematic and thorough proteomic characterization of highly pure human platelets (1) identifying  $\sim 4000$  platelet proteins, (2) estimating copy numbers per platelet for  $\sim 3700$  of those, relatively quantifying expression levels of (3) 1900 proteins between 4 healthy donors, and (4) 1500 proteins between 3 different blood samples of the same donor. Based on leukocount and quantitative proteome data, we conclude that the contamination with other blood components is negligible: for example, 1 leukocyte per  $10^6$  platelets and 1.0%-1.5% by volume plasma per platelet, which can be mainly attributed to the sponge-like structure of the open canalicular system.

To estimate copy numbers per platelet for the identified proteins, we used the established NSAF approach which we previously demonstrated as a robust method for quantifying proteins in complex mixtures. We related the obtained NSAF

values to a set of copy numbers found in the literature and found a surprisingly high degree of correlation ( $R^2 = 0.90$ ). Although absolute quantification based on spectral counting can suffer from errors up to 200% in individual cases (membrane proteins or proteins with an unusually frequent occurrence of PTM), known protein complex stoichiometries can be confirmed and unknown deduced.

To this end, our study provides a first comprehensive insight into the qualitative and quantitative platelet composition, allowing for a weighted appraisal of the physiologic relevance of distinct pathways and thus represents a rich source for the systematic search for novel platelet functions and mechanisms.

In addition, our quantitative proteomic comparisons between 4 healthy donors as well as 3 different blood samples from the same donor demonstrate that, despite the dynamics of the proteome and the high biologic variance between human beings, platelet formation from megakaryocytes is a highly regulated and reproducible process yielding only minor differences in protein expression patterns. As we demonstrate, thorough and monitored isolation of platelets from fresh blood donations, combined with quality controlled sample preparation and proteomic analysis, yields highly reproducible data. Thus, results and analytical strategy of the present study build a foundation for future investigations which can directly address issues of utmost clinical relevance such as differences in the response to antiplatelet treatment or in the pathologic potential for the genesis of cardiovascular diseases. Since between healthy donors  $> 85\%$  of the proteome show no variation, by combining an elaborate quantitative analytical strategy and the appropriate individual patient and/or patient cohort (and size) with the right scientific questions, novel biomarkers, drug targets or key mediators of platelet function can be identified starting from small blood donations. In principle, already less than 100  $\mu\text{g}$  of protein per donor ( $\sim 4 \times 10^7$  platelets, a few mL of blood) will be sufficient indicating that platelet proteome analyses of individual donors and/or patients is possible.

The quantitative results presented in our manuscript demonstrate the feasibility to assess on a global scale quantitative protein differences between (1) normal and well-defined dysfunctional platelets from individual persons, (2) platelets with normal and pathologic response to antiplatelet drugs, and (3) platelets obtained from patients at various stages of chronic cardiovascular and inflammatory diseases. Some such ambitious studies are currently initiated.

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J.M.B. and M.V. are PhD candidates at Universities of Dortmund, Germany and Ghent, Belgium and this work is submitted in partial fulfillment of the requirement of the PhD.

## Authorship

Contribution: J.M.B. designed and performed research, analyzed the data, and wrote the manuscript; M.V. developed the data



interpretation pipeline, analyzed the data, and wrote the manuscript; S.G. prepared and quality controlled platelet samples, and contributed to study design and the writing of the manuscript; S.R. conducted experiments and contributed to the writing of the manuscript; U.W. contributed to study design and the writing of the manuscript; L.M. contributed to data analysis; J.G. analyzed data, and contributed to study design and the writing of the manuscript; A.S. contributed to study design and the writing of the manuscript; and R.P.Z. conceived of the study, analyzed data, and wrote the manuscript.

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## References

- Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. *Nat Rev Drug Discov*. 2010;9(2):154-169.
- Gorog DA, Sweeny JM, Fuster V. Antiplatelet drug 'resistance'. Part 2: laboratory resistance to antiplatelet drugs—fact or artifact? *Nat Rev Cardiol*. 2009;6(5):365-373.
- Sweeny JM, Gorog DA, Fuster V. Antiplatelet drug 'resistance'. Part 1: mechanisms and clinical measurements. *Nat Rev Cardiol*. 2009;6(4):273-282.
- Spectre G, Mosseri M, Abdelrahman NM, et al. Clinical and prognostic implications of the initial response to aspirin in patients with acute coronary syndrome. *Am J Cardiol*. 2011;108(8):1112-1118.
- Pinto Slottow TL, Bonello L, Gavini R, et al. Prevalence of aspirin and clopidogrel resistance among patients with and without drug-eluting stent thrombosis. *Am J Cardiol*. 2009;104(4):525-530.
- Semple JW, Italiano JE Jr, Freedman J. Platelets and the immune continuum. *Nat Rev Immunol*. 2011;11(4):264-274.
- Garcia A, Prabhakar S, Hughan S, et al. Differential proteome analysis of TRAP-activated platelets: involvement of DOK-2 and phosphorylation of RGS proteins. *Blood*. 2004;103(6):2088-2095.
- Garcia A, Quinton TM, Dorsam RT, Kunapuli SP. Src family kinase-mediated and Erk-mediated thromboxane A2 generation are essential for VWF/GPIb-induced fibrinogen receptor activation in human platelets. *Blood*. 2005;106(10):3410-3414.
- Garcia A. Proteome analysis of signaling cascades in human platelets. *Blood Cells Mol Dis*. 2006;36(2):152-156.
- Senis YA, Tomlinson MG, Garcia A, et al. A comprehensive proteomics and genomics analysis reveals novel transmembrane proteins in human platelets and mouse megakaryocytes including G6b-B, a novel immunoreceptor tyrosine-based inhibitory motif protein. *Mol Cell Proteomics*. 2007;6(3):548-564.
- Lewandrowski U, Wortelkamp S, Lohrig K, et al. Platelet membrane proteomics: a novel repository for functional research. *Blood*. 2009;114(1):e10-e19.
- Qureshi AH, Chaoji V, Maiguel D, et al. Proteomic and phospho-proteomic profile of human platelets in basal, resting state: insights into integrin signaling. *PLoS One*. 2009;4(10):e7627.
- Dowal L, Yang W, Freeman MR, Steen H, Flaumenhaft R. Proteomic analysis of palmitoylated platelet proteins. *Blood*. 2011;118(13):e62-e73.
- Lewandrowski U, Zahedi RP, Moebius J, Walter U, Sickmann A. Enhanced N-glycosylation site analysis of sialoglycopeptides by strong cation exchange prefractionation applied to platelet plasma membranes. *Mol Cell Proteomics*. 2007;6(11):1933-1941.
- Zahedi RP, Lewandrowski U, Wiesner J, et al. Phosphoproteome of resting human platelets. *J Proteome Res*. 2008;7(2):526-534.
- Dittrich M, Birschmann I, Mietner S, Sickmann A, Walter U, Dandekar T. Platelet protein interactions: map, signaling components, and phosphorylation groundstate. *Arterioscler Thromb Vasc Biol*. 2008;28(7):1326-1331.
- Parguina AF, Grigorian-Shamajian L, Agra RM, et al. Proteins involved in platelet signaling are differentially regulated in acute coronary syndrome: a proteomic study. *PLoS One*. 2010;5(10):e13404.
- Nurden A, Nurden P. Advances in our understanding of the molecular basis of disorders of platelet function. *J Thromb Haemost*. 2011;9(Suppl 1):76-91.
- Parguina AF, Rosa I, Garcia A. Proteomics applied to the study of platelet-related diseases: Aiding the discovery of novel platelet biomarkers and drug targets [published online ahead of print May 8, 2012]. *J Proteomics*. doi:10.1016/j.jprot.2012.04.043.
- Gambaryan S, Kobsar A, Rukoyatkin N, et al. Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB-IkappaB complex. *J Biol Chem*. 2010;285(24):18352-18363.
- Burkhart JM, Schumbrutski C, Wortelkamp S, Sickmann A, Zahedi RP. Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *J Proteomics*. 2012;75(4):1454-1462.
- Ross PL, Huang YN, Marchese JN, et al. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics*. 2004;3(12):1154-1169.
- Paoletti AC, Parmely TJ, Tomomori-Sato C, et al. Quantitative proteomic analysis of distinct mammalian mediator complexes using normalized spectral abundance factors. *Proc Natl Acad Sci U S A*. 2006;103(50):18928-18933.
- Colaert N, Gevaert K, Martens L. RIBAR and xRIBAR: methods for reproducible relative MS/MS-based label-free protein quantification. *J Proteome Res*. 2011;10(7):3183-3189.
- Watson SP, Harrison P. The vascular function of platelets. In: Hoffbrand AV, Catovsky D, Tuddenham EGD, Green AR, eds. *Postgraduate Haematology*. Oxford, UK: Wiley-Blackwell; 2011.
- Fischer TH, Merricks EP, Bode AP, et al. Thrombus formation with rehydrated, lyophilized platelets. *Hematology*. 2002;7(6):359-369.
- Eigenthaler M, Nolte C, Halbrugge M, Walter U. Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets. Estimating the rate of cAMP-regulated and cGMP-regulated protein phosphorylation in intact cells. *Eur J Biochem*. 1992;205(2):471-481.
- Proty MB, Watkins NA, Colombo D, et al. Identification of Tspan9 as a novel platelet tetraspanin and the collagen receptor GPVI as a component of tetraspanin microdomains. *Biochem J*. 2009;417(1):391-400.
- López JA, Berndt MC. The GPIb-IX-V complex. In: Michelson AD, ed. *Platelets*. New York: Elsevier; 2002.
- Berndt MC, Gregory C, Kabral A, Zola H, Fournier D, Castaldi PA. Purification and preliminary characterization of the glycoprotein Ib complex in the human platelet membrane. *Eur J Biochem*. 1985;151(3):637-649.
- Hartwig JH. The platelet: form and function. *Semin Hematol*. 2006;43(1 Suppl 1):S94-S100.
- van Schravendijk MR, Handunnetti SM, Barnwell JW, Howard RJ. Normal human erythrocytes express CD36, an adhesion molecule of monocytes, platelets, and endothelial cells. *Blood*. 1992;80(8):2105-2114.
- Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol*. 2008;28(3):403-412.
- Shattil SJ, Ginsberg MH, Brugge JS. Adhesive signaling in platelets. *Curr Opin Cell Biol*. 1994;6(5):695-704.
- Best D, Senis YA, Jarvis GE, et al. GPVI levels in platelets: relationship to platelet function at high shear. *Blood*. 2003;102(8):2811-2818.
- Cossemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW. Multiple ways to switch platelet integrins on and off. *J Thromb Haemost*. 2008;8(8):1253-1261.
- Diacovo TG, deFougerolles AR, Bainton DF, Springer TA. A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. *J Clin Invest*. 1994;94(3):1243-1251.
- Holada K, Mondoro TH, Muller J, Vostal JG. Increased expression of phosphatidylinositol-specific phospholipase C resistant prion proteins on the surface of activated platelets. *Br J Haematol*. 1998;103(1):276-282.
- Kelton JG. The pathophysiology of heparin-induced thrombocytopenia: biological basis for treatment. *Chest*. 2005;127(2 Suppl):9S-20S.
- Geberhiwot T, Ingerpuu S, Pedraza C, et al. Blood platelets contain and secrete laminin-8 (alpha4beta1gamma1) and adhere to laminin-8 via alpha6beta1 integrin. *Exp Cell Res*. 1999;253(2):723-732.
- Rowley JW, Oler AJ, Tolley ND, et al. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*. 2011;118(14):E101-E111.
- Dittrich M, Birschmann I, Pfrang J, et al. Analysis of SAGE data in human platelets: features of the transcriptome in an anucleate cell. *Thromb Haemost*. 2006;95(4):643-651.
- Nagaraj N, Wisniewski JR, Geiger T, et al. Deep proteome and transcriptome mapping of a human cancer cell line. *Mol Syst Biol*. 2011;7:548.
- Beck M, Schmidt A, Malmstroem J, et al. The quantitative proteome of a human cell line. *Mol Syst Biol*. 2011;7:549.
- Santoni V, Molloy M, Rabilloud T. Membrane proteins and proteomics: an amour impossible? *Electrophoresis*. 2000;21(6):1054-1070.
- Bikoue A, George F, Poncelet P, Mutin M, Janossy G, Sampol J. Quantitative analysis of leukocyte membrane antigen expression: normal adult values. *Cytometry*. 1996;26(2):137-147.
- Amisten S, Braun OO, Bengtsson A, Erlinge D. Gene expression profiling for the identification of

- G-protein coupled receptors in human platelets. *Thromb Res*. 2008;122(1):47-57.
48. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol*. 2010;30(12):2341-2349.
49. Jardin I, Lopez JJ, Pariente JA, Salido GM, Rosado JA. Intracellular calcium release from human platelets: different messengers for multiple stores. *Trends Cardiovasc Med*. 2008;18(2):57-61.
50. Authi KS. TRP channels in platelet function. *Handb Exp Pharmacol*. 2007(179):425-443.
51. Cahalan MD. STIMulating store-operated Ca(2+) entry. *Nat Cell Biol*. 2009;11(6):669-677.
52. Bolotina VM, Orai, STIM1 and iPLA2beta: a view from a different perspective. *J Physiol*. 2008; 586(13):3035-3042.
53. Redondo PC, Salido GM, Pariente JA, Sage SO, Rosado JA. SERCA2b and 3 play a regulatory role in store-operated calcium entry in human platelets. *Cell Signal*. 2008;20(2):337-346.
54. Wangorsch G, Butt E, Mark R, et al. Time-resolved in silico modeling of fine-tuned cAMP signaling in platelets: feedback loops, titrated phosphorylations and pharmacological modulation. *BMC Syst Biol*. 2011;5:178.
55. Lundberg E, Gry M, Oksvold P, et al. The correlation between cellular size and protein expression levels—normalization for global protein profiling. *J Proteomics*. 2008;71(4):448-460.
56. Engelhard HH, 3rd Krupka JL, Bauer KD. Simultaneous quantification of c-myc oncoprotein, total cellular protein, and DNA content using multiparameter flow cytometry. *Cytometry*. 1991;12(1):68-76.
57. Adelson E, Rheingold JJ, Crosby WH. The platelet as a sponge: a review. *Blood*. 1961;17:767-774.
58. Farrah T, Deutsch EW, Omenn GS, et al. A high-confidence human plasma proteome reference set with estimated concentrations in PeptideAtlas. *Mol Cell Proteomics*. 2011;10(9):M110006353.
59. Hogh B, Petersen E, Crandall I, Gottschau A, Sherman IW. Immune responses to band 3 neoantigens on Plasmodium falciparum-infected erythrocytes in subjects living in an area of intense malaria transmission are associated with low parasite density and high hematocrit value. *Infect Immun*. 1994;62(10):4362-4366.
60. Winkler W, Zellner M, Diestinger M, et al. Biological variation of the platelet proteome in the elderly population and its implication for biomarker research. *Mol Cell Proteomics*. 2008;7(1):193-203.