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High Precision Platelet Releasate Definition by Quantitative Reversed Protein Profiling—Brief Report

Patrick Wijten, Thijs van Holten, Liy Liy Woo, Onno B. Bleijerveld, Mark Roest, Albert J.R. Heck, Arjen Scholten

Objective—Platelet activation and subsequent protein release play an important role in healthy hemostasis and inflammatory responses, yet the identity and quantity of proteins in the platelet releasate are still debated. Here, we present a reversed releasate proteomics approach to determine unambiguously and quantitatively proteins released from activated platelets.

Approach and Results—Isolated platelets were mock and fully stimulated after which the released proteins in the supernatant were removed. Using high-end proteomics technology (2D chromatography, stable isotope labeling, electron transfer dissociation, and high collision dissociation fragmentation) allowed us to quantitatively discriminate the released proteins from uncontrolled lysis products. Monitoring the copy numbers of ≈ 4500 platelet proteins, we observed that after stimulation via thrombin and collagen, only 124 ($<3\%$) proteins were significantly released ($P < 0.05$). The released proteins span a concentration range of ≥ 5 orders, as confirmed by ELISA. The released proteins were highly enriched in secretion tags and contained all known factors at high concentrations (>100 ng/mL, eg, thrombospondin, von Willebrand factor, and platelet factor 4). Interestingly, in the lower concentration range of the releasate many novel factors were identified.

Conclusions—Our reversed releasate dataset forms the first unambiguous, in depth repository for molecular factors released by platelets. (*Arterioscler Thromb Vasc Biol.* 2013;33:1635-1638.)

Key Words: mass spectrometry ■ platelet ■ platelet activation ■ proteomics

It is widely accepted that activated platelets release proteins into the circulation via various mechanisms, of which the α -granule route and shedding are the most established. The protein constituents of this releasate are associated with inflammation, coagulation, (tumor) angiogenesis, cell growth, and adhesion.¹⁻⁵ Although some of these factors are routinely assayed in the clinic, a comprehensive and unambiguous quantitative map of the platelet releasate is still at large. Previous releasate proteomics approaches relied on the isolation of α -granules from isolated platelets^{6,7} or the investigation of the isolated supernatant after in vitro platelet stimulation.^{8,9} However, these direct methods experience a high false-positive rate because of contamination by uncontrolled platelet lysis. Here, we take a reverse approach, monitoring quantitatively the concentration changes of all proteins in the platelets after platelet stimulation, whereby we assume that the released protein content should be detectable in the reduction of its level from the whole platelet proteome. For accuracy, our strategy uses stable isotope-labeling (Figure 1A) to discriminate the released proteins from uncontrolled lysis products. In other

words, most platelet proteins do not change except the ones that are significantly released on activation.

Materials and Methods

Materials and Methods are available in the online-only Supplement.¹⁰⁻¹³

Results

Performing the reversed releasate quantitative proteomics experiments on 3 individuals resulted in the identification of 4375 unique proteins, of which 2970 (68%) could be quantified in ≥ 2 of 3 individuals (Table I in the online-only Data Supplement).

Identity of the Platelet Releasate

First, we set out to identify the releasate on full platelet activation. The releasate was evaluated in each individual by the determination of the dimethyl intensity ratio between the light (resting) and intermediate (activated) labeled peptides (Figure 1A). As expected, the vast majority of

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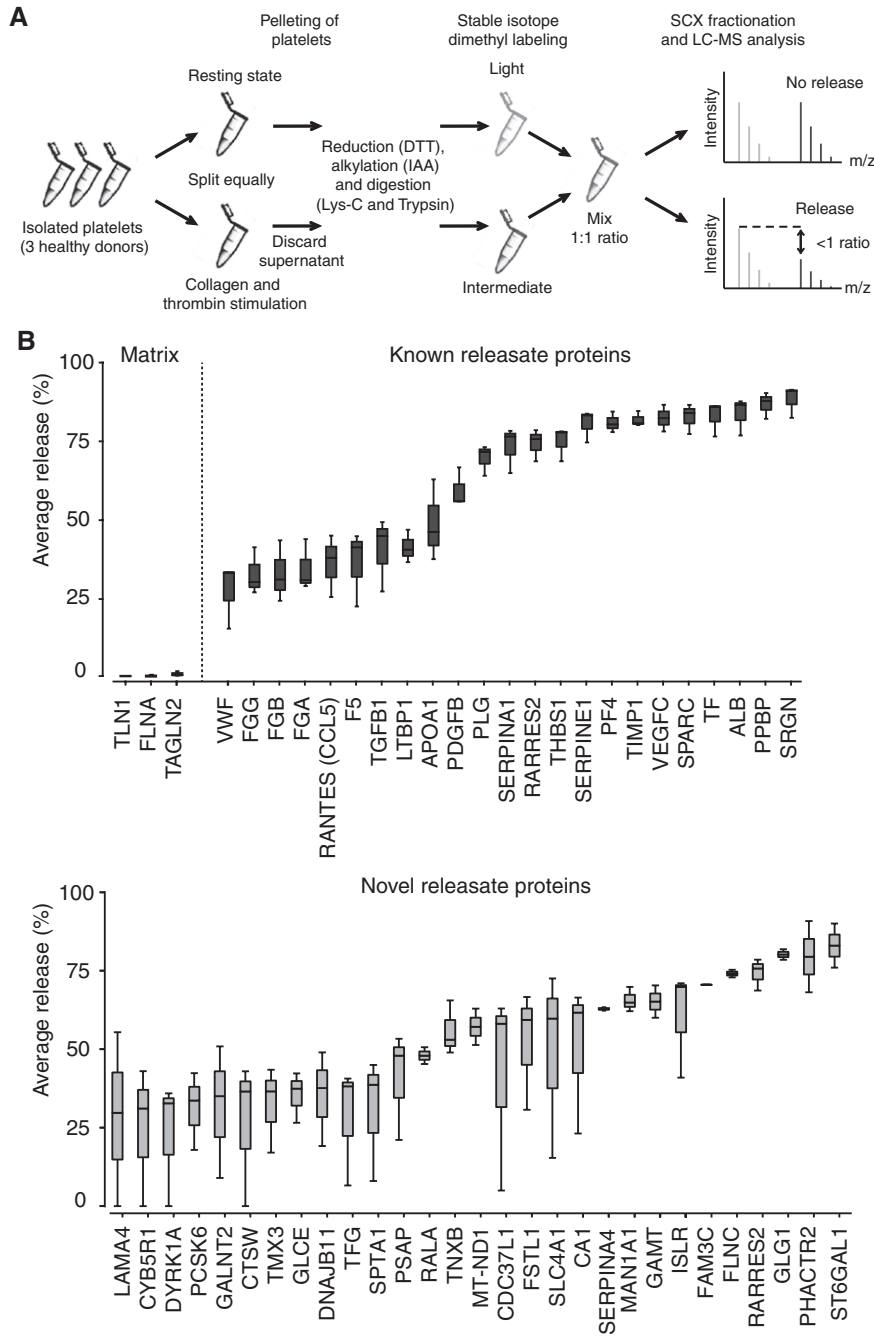


Figure 1. Quantitative profiling of the platelet reversed releasate. **A**, Flowchart depicting platelet reversed releasate analysis: platelets from 3 individual healthy donors were isolated and analyzed to exclude donor-specific anomalies. Half of each platelet sample was left untreated, whereas the other half was stimulated with a combination of thrombin and collagen. Both samples were thoroughly washed and subsequently, the supernatant was removed. The platelets were lysed, digested with Lys-C and trypsin, and differentially labeled with stable isotopes using dimethylation (light and intermediate), and subsequently mixed before strong cation exchange (SCX) chromatography and reversed phase chromatography-tandem mass spectrometry (LC-MS/MS) analysis. **B**, Selection of quantified proteins being strongly, weakly, or not released on platelet activation. Different releasate proteins release their copy number to a different extent as depicted by box-plots, which indicate the percentage of release (average and SD acquired from experiments with 3 donors). Displayed are nonreleased matrix proteins (black), a selection of known releasate proteins (dark gray), and novel releasate proteins (light gray). All 124 released proteins are depicted in Table II in the online-only Data Supplement. CCL5 indicates chemokine (C-C motif) ligand 5 (also known as RANTES). Depicted are the gene names as deposited in the uniprot database at www.uniprot.org.

proteins (>95%) had a resting/activated ratio close to 1.0. These ratios were highly reproducible between replicates with a median RSD between ratios of 9.6% (Table I and Figure I in the online-only Data Supplement). Using the statistical criteria described in the Methods section in the online-only Data Supplement, 124 proteins were observed to be significantly reduced in the activated platelets, in between 25% and 90% (Figure 1B; Table II in the online-only Data Supplement).

Measurement of the Releasate

Our approach allows us to quantify the full proteome of activated and mock-activated platelets and consequently the releasate. We semiquantitatively quantified¹⁴⁻¹⁸ the entire

detected proteome using a robust nonlinear fit of the molecular weight corrected spectral counts to a set of proteins with a known copy number per cell.¹⁸ As we determined the fraction released for each protein in the previous section, we can use the average number of 2×10^8 platelets per milliliter blood to provide the concentration of each protein in the releasate in nanogram per milliliter blood (see Methods section in the online-only Data Supplement). We observed that the protein concentrations in the releasate span >5 orders of magnitude, ranging from highly abundant ($\mu\text{g/mL}$) hallmark releasate proteins, such as thrombospondin-1, platelet basic protein, and platelet factor 4, to lower abundant (pg/mL) novel releasate proteins, such as Tenascin-X and Kallistatin (Table II in the online-only Data Supplement). To assess whether

these numbers make sense, we performed ELISA experiments for several releasate proteins within the supernatant of the stimulated platelets (Figure 2A). Finally, we classified the releasate according to major functional categories associated with platelet activation (Figure 2B).

Discussion

Overall, this comprehensive platelet proteome compares both qualitatively and quantitatively very well with the 4116 unique proteins identified by Burkhart et al¹⁸ recently (Figures II and III in the online-only Data Supplement). The releasate as determined in our study is remarkably small in relation to previous reports,^{8,9} which by using a qualitative, instead of a quantitative, method probably contains many proteins originating from undesired platelet lysis (Actins, Talins, etc). As a benchmark for our approach, all currently known, important releasate proteins are present in our dataset, such as platelet factor 4, chemokine ligand 5, von Willebrand factor etc (Figure 1B; Figure IV in the online-only Data Supplement). Convincingly, 80% of the proteins identified in our releasate are documented, or predicted, to be secreted as based on SignalP¹⁹ and Uniprot (Figure 2B; Figure V in the online-only Data Supplement). Closer

inspection revealed that proteins involved in cell and matrix interactions, which is associated with wound healing, were among the highly released proteins ($\approx 2 \mu\text{g/mL}$). There are also several novel releasate proteins found in this study, as determined by String network analysis (Figure 2B; Figure IV in the online-only Data Supplement). For example, Follistatin-like protein 1 has been found in osteoblasts and fibroblasts and characterized to play a role in the inflammatory pathway²⁰; filamin C plays a role in the crosslinking of actin in muscle cells²¹; and tenascin-X binds to several collagen subtypes and plays a role in wound healing, as shown in patients with Ehlers–Danlos syndrome.²²

The calculated released concentrations match with the concentration determined by ELISA within a margin of 1 order, establishing that our calculated proteomics-based protein abundance is a good proxy for the actual content that is released.

In conclusion, we have developed a robust reversed releasate quantitative protein profiling method to more reliably identify and semiquantify the platelet releasate with high accuracy. It covers an unprecedented depth of 5 orders of magnitude, exposing many novel molecular determinants of the platelet releasate to inspire further investigations into their roles.

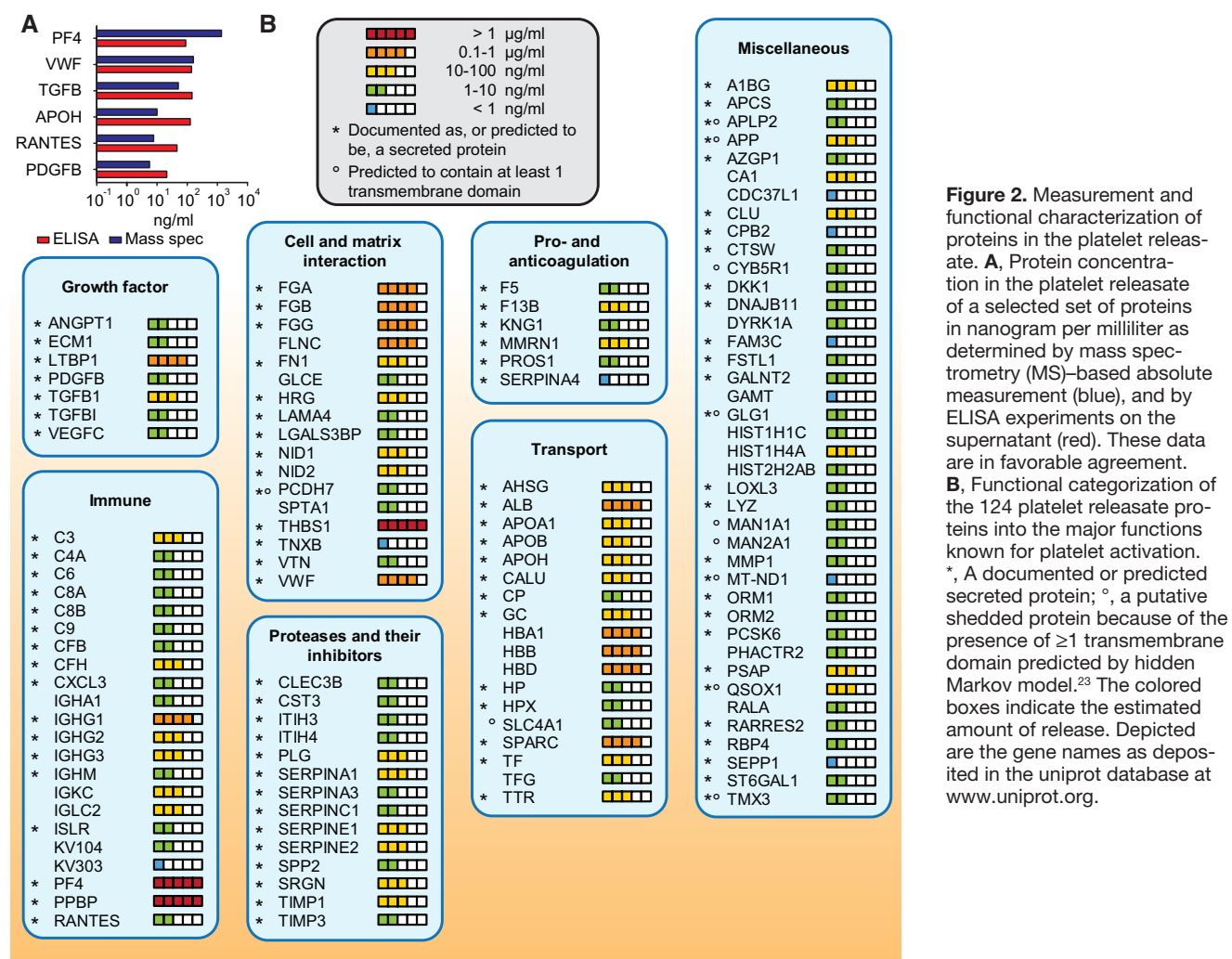


Figure 2. Measurement and functional characterization of proteins in the platelet releasate. **A**, Protein concentration in the platelet releasate of a selected set of proteins in nanogram per milliliter as determined by mass spectrometry (MS)-based absolute measurement (blue), and by ELISA experiments on the supernatant (red). These data are in favorable agreement. **B**, Functional categorization of the 124 platelet releasate proteins into the major functions known for platelet activation. *, A documented or predicted secreted protein; °, a putative shedded protein because of the presence of ≥ 1 transmembrane domain predicted by hidden Markov model.²³ The colored boxes indicate the estimated amount of release. Depicted are the gene names as deposited in the uniprot database at www.uniprot.org.

Acknowledgments

A. Scholten designed the study; P. Wijten, L.L. Woo, T. van Holten, and O.B. Bleijerveld performed experiments; P. Wijten and L.L. Woo analyzed data; M. Roest, A.J.R. Heck, and A. Scholten provided supervision; and P. Wijten, M. Roest, A.J.R. Heck, and A. Scholten wrote the article. Mirjam Damen is acknowledged for technical assistance. This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (<http://www.ctmm.nl>), project CIRCULATING CELLS (grant 01C-102), and supported by the Dutch Heart Foundation. The Netherlands Proteomics Center embedded in The Netherlands Genomics Initiative is kindly acknowledged for financial support (P. Wijten, A.J.R. Heck, and A. Scholten). The Cardiovascular "Focus en Massa" program at Utrecht University is acknowledged for additional financial support (A. Scholten).

Disclosures

None.

References

- Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost.* 2002;88:186–193.
- Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol.* 2010;30:2341–2349.
- Semple JW, Italiano JE Jr, Freedman J. Platelets and the immune continuum. *Nat Rev Immunol.* 2011;11:264–274.
- Peterson JE, Zurakowski D, Italiano JE Jr, Michel LV, Connors S, Oenick M, D'Amato RJ, Klement GL, Folkman J. VEGF, PF4 and PDGF are elevated in platelets of colorectal cancer patients. *Angiogenesis.* 2012;15:265–273.
- de Groot PG, Urbanus RT, Roest M. Platelet interaction with the vessel wall. *Handb Exp Pharmacol.* 2012:87–110.
- Maynard DM, Heijnen HF, Gahl WA, Gunay-Aygun M. The α -granule proteome: novel proteins in normal and ghost granules in gray platelet syndrome. *J Thromb Haemost.* 2010;8:1786–1796.
- Maynard DM, Heijnen HF, Horne MK, White JG, Gahl WA. Proteomic analysis of platelet alpha-granules using mass spectrometry. *J Thromb Haemost.* 2007;5:1945–1955.
- Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond JP, Cahill DJ, Emili A, Fitzgerald DJ, Maguire PB. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood.* 2004;103:2096–2104.
- Piersma SR, Broxterman HJ, Kapci M, de Haas RR, Hoekman K, Verheul HM, Jiménez CR. Proteomics of the TRAP-induced platelet releasate. *J Proteomics.* 2009;72:91–109.
- Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods.* 2009;6:359–362.
- Boerema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc.* 2009;4:484–494.
- Boerema PJ, Aye TT, van Veen TA, Heck AJ, Mohammed S. Triplex protein quantification based on stable isotope labeling by peptide dimethylation applied to cell and tissue lysates. *Proteomics.* 2008;8:4624–4632.
- Frese CK, Altelaar AF, Hennrich ML, Nolting D, Zeller M, Griep-Raming J, Heck AJ, Mohammed S. Improved peptide identification by targeted fragmentation using CID, HCD and ETD on an LTQ-Orbitrap Velos. *J Proteome Res.* 2011;10:2377–2388.
- Peng M, Taouatas N, Cappadona S, van Breukelen B, Mohammed S, Scholten A, Heck AJ. Protease bias in absolute protein quantitation. *Nat Methods.* 2012;9:524–525.
- Aye TT, Scholten A, Taouatas N, Varro A, Van Veen TA, Vos MA, Heck AJ. Proteome-wide protein concentrations in the human heart. *Mol Biosyst.* 2010;6:1917–1927.
- Lu P, Vogel C, Wang R, Yao X, Marcotte EM. Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nat Biotechnol.* 2007;25:117–124.
- Malmström J, Beck M, Schmidt A, Lange V, Deutsch EW, Aebersold R. Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. *Nature.* 2009;460:762–765.
- Burkhardt JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood.* 2012;120:e73–e82.
- Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods.* 2011;8:785–786.
- Wilson DC, Marinov AD, Blair HC, Bushnell DS, Thompson SD, Chaly Y, Hirsch R. Follistatin-like protein 1 is a mesenchyme-derived inflammatory protein and may represent a biomarker for systemic-onset juvenile rheumatoid arthritis. *Arthritis Rheum.* 2010;62:2510–2516.
- Fujita M, Mitsuhashi H, Isogai S, Nakata T, Kawakami A, Nonaka I, Noguchi S, Hayashi YK, Nishino I, Kudo A. Filamin C plays an essential role in the maintenance of the structural integrity of cardiac and skeletal muscles, revealed by the medaka mutant zacro. *Dev Biol.* 2012;361:79–89.
- Burch GH, Gong Y, Liu W, Dettman RW, Curry CJ, Smith L, Miller WL, Bristow J. Tenascin-X deficiency is associated with Ehlers-Danlos syndrome. *Nat Genet.* 1997;17:104–108.
- Sonnhammer EL, von Heijne G, Krogh A. A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc Int Conf Intell Syst Mol Biol.* 1998;6:175–182.

Significance

In this large-scale proteomics study, we have developed a novel method to identify and quantify the platelet releasate using a reversed protein profiling approach. In contrast to previous approaches, this method is able to distinguish between releasate proteins and false positives with high accuracy. This approach yielded the first semiquantitative map of the platelet releasate which turned out to be surprisingly small.

Supplemental Methods belonging to:

High precision platelet releasate definition by quantitative reversed protein profiling

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Platelet Isolation and Stimulation

Blood was collected with an open system, anticoagulated with 3.2% tri-sodium citrate (Merck). Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 160g for 15 minutes room temperature, no brake. Washed platelets were prepared by adding PRP with ACD (8.5mM tri-sodium citrate, 7.1mM citric acid, 5.5mM D-glucose; final concentrations) and centrifugation for 15 minutes at 340g at room temperature, no brake. The platelet pellet was resuspended in Tris-buffer (145mM NaCl, 5mM KCl, 260nM NaH₂PO₄, 1mM MgSO₄, 100mM Tris, 5.5mM D-glucose, pH 6.5) with 10ng/mL prostacyclin. Platelets were centrifuged for 15 minutes at 340g at room temperature and resuspended up to 200 x 10⁹/L in Tris-buffer (pH 7.4). Platelets were not used until 30 minutes after isolation. After stimulation with 5 µg/mL collagen and 1U/mL thrombin, platelet suspensions were centrifuged at 4000g for 2 minutes, the supernatant was aspirated and the platelet pellet was snap-frozen in liquid nitrogen.

Sample preparation

The stored platelet pellets were thawed and centrifuged at 14,000 g for 10 min at 4 °C. The pellet was reconstituted in a buffer containing 100 mM Tris, 10 mM DTT, 2% SDS at pH 8.0 with Complete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany) and PhosSTOP phosphatase inhibitor cocktail (Roche Diagnostics). The cell suspension was subsequently subjected to ultra-sonication as follows: (samples were pulsed for 30 seconds at 100% amplitude, 80% interval followed by 30 seconds rest in 3 cycles; subsequently, a final pulse of 60 seconds at 100% amplitude, 100% interval was performed. The remaining cell debris was removed by centrifugation at 14,000 g for 5 minutes at room temperature. Proteins were then reduced, alkylated and digested using the FASP approach, in which the buffer is exchanged to 8 M urea pH 8.0 in order to remove the SDS present in the sample, as described previously¹. Digestion was performed for 4 hours with Lys-C (Wako, Richmond, VA, USA) after which the mixture was diluted 4-fold to 2M urea and digested with trypsin (Promega, Madison, WI, USA) at 37 °C overnight. Finally the sample was acidified with formic acid to a final concentration of 5%. Tryptic peptides were desalted using Sep-Pak C18 cartridges (Waters Corporation, Milford, MA). The peptides were subsequently labeled on-column with stable isotope dimethyl labeling as described previously², the resting state platelets were labeled 'light', whereas the stimulated platelets received the 'intermediate' label. Labeling efficiency was checked by LC-MS/MS before mixing the pools in a 1:1 ratio.

Strong Cation Exchange (SCX) Chromatography

The samples were dried *in vacuo*, and re-suspended in 10% formic acid. SCX was performed on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a nanopump, a micro well plate autosampler, a multiple wavelength detector and a fraction collector. The columns used were a Opti Lynx C18, 40 µm, 100 Å, 2.1 mm x 15 mm (Optimize Technologies, Oregon, OR) for trapping and a Zorbax BioSCX-series II (Agilent Technologies, Waldbronn, Germany) 0.8 mm x 50 mm analytical column with 3.5 µm particles. A total of 250 µg of protein digest was loaded on the trapping column using a buffer containing 0.05% formic acid, pH 2.9 onto the trap column and subsequently eluted using a buffer containing 0.05% formic acid, 80% acetonitrile, pH 2.9 (buffer A) onto the analytical column. For SCX separation, buffer A and a buffer containing 0.05% formic acid, 80% acetonitrile, pH 2.9 and 500 mM NaCl (buffer B) were used. The separation was performed by a nonlinear 65 min elution gradient: from 0-17 min, 0% buffer B; from 17-22 min, from 2-3% buffer B; from 22-24 min, 3-5% buffer B; from 24-32 min, 5-8% buffer B; from 32-40 min, 8-20% buffer B; from 40-48 min, 20- 40% buffer B; from 48- 53 min, 40-90% buffer B. The column was subsequently washed for 3 min with 90% buffer B and finally equilibrated with 100% buffer A again for 9 mins. A total number of 50 SCX fractions were collected and dried in a vacuum centrifuge. Fractions 7-30 were reconstituted in 10% formic acid for further analysis.

Liquid chromatography and tandem mass spectrometry

LC-MS/MS was performed with a nano-LC coupled to an LTQ-Orbitrap Velos (Thermo Scientific, Bremen, Germany). The nano-LC consists of an Agilent 1200 series LC system equipped with a 20 mm ReproSil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) trapping column (packed in-house, i.d., 100 μ m; resin, 5 μ m) and a 400 mm ReproSil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) analytical column (packed in-house, i.d., 50 μ m; resin, 3 μ m) arranged in a vented-column configuration. The flow was passively split to 100 nl/min. A 3 hour elution profile consisting of: 0-10 min isocratic solvent A (0.1 M acetic acid) at 5 μ L/min for sample trapping, followed by a gradient of 10.1-117 min, 10-25% solvent B (0.1 M acetic acid in 80% acetonitrile); 117.1-152 min, 25-50% solvent B; 152.1-154 min, 100% solvent B, 154.1-169, 0% solvent B. Nanospray was achieved using a distally coated fused silica emitter (made in-house, o.d. 375 μ m; i.d. 20 μ m) biased to 1.7 kV. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS. The high resolution survey full scan was acquired in the orbitrap from m/z 350 to m/z 1500 with a resolution of 30,000 (FHMW) whereas the MS² scan is acquired at a resolution of 7500. The ten most intense precursors were isolated with an isolation width of 1.5 m/z and fragmented using a data-dependent decision tree method utilizing HCD, ETD-IT (ion trap read-out) and ETD-FT (orbitrap read-out)³. In brief, doubly charged peptides were always subjected to HCD fragmentation as well as triply charged peptides with an m/z >750 Th and are analyzed by the orbitrap. Triply charged peptides were fragmented by ETD when m/z < 750 Th. Analysis of ETD fragment ions was performed in the ion trap (ETD-IT). Quadruply charged ions were all fragmented with ETD and the fragments were analyzed either by ETD-IT (m/z >1000 Th) or the orbitrap (ETD-FT, m/z <1000 Th). Higher charged species were always analyzed by ETD-IT. The normalized collision energy for HCD was set to 35%. ETD reaction time was set to 50 ms for doubly charged precursors. Supplemental activation was enabled.

Data analysis: Identification and Quantitation

Peak lists were generated from the raw data files using the Proteome Discoverer software package version 1.3.339 (Thermo Scientific, Bremen, Germany). Peptide identification was performed by searching the individual peak lists (HCD, ETD-IT and ETD-FT) against a concatenated target-decoy database containing the human sequences in the Uniprot database (release 2012_06) supplemented with a common contaminants database using the Mascot search engine version 2.3 (Matrix Science, London, United Kingdom) via the Proteome Discoverer interface. The search parameters included the use of semitrypsin as proteolytic enzyme allowing up to a maximum of 2 missed cleavages. Carbamidomethylation of cysteines was set as a fixed modification whereas oxidation of methionines and the dimethyl "light" and "intermediate" labels on N-termini and lysine residues were set as variable modifications. Precursor mass tolerance was initially set at 50 ppm, while fragment mass tolerance was set at 0.6 Da for ETD-IT fragmentation and 0.05 Da for HCD and ETD-FT fragmentation. Subsequently, the peptide identifications were filtered for true mass accuracy <4 ppm and an ion score of 40 until an FDR <1% at peptide level was achieved. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository⁴ with the dataset identifier PXD000072 and DOI 10.6019/PXD000072.

Peptide and protein quantification were performed using Proteome Discoverer as described previously⁵. Protein ratios were normalized per replicate based on the average ratio of the top 3 cytoskeletal platelet proteins based on the number of PSMs: Talin-1, Filamin-A and Myosin-9.

Releasate determination

For each biological replicate a 95% confidence interval was used to determine the outliers. The ratios were binned according to the number of quantification counts in such a fashion that each bin consists of at least 200 proteins and proteins with an identical quantification count can only be contributed to 1 bin. Proteins that were determined as outlier with a downregulated resting / activation ratio in at least 2 of 3 biological replicates are contributed to the releasate. The % release was subsequently calculated by $(1 - \text{the average ratio of the 3 replicates}) \times 100\%$, only proteins with a positive % were considered.

Release concentration calculation

The number of spectral counts for each protein was corrected for its respective molecular weight and served as a proxy for the protein abundance, as described previously by us and others⁶⁻¹⁰. Next, the proteome was calibrated using a set of proteins for which the copy numbers per platelet is known and interpolating the unknown proteins using a robust non-linear fit, similar to Burkhardt et al⁶. Assuming 200 million platelets per ml blood, the copy number per cell could be expressed as ng protein per ml blood by multiplying the copy number of a particular protein with the amount of platelets per ml blood and the molecular weight of the protein, divided by Avogadro's number. Finally we were able to calculate the amount of protein released into the bloodstream (if all platelets are assumed to be fully activated), by multiplying the ng/ml concentration of a particular protein with its respective % release as determined from the resting / activated ratio.

Enzyme linked immunosorbent assays (ELISA's) on releasate

Washed platelets were stimulated with using 5 µg/mL collagen and 1U/mL thrombin for 5 minutes. Platelet suspensions were centrifuged at 4000g for 2 minutes and the supernatant was collected. Nunc (Greiner) plates were coated with mouse anti-human β-TG (R&D, MAB393), mouse anti-human RANTES (R&D), recombinant PDGF-Rβ (R&D, DY220), mouse anti-human PF-4 (R&D, MAB7951), or rabbit anti-human vWF (DAKO, A0082). Standards for intrapolation of protein concentrations were prepared with normal pool serum, except for PDGF-BB that was prepared with recombinant protein. Plates were blocked with 1% bovine serum albumin, and subsequently incubated with supernatants and standards. Plates were washed with phosphate buffered saline (PBS) pH 7.4 with 0.05% Tween 20. Bound factors were detected with biotin coupled goat anti-human β-TG (R&D, BAF393), goat anti-human RANTES (R&D, AB287-NA), biotin coupled goat anti-human PDGF-BB (R&D, DY220), goat anti-human PF-4 (R&D, AF795), or horse radish peroxidase (HRP) conjugated rabbit anti-human vWF (DAKO, P0226). Plates were washed with PBS pH 7.4 with 0.05% Tween 20. Biotin coupled antibodies were detected with streptavidin-HRP (DAKO, P0397), goat anti-human antibodies with rabbit anti-goat HRP antibodies (DAKO, P0449).

Supplemental References

1. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009;6:359-362
2. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc*. 2009;4:484-494
3. Frese CK, Altelaar AF, Hennrich ML, Nolting D, Zeller M, Griep-Raming J, Heck AJ, Mohammed S. Improved peptide identification by targeted fragmentation using cid, hcd and etd on an Itq-orbitrap velos. *J Proteome Res*. 2011;10:2377-2388
4. Vizcaino JA, Cote R, Reisinger F, Barsnes H, Foster JM, Rameseder J, Hermjakob H, Martens L. The proteomics identifications database: 2010 update. *Nucleic Acids Res*. 2010;38:D736-742
5. Scholten A, Mohammed S, Low TY, Zanivan S, van Veen TA, Delanghe B, Heck AJ. In-depth quantitative cardiac proteomics combining electron transfer dissociation and the metalloendopeptidase lys-n with the silac mouse. *Mol Cell Proteomics*. 2011;10:O111 008474
6. Burkhardt JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;120:e73-82
7. Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW, Aebersold R. Proteome-wide cellular protein concentrations of the human pathogen leptospira interrogans. *Nature*. 2009;460:762-765
8. Peng M, Taouatas N, Cappadona S, van Breukelen B, Mohammed S, Scholten A, Heck AJ. Protease bias in absolute protein quantitation. *Nat Methods*. 2012;9:524-525
9. Lu P, Vogel C, Wang R, Yao X, Marcotte EM. Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nat Biotechnol*. 2007;25:117-124
10. Aye TT, Scholten A, Taouatas N, Varro A, Van Veen TA, Vos MA, Heck AJ. Proteome-wide protein concentrations in the human heart. *Mol Biosyst*. 2010;6:1917-1927

Supplemental Material belonging to:

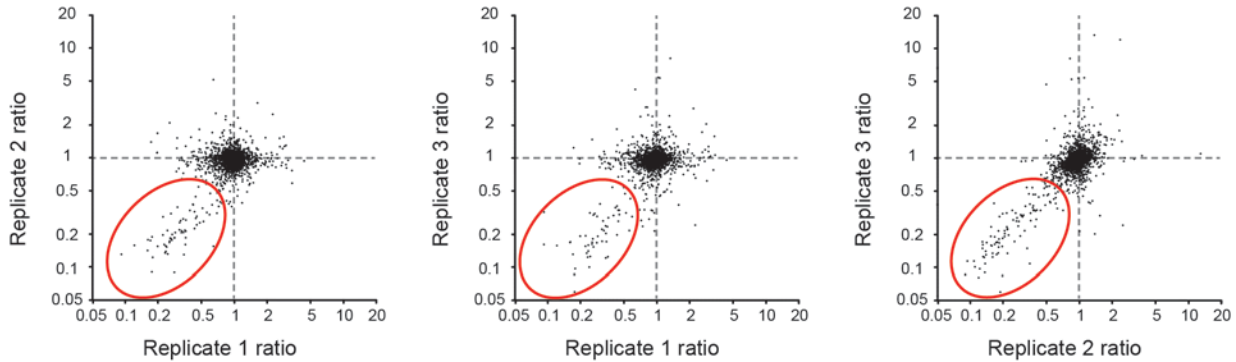
**High precision platelet releasate definition by
quantitative reversed protein profiling**

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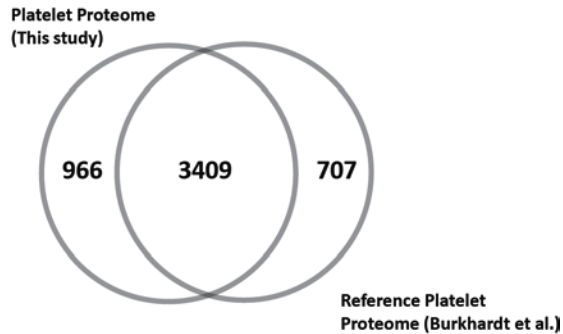
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Supplemental Figures

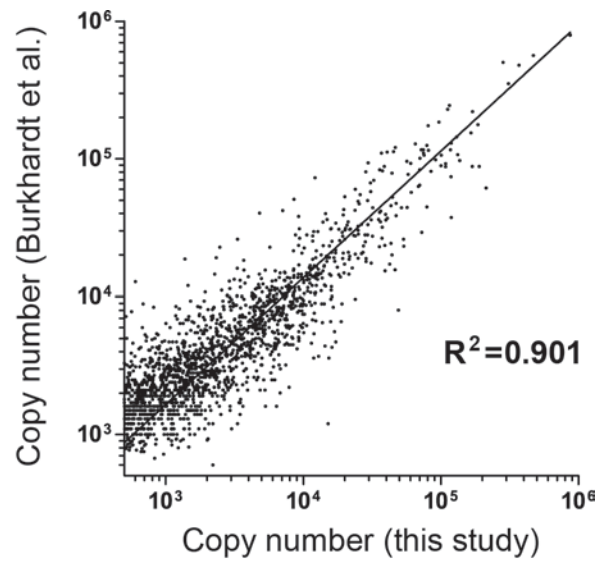


Supplemental Figure I. Reproducibility of the obtained quantitative release ratios. Correlation diagrams between the 3 biological replicates. The vast majority of the detected protein abundance ratios are close to 1 (high clustering of data points near the origin), the median coefficient of variation between the 3 replicas is 9.6% indicating the reproducibility of the measurements. Consistency within ratios of released proteins is revealed in the bottom left quadrant, as annotated by the red ovals.



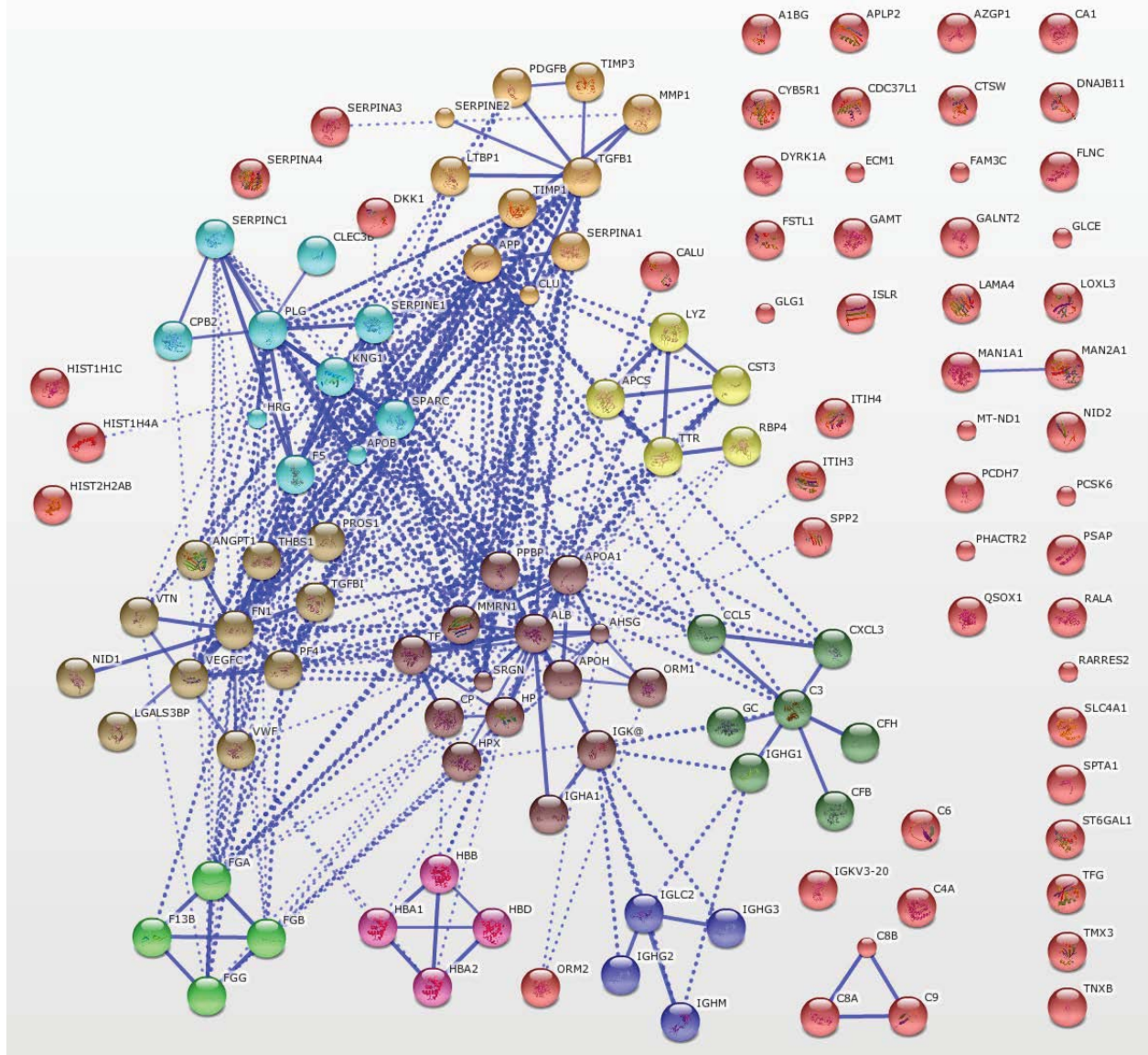
Supplemental Figure II. A comprehensive platelet proteome.

Overlap in protein identifications between the platelet proteomes determined in this study (left) and recently by Burkhardt et al.¹ (right). Both studies used a similar LC-MS/MS approach leading to similar numbers of identifications with a good overlap in protein identifications.



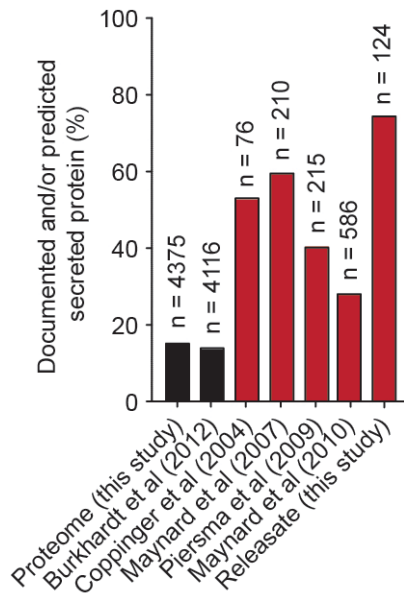
Supplemental Figure III. Strong correlation in platelet protein copy numbers.

The correlation between the copy number as determined in this study (x-axis) and that from the data of Burkhardt et al¹ (y-axis) shows strong correlation as represented by the Pearson coefficient (R^2).



Supplemental Figure IV. Network analysis of releasate proteins.

Network analysis of the releasate proteins using String version 9.05 in which the line thickness connecting the nodes indicate the confidence of the interaction. Proteins are clustered into 10 clusters using KMEANS based on the String global scores. The unconnected nodes are candidate novel releasate proteins that require further evaluation.



Supplemental Figure V. Comparison of secretion tag prevalence in releasate studies.

The prevalence of signal/secretion tags (using SignalP² and Uniprot) is highest in our reversed releasate dataset, when compared to previously published releasate proteomes³⁻⁶ (red) and the full platelet proteomes¹ (black) of Burkhardt et al.¹ and this study. The total number of non-redundant proteins identified in each of the releasate studies (n) is indicated above the bar.

Supplemental References

1. Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;120:e73-82
2. Petersen TN, Brunak S, von Heijne G, Nielsen H. Signalp 4.0: Discriminating signal peptides from transmembrane regions. *Nat Methods*. 2011;8:785-786
3. Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond JP, Cahill DJ, Emili A, Fitzgerald DJ, Maguire PB. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood*. 2004;103:2096-2104
4. Maynard DM, Heijnen HF, Gahl WA, Gunay-Aygun M. The alpha-granule proteome: Novel proteins in normal and ghost granules in gray platelet syndrome. *J Thromb Haemost*. 2010;8:1786-1796
5. Maynard DM, Heijnen HF, Horne MK, White JG, Gahl WA. Proteomic analysis of platelet alpha-granules using mass spectrometry. *J Thromb Haemost*. 2007;5:1945-1955
6. Piersma SR, Broxterman HJ, Kapci M, de Haas RR, Hoekman K, Verheul HM, Jimenez CR. Proteomics of the trap-induced platelet releasate. *J Proteomics*. 2009;72:91-109