Supplementary Material to Loroch et al. “Alterations of the platelet proteome in type I Glanzmann thrombasthenia caused by different homozygous delG frameshift mutations in ITGA2B” (Thromb Haemost 2017; 117.3)

S1: Suppl. Materials and methods

Materials

α-thrombin, apyrase grade-VII, adenosine diphosphate (ADP), arachidonic acid, epinephrine, EGTA, mouse IgG-FITC, rabbit anti-mouse IgG-FITC (Sigma-Aldrich, St. Louis, USA); Gly-Pro-Arg-Pro (Bachem Biochemica GmbH, Heidelberg, Germany); convulxin (pEnzo-Life-Sciences, Lörrach, Germany); Willfact® (LFB-Biotechnologies, Courtaboeuf Cedex, France); ristocetin-A-SO₄ (American Biochemical & Pharmaceuticals, London, UK); human fibrinogen-Alexa-Fluor®-488-conjugate, calcein-AM (Life Technologies GmbH, Carlsbad, USA); anti-α_{IIIbβ3}-FITC clone P2, anti-integrin-β1-FITC clone K20, anti-GPIbα clone SZ2 (Beckman-Coulter, Krefeld, Germany); anti-CD62P-FITC clone AK-4, anti-CD63-FITC clone H5C6, anti-GPIX-FITC clone Beb-1, anti-LAMP-1-PE clone H4A3 (Becton Dickinson, Heidelberg, Germany); anti-human GPVI-eFluorR-660 clone HY101 (eBioscience, Frankfurt, Germany); Quantum™ Simply Cellular® anti-mouse IgG (Bangs-Laboratories, Fishers, USA); anti-human carboxypeptidase-B2 clone #650801, goat anti-human FcγRIIa (R&D Systems, Minneapolis, Canada); goat anti-human plasminogen (Abcam, Cambridge, UK), rabbit anti-human α-actinin (Cell-Signaling-Technology, Denvers/Boston, USA); horse radish phosphatase-conjugated goat anti-mouse IgG, anti-rabbit IgG, Western-ECL-Blotting-Substrate (Bio-Rad Laboratories, Munich, Germany); horseradish peroxidase-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology, Dallas, USA); anti-α_{IIIbβ3} antibody abciximab (ReoPro®) was from Eli Lilly (Bad Homburg, Germany); mouse anti-FcγRIIa antibody (clone IV.3) was from Stemcell Techonologies (Vancouver, Canada); rabbit anti-mouse IgG F(ab’)2-fragment was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA);
IMUBIND® Vitronectin ELISA was from Sekisui Diagnostics (Pfungstadt, Germany); sequencing grade modified trypsin (Promega); Acclaim-PepMap columns (Thermo Scientific, Bremen, Germany); highrom-AEC-C18 bulk material (Advanced Chromatography Technologies, Aberdeen, Scotland); metal-coated silica emitters (New Objective, Woburn, MA, USA); solvents ULC/MS-grade (Biosolve, Valkenswaard, Netherlands); iTRAQ 8-plex-kits (ABSciex, Darmstadt, Germany).

Methods

Filter-aided sample preparation

Sample preparation of human platelets for proteome analysis was conducted as described by Beck et al. (1), with slight modifications. Platelets were lysed by adding 4x lysis buffer to a final concentration of 1% SDS, 150 mM NaCl, 1 tablet PhosStop/10 mL, pH 7.8. All samples were immediately shock-frozen and stored at -80°C until further usage. Protein concentration was determined by BCA and equal amounts of protein were processed in parallel. Protein concentration was determined and equal amounts were processed in parallel. Disulfide bonds were reduced and alkylated and samples were digested by filter-aided sample preparation (2, 3). After removing the lysis buffer by centrifugation (13,800 x g, 45 min), filters were washed three times with 100 µL 8 M urea in 150 mM Tris, pH 7.8 (13,800 x g, 20 min). Next, filters were washed three times with 100 µL 50 mM triethylammonium bicarbonate (TEAB; for iTRAQ experiments) / 50 mM ammonium bicarbonate (ABC; label free and targeted experiments). Tryptic digestion was performed by adding trypsin 1:30 (enzyme : sample ratio) in 100 µL digestion buffer (0.1 M urea in 150 mM Tris, pH 7.8, 1 mM CaCl₂, 50 mM TEAB or 50 mM ABC) followed by incubation for 15 h at 37°C. Peptides were collected by centrifugation at 13,800 x g for 30 min. Subsequently, the filters were washed with 50 µL of 50 mM TEAB/ABC and another 50 µL of ultra-pure water. All samples were adjusted to an
equal volume of ~180 µL. Quality control of all samples was performed using a monolithic HPLC system (digestion control) as described by Burkhart et al. (4).

**LC-MS/MS for label-free analysis**

Label-free analysis of the samples was conducted using an LTQ Orbitrap Velos Pro mass spectrometer online coupled to a U3000 HPLC equipped with in-house packed Hichrom ACE C18 columns (precolumn: 100 µm x 2 cm, 5 µm particles, 100 Å pore size; main column: 75 µm x 24 cm, 3 µm particles, 100 Å pore size). 400 ng of peptides were loaded in 0.1 % trifluoroacetic acid (TFA) onto the precolumn at a flow rate of 20 µl/min. After 10 minutes the precolumn was switched in-line with the main column and peptides were separated using a 60-minutes linear gradient from 3 to 35 % acetonitrile (ACN) in 0.1 % formic acid (FA) at a flow rate of 270 nL/min. A survey scan was acquired in the Orbitrap at a resolution of 60,000 and the 15 most intense peptide ions with charge states +2-4 were isolated, fragmented by collision-induced dissociation and MS/MS spectra were acquired in the linear ion trap. AGC target values were $10^6$ for MS and $10^4$ for MS/MS scans, maximum injection times were 100 ms for both MS and MS/MS. The dynamic exclusion was set to 10 seconds. All samples were analyzed in technical triplicates.

**iTRAQ labelling of peptides and high-pH reversed phase prefractionation**

30 µg of each sample were acidified with TFA and labelled with an iTRAQ 8-plex kit according to the manufacturer’s instruction. Samples were pooled, dried in a vacuum centrifuge, re-dissolved in 0.1% TFA and desalted using SPEC C18-AR 4 mg cartridges (Agilent).

30 µg of iTRAQ labelled peptides were loaded 15 µL of sample in buffer A (10 mM ammonium acetate, pH 6) onto a reversed phase chromatography system equipped with a Zorbax C18 column (0.5 mm x 150 mm, 5 µm particles, 300 Å pore size, Agilent Technologies). After 15 minutes of isocratic washing with 3 % B (10 mM ammonium acetate,
pH 6, 84 % ACN) peptides were separated using a linear gradient from 3 % to 45 % B in 60 minutes. Afterwards, the column was washed with 95 % B for 5 minutes. 12 fractions were collected from 15 to 75 minutes by concatenating 30 sec elution windows. All fractions were dried in a vacuum centrifuge and re-dissolved in 0.1 % TFA for LC-MS analysis.

**LC-MS/MS analysis of iTRAQ labelled peptides**

50% of each fraction were used for subsequent LC-MS/MS analysis using a Q-Exactive Plus mass spectrometer online coupled to a U3000 RSLC nanoHPLC equipped with an Acclaim PepMap trap-column (100 µm x 2 cm, 5 µm particles, 100 Å pores) and Acclaim PepMap main column (75 µm x 50 cm, 3 µm particles, 100 Å pores). Peptides were injected onto the trap column in 0.1% TFA using a flow of 20 µL / min. After 10 min the trap column was switched in-line and separation was conducted using a 115 min gradient ranging from 2.5 to 35 % ACN in 0.1% FA at a flow rate of 250 nL / min. A survey scan was acquired at a resolution of 70,000 and the 15 most intense peptide ions with a charge states +2-4 were isolated (2 m/z isolation window) and fragmented using higher-energy collision-induced dissociation (normalized collision energy of 30). AGC target values were $10^6$ for MS and $2 \times 10^5$ for MS/MS; maximum injection times were 120 ms for both. Fragment ion spectra were acquired at a resolution of 17,500. The dynamic exclusion was set to 12 seconds.

**Parallel reaction monitoring (PRM)**

PRM analyses were conducted using the same LC-MS equipment as for iTRAQ samples and monitoring a total of 57 peptides derived from 14 proteins: Coagulation factor XIII B chain (F13B, P05160); Plasminogen activator inhibitor 1 (PAI1, P05121); Heat shock protein beta-1 (HSPB1, P04792); Catalase (CATA, P04040); Plasminogen (PLMN, P00747); cAMP-dependent protein kinase type II-beta regulatory subunit (PRKAR2B, P31323); C-X-C motif chemokine 5 (CXCL5, P42830); Protein disulfide-isomerase A5 (PDIA5, Q14554); Laminin subunit alpha-4 (LAMA4, Q16363); Fermitin family homolog 2 (FERMT2, Q96AC1);
Carboxypeptidase B2 (CPB2, Q96IY4); Heparanase (HPSE, Q9Y251); Low affinity immunoglobulin gamma Fc region receptor II-a / Low affinity immunoglobulin gamma Fc region receptor II-c (FCGR2A/FCGR2C, P12318/P31995); and Tropomyosin beta chain (TPM2, P07951). Precursors were isolated using a 0.4 Da window and fragmented using a normalized collision energy of 27, considering an 8 minute elution window around the expected retention time. Automatic gain control was set to a target value of $10^6$ with a maximum injection time of 100 ms. Peptides were separated using a 110-minute LC gradient from 2.5-38 % ACN in 0.1% FA. All samples were analyzed in technical triplicates.

**Data processing**

Label-free data were processed with Progenesis (NonLinear Dynamics). Peak lists of feature MS/MS spectra were exported and searched using Mascot v.2.4.0 against a concatenated target/decoy database (UniProt, taxonomy human, 2013-11-12, 20,273 target sequences). Search settings were as follows: trypsin as enzyme with a maximum of two missed cleavages; oxidation of methionines as variable modification and carbamidomethylation of cysteines as fixed modification. Error tolerances were set to 10 ppm and 0.8 Da for MS and MS/MS, respectively. Mascot search result files were imported to Peptide Shaker 0.28.0 for calculating a 1% false discovery rate (5). After import of search results into Progenesis, normalized protein abundances as calculated by the software were used for relative protein quantification.

iTRAQ data were processed with Proteome Discoverer 1.3 (PD, Thermo Scientific) and searched using Mascot v2.4.0 and Sequest against a human Uniprot database (2013-11-12, 20,273 target sequences) in a target/decoy search. Search settings were as follows: trypsin as enzyme with a maximum of two missed cleavages; oxidation of methionines as variable modifications; carbamidomethylation of cysteines and iTRAQ 8-plex labelling of lysines and N-terminii as static modifications. Error tolerances were set to 10 ppm and 0.02 Da for MS and MS/MS, respectively. For quantification we used the iTRAQ 8-plex custom setup in the
reporter ion quantifier-node of PD and disabled normalization. Result files were exported to Excel and reporter ion ratios were log2-transformed and median-centered. Proteins were selected for targeted analysis if (I) either the index patient significantly differed from controls, i.e. on average at least 1.33-fold regulated compared to controls; or (II) if patient and heterozygote family members significantly differed from healthy controls (on average at least 1.33-fold up/down-regulation).

For targeted quantification we set up a parallel reaction monitoring (PRM) method for a Q-Exactive mass spectrometer using in-house-built spectral libraries and Skyline 2.6.0 (6). For data analysis we only considered (I) peptides which were detected in every sample and (II) proteins quantified by at least two proteotypic peptides in each of three replicate measurements. Areas of peptide transitions were summed using in-house-build R v.3.0.3 scripts and exported for further processing into Excel. We calculated relative protein abundances by the sum of relative peptide abundances. For each donor, based on signal intensities intra-individual standard deviations and average values were calculated per protein over all replicates. Proteins were considered regulated between two donors, if their average intensities ± standard deviation did not overlap. For barplots, relative protein levels per donor were scaled to the respective control (control = 100%). A list of peptides used for PRM is given in the supplemental data file S2.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD003912 (7).

**NSAF values for grouped individuals (NSAF calculated for top 675 proteins)(8):**

For validation of key-platelet proteins, the normalised spectral abundance factor (NSAF) was calculated for all LC-MS analyses of patients, heterozygous family members, healthy day controls and 4 additional controls which were taken from independent studies. NSAFs were calculated for each protein using:

\[
(\text{NSAF})_k = \frac{(\text{PSM}/L)_k}{\sum_{i=1}^{N}(\text{PSM}/L)_i} \\
\text{PSM: peptide spectrum matches, } L: \text{protein length (amino acids)}
\]
From these samples, the smallest number of quantifiable proteins was 675, consequently NSAF values were determined only for the top 675 proteins of each sample, for a better comparison. For bar plots, values were scaled to the controls (100%).

*Flow cytometric analysis of platelet binding of soluble fibrinogen and platelet granule exocytosis*

ADP-, convulxin- or thrombin-induced exogenous binding of soluble fibrinogen to platelets, platelet P-selectin (CD62P) and CD63-surface expression were detected by flow cytometry (FACS Canto II flow cytometer with FACS Diva software, Becton Dickinson Biosciences, Heidelberg, Germany) as described previously (9, 10).

*Light transmission aggregometry*

Platelet aggregation was determined in native PRP (11), according to the method of Born (12) using a four channel light transmission aggregometer (APACT 4S Plus, DiaSys, Flacht, Germany). ADP (10 µM), convulxin (25 nM), thrombin (0.1 U/ml), arachidonic acid (0.5 mg/ml) and epinephrine (10 µM), respectively were used as platelet agonists. Ristocetin (1.25 mg/ml) was used to induce VWF-mediated platelet agglutination and subsequent platelet aggregation. Platelet aggregation was monitored under continuous stirring at 1000 rpm at 37 °C for 5 minutes.

*Platelet adhesion to and aggregation on von Willebrand factor under flow conditions*

The flow chamber system BioFlux 1000Z (IUL, Königswinter, Germany) was used according to manufacturer’s recommendations and as described previously (13). Briefly, channels of a 48-well low shear plate (0-20 dynes/cm²) were coated with 12 U/ml VWF (Willfact®) over night at 4 °C. Platelets in citrated-whole blood were stained with calcein-AM (5 µM final concentration) for 25 min at room temperature. A shear stress of 10 dynes/cm² was applied for 10 min. Channels were washed three-times with PBS, pH 7.4 and adhered platelets were
analyzed by capturing 5 fluorescence images per channel by using an inverted microscope (Zeiss Observer), CCD camera (QICam, QImaging, Surrey, BC, Canada) and BioFlux Montage software. Platelet adhesion to VWF was expressed as percentage of area covered by platelets using BioFlux Montage software.

References


