Supplemental Table of Contents

Supplementary Materials

Methods

Patients and Controls

The study included 27 ESRD patients (15 men and 12 women, 27 to 82 years) receiving HD treatment 2 to 4 times weekly at Haukeland University Hospital, Bergen, Norway. Collection of blood samples from the patients was approved by the Regional Committee for Medical and Health Research Ethics (2013/2320/REK vest) and written informed consent was obtained from all participants prior to enrollment. Blood samples were drawn from the arterial needle or the outflow line of the dialysis catheter at the start of dialysis prior to administration of fragmin (LMW heparin). Samples were collected in ACD-A tubes (acid citrate dextrose, Vacuette, Greiner Bio-one) with the first one being discarded to avoid unwanted platelet activation. From the 19 patients included in the aggregation experiments 7 were on regular Albyl-E (acetylsalicylic acid) therapy (75 mg per day), while 12 patients did not receive any medication that affects platelet function. All handling of patient material and data was conducted in compliance with the regulations set up by REK Vest. Control samples were obtained from regular blood donors at the blood bank (Department of Immunology and Transfusion Medicine, Haukeland University Hospital). Control subjects had not taken any medication for at least 14 days prior to sampling.

Platelet isolation and carbamylation

Blood samples were centrifuged for 20 minutes at 200 x g before platelet rich plasma (PRP) was collected and diluted with an equal volume of HEP buffer (140mM NaCl, 2.7mM KCl, 3.8mM HEPES, 5mM EDTA, 1μM prostaglandin E1, pH 7.4). After centrifugation for 20 minutes at 100 x g to remove residual red and white blood cells, platelets were pelleted at 300 x g for 20 minutes and resuspended in platelet buffer (145mM NaCl, 5mM KCl, 10mM HEPES, 0.5mM Na_2HPO_4, 6mM glucose, pH 7.4). All centrifugations were carried out at room temperature (RT). For experiments investigating activation dependent responses, platelets were allowed to rest for 30 to 60 minutes at RT to ensure that they were in the resting state.

Platelets were carbamylated by treatment with the indicated concentrations of potassium cyanate (KOCN, Sigma Aldrich, Oslo, Norway) for 30 minutes at 37°C. Control platelets were incubated in pure platelet buffer. To test whether free amino acids can protect platelets from carbamylation, platelets were incubated with KOCN in the presence of the amino acid infusions Glavamin or Vamin (Fresenius Kabi, Halden, Norway) diluted 1:4 in reaction buffer. Platelets were then centrifuged at 300 x g for 5 minutes, resuspended in binding buffer (140mM NaCl, 2.5mM $CaCl_2$, 10mM Hepes) and subjected to subsequent analyses.

For platelet activation in plasma, platelet rich plasma (PRP) was used after removal of residual red and white blood cells. Plasma was diluted in a ratio 1:1 with HEP buffer and carbamylated with 5mM or 10mM KOCN for 30 minutes at 37°C. After carbamylation, the calcium concentration in plasma was adjusted to 2.5mM by using $CaCl_2$.

Isolation of total platelet proteins

Platelets were isolated from ACD-A tubes as described, frozen to -70°C and after addition of protease inhibitor (Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Mannheim, Germany) thawed in a water bath at 37°C. Freezing and thawing was repeated 2 times. Platelets were disrupted by sonication, centrifuged for 5 minutes at 4500 x g and 4°C and the supernatants were collected. Protein extraction was repeated once, the supernatants were pooled and the protein concentration in the resulting solution was determined by using a BCA Protein Assay Kit (abcam, Cambridge, UK).

Quantification of HCit in platelet protein preparations by LC-MS/MS

Platelet proteins were diluted to a concentration of 4 mg/mL in 150mM NaCl and subjected to acid hydrolysis prior to LC-MS/MS quantification of HCit. For that purpose, 600 µL platelet proteins were mixed with 600 µL 12M HCl and incubated in closed glass tubes at 110°C for 18 hours. Hydrolysates were twice evaporated to dryness under a stream of nitrogen. Dried samples were resuspended in 100 µL of 125mM ammonium formate containing 1µM d_7-citrulline and 65µM $d_8$lysine (used as internal standards) and filtered using Uptidisc PTFE filters (4 mm, 0.45 µm, Interchim, Mannheim, Germany). Diluted hydrolysates were subjected to LC-MS/MS analysis (API4000, ABSciex) using the same conditions as described in previous studies[1,2]. HCit values were expressed relative to the lysine content in the hydrolysates.

Platelet protein fractionation

Platelets were isolated from a platelet concentrate and carbamylated using 5mM and 10 mM KOCN for 1 hour at 37°C. Control platelets were incubated under the same conditions in pure buffer without KOCN. Platelets were frozen to -70°C and, after addition of protease inhibitors, thawed at 37°C in a water bath. Freezing and thawing was repeated 2 times. Platelets were disrupted by sonication, centrifuged for 5 minutes at 4500 x g and 4°C and the supernatant was collected. The pellet was resuspended in platelet buffer containing protease inhibitors, sonicated and centrifuged a second time as described. Supernatants were combined in centrifuge tubes and after addition of protease inhibitors centrifuged for 1 hour at 47800 x g and 4°C. After removal of the supernatant containing the cytosolic protein fraction, the pellets were washed once with cold platelet buffer and

resuspended in 2 mL cold 100mM sodium carbonate (Na_2CO_3) buffer pH 11.5. Samples were transferred into small beakers, stirred on ice for 1 hour and centrifuged for 1 hour at 47800 x g and 4°C to obtain the peripheral membrane proteins (PMPs) in the supernatant. The pelleted integral membrane proteins (IMPs) were resuspended in NP40 buffer (1% NP40, 150mM NaCl, 10mM Tris, 5mM EDTA). Protein concentrations in the individual fractions were determined by using the BCA Protein Assay Kit.

For biotin-PG labeling and the analysis of $\alpha_{IIb}\beta_3$ carbamylation, plasma from 4 to 5 patients or controls was pooled generating 3 patient and 3 control pools. Patient samples were assigned to a pool according to their blood urea concentrations (Supplementary table 1).

Platelets were isolated from the pooled plasma samples and the platelet proteins fractionated as described above with the difference that only the entire membrane proteins were separated from the cytosolic proteins but not PMPs and IMPs from each other. Membrane proteins were solubilized in 100mM sodium carbonate buffer pH 11.5 and the protein concentrations were determined by using the BCA Protein Assay Kit.

SDS polyacrylamide gel electrophoresis and Western Blot of peripheral and integral membrane proteins

Proteins (2 µg PMPs and IMPs, respectively) were mixed with 6x sample buffer and denatured for 10 minutes at 75°C. The complete sample volume (30 µL) was loaded into Novex 10% Wedge well Tris Glycine Mini Gels (Life Technologies, Oslo, Norway) and separation was accomplished using Tris Glycine running buffer at an electrical potential of 100 V. Protein bands were visualized using Coomassie Stain for 1 hour at RT followed by overnight destaining in a solution consisting of 140 mL acetic acid, 100 mL ethanol and 1760 mL dH_2O.

For Western Blot, proteins were transferred onto nitrocellulose membranes (BioRad, Oslo, Norway) at 100 V and 4°C for 1 hour. Membranes were blocked with 5% non-fat dried milk in TBST for 1 hour before primary antibodies were applied in blocking buffer at 4°C overnight. The primary antibodies used were sheep anti-human platelet $\alpha_{IIb}\beta_3$ (Affinity Biologicals, Ancaster, ON, Canada; 1:5000 v/v) and rabbit anti-CBL (Cell Biolabs, San Diego, CA, USA; 1:2500 v/v). After washing, the membranes were incubated with HRP-conjugated secondary antibodies, rabbit antisheep IgG (abcam, Cambridge, UK; 1:20000 v/v) and mouse anti-rabbit IgG (Jackson ImmunoResearch, Cambridge, UK; 1:10000 v/v) respectively, for 2 hours at RT. For development, Super Signal Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Oslo, Norway) was used according to the manufacturer's instructions and pictures were recorded in a ChemiDoc XRS+ system (BioRad, Oslo, Norway).

Biotin-PG labeling of *in vitro* samples

Biotin-PG labeling was performed as previously described[3]. Briefly, each sample 0.5 mg/mL (reaction volume 600 µL) was incubated with 20% (v/v) trichloroacetic acid (120 µL of 100% TCA) and 0.2mM biotin-PG (24 µL of 5mM stock) for 30 minutes at 37˚C. After a 30 minutes incubation, the reaction was quenched with 150 µL of 0.1M citrulline dissolved in 50mM HEPES pH 7.6. Proteins were precipitated by placing the solutions on ice for 30 minutes followed by centrifugation (13500 rpm, 15 min) at 4˚C. The supernatants were discarded, and the protein pellets were washed twice with cold acetone and dried. To eliminate the labeling of arginine residues after neutralization, the pellet was dissolved in a buffer containing 1% SDS, 7% β-mercaptoethanol, 20mM HEPES pH 8.0, 100mM arginine and 100mM NaCl (50 µL). The sample was then boiled for 10 minutes at 95˚C and further sonicated for 15 minutes twice to completely resolubilize the pellets. The biotin-PG labeled proteins were separated by SDS-PAGE (gradient 4-12%, Bio-Rad) and transferred to membranes. The blots were blocked with 5% BSA in PBST for 1 hour at room temperature and further probed with sheep anti-human $\alpha_{IIb}\beta_3$ primary antibody diluted in 5% BSA overnight at 4˚C. The blots were then washed with PBST (3x) and probed with donkey anti-goat 680RD secondary antibody and streptavidin 800CW as a surrogate antibody diluted in 5% BSA for 1 hour at RT. The blots were further washed with PBST (3x) and images were taken using a Licor Imager (700 nm and 800 nm). The band intensities were quantified using Image J software and p-values between control and carbamylated samples were calculated.

Biotin-PG labeling of patient and control samples

Samples were labeled with biotin-PG similarly to previously described methods[4,5]. Briefly, samples (300 µg) were diluted in buffer (100mM HEPES pH 7.6 to a final concentration of 1 mg/mL in a reaction volume of 300 µL) and incubated with 20% trichloroacetic acid (60 µL of 100% TCA) and 0.2mM biotin-PG (12 µL of 5mM stock) for 30 minutes at 37˚C. After a 30 minutes incubation, the reaction was quenched with 60 µL of 0.1M citrulline dissolved in 50mM HEPES pH 7.6. Proteins were precipitated by placing the reaction mixtures on ice for 30 minutes followed by centrifugation (13500 rpm, 15 minutes) at 4˚C. The supernatants were discarded, and the protein pellet was washed twice with cold acetone and dried. To eliminate the labeling of arginine residues after neutralization, the pellet was dissolved in a buffer containing 1% SDS, 7% β-mercaptoethanol, 20mM HEPES pH 8.0, 100mM arginine and 100mM NaCl (50 µL). The sample was then boiled for 10 minutes at 95˚C and further sonicated for 15 minutes twice to completely resolubilize the pellets. The biotin-PG labeled proteins were separated by SDS-PAGE (gradient 4-12%, Bio-Rad) and transferred to membranes. The blots were developed as described above.

Statistical analysis of $\alpha_{IIb}\beta_3$ carbamylation

The plasma [healthy donors (n=12) and HD patients (n=14)] samples were pooled together according to the blood urea concentrations of the patients to generate 3 representative samples (Supplementary table 1). The labeling experiments were performed with the platelet membrane proteins isolated from these samples in a blinded fashion with three technical replicates. The band intensities in the blots were quantified using Image J software and averaged. The relative levels of carbamylation of the α_{IIb} and β_3 subunits were calculated by dividing the band intensities of carbamylated fractions by that of the total input of the respective receptor bands (Figure 3B). The relative levels of carbamylated $\alpha_{IIb}\beta_3$ in healthy control and HD patient samples were plotted as the average of the three individual patient and control pools (Figure 3C). The error bars represent standard errors of the mean.

Platelet adhesion assay

Wells were coated with 100 µL human fibrinogen or albumin (2.5 mg/mL in 0.1M carbonate buffer, pH 9.5) for 3 hours at 37°C. The coated plates were washed twice with 0.9% NaCl, $5*10^6$ platelets in a volume of 100 µL platelet buffer were added to each well and incubated in the plates for 3 hours at 37°C in a humidified atmosphere. Plates were washed twice with PBS and adhered platelets fixed with 1% glutaraldehyde in PBS for 10 minutes at RT. Plates were washed twice with PBS and the cells stained with 0.5% crystal violet in dH_2O for 10 minutes at RT. Plates were washed 3 times with dH_2O and thoroughly drained on paper towels before 100 µL 1% SDS was added to each well. The plates were briefly agitated on a plate shaker to allow for uniform development of the color. Absorbance was measured at 570 nm on a Synergy H1 Hybrid MultiMode Reader (Biotek Bad Friedrichshall, Germany). Binding of platelets to albumin was used as a reference.

Platelet aggregation microplate assay

Platelets were isolated from ACD-A tubes and carbamylated as described under platelet isolation. 5.6 µL pure platelet buffer (blank) or human thrombin (10 U/mL; Sigma Aldrich, Norway) were pipetted into the wells of a 96-well non-binding microplate (Greiner bio-one, Monroe, NC, USA). $3*10^7$ carbamylated or control platelets in a volume of 145 µL platelet buffer were added to the wells and mixed with thrombin or buffer by pipetting once up and down before aggregation kinetics were recorded at 37°C and 405 nm for 30 minutes using continuous double orbital shaking. Light transmission was converted into percentage aggregation based on the blank wells containing buffer instead of thrombin as 0% and pure buffer as 100% aggregation.

Light Transmission Aggregometry

Light transmission aggregometry (LTA) was performed in a Chrono-log model 700 whole blood lumi-aggregometer (Chrono-log corporation, Havertown, PA, USA). 240 µL washed platelets ($1.8*10^8$/mL) were pipetted in glass cuvettes containing a magnetic stirrer bar and pre-warmed to 37°C in the aggregometer. After 3 minutes the samples were transferred into the PRP positions of the aggregometer channels using rubber spacers to be able to measure in a total volume of 250 µL. A cuvette with 500 µL pure platelet buffer was placed as blank in the position for platelet poor plasma (PPP) of channel 1 and the baseline in both channels was set to 0% aggregation. 10 µL thrombin (10 U/mL) containing 2.5mM $CaCl_2$ was added, and aggregation was recorded for 7 minutes at 37°C and 1000 rpm. Measurements and slope calculations were performed in AGGRO/LINK8™ for windows. For analysis and graphical representation, data were exported in ASCII format and processed in Microsoft Excel version 14.0.0 and GraphPad Prism version 7.0 for Mac.

The same experimental procedure was used to record aggregation curves of control platelets and platelets isolated from blood samples of HD patients.

Ristocetin induced platelet agglutination

Blood was taken in 3.2% sodium citrate tubes (Vacuette, Greiner Bio-one) and centrifuged for 20 minutes at 100 x g and RT to obtain PRP. 600 µL PRP were centrifuged for 10 minutes at 3500 x g and RT to generate PPP which was used as blank.

PRP was carbamylated by incubation with 1mM, 5mM or 10mM KOCN for 30 minutes at 37°C. Control plasma was incubated with buffer. After carbamylation, the calcium concentration in PRP was adjusted to 2.5mM using 100mM $CaCl_2$.

Platelet agglutination was analyzed in a Chrono-log model 700 whole blood lumi-aggregometer (Chrono-log corporation, Havertown, PA, USA). Therefore, 250 µL PRP was pipetted in glass cuvettes containing a magnetic stirrer bar and pre-warmed to 37°C in the aggregometer. After 3 minutes the samples were transferred into the PRP positions of the aggregometer channels using rubber spacers. A cuvette with 500 µL PPP was placed as blank in the PPP position of channel 1 and the baseline in both channels was set to 0% aggregation. 62.5 µL ristocetin (10 mg/mL; MP Biomedicals LLC, Irvine, CA, USA) was added, and platelet agglutination was recorded for 7 minutes at 37°C and 1000 rpm. Measurements were performed in AGGRO/LINK8™ for windows. For analysis and graphical representation, data were exported in ASCII format and processed in Microsoft Excel version 14.0.0 and GraphPad Prism version 7.0 for Mac.

Flow cytometry aggregation assay

Platelets were labeled with 0.25µM CellTrace carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA) or 0.25µM CellTrace Violet (Invitrogen) for 20 minutes at 37°C. Differently labeled platelets were mixed 1:1, activated with thrombin (0.4 U/mL) and incubated for 15 minutes at 37°C and 14000 rpm on a thermocycler. Samples were diluted with 400 µL PBS (100 mg/L Mg^{2+}, 100 mg/L Ca^{2+}) and aggregation was evaluated based on the increase in formation of double-colored events after platelet activation.

Assessment of $\alpha_{IIb}\beta_3$ and PAR-1 activation by Flow Cytometry

Carbamylated and untreated or patient and control platelets (95 µL, $5*10^7$ platelets/mL binding buffer) were activated with 5 µL human thrombin (0.4 U/mL) for 15 minutes at 37°C. To resting platelets 5 µL pure binding buffer was added.

For experiments in plasma, 95 µL control or carbamylated PRP was activated with 100 ng/mL convulxin (Cayman Chemical, Ann Arbor, Michigan, USA) for 15 minutes at 37°C.

Activated and resting platelets or PRP were stained with 20 µL FITC mouse anti-human PAC-1 antibody (BD Biosciences, Franklin Lakes, NJ, USA), 10 µL AF488 conjugated fibrinogen from human plasma (Thermo Fisher Scientific, Oslo, Norway) or 20 µL PE conjugated anti-human PAR1 antibody (Beckman Coulter, Brea, CA, USA) for 20 minutes at RT in the dark. Optimal antibody dilutions to generate specific staining were determined beforehand by titrating the antibodies in resting and activated cells under the conditions set for the experiment. To avoid fibrin polymerization, thrombin was inactivated with hirudin (3.125 U/U thrombin; Sarstedt, Nümbrecht, Germany) when binding of fibrinogen to activated $\alpha_{IIb}\beta_3$ was analyzed.

After staining, samples were diluted with 400 µL PBS (100 mg/L Mg^{2+}, 100 mg/L Ca^{2+}) and $\alpha_{IIb}\beta_3$ activation was analyzed by measurement of antibody binding to resting and activated platelets by flow cytometry on a BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA) equipped with BD FACSDivaÔ software. Light scatter and fluorescence data were obtained with gain settings in logarithmic mode and 50000 events were acquired for each sample. The median fluorescence intensity (MFI) or the percentage of positive cells was used as a quantitative measure for antibody binding. Data analysis and preparation of figures was performed in FlowJo version 10.4.1 for Mac OS X.

Preparation of DNA constructs

The expression vectors encoding human α_{IIb} sequence (pCMV6-A-Hygro-α_{IIb}) and human β_3 sequence (pCMV6-Entry-β_3) were purchased from OriGene (Technologies GmbH, Herford, Germany). To obtain mutated $\alpha_{IIb}\beta_3$, 5 lysine residues located within the fibrinogen biding site of the β_3 subunit were replaced by alanine (K163A, K170A, K185A, K207A or K217A). Single mutations were introduced into the β_3 sequence using the QuikChange Lightning Site-Directed Mutagenesis Kit and two specific overlapping oligonucleotide primers incorporating two base pair substitution (Table 1). An expression vector containing all five mutations in the β_3 sequence was generated with the use of the QuikChange Lightning Multi Site-Directed Kit (Stratagene, Agilent Technologies, La Jolla, CA, USA) and all oligonucleotide primer pairs. All generated vectors were confirmed by sequencing (Eurofins Genomics, Ebersberg, Germany) and used for transfection of human embryonic kidney (HEK293) cells (American Type Culture Collection, Manassas, VA, USA).

Transfection of HEK293 cells with $\alpha_{IIb}\beta_3$ sequences

HEK293 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and maintained at 37°C in a humidified atmosphere with 5% CO_2. In order to obtain HEK293 cells with transient expression of wild-type and mutated $\alpha_{IIb}\beta_3$ complexes, the expression vector encoding the α_{IIb} sequence (pCMV6-A-Hygro-α_{IIb}) was co-transfected with the expression vector encoding the wild-type β_3 sequence (pCMV6-Entry- β_3_WT) or mutated β_3 cDNAs (pCMV6-Entry- β_3_K→A) using the FuGene HD transfection reagent according to the manufacturer's instruction (Promega, Madison, WI, USA). Cells transiently expressing $\alpha_{IIb}\beta_3$ were analyzed 2 days after transfection.

Assessment of $\alpha_{IIb}\beta_3$ activation and fibrinogen binding in HEK293 cells by Flow Cytometry

HEK293 cells transiently expressing wild-type and mutated $\alpha_{IIb}\beta_3$ complexes were harvested with 2mM EDTA in PBS and washed with PBS containing 2.5mM $CaCl_2$. To assess $\alpha_{IIb}\beta_3$ activation and fibrinogen binding HEK293 cells were incubated with 10mM DTT in PBS containing 2.5mM $CaCl_2$ for 20 minutes at 4°C. The high affinity state of $\alpha_{IIb}\beta_3$ complexes was assessed by staining with FITC conjugated anti-PAC-1 antibodies (BD Biosciences, Franklin Lakes, NJ, USA) for 30 minutes at 4°C in the dark. Fibrinogen binding was assessed by incubation of cells in activation buffer supplemented with 2% BSA followed by staining with 50 µg/ml AF488 conjugated fibrinogen from human plasma (Thermo Fisher Scientific, Oslo, Norway). After staining all samples were centrifuged, diluted in PBS containing 2.5mM $CaCl_2$ and subjected to flow cytometric analysis using a BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA).

Quantitative analysis of $\alpha_{IIb}\beta_3$ activation and fibrinogen binding was determined by measuring the percentage of FITC or AF488 positive cells.Non-transfected HEK293 cells were used as a negative control. The same number of cells (10^4) was recorded for each sample. Data were analyzed only for living cells (PI negative cells) using FlowJo version 10.7.1.

Assessment of α_{IIb} and β_3 surface expression in HEK293 cells followed by cell surface biotinylation

HEK293 cells transiently expressing wild-type and mutated $\alpha_{IIb}\beta_3$ complexes were washed with cold PBS and treated with 0.1 mg/mL EZ-link™ Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour at 4°C. After incubation cells were washed two times with PBS containing 100mM glycine, lysed with 300 µL RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing Complete EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany) and 2.5mM EDTA, and centrifuged at 16000 x g for 15 minutes at 4°C. The protein concentration of the lysate was determined using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). A total of 200-250 µg was loaded onto streptavidin magnetic beads (GE Healthcare, Chicago, Illinois, USA) and incubated overnight at 4°C. After incubation the beads were washed three times in RIPA buffer and suspended in 4x reducing sample buffer diluted in RIPA buffer to a final volume of 60 µL. Samples were then boiled at 95°C for 5 minutes, resolved on SDS-PAGE, and electrotransferred onto a PVDF membrane. The membrane was subsequently blocked with 5% skim milk in TTBS (50mM TrisHCl, 500mM NaCl, 0.1% Tween-20, pH 7.5) for 2 hours at RT. Next, primary anti-α_{IIb} and anti-β_3 (Bio-Techne, Minneapolis, MN, USA) in 5% skim milk was incubated overnight at 4°C. In the next step the membrane was washed three times with TTBS and incubated with secondary HRP conjugated antibodies for 1 hour at RT (Sigma-Aldrich, St. Louis, MO, USA). After washing three times in TTBS the membrane was developed with ECL Western Blotting Substrate. Detection of the luminescence signal was performed using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Clot Retraction Assay

Blood was taken in 3.2% sodium citrate tubes (Vacuette, Greiner Bio-one) and centrifuged for 20 minutes at 100 x g and RT to obtain PRP. PRP was rested for 30 minutes in a warm water bath (3035°C). Glass tubes were filled with 745 µL prewarmed Tyrodes-Hepes buffer (134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM Hepes, 5mM Glucose, 1mM $MgCl_2$, pH 7.3) and 5 µL red blood cells (from the bottom layer of the tubes after centrifugation) and mixed by once pipetting up and down. To each sample, 200 µL PRP and 2 µL KOCN (2.5M or 5M) were added, the samples were sealed with parafilm and incubated for 30 minutes at 37°C. After

carbamylation, 50 µL thrombin (10 U/mL) were added to initiate coagulation, and the samples were sealed again with parafilm. The samples were left undisturbed at RT for the duration of the experiment.

For the assessment of clot retraction, pictures were taken at the start of the experiment and then every 30 minutes from the time the first signs of clot retraction were observed. After complete retraction in the control sample, the weight of the clots was determined.

Statistics

Group means were compared using the Mann-Whitney U test or ANOVA. Correlations between fibrinogen binding to $\alpha_{IIb}\beta_3$ and aggregation were assessed by determining Spearman's rank correlation coefficients. All statistical analyses were performed using GraphPad Prism, version 7.0 for Mac, with P values < 0.05 considered statistically significant.

References

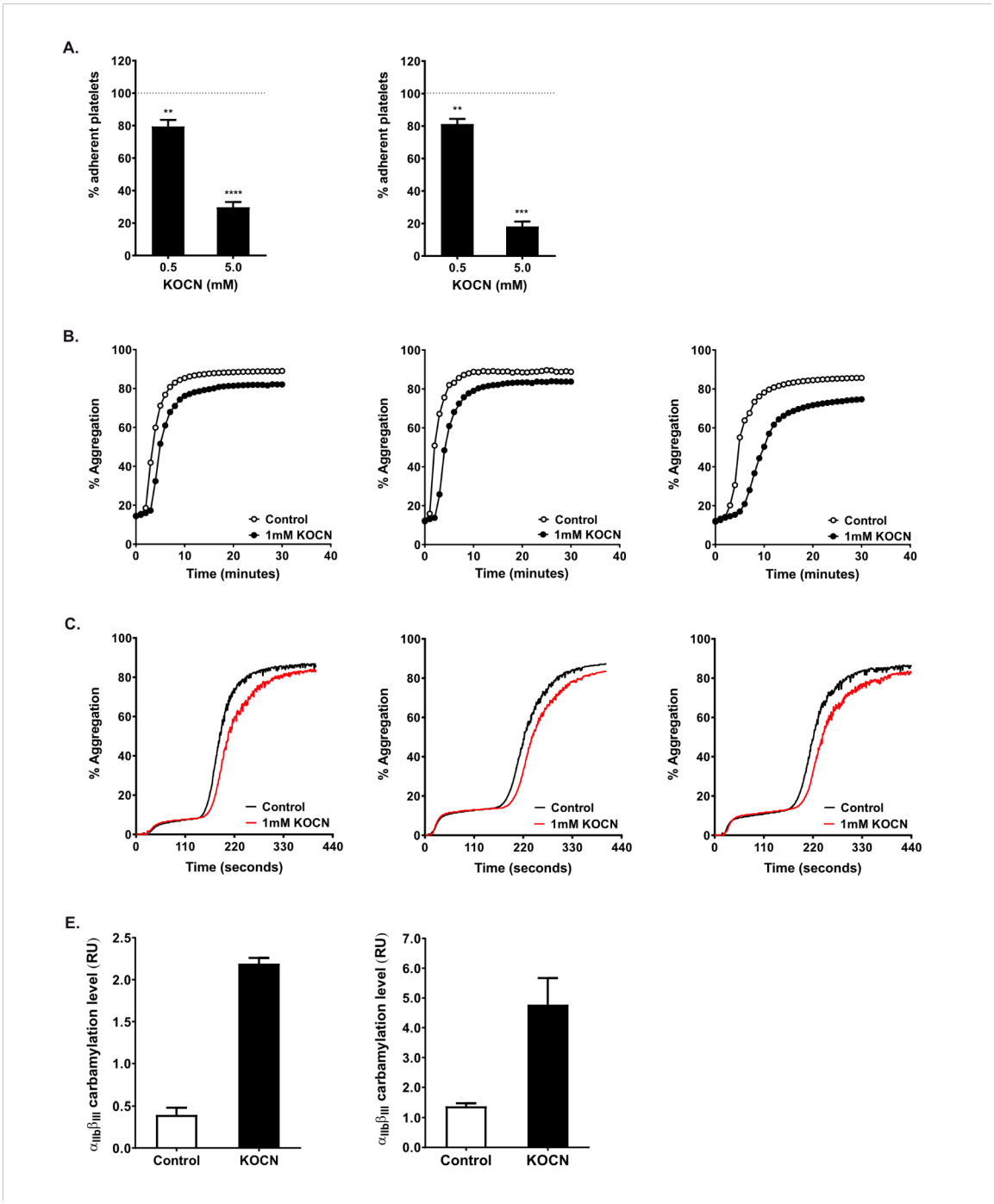
1. Binder V, Bergum B, Jaisson S, et al. Impact of fibrinogen carbamylation on fibrin clot formation and stability. *Thromb Haemost*. 2017;117(5):899–910.
2. Jaisson S, Desmons A, Doue M, et al. Measurement of Homocitrulline, A Carbamylation-derived Product, in Serum and Tissues by LC-MS/MS. 2018;92(1):.
3. Bicker KL, Subramanian V, Chumanevich AA, Hofseth LJ, Thompson PR. Seeing Citrulline: Development of a phenylglyoxal-based probe to visualize protein citrullination. *J Am Chem Soc*. 2012;134(41):17015–17018.
4. Lewallen DM, Bicker KL, Subramanian V, et al. Chemical Proteomic Platform To Identify Citrullinated Proteins. *ACS Chem Biol*. 2015;10(11):2520–2528.
5. Tilvawala R, Nguyen SH, Maurais AJ, et al. The Rheumatoid Arthritis-Associated Citrullinome. *Cell Chem Biol*. 2018;25(6):691–704.

Supplementary Tables

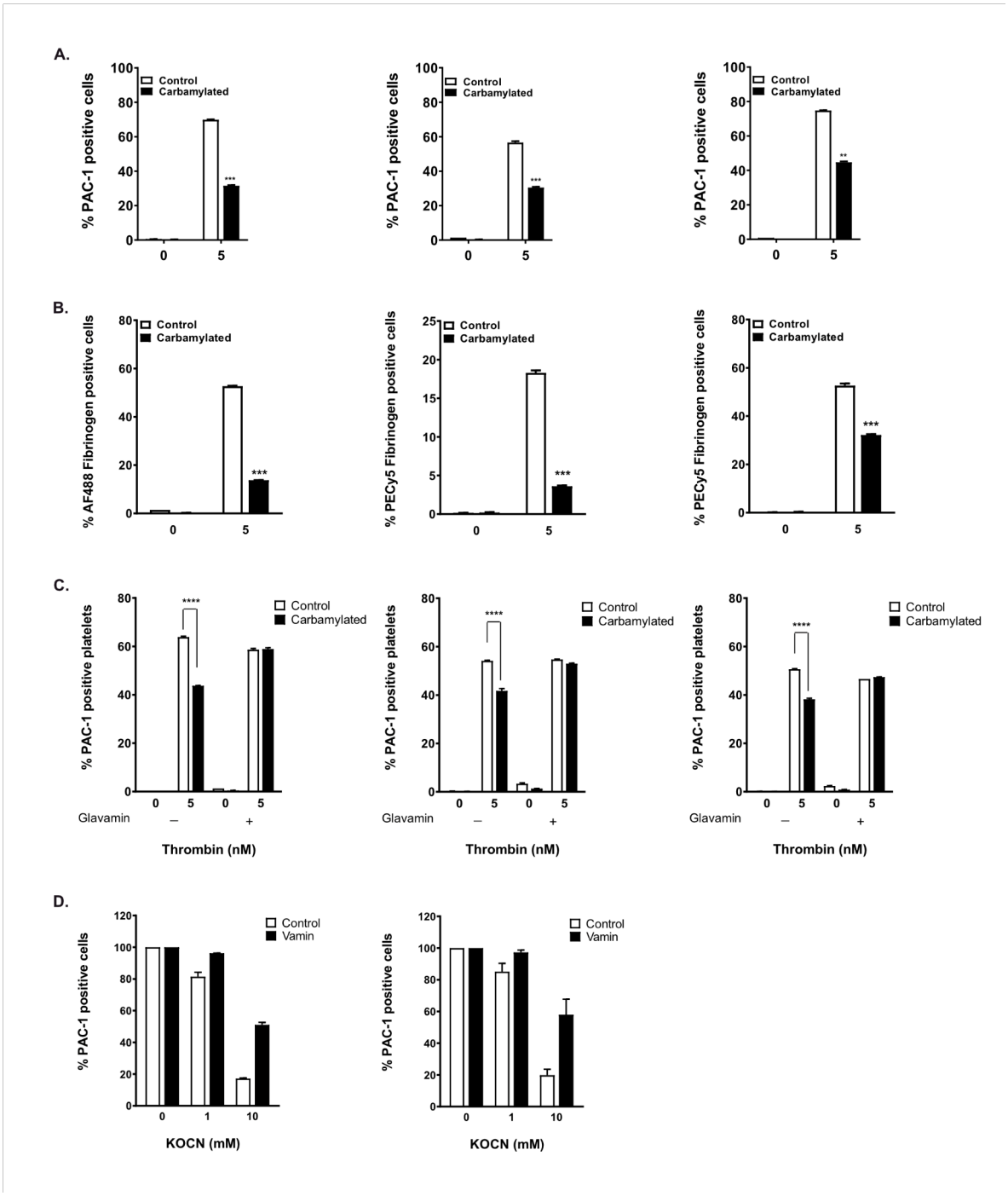
Supplementary Table 1. Blood urea concentrations of individual patients and patient pools.

POOL NUMBER	FINAL UREA CONCENTRATION	INDIVIDUAL PATIENT UREA CONCENTRATION
1	16.3mM	13.9mM; 17.5mM; 18.6mM; 17.4mM; 13.9mM
2	22.7mM	21.4mM; 20.2mM; 20.4mM; 24.9mM; 26.7mM
3	30.2mM	33.2mM; 30.3mM; 28.8mM; 28.4mM

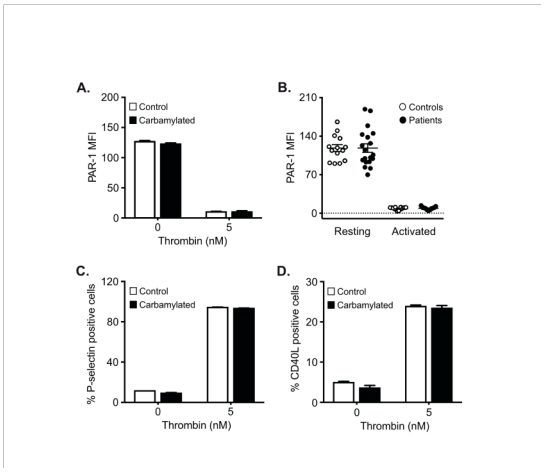
Supplementary Figures



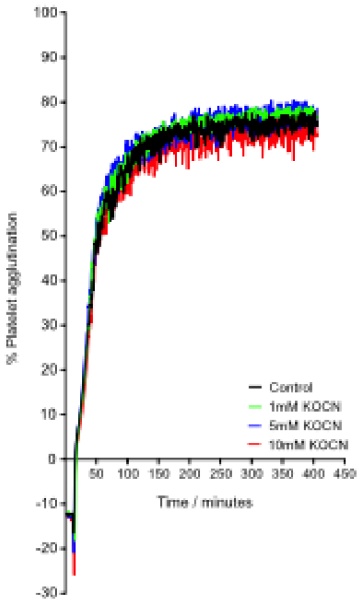
Supplementary Figure 1. Biological replicates to Figure 1. Biological replicates to figure 1A **(A)**. Biological replicates to figure 1B **(B)**. Biological replicates to figure 1C **(C)**. Biological replicates to figure 1E **(E)**.



Supplementary Figure 2. Biological replicates to Figure 2. Biological replicates to figure 2A **(A)**. Biological replicates to figure 2B. Fibrinogen was labeled with AF488 or PECy5. Labeling with PECy5 was done by using a labeling kit resulting in large differences in fluorescent intensity. Therefore, no average was calculated for fibrinogen **(B)**. Biological replicates to figure 2C **(C)**. Biological replicates to figure 2D **(D)**.

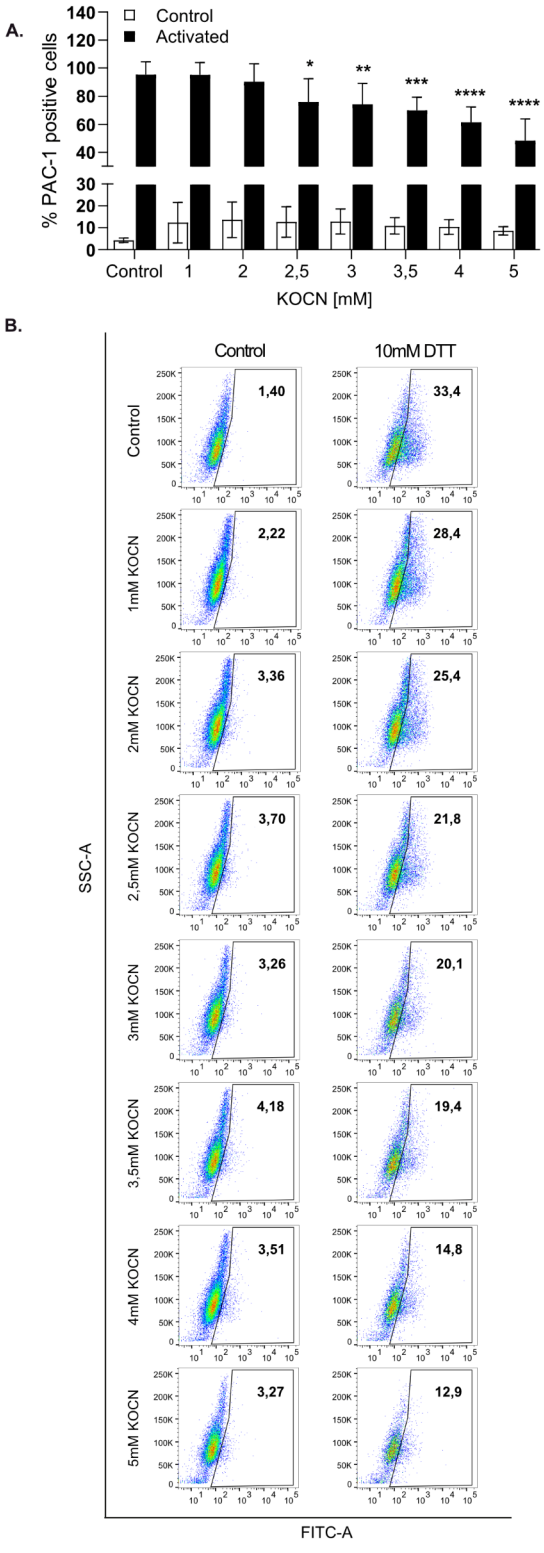


Supplementary Figure 3. Platelet carbamylation does not affect PAR-1 cleavage and agranule secretion. Carbamylated and control platelets were left in the resting state or activated with 5nM thrombin for 15 minutes at 37°C. PAR-1 activation **(A)** and CD62P **(C)** and CD40L **(D)** translocation to the platelet surface were analyzed by flow cytometry. The anti-thrombin receptor antibody used is cleavage sensitive and reacts with uncleaved thrombin receptors only. A reduction in anti-PAR-1 antibody binding indicates platelet activation. **(B)** Patient and control platelets were left in the resting state or activated with 5nM thrombin for 15 minutes at 37°C. PAR-1 activation was analyzed as described for **(A)**.



Supplementary Figure 4. Platelet carbamylation has no impact on GpIb function. PRP was carbamylated for 30 minutes at 37°C using 1mM, 5mM or 10mM KOCN. Carbamylated and control PRP was activated using 2 mg/mL Ristocetin and Ristocetin induced platelet agglutination

was measured in a lumi-aggregometer for 7 minutes at 1000 rpm and 37°C. Results are shown from one representative experiment (n = 3). Since carbamylation had no impact on GpIb mediated platelet agglutination after activation with Ristocetin, the experiment was not performed in the presence of free amino acids.



Supplementary Figure 5. Carbamylation affects activation of $\alpha_{IIb}\beta_3$ expressed in HEK293 cells. HEK293 cells were transiently transfected with wild-type $\alpha_{IIb}\beta_3$ complex following treatment with different concentrations of KOCN overnight in standard culture conditions. After treatment cells were activated with 10mM DTT for 20 minutes at 4°C. Activation was analyzed by flow cytometry using an activation-dependent anti-PAC-1 antibody. Percentages of activated cells were compared using two-way analysis of variance ANOVA with follow-up Bonferroni multiple comparison test (n = 4). * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. Flow data are shown for representative experiment.