Unfolded protein response differentially modulates the platelet phenotype

Online Methods

Study approval

All human blood studies were approved by the Yale Human Investigation Committee (HIC) (IRB #1005006865). Informed consent was obtained from each subject and conformed to the principles set out in the WMA Declaration of Helsinki and the Belmont Report.

Human platelet studies

Venous blood was drawn from patients or healthy adult volunteers (Yale HIC approval #1005006865). Platelets were isolated as described previously (Jain et al. 2019). Venous blood was drawn from patients or healthy adult volunteers. Platelet-rich plasma (PRP) was prepared from citrated freshly drawn blood by centrifugation at 220 *g* for 12 min at 25°C. White blood cell exclusion was confirmed by viewing under phase contrast microscope. Washed platelets (WB) were prepared by centrifugation of PRP (upper 2/3 of supernatant) at 700 *g* for 8 min. To avoid activation due to processing, PGI_2 (20 ng/ml; BML-PG011-0001, Enzo Life Sciences) and apyrase (A6535; Sigma-Aldrich) were added to the PRP. Platelet pellet was resuspended in platelet wash buffer (103 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgCl2, 5 mmol/l glucose, 36 mmol/l citric acid, 0.35% BSA, pH 6.5). Platelet counts were measured manually and were diluted with same washing buffer as needed. Washed platelets were either used for ex vivo studies or processed further for experiments.

Ex vivo human platelet studies

For tunicamycin (Tm) studies, washed platelet samples, normalized to platelet counts (~3×10^8 platelets per mL) were either treated with Tm (5 µg/ml, ab120296, Abcam) or vehicle (saline) for 3 h at 37°C (Abdullahi et al. 2017). For oxidative stress induction, the washed platelet samples were incubated in the presence of H_2O_2 (50 µM) or vehicle (saline) as described previously (Jain et al. 2019). For DTT studies, washed platelet samples were incubated with DTT (2 mM) or vehicle (saline) for 30 min at 37°C. The concentration and time for incubation were pre-optimized for platelets. For hyperglycemia studies, the washed platelets were incubated with 5.5 (NG) or 25 mmol/l glucose (HG) for 2 hours at 37°C as described previously (Tang et al. 2011). Following incubation with the respective inducer, the samples were processed for biochemical assays, confocal microscopy, transmission electron microscopy, western blotting and RNA studies.

Mouse platelet studies

All mice procedures were approved under Institutional Animal Care and Use Committee at Yale (#2017-11413) and all guidelines duly followed. C57BL/6 mice were purchased from Jackson Laboratory and housed

in Yale vivarium. Mice were housed in a specified pathogen-free facility in a room on 12 hr light/dark cycle. Mice were fed normal rodent chow unless otherwise specified and kept in cages with regular paper pulp bedding. Mice which were part of breeder pairs, were excluded from the study. Collection of mouse blood and tissues and sample preparation was blinded through numerical coding of samples. A prior defined criteria was used. Samples in which the blood draw volume was insufficient or with hemolysis, were excluded from platelet studies. Data and image acquisition was done using the numerical codes to further ensure that the selection of representative images was done in a blinded manner. Both male and female mice were included in this study.

Tunicamycin (Tm) (Sigma Aldrich) was diluted in 150 mM dextrose at a concentration of 1 μg/ml, based on previous studies of Tm injection in adult animals (Abdullahi et al. 2017). Mice, aged 10-14 weeks, were injected intraperitoneally with Tm. Age matched control mice were injected with dextrose vehicle solution. Mice were sacrificed 24 h after injection and blood and tissues were collected.

8 week-old mice were injected with streptozotocin (STZ, 50 mg/ml) intraperitoneally for 5 consecutive days to induce diabetes (DM) as described earlier (Lee et al. 2016b; Tang et al. 2014)

Generation of platelet-specific knockout mice

Mice containing *Perk* (*Perk*$^{f/f}$ mice) and *Atf6* (*Atf6*$^{f/f}$) flanked by loxP conditional allele were obtained from the Jackson Laboratory (Bar Harbour, ME, USA). Mice with the *Xbp1* floxed allele (*Xbp1*$^{f/f}$) were obtained from the Glimcher Lab. Platelet-specific deletion of the floxed region was then accomplished by breeding of each of the floxed mice for the UPR genes with the *Pf4 iCre* recombinase transgenic mice (obtained from the Jackson Laboratory). Wild-type littermates for each strain were used as controls for all experiments. Genotyping for each strain was carried out as previously described for *Perk* (Zhang et al. 2002), *Atf6* (Engin et al. 2013) and *Xbp1* (Lee et al. 2008) and the Cre-mediated excision (Tiedt et al. 2007) using polymerase chain reaction (PCR)–based genotyping assays of tissue biopsies. Mice heterozygous for the UPR gene of interest were excluded from the study. Both male and female mice, age 10-14 weeks, were randomly selected and used for the experiments.

Isolation of mouse platelets

As described previously (Jain et al. 2019), following euthanasia, blood was collected by cardiac puncture, directly into 1.8% sodium citrate (pH 7.4). The blood was centrifuged at 200 *g* to obtain PRP and then PRP was again centrifuged at 100 *g* for 5 min to settle the contaminating white blood cells. The purity was checked manually under a microscope using a hemocytometer. The PRP was diluted with platelet wash buffer (calcium free) and was either used for further assays or centrifuged again to obtain pellet. The pellet was resuspended to obtain washed platelets and appropriately diluted to equate counts between treated and vehicle groups. Washed platelets were lysed in NP-40 lysis buffer or RIPA buffer with the addition of protease and

phosphatase inhibitors (Sigma Aldrich) as per the assay. For mouse aggregation, ex vivo treatment and western blotting studies, bloods from n=3 mice were pooled to obtain the appropriate platelet count (as specified) for the assay.

Soluble P-selectin measurement

Soluble platelet activation markers were assayed by commercially available enzyme immunoassays specific for soluble P-selectin (human, BMS219-4; mouse, EMSELP, Thermo Scientific) as per the manufacturer's guidelines in human and mouse plasma samples respectively. Results are expressed as ng/mg protein.

Platelet surface P selectin by flow cytometry

The washed platelets (human or mice) were prepared as described above and counts were adjusted by dilution in platelet wash buffer. Surface P-selectin was measured by flow cytometry-based assay. The primary conjugated antibodies (anti-human CD62P-APC, 304910; or anti-mouse CD62P-APC, 148304, Biolegend) , were used as per manufacturer's instructions. Platelets were fixed in 1% PFA and washed followed by incubation with antibodies for 45 min at RT in dark. This was followed by washing in staining buffer. Platelet purity was confirmed by anti-CD41 (anti-human Biolegend 303704; anti-mouse, Biolegend 133912) antibody and were gated on log scale scatter plot. A total of 3×10^4 events per sample were acquired within 1 hour using BD LSR II Cell Analyzer and data were analyzed by FlowJo v10.4.

Protein carbonylation assay

Protein carbonyl content is the most common indicator of protein oxidation. The levels of protein carbonyl in platelets were determined using a Protein Carbonyl Content Assay Kit (ab126287, Abcam) and all the procedures were performed as per the manufacturer's instructions. Results were expressed as nmol carbonyls/mg protein.

Protein aggregation assay

Protein aggregation was measured in the platelet samples using the ProteoStat Protein Aggregation Assay Kit (ENZ-51023, Enzo Life Sciences) according to the manufacturer's instructions. Capable of detecting even small aggregates, the dye displays increased fluorescence on constrained rotation as it gets captured in the cavities of protein aggregates. In brief, the ProteoStat® Detection Reagent Loading Solution was dispensed into a black 96-well microplate, and platelet lysate (from ~3×10^8 platelets per mL) was added. The microplate was incubated for 15 min in the dark at room temperature, and fluorescence was measured using a fluorescence plate reader with a filter set of an excitation at 544 nm and an emission at 646 nm. The relative level of protein aggregates was calculated based on the protein aggregate standards provided (reconstituted aggregated and

monomeric lysozyme; ENZ-51039, Enzo Life Sciences) and the amount of protein aggregates expressed as percentage.

Transmission electron microscopy

Immediately after incubation under various experimental conditions, the platelet suspension was fixed by 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1.5 h at room temperature and then centrifuged at 1500 *g* for 5 min. The precipitate was washed with 0.1 M cacodylate buffer (pH 7.2) and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer supplemented with sucrose (25 mg/ml) for 2 h. The samples were dehydrated in ascending ethanol concentrations, acetone, propylene oxide, and embedded into Epon 812. The ultrathin sections were cut using an Ultramicrotome-III and stained with saturated aqueous uranyl acetate and lead citrate. The sections were examined under FEI Tecnai Biotwin (LaB6) transmission electron microscope at Yale's EM facility. A minimum of 15 images were collected for each sample in a total of n=3 independent experiments.

Caspase-3 activity assay

Caspase-3 activity in the platelets was measured colorimetrically at 405 nm by degradation of the specific substrate Ac-DEVD-pNA as per the manufacturer's instructions (Abcam, ab39401). The specific caspase-3 activity, normalized to total proteins of platelet lysates, was expressed as µmol pNA/min/mg protein.

TMRE assay

For apoptosis assay, the pretreated washed platelets were incubated with 1 µM tetramethylrhodamine methyl ester (TMRE) reagent (T669, Invitrogen) at 37°C for 20 min in TMRE assay buffer (Wang et al. 2013). After one wash, platelets were resuspended in assay buffer and acquired. This assay quantifies the cells with depolarized mitochondrial membrane. The mitochondrial depolarization precedes other apoptotic events like caspase 3 activation (Leytin et al. 2004). For anucleate platelets, unlike nucleated cells, this forms a reliable method to measure platelet apoptotic tendency. High calcium (2mM $CaCl_2$) was also used to activate one set of mouse platelets from Tm injected mice. A total of 3 x10^4 events were acquired within 1 hour using BD LSR II Cell Analyzer and data were analyzed by FlowJo v10.4.

SDS PAGE and western blotting

Washed platelets (~3 x 10^8 per mL) were lysed in RIPA buffer (9806S, Cell Signaling Technology) with the addition of protease (P8340, Sigma Aldrich) and phosphatase inhibitors (P5726, Sigma Aldrich). Western blot analysis was performed using 20 µg samples of protein to evaluate expression of ER stress and UPR associated proteins. The platelets were resolved by SDS-polyacrylamide gel electrophoresis using precast gels (Biorad). The protein was electrophoretically transferred to nitrocellulose membrane and then blocked with 5% BSA in

Tris-buffered saline solution containing 0.5% Tween-20. The membranes were probed using the respective antibodies and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (#7074, CST), anti-mouse IgG (#7076, CST). For immunoblotting, the following primary antibodies were used GRP78 (ab21685, Abcam) , GRP94 (#20292, Cell Signaling (CST)), PDI (#2466, CST), Calreticulin (#12238, CST), IRE1α (MA5-14991, Invitrogen), p-IRE1α (PA5-105424, Invitrogen), spliced XBP1 (human: #12782; mouse: #82914, CST), PERK (20582-1-AP, Proteintech), p-PERK (MA5-15033, Invitrogen), ATF6 (PA5-20216, Invitrogen), p-eIF2α (ab32157, Abcam), eIF2α (MA5-31724, Invitrogen), p-PLCγ2 (#3871, CST), PLCγ2 (#3872, CST), p-38 (#14451, CST), phospho p-38 (#4511, CST), p-PLCβ3 (#29021, CST), p-PLCβ3 (#14247, CST), PKCδ (#2508, CST) , p- PKCδ (#9374, CST), PKACα (#4781, CST), p-PKACα (#4782, CST), p-Akt (#9018, CST), Akt (#2938, CST), CHOP (#2895, CST), ATF4 (#11815, CST), cleaved caspase-3 (#9664, CST), Bcl-2 (#3498, CST), Bax (#2772, CST) and GAPDH (#2118, CST). Bands were developed using the enzyme-linked chemiluminescence detection reagents (#34096 and #34577, Thermo Scientific). Membranes were stripped (RestorePlus Stripping Buffer, #46430, Thermo Scientific) to re-probe for different proteins as appropriate. For quantification, the loading control GAPDH was measured for each blot and used for the normalization calculations of the respective protein. For phosphorylated proteins, the quantification represents the ratio of phosphorylated to total protein normalized to the respective loading control and presented as fold change over the experimental control/vehicle. Representative images are shown in the figure and the observed approximate molecular weights are indicated in kDa.

XBP1 splicing assay

Total RNA from platelets was extracted using TRIzol reagent (Invitrogen). RNA was isolated from platelets using the Zymograph Direct-zol kit (R2050; with DNase treatment and A260/A280 > 1.8). The purity of each platelet RNA preparation was assessed by PCR analysis of platelet (GPIIIa) and leukocyte (CD45) markers, as described previously (Landry et al. 2009). 1 µg of RNA isolated from platelets, treated or otherwise, was reverse transcribed using an iScript cDNA synthesis kit (1708891, Bio-Rad). Primers were designed to span the 26 base pair intron that is removed by IRE1 to obtain the spliced XBP1 mRNA (*XBP1s*), forward 5′-TCGCTGTCTTAACTCCTGGTTC-3′ and reverse 5′-CTGGAACAGCAAGTGGTAGA-3′; and the unspliced region (*XBP1u*) 5′-AATCTACCACTTGCTGTTCCA-3′ and reverse 5′-GAGAAGGCGCTGAGGAG-3′. Total XBP1 primers for humans were used as described previously (Yoon et al. 2019). qPCR was performed with SSoAdvanced™ Universal SYBR® Green Supermix (1725271, Biorad). The PCR was performed using Bio-Rad CFX Real-time PCR system in triplicates and arbitrary mRNA concentrations were calculated by the Bio-Rad software, using the relative standard curve method. Purity of the platelet RNA was validated using CD45 as a marker for leucocyte contamination. Fold changes

were calculated relative to the control untreated samples, utilizing the ΔΔCT method by using of tXBP1 as an adequate endogenous control for normalization of sXBP1 and uXBP1 expression (Yoon et al. 2019).

Confocal microscopy

As described previously (Lee et al. 2016a), washed platelets (counts equalized between groups) were allowed to settle on glass-bottomed dishes for 1 h at 37°C and then fixed with 4% paraformaldehyde solution. The platelets were then washed 2 × 5 min in PBS to remove fixative and blocked for 30 min in 10% bovine serum albumin (BSA)/PBS at 37°C. Following blocking, the platelets were incubated in 3% BSA/PBS/primary antibody for 30-60 min at 37°C and then washed with PBS, followed by an additional incubation for 30 min at 37°C in secondary antibody/3% BSA/PBS. For microscopy experiments, primary unconjugated GRP78 (ab21685, abcam), IRE1α (I6785, Sigma Aldrich), PERK (20582-1-AP) and active caspase-3 antibodies (9664, CST) were used as specified in the respective experiments. FITC conjugated CD41 (human: 303704; mouse: 133903 Biolegend; for staining platelets) and Alexa Fluor 647 conjugated CD62p (304918, human specific) specific for human, were purchased from BioLegend. After washing away the excess antibody, the stained platelets were imaged using Perkin-Elmer 5-Laser Spinning Disk Confocal Microscope (equipped with Nikon Ti-E Eclipse inverted microscope) with 100× oil immersion lens. Isotype controls (human: 400108; mouse: 400406, from Biolegend) and secondary antibody controls (anti mouse, A-31571 and anti rabbit, 21207, Invitrogen) were used as negative controls to help differentiate non-specific background signal from specific antibody signal in the platelet samples from both human and mouse. Secondary antibody only controls yielded negative results (data not shown) confirming genuine staining for the target protein of interest. Images were collected for each sample group in n=3 independent experiments. Colocalization between respective proteins was assessed using parameters set in the Volocity software (PerkinElmer, USA) and image analysis done by ImageJ.

Mice platelet aggregation

Mice platelet aggregation was tested in whole blood using aggregometer (Chrono-log model 700) by a modified method. Citrated whole blood from mice from each strain was utilized aggregation with ADP (20µM; P/N 384, ChronoLog) as the agonist and the assay performed as per the manufacturer's instructions. The aggregation was measured as impedance (ohms), which was converted to % aggregation for quantitation comparisons between groups. For comparative quantitation, the maximum level of 20 ohms was taken as 100% aggregation.

Mouse washed platelet aggregation was performed in freshly isolated samples as described previously (Severin et al. 2011). Briefly, washed platelets in Tyrode's buffer were adjusted for platelet counts ($2x10^8$/ml) and platelet aggregation was performed on dual channel aggregometer (Chrono-log model 700) in optical mode as

per manufacturer's instructions. The agonists – Clec-2 IgG (17D9, Biolegend, 5µg/mL) and equine tendon collagen (5µg/mL, P/N 385 Chrono-log) were used as described previously (Severin et al. 2011; Jarvis et al. 2002). The maximum amplitude of percent light transmission was recorded for quantitation.

Determination of reticulated platelet abundance

For quantitating reticulated or immature platelet fractions in mice, washed platelets freshly prepared from control and the respective cKO mice were stained with Thiazole Orange dye (1.0 µM, 20 min; ab145287, Abcam). The flow cytometry-based method was performed as described previously (Angenieux et al. 2016). The TO bright or TO+ve (immature platelets) and TO dim platelet subpopulations (CD41 parent gate) were identifiable in scatter dot plots. The data were analyzed on FlowJo v10.6.

Mouse In-vivo arterial thrombosis model

The $FeCl_3$ (Sigma) induced arterial thrombosis model was used as described previously (Heidt et al. 2011). Briefly, mice (12 to 14 weeks old) were anesthetized by intraperitoneal cocktail injection of ketamine (100mg/kg) and xylazine (10mg/kg). A superficial incision was made into skin and blunt dissection of the fascia was done over the vessel to expose a segment of the right common carotid artery. Whatman filter paper no. 1 (3x2 mm) saturated with 7.5% ferric chloride was placed on the surface of the carotid artery for inducing intravascular thrombosis (semi occlusive). After 5 minutes, the filter paper was removed from the artery surface and saline solution was used to gently wipe the excess ferric chloride. The whole surgical procedure was performed under a dissecting microscope under close observation and the injured artery was closely observed. After 15 min, the mice were terminally anesthetized, and the artery was excised carefully and fixed using 4% PFA. An uninjured artery was also excised from mice and served as a negative control. The arterial tissue was embedded in optimal cutting temperature compound from Tissue-Tek (Sakura Finetek), frozen with dry ice, and then stored at −80°C until sectioned. To determine the composition of the FeCl3-induced thrombus, 8uM thin cryo-sections were cut and stained using Carstairs Staining (#26381, Electron Microscopy Sciences) as described previously (Carstairs 1965) for visualizing platelet-fibrin rich thrombi and vessel wall. The Carstairs' method stains platelets grey blue /navy, fibrin red, collagen bright blue, and red blood cells yellow to clear. Micrographs of stained tissues were visualized on Nikon 80i microscope . The quantitation of thrombus size was done by measuring platelet-fibrin rich thrombus area of the total vessel area (percentage) by ImageJ. The slides were evaluated by an independent observer who was blinded to the sample identity.

Pathway focused PCR array

The differences in the expression levels of 84 key genes in the Unfolded Protein Response (UPR) were analyzed using Human Unfolded Protein Response RT2 Profiler PCR Array System (PAHS-089Z;

SABiosciences, Qiagen). Platelet RNA from randomly selected healthy control (n=6) and diabetes patients (n=6) were used for the array. RNA was extracted using TRIzol reagent (Invitrogen) and RNA isolated using the Zymograph Direct-zol kit (with DNase treatment and A260/A280 > 1.8). Purity was confirmed by the absence of CD45. The platelet RNA samples (1 µg) were reverse transcribed to synthesize the cDNA using the RT^2 First Strand Kit (#330404, Qiagen). The cDNA template was combined with RT2 SYBR Green qPCR Master Mix (Qiagen) and equal amounts of this mixture (25 µl) were added to each well of the PCR array plate precoated with the gene-specific primer sets and the plate was run on the Biorad CFX96 Real Time PCR as per the manufacturer's instructions. Briefly, activation of the HotStart DNA Taq Polymerase was done at 95°C for 10 mins following by 40 cycles of denaturation, annealing and extension at 95°C for 15 secs and 60°C for 1 min. Using an integrated web-based software package for the PCR Array System at Qiagen, the ΔΔCt-based fold-change calculations from the uploaded raw threshold cycle data were obtained. The relative quantitation for each gene was normalized for the panel of housekeeping genes. Only genes that showed statistically significant differences from the control group (Student's t-test and $P < 0.05$) and presented a fold regulation of ≥1.5 over the control group were considered for analysis. Following the blinded data analysis, the DM platelet results samples were further categorized into HbA1C< 8.5 (less severe) and HbA1C> 8.5 (more severe) to represent the difference in the gene expression as fold regulation over control.

Quantitative RT-PCR

Quantitative Real-time PCR (qRT-PCR) was used to measure mRNA expression levels of selected genes as a validation of the array data. 1 µg of RNA isolated from platelets was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad). qPCR was performed as per manufacturer's instructions with SSoAdvanced™ Universal SYBR® Green Supermix (#1725271, Biorad) and sequence-specific primers (purchased from IDT) using a Bio-Rad CFX96 Real Time PCR System. The PCR conditions were as follows: 95°C for 30 s (polymerase activation and DNA denaturation), 95°C for 5s (denaturation), 60°C for 30 s (annealing and extension) (45 cycles) , followed by melt curve analysis at 65–95°C (0.5°C increment 2–5 sec/step).

Primers used were as follows: GRP78 *(coded by hspa5)* 5'-CCAGTCAGATCAAATGTACCCA-3' and 5-GTGCCTACCAAGAAGTCTCAG-3'; IRE1α *(coded by ern1)* 5'- CCCATGCCGAAGTTCAGAT -3' and 5'- ACAAAGTCTGCTGCTTCTCTC -3'; PERK *(coded by eif2ak3)* 5'-CTAACCCAAAGTCTCCAACCT-3' and 5'- GATCGCACATCTTCCTGCA-3'; *ATF6* 5'- CTTGGTCCTTTCTACTTCATGTCT-3' and 5'-CCCTGATGGTGCTAACTGAA-3'; *p-eIF2α* 5'- GTCCCCAGAAATTGACTGAGA-3' and 5'-GGAAGCATGAAGCTAAGAAAGC-3'; *ATF4* 5'- GCATCCAAGTCGAACTCCTT-3' and 5'-GTCAGTCCCTCCAACAACAG-*3'; β-Tubulin* 5'- TTCCGTGAAGATCTCAGACTTG -3' and 5'-TGTTCCTCACCAATCATCTCC -3'. Purity of the platelet RNA was validated using *CD45* as a marker for

leucocyte contamination. Fold changes were calculated relative to the young healthy control, using the ΔΔCT method by normalizing to the housekeeping genes included on each plate (Livak and Schmittgen 2001). Values normalized to *tubulin* and relative to age-matched healthy control samples are shown.

UPR inhibition Studies

Age and gender matched healthy control (HC, with no history of cardiovascular disease) and diabetes mellitus (DM) patients were randomly selected for ex vivo treatment to study the effect of UPR modulation on the platelet phenotype. Briefly, freshly isolated platelet rich plasma (PRP) from either HC or DM patients was incubated with the chemical chaperone, sodium 4- phenylbutyric acid (PBA; ab141253, Abcam), 5mM or vehicle (PBS) for 3 hr at 37°C. For IRE1α inhibition, freshly isolated PRP from both HC and DM patients was incubated with STF-083010 (STF, 30μM; ab146176, Abcam) or vehicle (DMSO) for 1 hr at room temperature. PRP, in part, was used immediately following treatment for platelet aggregation studies. Platelets were isolated from the remaining PRP and used fresh for biochemical assays.

Human Platelet Aggregation

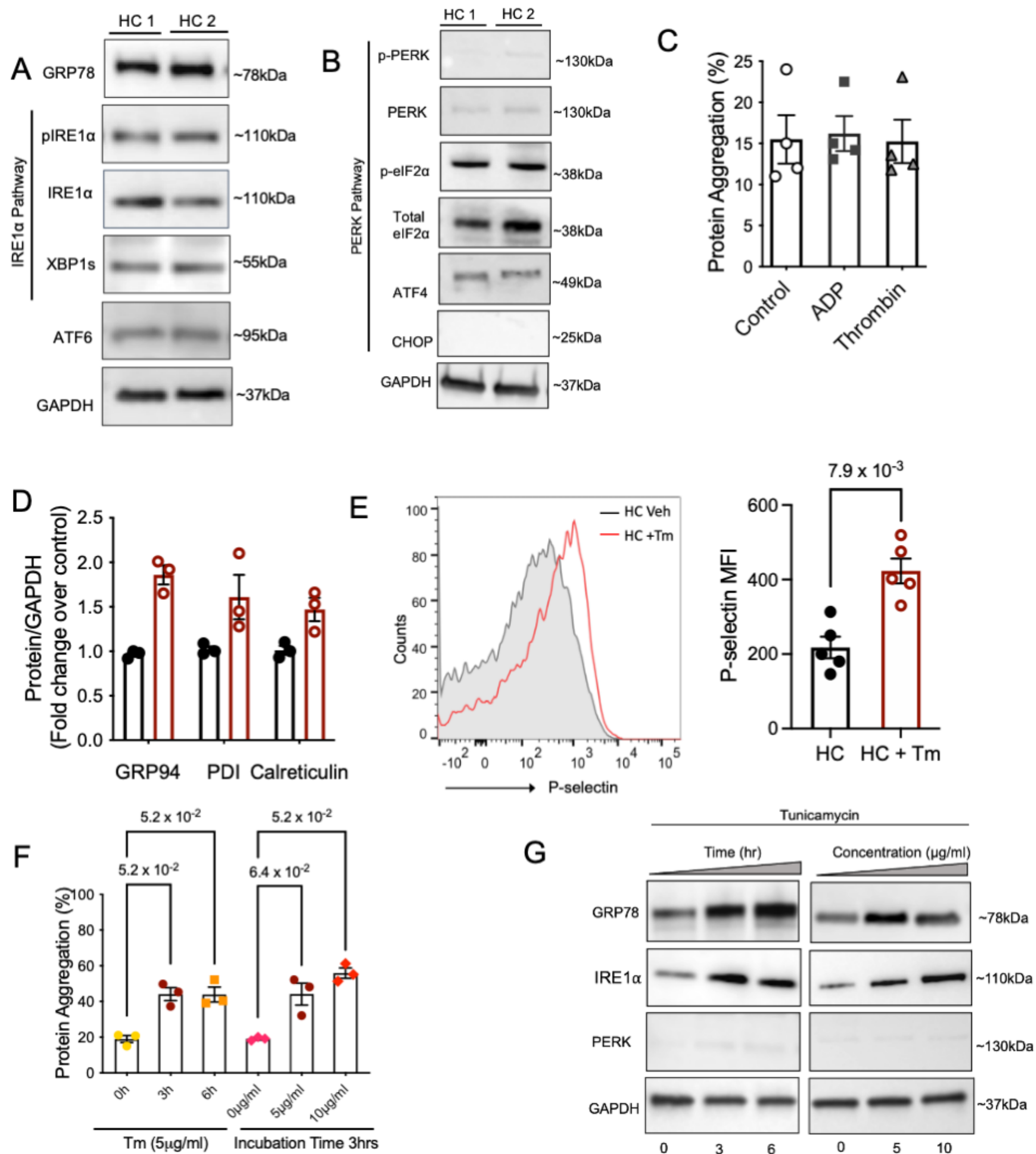
Platelet aggregation was monitored in PRP by using a Aggregometer (Chrono-log model 700, USA) as per manufacturer's instructions. The aggregation assay was performed at 37°C with continuous stirring at 1200 rpm. ADP (2.5μM, ChronoLog) and collagen (0.8μg/mL, Chrono Log) were used as agonist. The baseline value was set with platelet poor plasma. The maximum transmittance (percent) of light was recorded as a measure of aggregation.

Statistical analysis

Values are presented as mean ± standard error of the mean (SEM) and 'n' is the number of biological replicates. A minimum of 3 independent biological replicates were used for each assay. The webtool (https://clincalc.com/stats/samplesize.aspx), which utilizes targeted experimental sample size and variability estimated based on available preliminary data and/or extensive literature search, was used to determine the sample size for adequate study power. GraphPad Prism v9 (GraphPad Software, Inc.) was used for statistical analyses. For sample sets larger than ≤6, data were tested for normality using the Shapiro-Wilk test. For normally distributed data, student's unpaired two tailed *t*-test was used for two groups; one way ANOVA, with Tukey multiple comparison test, was used for comparison between multiple groups. For data sets that did not follow a normal distribution or sample size < 6, unpaired two tailed Mann-Whitney *U* test was used for two groups and Kruskal-Wallis non-parametric test, with Dunn's multiple comparisons, was used for comparison between multiple groups. Wilcoxon matched-pairs test was used for before-after studies with same subjects. Two-sided $P<0.05$ was considered statistically significant. Flow cytometry and western data were

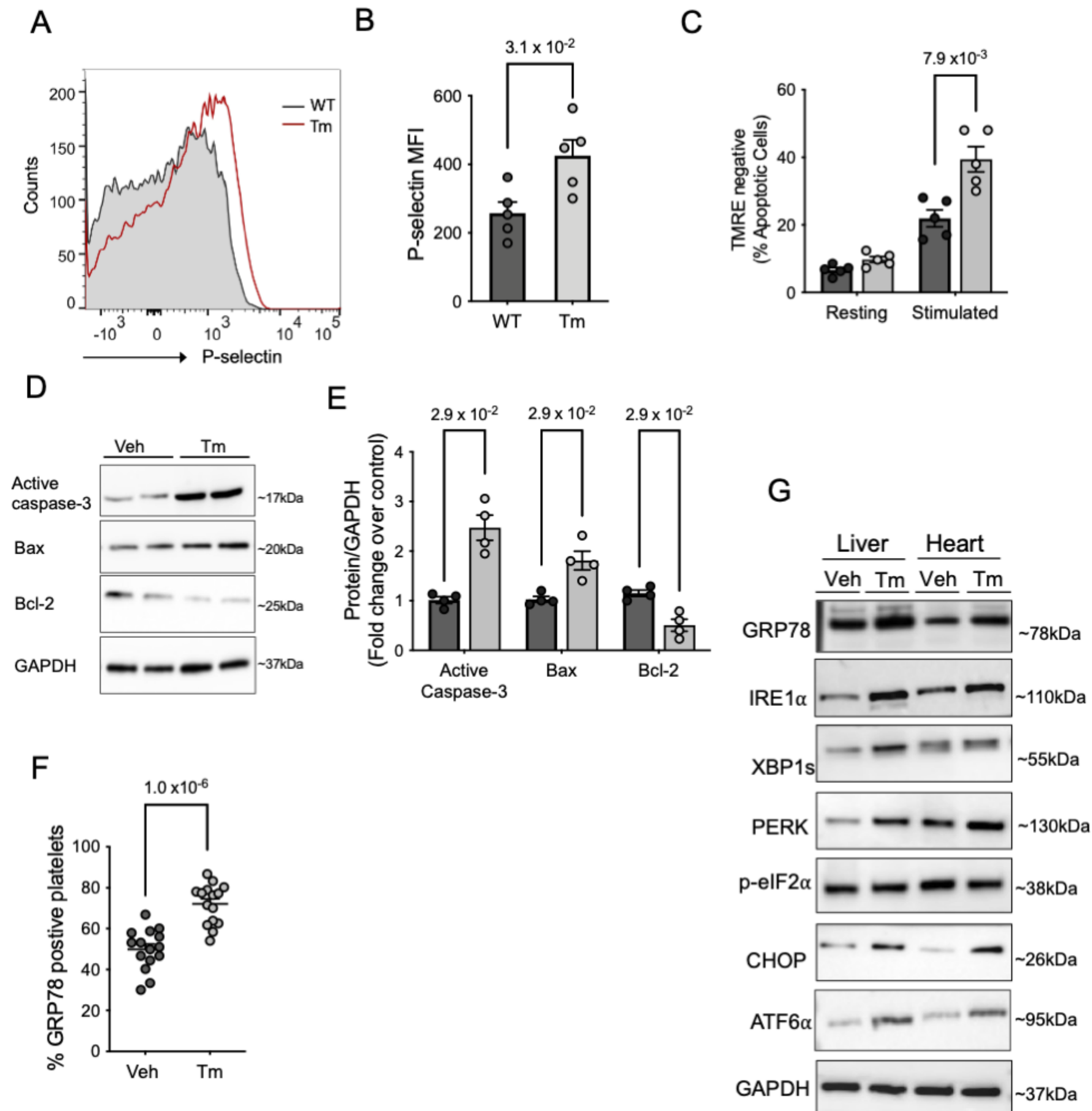
analyzed using FlowJo and ImageJ, as indicated. The analysis of the gene expression data from the RT^2 Profiler PCR Arrays was performed using RT^2 Profiler PCR Array Data Analysis v.3.5 online software (Qiagen). Data were calculated using values for n=6 samples in both HC and DM group. Only genes that showed statistically significant differences from the control group and presented a fold regulation of ≥1.5 over the control group were considered for analysis. For correlation, Pearson's correlation was used. Representative images were selected based on the mean quantified values for experimental group for immunoblotting, immunofluorescence and histology. The graphical abstract was created using BioRender.

Supplemental Figures



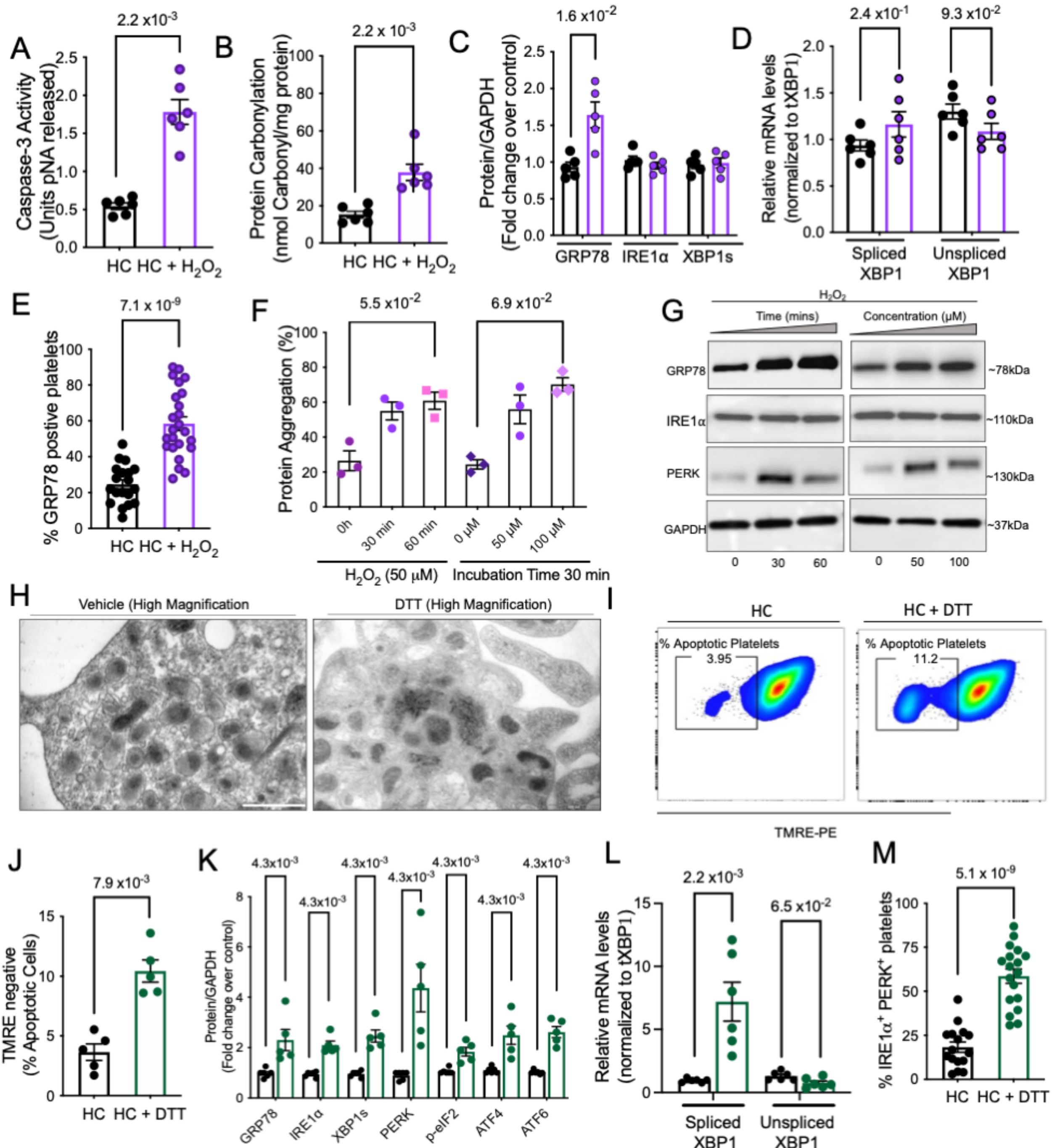
Supplementary Figure S1: **Presence of UPR in human platelets and the effect of Tunicamycin on human platelets, ex vivo**. **A-B**. Expression of UPR proteins,IRE1α, ATF6 and PERK and their signaling in resting healthy control (HC) platelets. **C**. Effect of platelet agonists (ADP 20µM, weak agonist; Thrombin 0.2U, Strong agonist) on intracellular protein aggregate formation within platelets. **D**. Quantification for protein expression changes seen by immunoblotting between HC (black) and tunicamycin (Tm, red) treated platelets (n=3/group). **E.** Histogram and quantification for surface P-selectin levels measured using CD62-APC

antibody by flow cytometry in HC and Tm platelets (n=5/group). **F**. Concentration (5 μg/ml and 10 μg/ml) and incubation time (3 hrs, 6 hrs) dependent changes in Tm induced formation of protein aggregates within platelets. **G**. Effect of different concentration and incubation time of Tm on UPR protein expression in platelets. For incubation time studies, concentration of Tm was 5 μg/ml. For concentration studies, incubation time was 3hrs. n=3 samples for each time and concentration were used. Values expressed as Mean ± SEM and exact *p*-values correspond to Mann Whitney test.



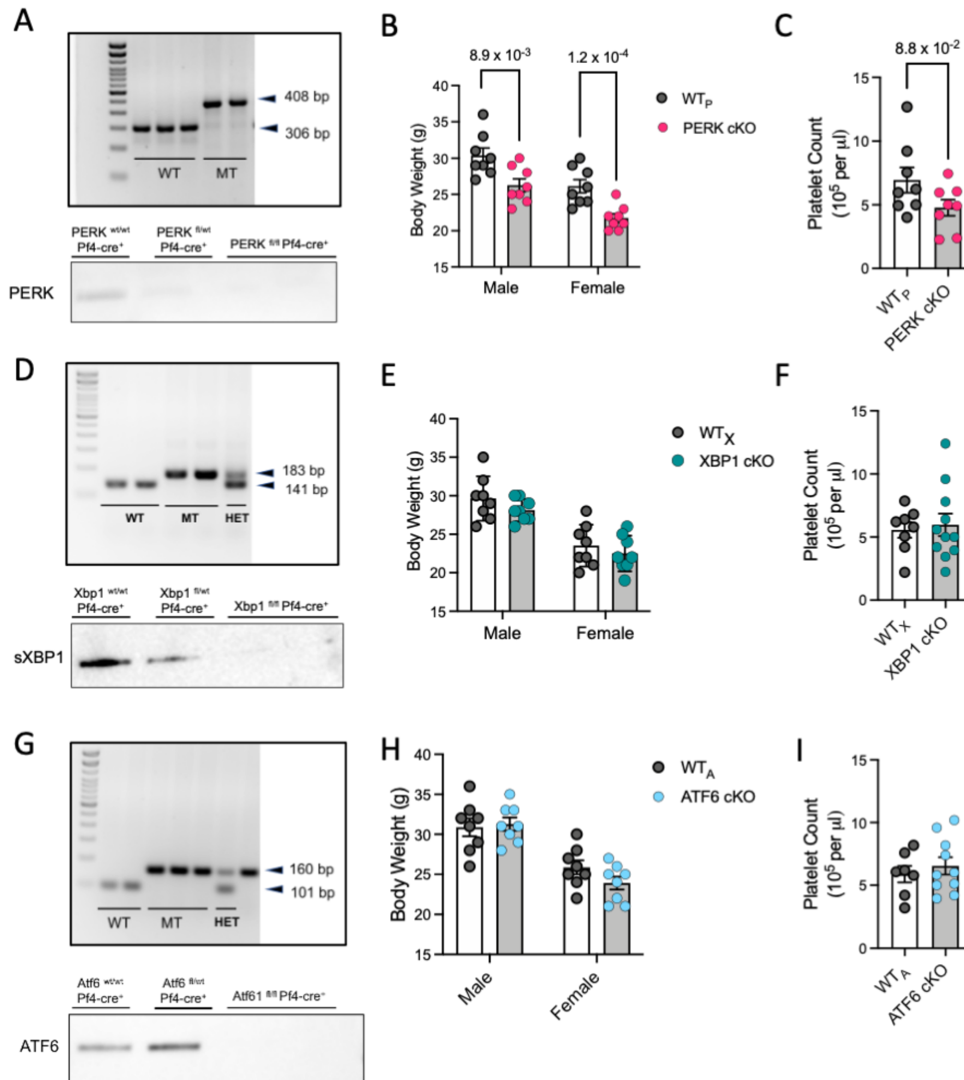
Supplementary Figure S2: **Effect of *in vivo* tunicamycin injections in mice on platelets and tissue**. WT mice were injected i.p with Tunicamycin (Tm) or vehicle (dextrose); n=15 per group, 8 males/7 females. **A-B.** Surface P-selectin levels were measured by flow cytometry in mouse platelets. Shown are histogram and quantification from n=5 independent biological replicates/group. **C**. Quantification of the percentage of apoptotic cells as measured using TMRE dye in both resting and calcium stimulated platelets (n=5/group). **D-E**. Representative immunoblots and quantitation for the expression of pro-apoptotic proteins in lysed mice

platelets (3x 10^8 per mL) following 24 hr Tm injection (n=4/ group). **F**. Quantification for confocal microscopy staining for GRP78 positive platelets. **G**. Representative immunoblots for the expression of UPR proteins in the heart and liver tissues isolated from Tunicamycin injected mice and respective controls (n=3 mice/ group). Values expressed as Mean ± SEM and precise *p*-values were calculated from Mann Whitney test (**B, C, E**) or Student's *t*-test (**F**).

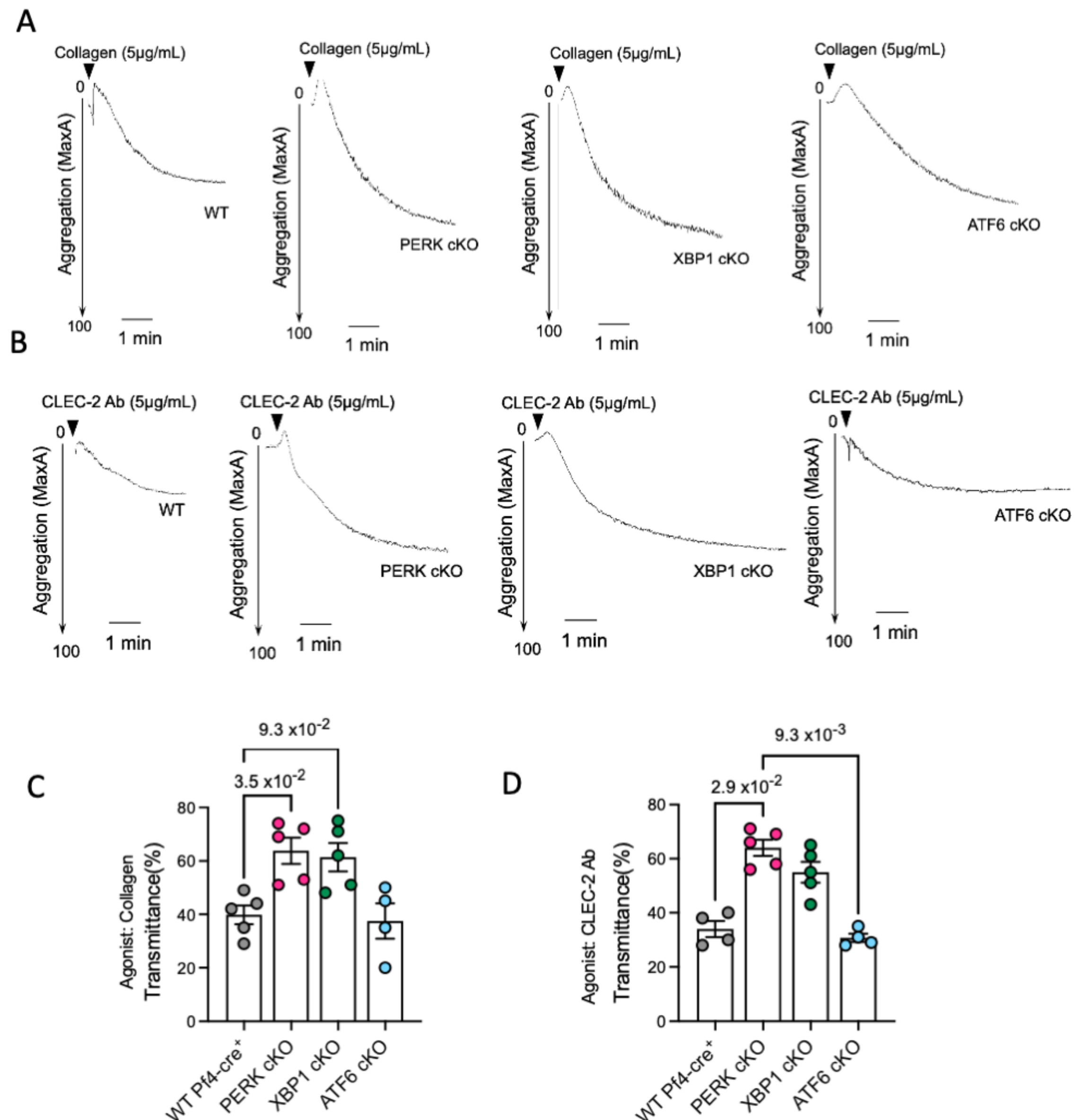


Supplementary Figure S3: **Effect of H_2O_2 induced oxidative stress and DTT induced protein misfolding on human platelets, ex vivo**. **A-F**. Freshly isolated human platelets were treated with H_2O_2 (50 μM) or vehicle (saline) for 30 min at 37°C. **A**. Intraplatelet caspase-3 activity in platelets. **B**. Protein carbonylation as a marker of oxidative protein modifications (n=6/group). **C**. Quantitation for the expression of GRP78, IRE1α and

sXBP1 following H_2O_2 treatment (n=5/ group). **D**. Spliced and unspliced *XBP1* mRNA levels measured by qPCR in vehicle and treated samples (n=6/ group). **E.** Quantification for percentage of GRP78 positive platelets as observed by immunofluorescence microscopy. **F.** Concentration (50 µM and 100 µM) and incubation time (30 min, 60 min) dependent effect of H_2O_2 treatment on intraplatelet protein aggregate formation. **G.** Effect of H_2O_2 concentration and incubation time on the expression of UPR proteins within platelets. For time course, concentration was kept constant at 50 µM ; for concentration studies, time was 30 min. For concentration and incubation time studies, n=3 samples per respective groups. **H-M**. Freshly isolated human platelets were treated with DTT (2 mM) or vehicle (saline) for 30 min at 37°C. **H**. TEM of vehicle and DTT treated platelets acquired on the FEI Tecnai T12 (LaB6, 120 kV). High magnification for HC vehicle and DTT treated samples (low magnification provided in Figure 2 of manuscript). Scale bar, 500nm. **I-J**. Flow cytometry based TMRE assay for mitochondrial apoptosis in platelets treated with DTT or vehicle (n=5/ group). The gated events represent TMRE negative platelets; purity of platelet population confirmed by CD41 staining. **K**. Quantitation for expression of major UPR proteins following DTT treatment (n=4/ group). For phosphorylated proteins, the quantification represents the ratio of phosphorylated to total protein normalized to the respective loading control, GAPDH and presented as fold change over experimental control/vehicle. **L**. Spliced and unspliced *XBP1* mRNA levels normalized to tXBP1. **M**. Quantification for percentage of platelets positive for both IRE1α and PERK expression in confocal microscopy. Values expressed as Mean ± SEM and exact *p*-values calculated from Mann Whitney test (**A-F, J-L**) or Student *t* test (**E, M**).



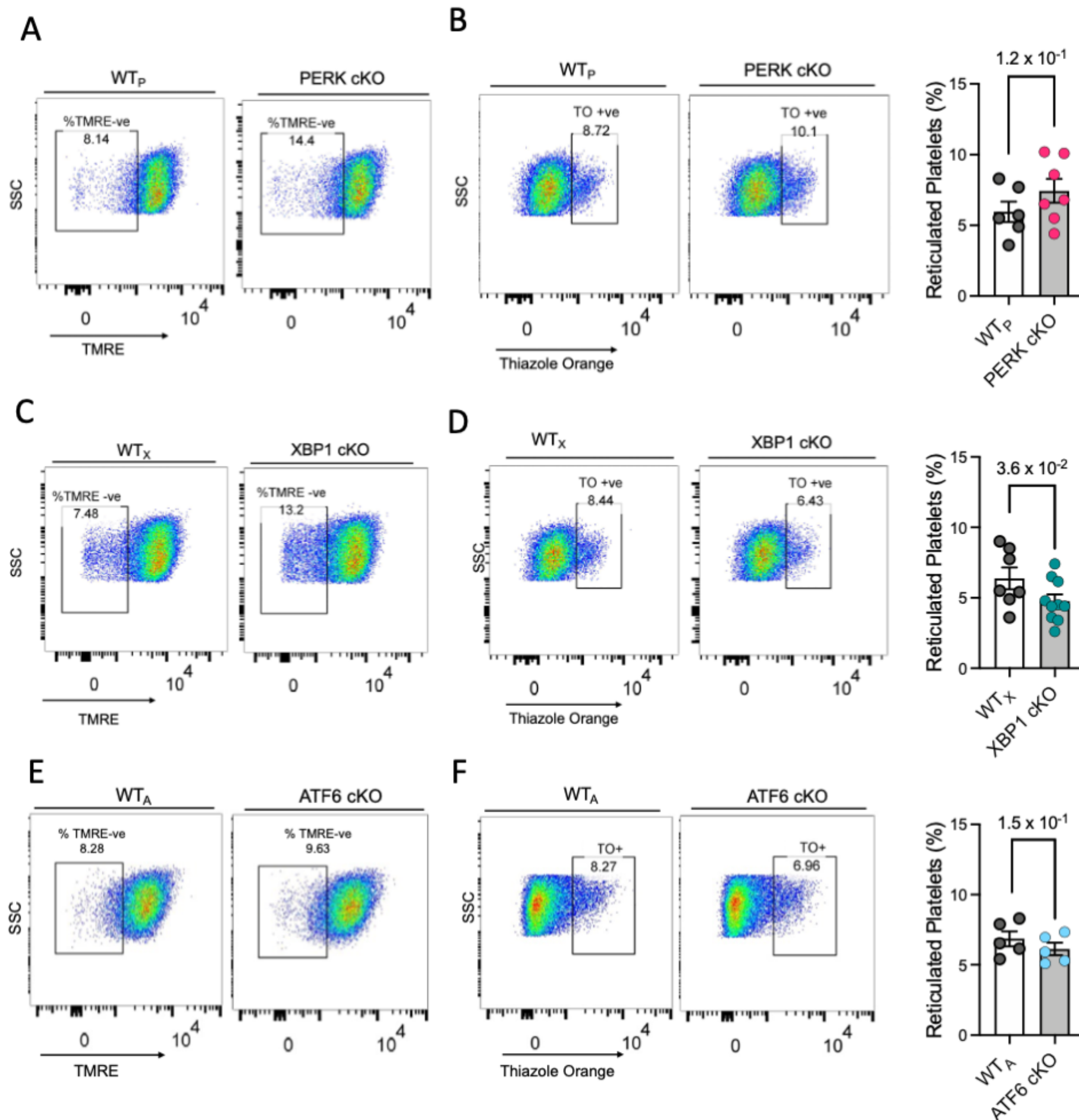
Supplementary Figure S4: **Effect of platelet specific UPR deletion on body weight and platelet counts**. **Panel A-C** is representative for PERK cKO mice, **Panel D-F** displays XBP1 cKO mice and **Panel G-I** shows results of ATF6 cKO mice and their respective littermate controls. Upper panels in **Figure A, D, G** are the genotyping results for each UPR gene following the cross between respective floxed mice and Pf4 iCre mice. WT refers to wild type littermate controls (wt/wt cre^+); MT refers to experimental homozygous mutant mice (fl/fl cre^+) mice and HET refers to the heterozygous (fl/+ cre^+) mice. Bottom Panel shows the immunoblotting for the respective deleted UPR gene. **B, E, F**. Body weights of male and female mice measured at 10 weeks of age expressed in grams. n=8 mice/ gender for each strain. **C, F, I.** Platelet counts for each strain of mice expressed as 10^5 platelets per µL. Results shown from n=8 WT_P and n=8 PERK cKO, n=8 WT_X and n=11 XBP1 cKO , n=7 WT_A and n=10 ATF6 cKO mice respectively. Values expressed as Mean ± SEM and *p*-values calculated from Student *t* test.



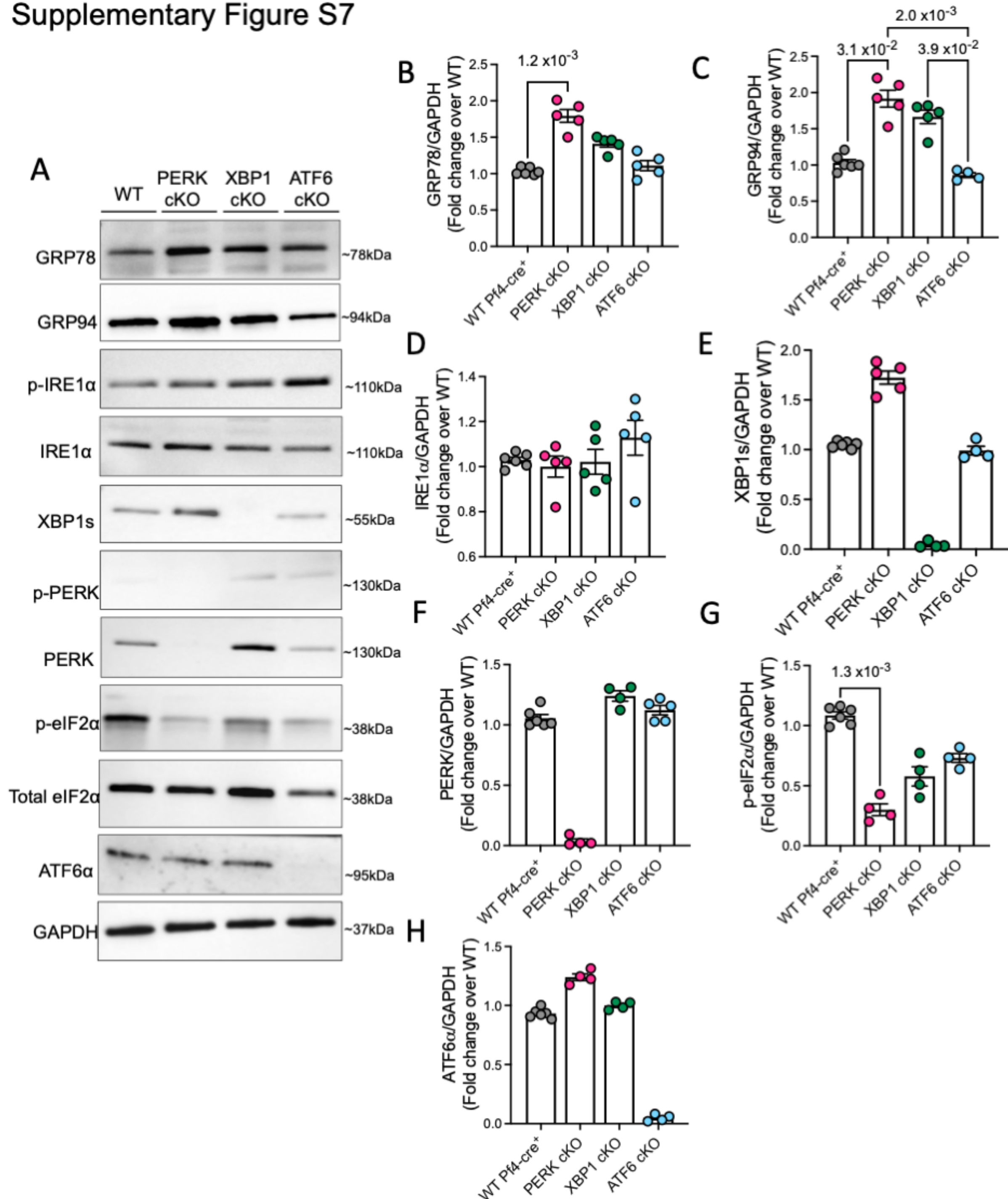
Supplementary Figure S5: **Effect of UPR gene deletion on ex-vivo platelet aggregation.** Mice blood samples were pooled from n=3 mice and washed platelets prepared in Tyrode's buffer as described. Aggregation curves reflect results from n=5 WT, PERK cKO and XBP1 cKO mice and n=4 ATF6 cKO pooled biological replicates. Washed platelets were adjusted for platelet counts ($2x10^8$/ml) and platelet aggregation was performed using the agonists, collagen and CLEC-2 stimulatory antibody at the indicated concentrations. **Panel A**. Representative aggregation curve for the response of platelets from the respective conditional knockout mice and wild type (WT) controls to collagen (5µg/mL) showing % increase in light transmission through washed platelets upon addition of agonist at indicated points (arrowhead). **Panel B**. Representative

aggregation curve for the response of platelets from respective cKO mice and WT controls to CLEC-2 stimulatory antibody (5µg/mL). The maximum amplitude of percent light transmission was recorded for quantitation. **C-D**. Quantification for the mouse aggregation for collagen **(C)** and CLEC-2 **(D)**. Values expressed as Mean ± SEM. Exact *p* value shown corresponds to Kruskal-Wallis test with Dunn's multiple comparisons.

Supplementary Figure S6

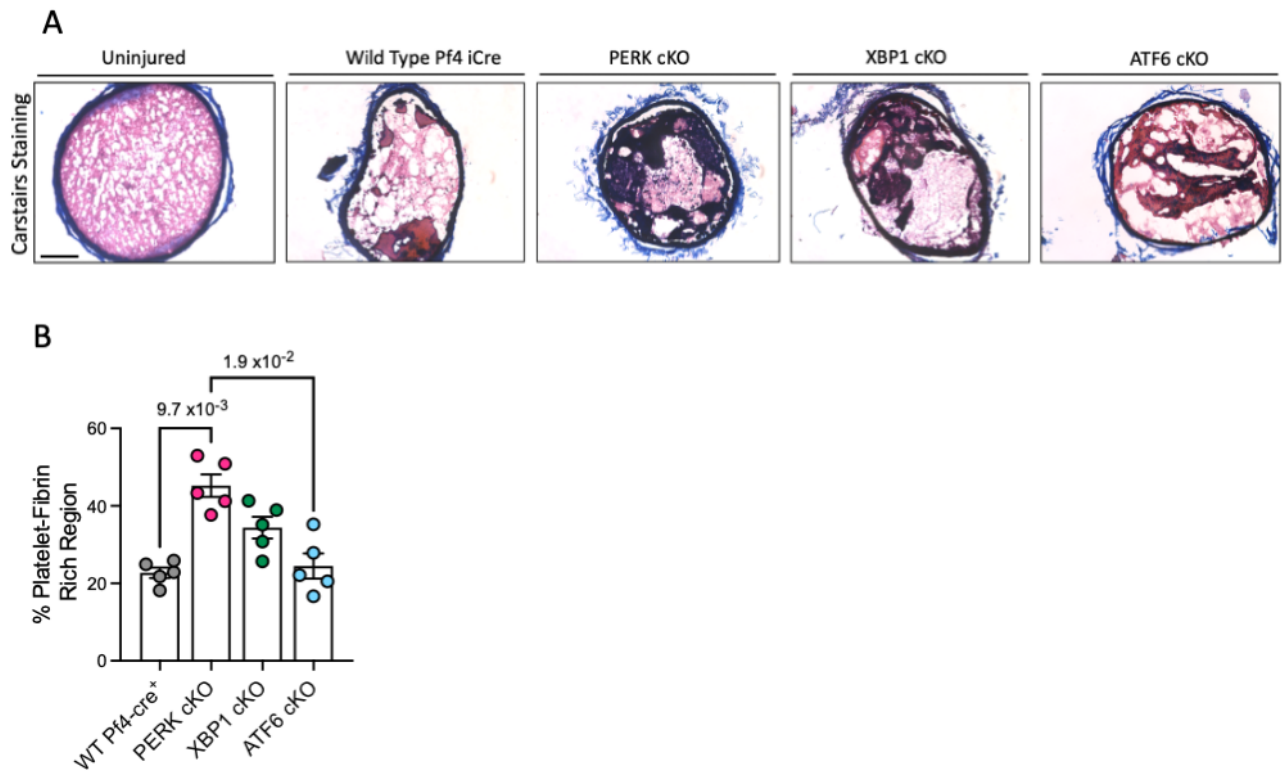


Supplementary Figure S6: **Effect of platelet specific UPR deletion on apoptosis and platelet turnover. Panel A, C, E.** Representative images for FACS based TMRE assay for mitochondrial apoptosis. **Panel B, D, F**. Representative flow images and quantification for measurement of reticulated platelets (thiazole orange-based assay) using flow cytometry in respective KO mice strains. Mouse platelets were gated on FSC/SSC, excluding debris and aggregates and purity of platelet population confirmed by CD41 staining. Quantification of thiazole orange staining reflects the results from n=6 WT_P and n=7 PERK cKO, n=7 WT_X and n=10 XBP1 cKO , n=5 WT_A and n=5 ATF6 cKO mice. **A-B**. Results from PERK cKO mice. **C-D.** Results from the XBP1 cKO mice. **E-F**. Results from the ATF6 cKO mice. Values expressed as Mean ± SEM and *p*-values correspond to Mann Whitney test.



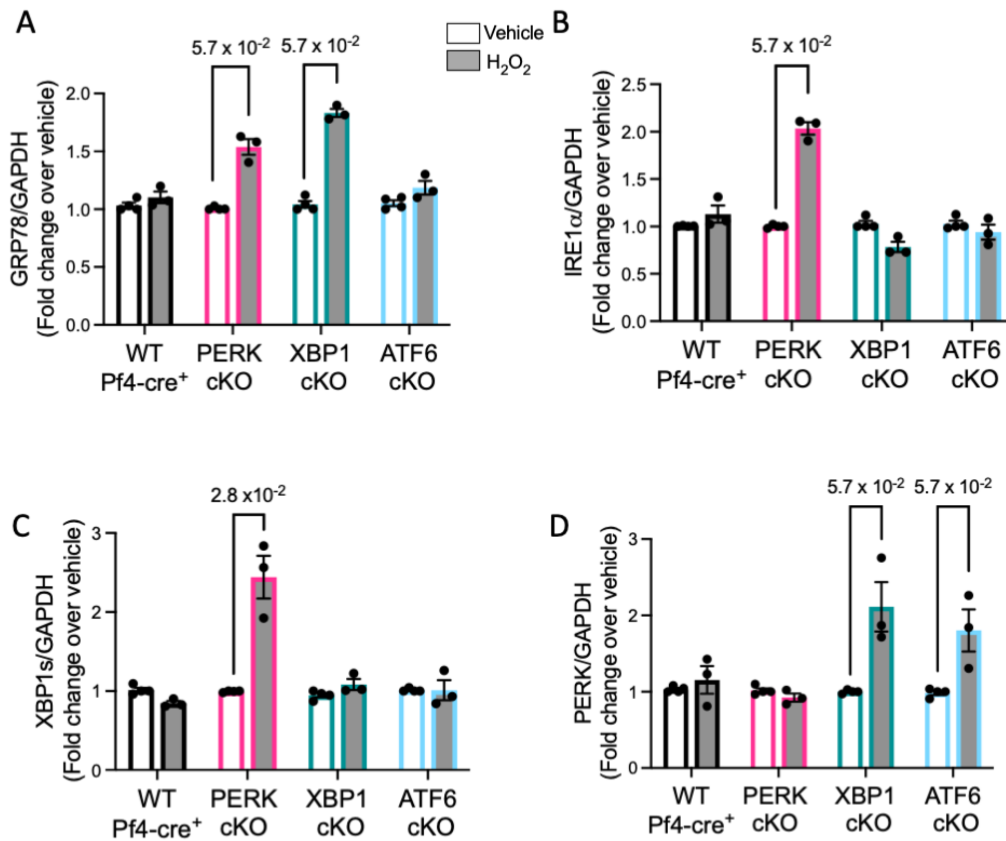
Supplementary Figure S7: **Effect of platelet specific UPR gene deletion on the UPR pathways, under physiological conditions.** Mice blood samples were pooled from n=2 mice for each of the conditional knockout strains and littermate controls and isolated platelets ($3x10^8$ per mL) lysed in RIPA buffer and resolved on SDS PAGE gel. **A**. Representative immunoblots for the expression of the UPR pathway proteins. **B-H**. Quantitation for the relative expression of UPR proteins in the cKO mice, as compared to WT platelets.

For phosphorylated proteins, the quantification represents the ratio of phosphorylated to total protein normalized to respective loading control, GAPDH and presented as fold change over WT control. Values expressed as Mean ± SEM. The exact *p* value shown corresponds to Kruskal-Wallis test with Dunn's multiple comparisons.

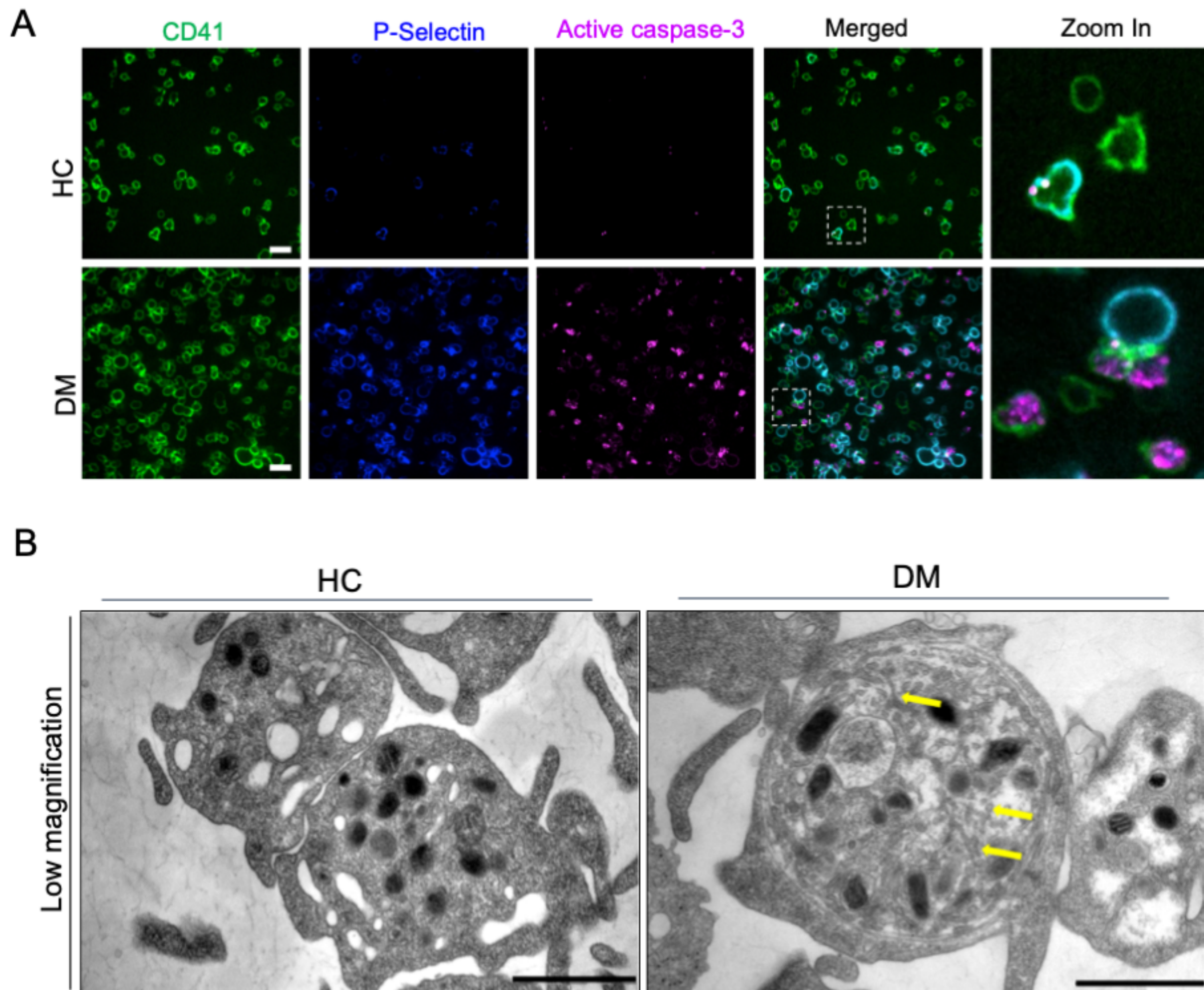


Supplementary Figure S8: **Effect of platelet specific UPR gene deletion on in-vivo thrombus formation.** **A**. Representative panels for Carstairs Staining for thrombus composition in carotid arteries from the cKO and WT mice following FeCl3 induced injury (n=5/ group). Different components of thrombus show different colors in Carstairs staining (bright red for fibrin, gray-blue to navy blue for platelets, bright blue for collagen, red for muscle, and clear yellow for red blood cells). Original magnification 20X. Scale bar, 0.1mM. **B**. Quantitation of thrombus size presented as the percentage of platelet-fibrin rich thrombus area of the total vessel area by ImageJ. Slides evaluated by an independent observer, blinded to the sample identity. Values expressed as Mean ± SEM and *p* value shown corresponds to Kruskal-Wallis test with Dunn's multiple comparisons.

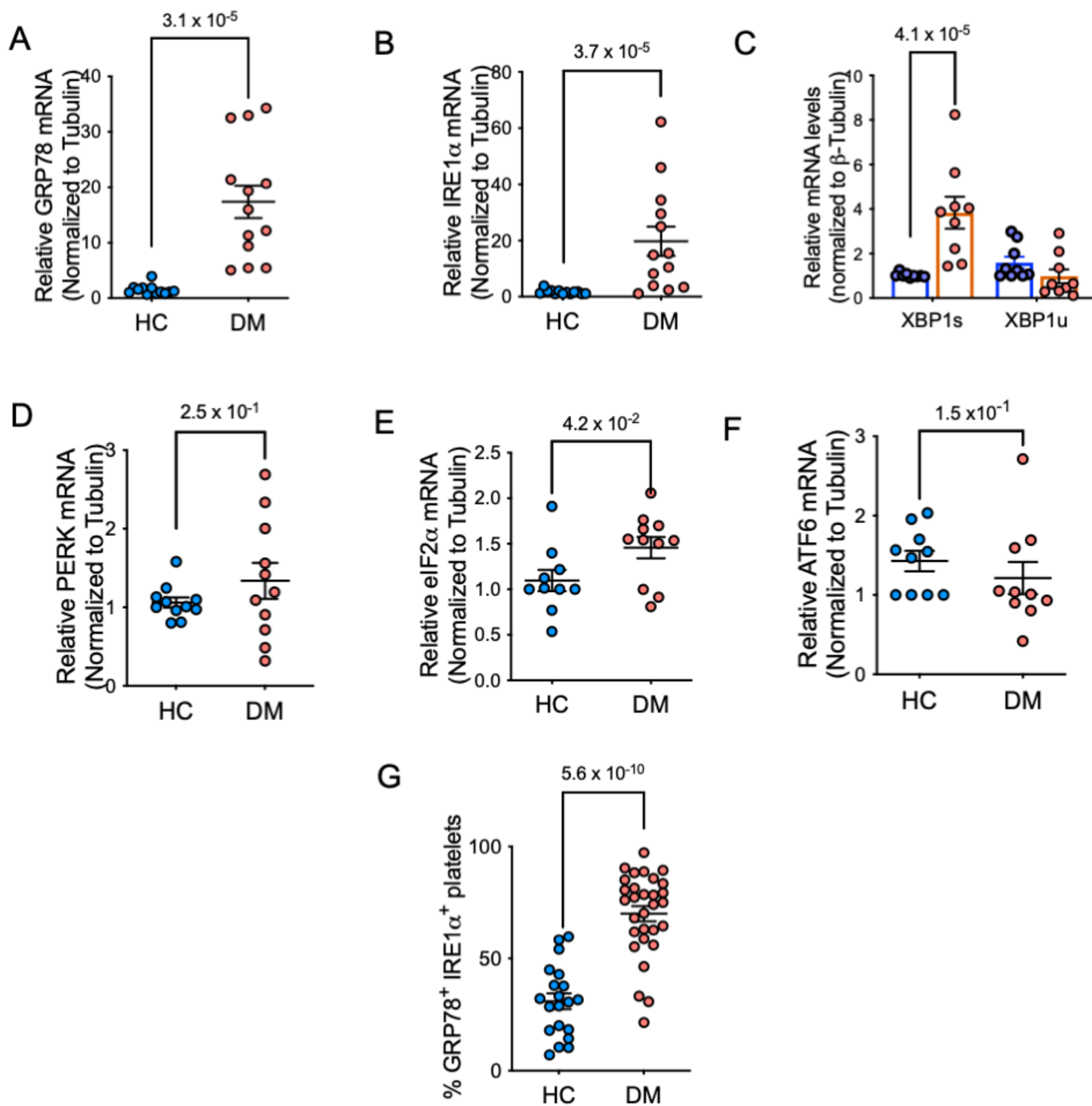
Supplementary Figure S9



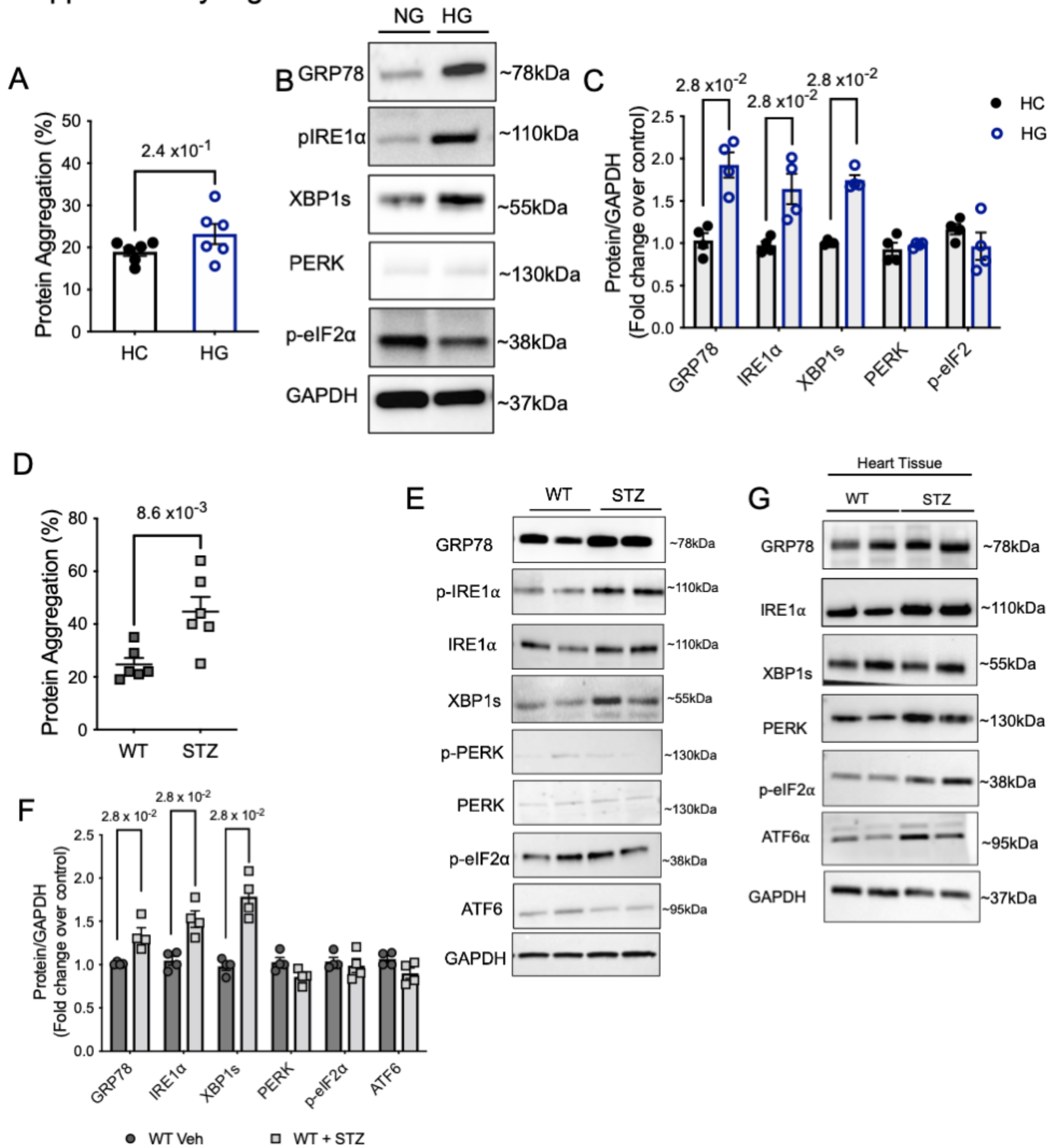
Supplementary Figure S9: **Effect of ex vivo oxidative stress on the expression of UPR proteins in the platelet specific UPR knockout mice.** Freshly isolated platelets (3×10^8 platelets per mL, n=3 samples pooled to obtain the appropriate platelet counts) from each mouse KO strain and their respective littermate controls (n=4 per WT littermate control and n=3 per KO strain) were incubated for 30 min with 50 µM H_2O_2. **A-D**. Quantitation for the relative expression of the UPR proteins in the cKO mice, as compared to the wild type platelets. Values expressed as Mean ± SEM. Data presented as scatter plots with mean ± SEM and *p* value corresponds to Mann Whitney tests for comparison between vehicle and H_2O_2 treated groups.



Supplementary Figure S10: **Platelet phenotype in Type II diabetic patients**. **A**. Panel showing confocal microscopy images showing the localization of active caspase -3 (magenta) and p-selectin (blue) in platelets (green, CD41). Dotted box shows the Zoom-in on individual platelets. Scale Bar is 10 µm. **B.** TEM images of the control and DM platelets. Bar represents 1 µm. Electron and confocal microscopy performed for a total of n=5 independent experiments.

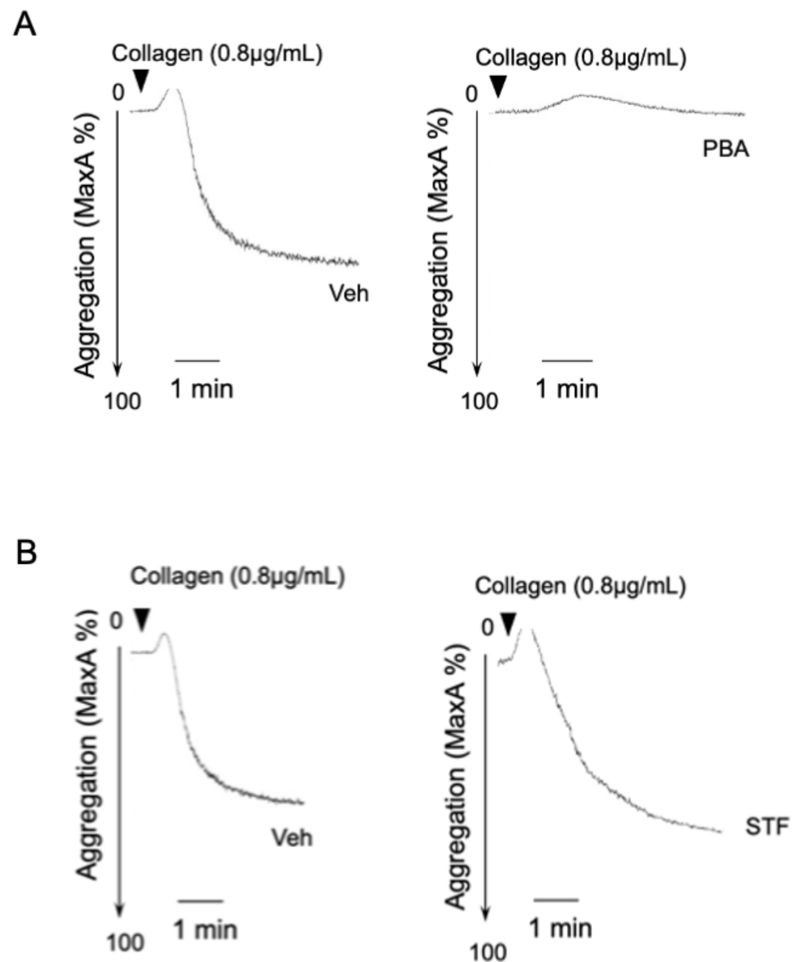


Supplementary Figure S11: Expression of UPR genes in the platelets. **A-F**. Platelet mRNA was isolated and qPCR experiments performed for the respective genes. **A**. GRP78 (*hspa5*) (n=12 HC, n=13 DM). **B.** IRE1α (*ern1*) (n=13 for HC and DM). **C**. *XBP1* (spliced and unspliced) (n=9 for HC and DM). **D**. PERK (*eif2ak*) (n=11 for HC and DM). **E**. *p-eIF2-α* (n=10 for HC, n=11 DM). **F**. *ATF6* (n=10 for HC and DM). **G**. Quantification of platelets positive for both GRP78 and IRE1α. Experiments performed in a minimum of n≥10 samples for gene expression studies and n=5 samples for confocal imaging. Values expressed as Mean ± SEM. *p*-values correspond to Student *t* test (**A, D-E, G**) or Mann Whitney test **(B, C, F)**.



Supplementary Figure S12: **Effect of ex vivo hyperglycemic experiments and STZ induced diabetes in mice on the UPR**. **A-C**. Freshly isolated human platelets (n=6/group) incubated with 5.5 mmol/l glucose (normoglycemic, NG) or 25 mmol/l glucose (hyperglycemic, HG) for 120 minutes at 37°C. **A**. Protein aggregate formation within platelets. **B-C**. Representative immunoblots and quantification for expression of UPR proteins in platelets following hyperglycemia (n=4/group). **D-F**. Platelets isolated from STZ induced

diabetes mice (n=6; 3 males/3 females). **D**. Protein aggregation in WT and STZ mice. **E-F**. Representative immunoblots and quantification for expression of UPR proteins (n=4/group). **G**. Representative immunoblots for the expression of UPR proteins in heart tissues isolated from STZ injected diabetic mice and controls (n=3 heart samples/ group). Values expressed as Mean ± SEM. *p*-values correspond to Mann Whitney test.



Supplementary Figure S13: Effect of chemical chaperone 4-PBA and IRE1α inhibition on human platelet aggregation. Healthy control (HC) samples (n=3 per group) randomly selected for ex vivo interventions to study the effect of UPR modulation on platelet phenotype. **Panel A.** Washed platelets were incubated with 4- phenylbutyric acid (PBA), a chemical chaperone (5mM) or vehicle (PBS) for 3 hr at 37°C. **Panel B**. Washed platelets were incubated with STF-083010 (STF, 30μM) or vehicle. **A-B**. Representative platelet aggregation tracings to collagen (5μg/mL) showing % increase in light transmission through washed platelets upon addition of agonist at indicated points (arrowhead).

Supplemental Tables

Supplemental Table S1: Clinical Characteristics of Diabetes Mellitus Type II patients and healthy controls.

	Healthy Control (HC) n=45	**Diabetes Mellitus Type II (DM)** n=102
Age (Mean ± SD)	42.05 ± 8	55.98 ± 10
Males, n (%)	29 (64)	68 (67)
BMI (kg/m^2 ,Mean ± SD)	25.72 ± 4	34.29 ± 7
Blood Glucose (Mean ± SD)	81.12 ± 17	191.4 ± 78
HbA1c (%)	NA	8.39 ± 2
Platelet Count (x 10^3per µl, Mean ± SD)	250 ± 30	227 ± 67
Smokers, n (%)	3 (6)	15 (15)
Co-Morbidities		
Hypertension, n (%)	0/45 (0)	76 (75)
CAD, n (%)	0/45 (0)	21 (21)
Retinopathy, n (%)	0/45 (0)	18 (18)
Neuropathy, n (%)	0/45 (0)	15 (15)
Nephropathy, n (%)	0/45 (0)	13 (13)
Medications		
Aspirin, n (%)	0/45 (0)	47 (46)
Statin, n (%)	0/45 (0)	52 (51)
Beta-Blocker, n (%)	0/45 (0)	41 (40)
ACE inhibitor, n (%)	0/45 (0)	28 (27)
Insulin, n (%)	0/45 (0)	58 (57)
Metformin, n (%)	0/45 (0)	77 (75)
Other DM Drugs, n (%)	0/45 (0)	48 (47)