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Platelet dysfunction during trauma involves diverse signaling pathways and an inhibitory activity in patient-derived plasma

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Abstract

Background—Trauma-induced coagulopathy (TIC) occurs in about 25% of injured patients and accounts for about 10% of deaths worldwide. Upon injury, hemostatic function may decline due to vascular dysfunction, clotting factor deficiencies, hyperfibrinolysis, and/or platelet dysfunction. We investigated agonist-induced calcium signaling in platelets obtained over time from trauma patients.

Methods: Platelets from trauma patients and healthy donors were monitored via intracellular calcium mobilization and flow cytometry markers ($\alpha_2\beta_3$ activation, P-selectin display, and phosphatidylserine exposure) following stimulation with a panel of agonists [ADP, U46619, convulxin, PAR-1/4 activating peptides, iloprost] used in isolation or in pairwise tests. Furthermore, healthy donor platelets were tested in heterologous plasma isolated from healthy subjects and trauma patients.

Results: When exposed to agonists over the first 24 hr post-injury, trauma patient platelets mobilized less calcium in comparison to healthy platelets. Partial recovery of platelet activity was observed in about a third of patients after 120 hr, although not fully obtaining healthy baseline function. Flow cytometry markers of trauma platelets were similar to healthy platelets prior to stimulation, but were depressed in trauma platelets stimulated with ADP or convulxin. Also, washed healthy platelets showed a significant reduction in calcium mobilization when reconstituted in plasma from trauma patients, relative to healthy plasma, at all plasma doses tested.

Conclusion: Platelet dysfunction in trauma patients included poor response to multiple agonists relevant to hemostatic function. Furthermore, the inhibitor effect of patient plasma on healthy

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AUTHORSHIPS

Contributions: C.C.V and S.L.D. designed research, analyzed and interpreted the data, and wrote the manuscript; C.C.V. performed the research. A.D. conducted TEG experiments and compiled the data. S.B. organized patient demographic and health records on electronic database. C.A.S. served as the primary P.I. for the TrICI study.

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platelets suggests that soluble plasma species may downregulate endogenous or transfused platelets during trauma.

Level of evidence—Prognostic/Epidemiological, Level III

Keywords

coagulopathy; hemostasis; trauma

BACKGROUND

Traumatic injuries are one of the most common causes of death in the United States and abroad, accounting for about 10% of fatalities worldwide (1). Trauma is well recognized as the leading cause of death for younger populations (persons aged 30–40) (1,2). Upon admission to trauma centers for a wide range of mechanisms of injury (MOI), approximately 25% of patients will present with a condition known as trauma-induced coagulopathy (TIC) (1,3), a complex clinical state highlighted by impaired activity of important blood clotting proteins, excessive fibrinolysis, and significant platelet dysfunction (1). The intersection of these phenomena tends to lead to a coagulopathic bleeding phenotype which becomes difficult to treat and increases the chance of mortality by more than 4-fold (4).

Several events may combine to drive TIC. For example, perturbation of the endothelium during a response to a traumatic episode can lead to generation of activated protein C (APC) and glycocalyx shedding, both of which serve anticoagulant and profibrinolytic roles (1). Other biochemical changes also occur in concert with hemorrhagic shock, including release of tissue factor (TF) and tissue plasminogen activator (tPA), which lead to thrombin and plasmin generation in the circulation (2,5,6). The coinciding coagulant and lytic processes are responsible for elevated levels of soluble fibrin species, which have traditionally been quantified by plasma levels of fibrin degradation products such as D-dimer (7–10). However, the full extent and mechanisms of acquired platelet dysfunction remain poorly understood.

Platelet dysfunction in trauma patients has been reported in several studies through the use of traditional assays like aggregometry, thromboelastography (TEG), and microfluidics (11–14). Key findings include decreased aggregation responses to an array of platelet agonists in about half of the tested subjects (12), 80% inhibition of ADP-induced platelet function (13), and defects in deposition to collagen under flow in two-thirds of the patient cohort (14). The concept of an “exhausted” platelet arises from initial hyperstimulation in response to tissue damage and eventual tiring of the cell as it continues in circulation (13). Proteolysis or internalization of platelet receptors, including glycoproteins VI (GPVI) or Ib (GP1b), might also play a role in downregulation of platelet function (15). Soluble fibrin has been shown to cause a signaling defect in GPVI (16), possibly contributing to impaired initial platelet adhesion to exposed collagen at the site of vascular injury if platelets interact with circulating fibrin species prior to encountering a wound site in need of hemostatic response.

Studies of platelets from trauma patients have typically only used one or two major agonists. With technologies for high dimensional phenotyping of calcium signaling pathways (17–19), building patient-specific phenotypic profiles of blood cell function may provide more

information for treatment decisions. This work focuses on assessing platelet function in trauma patients using a high-throughput technique to interrogate up to six platelet signaling pathways via combinatorial stimulation of surface receptors and using intracellular calcium mobilization as a dynamic readout. Additionally, we investigated platelet dysfunction during trauma by examining the effects of trauma patient plasma on healthy platelets. By all metrics, platelets from trauma patients frequently display a defect in agonist response and the plasma from trauma patients may influence this dysfunctional platelet phenotype.

METHODS

Reagents

Apixaban (SelleckChem, Houston, TX, USA), D-phenylalanyl-prolyl-arginyl chloromethylketone (PPACK; Essex Junction, VT, USA), adenosine 5'-diphosphate sodium salt (ADP), prostaglandin E₁ (PGE₁), citrate concentrated solution, and apyrase (Sigma-Aldrich, St. Louis, MO, USA), the GPVI agonist convulxin (Cayman Chemical, Ann Arbor, MI, USA), the TP agonist U46619, the IP agonist iloprost (Tocris Bioscience, Bristol, UK), the PAR4 agonist peptide H-Ala-Tyr-Pro-Gly-Lys-Phe-NH₂ trifluoroacetate salt (AYPGKF) and the PAR1 agonist peptide H-Ser-Phe-Leu-Leu-Arg-Asn-OH trifluoroacetate salt (SFLLRN) (Bachem, Torrance, CA, USA), Fluo-4 NW calcium dye and probenecid (Invitrogen, Carlsbad, CA, USA), and FITC mouse anti-human PAC-1, PE mouse anti-human CD62P, and Cy5 Annexin V fluorescent antibodies (BD Biosciences, San Jose, CA, USA) were used and stored according to manufacturers' instructions. HEPES-buffered saline (HBS, pH 7.4) was prepared with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Fisher Scientific, Fair Lawn, NJ, USA) and 150 mM NaCl (Fisher); Tyrode's buffer (pH 7.4) was prepared with 10 mM HEPES, 127.2 mM NaCl, 11.9 mM NaHCO₃ (Fisher), 5 mM KCl (Fisher), 0.4 mM NaH₂PO₄ (Fisher), 1 mM MgCl₂ · 6 H₂O (Sigma), and 5 mM D-glucose (Sigma). Where specified, Ca²⁺ HBS was prepared by dissolving CaCl₂ · 2 H₂O in HBS to achieve a calcium concentration of 2 mM.

Study design

Under Institutional Review Board approval, blood was obtained from trauma patients (n=16) who had been admitted to the Penn Presbyterian Medical Center Level 1 Trauma Center following a severe injury. Patients who are 18-years-old or older, present as a trauma alert, and are admitted to the Trauma and Surgical Intensive Care Unit (TSICU) were eligible for the study. Upon arrival to the trauma bay, a research blood sample (15 mL) and clinical data were collected by Penn Acute Research Collaboration (PARC) research staff. The legal authorized representative and/or patient is consented once available and after being debriefed by the clinical care team. Descriptions of patient demographics and clinical presentations are outlined in Table 1.

For each enrolled patient, blood samples were taken at specific time points (0, 3, 6, 12, 24, 48, and 120 h) after initial admission. Several aliquots with specific anticoagulants were prepared for various clinical tests according to the previously established protocol; one such tube pre-loaded with apixaban (1.25 µM, final concentration) and PPACK (100 µM, final concentration) was prepared for the studies in this paper. Collecting blood (2 mL) into two

anticoagulants ensured inhibition of factor Xa-mediated thrombin generation and thrombin activity, respectively. The sample was split into two microcentrifuge tubes to isolate platelet-rich plasma (PRP) for immediate platelet function studies and platelet-poor plasma (PPP) to be frozen for future analysis.

In addition to the patient cohort, a set of healthy donors (n=11) was recruited to donate blood according to University of Pennsylvania Institutional Review Board approval. Donors self-reported to being free of any medication or alcohol use for 3 days leading up to the blood draw. Additionally, female donors self-reported to not using oral contraceptives.

Intracellular calcium mobilization assays

Platelet calcium measurements were conducted using a 384-well plate format as previously described (16). Briefly, PRP was prepared via centrifugation of whole blood (120g, 10 min) and incubated with Fluo-4 NW calcium dye for 30 min. The dye was reconstituted with 5 mL HBS and 200 μ L probenecid (77 mg/mL) to prevent dye leakage from cells during incubation. One 384-well plate was filled with dilute, dye-loaded PRP (12% final concentration after agonist dispense), while a second plate was prepared with platelet agonists at specified concentrations. Using the pairwise agonist scanning (PAS) method as motivation (17,19), an array of combinatorial stimuli (platelet agonists/antagonists) was used to develop subject-specific phenotypic profiles of platelet activity. As reported previously, PAS involves exposing dilute PRP to all single and pairwise combinations of up to six stimuli at low, medium, and high concentrations, resulting in 154 unique conditions. In order to complete two replicates/condition, ~15–20 mL of whole blood is required to isolate the necessary amount of PRP. However, due to volume constraints in the protocol (2 mL whole blood per patient sample), the combinatorial space was restricted to 31 unique conditions – single combinations of five agonists (ADP, EC_{50} =1 μ M; U46619, EC_{50} =1 μ M; convulxin, EC_{50} =2nM; SFLLRN, EC_{50} =10 μ M; and AYPGKF, EC_{50} =300 μ M) at three dosage levels each (0.1x, 1x, 10x EC_{50}), and pairwise combinations of the same five agonists as well as one antagonist (iloprost, EC_{50} =0.2 μ M) with each component present at a medium dose (1x EC_{50}) (Supp. Fig. S1). The two well plates were then inserted into a FlexStation 3 automated plate reader (Molecular Devices, Sunnyvale, CA, USA) and liquid handling functions were used to dispense 20 μ L of agonist(s) directly into each well of 30 μ L PRP. The fluorescence of the plate was read column-wise (Ex: 485 nm; Em: 525 nm) for 20 s prior to dispense and then for an additional 4 min; the dynamic signal $F(t)$ was also normalized to the pre-dispense baseline F_0 . As a metric of each platelet sample, the amount of calcium mobilized for all stimuli can be calculated as the Total Platelet Calcium Mobilization (TPCM) by Eqn. 1:

$$TotalPlateletCalciumMobilization = \sum_{i = PAScondition} (Ca^{2+}AUC(i) - Ca^{2+}AUC(buffer)) \quad (1)$$

where AUC is area-under-the-curve for calcium trace obtained for the i^{th} -condition.

Flow cytometry assays

Monitoring platelet activation via flow cytometry was conducted as documented previously (20). In short, PRP was isolated as described above and diluted to 10% v/v with Ca^{2+} HBS. In each test tube, 10 μL dilute PRP was added to 74 μL Ca^{2+} HBS and 2 μL each of three fluorescently-labeled antibodies (FITC anti-PAC-1 for $\alpha_{2b}\beta_3$ activation, PE anti-CD62P for P-selectin expression, and Cy5 Annexin V for PS exposure). Prior to analysis, 10 μL of platelet agonists were added to the tubes and allowed to incubate in the dark for 10 min. Each sample was analyzed using an Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA), setting the flow rate to low (14 $\mu\text{L}/\text{min}$, 10 μm core) and reading for 60 s. Data were collected and reported as mean fluorescence intensity (MFI) for activated $\alpha_{2b}\beta_3$ and P-selectin, or % PS-positive cells as determined by pre-set gating techniques.

Washed platelet preparation for plasma reconstitution experiments

Plasma samples were collected from healthy/patient whole blood and stored at -80°C for future analysis. Healthy washed platelets (WP) were prepared fresh following standard procedures. Specifically, whole blood was drawn into syringes containing citrate concentrated solution (4% w/v) in a 1:9 citrate:whole blood ratio and apixaban (1.25 μM). PRP was isolated as described above, incubated in calcium dye for 30 min, and recalcified with 2 mM CaCl_2 . The PRP mixture was diluted into Tyrode's buffer supplemented with 1 μM PGE_1 and 1 U/mL apyrase prior to further centrifugation (1200g, 14 min). The supernatant was removed and the resulting platelet pellet was resuspended in Ca^{2+} HBS. Washed platelets were combined with plasma samples at specified volume fractions and incubated for approximately 30 min prior to platelet function challenge with various agonists (Supp. Fig. S2). For example, a final plasma concentration of 12% is achieved by addition of 6 μL PPP to 24 μL WP and 20 μL agonist. Calcium mobilization measurements were recorded following the same procedure detailed in the previous section.

Thromboelastography

Blood samples were collected on arrival to the trauma bay (T0), and at 3h, 6h, 12h, 24h, 48h, and 120h and immediately aliquoted into tubes containing distinct anticoagulants for assessment of platelet function or hemostasis state using the TEG 6s Hemostasis system (Haemonetics Corp., Braintree, MA, USA) as per the manufacturer's instructions. To monitor the kinetics of patient blood clotting, one mL of blood was mixed with 15.87 units of heparin (Sagent Pharmaceuticals, Schaumburg, IL, USA) and was pipetted into the PlateletMapping ADP cartridge (Haemonetics, 07-615-US). Separately, whole blood was anticoagulated in 3.2% buffered sodium citrate (BD Vacutainer, Becton, Dickson & Co., Franklin Lakes, NJ, USA) and pipetted into the Citrated Multichannel Cartridge (Haemonetics, 07-601-US) which provides clotting characteristics data simultaneously from four independent assays. The de-identified data was stored in a secure server.

Statistical analysis

Analysis of raw calcium fluorescence data was performed by MATLAB version R2016a (MathWorks, Natick, MA). Statistical significance tests of results based on experimental

condition were conducted using a two-tailed unpaired Student's t-test in Prism version 5.03 (GraphPad Software, San Diego, CA).

RESULTS

Calcium mobilization measurements indicate global platelet dysfunction in trauma patients

A total of 31 blank, single, and pairwise mixtures of platelet agonists were prepared as shown in the color-coded concentration map (Fig. 1A) where each row corresponds to a specific input agonist mixture (dark blue = no agonist; light blue = low concentration; yellow = medium concentration; red = high concentration). Each stimulation condition yields a normalized calcium trace $[F(t)/F_0]$ and all 31 traces are shown as 31 rows in a heatmap (Fig. 1B-C). Comparison of the platelets from healthy donors (Fig. 1B) and trauma patients (Fig. 1C) demonstrate a markedly dysfunctional phenotype in the trauma patient platelets across all types of agonist stimuli. While trauma platelets responded best to the highest dose of convulxin, this response was greatly attenuated compared to healthy platelets. Overall, the healthy donor pool exhibited a significantly greater extent of TPCM than the trauma patient cohort (Fig. 1D-E). The average TPCM for healthy platelets was 220 ± 62 a.u. (Fig. 1D), greater than that found for every trauma patient at the initial time point of the test 0-hr TPCM (Fig. 1E). TPCM was depressed for every patient in blood samples tested between 0 and 12 hr. In general, TPCM displayed an increasing trend with time in several patients, but only 4 patients (Patients 014, 019, 020, 038) had a single reading in the healthy range at times between 12 and 120 hr, potentially due to platelet transfusion.

Trauma patient platelets exhibit decreased $\alpha_{2b}\beta_3$ activation, P-selectin expression, and phosphatidylserine exposure upon stimulation in flow cytometry

Beyond calcium mobilization, platelet activation also can drive integrin activation, granule release and phosphatidylserine exposure (especially with collagen/thrombin stimulation). Fluorescently activated $\alpha_{2b}\beta_3$ antibody (PAC1), P-selectin antibody, and annexin V were used in a flow cytometry assay to measure platelet activation in the healthy and patient cohorts. Dilute (1%) PRP was stimulated with calcium-containing HBS, ADP (2 μ M), or convulxin (4 nM). Unstimulated trauma platelets displayed low levels of activated $\alpha_{2b}\beta_3$, P-selectin, and PS indicating that they were not in a classically "activated" state at the start of the assay. The trauma platelets, however, were able to display stimulated levels of $\alpha_{2b}\beta_3$, P-selectin, and PS, but to an extent much lower than that seen with healthy platelets (Fig. 2A-C). As was observed in the calcium mobilization assay, addition of convulxin caused the greatest response in both healthy subjects and trauma patients. Significant PS exposure was only observed in healthy donors when subjected to convulxin; the other data showed no detectable difference above the buffer control response (Fig. 2C). A representative set of results for a healthy donor is shown in Supp. Fig. S3.

Thromboelastography (TEG) data reveals patient platelet defects in whole blood samples

Whole blood samples from trauma patients were anticoagulated with sodium citrate or heparin and dispensed into TEG cartridges to measure platelet function and/or hemostatic state. For each test using citrated blood, the machine generates an output of five parameters

describing different aspects of the clot formation process: the time to initial fibrin formation (R), the time to achieve a certain level of clot strength (K), the rate of clot generation (angle), the strongest point of fibrin clot (MA), and the extent of fibrinolysis 30 min after achieving MA (LY30). Using the RapidTEG reagent to interrogate both the intrinsic and extrinsic coagulation pathways with kaolin and tissue factor, respectively, patient blood samples exhibit relatively accelerated coagulation characteristics, namely clotting times and rates, with little to no observed lysis (Fig. 3A-D). However, platelet-driven clot development and strength appear to be diminished during the first six hours post-trauma, as suggested by the MA parameter (Fig. 3E), typically most indicative of platelet function (21). Further, ADP stimulation of heparinized whole blood reveals a distinct platelet aggregation defect on par with the results from calcium mobilization and flow cytometry experiments (Fig. 3F).

Healthy human platelets exhibit impaired function in plasma from trauma patients

Freshly prepared washed platelets reconstituted with plasma (12% v/v plasma, final concentration) were challenged with agonists at high doses (10xEC₅₀). Responses to convulxin, ADP, U46619, and SFLLRN were significantly reduced when platelets were in the presence of plasma isolated from trauma patient blood samples (Fig. 4A-D). Specifically, the signals were 54%, 56%, 59%, and 54% lower in terms of area-under-the-curve (AUC) when the system contained patient plasma compared to healthy plasma. These data indicate a clear role for a plasma component on platelet function, perhaps polymerized fibrin or its degradation products as suggested in other work (16,22,23). ELISA for D-dimer, the smallest fibrin degradation product (FDP) in terms of size, showed ~30-fold increase in D-dimer concentration in trauma patient plasma in comparison to healthy plasma (Supp. Fig. S4). The patient and healthy plasma samples used in the experiment shown in Fig. 4 contained 21.9 nM and <1 nM D-dimer, respectively.

Healthy plasma imparts a detectable inhibition of washed platelet activation but patient plasma samples result in more potent effect

The previous experiment was repeated with a screen of plasma samples from several healthy donors and trauma patients to detect the range of effects of each class of plasma. A control condition (no plasma addition) served as a baseline response to which wells containing healthy or trauma plasma were normalized. High concentrations of convulxin (20 nM) or ADP (10 μ M) were used as challenging stimuli and the calcium mobilization AUC result for each platelet/plasma pair are plotted as percent of the control response (Fig. 5A-B). In the case of each stimulating agonist, presence of healthy plasma resulted in ~25% reduction of the control response, whereas addition of patient plasma samples further decreased the observed signal to ~50% on average. Each dataset shows statistical significance from the others, with the difference between the control condition and the trauma plasma samples being the most significant for both agonists.

Plasma concentration is correlated with decreased platelet function

All platelet-plasma combination experiments in previous sections were comprised of 12% plasma and a clear down-regulatory effect was observed. The effect of final plasma content was studied by titrating a range of concentrations (1–30%) and challenging platelet function with 20 nM convulxin, 100 μ M SFLLRN, or 10 μ M ADP. For each of the agonists, a

negatively-sloping dose-response curve was created in which platelets incubated in increasing levels of healthy donor-derived or trauma patient-derived plasma steadily became hypofunctional (Fig. 6A-C). The negative correlation between platelet calcium mobilization and plasma concentration was evident in both healthy and patient plasma settings, though there was clear parity between the two plasma classes at each concentration to an extent comparable to the results in Figs. 4–5.

DISCUSSION

In an effort to better understand platelet dysfunction in trauma patients, we present new results confirming the phenotype in several functional assays. Combinatorial agonist stimulation revealed significant depression of platelet calcium mobilization in patient samples compared to healthy controls, though it appeared the two populations show similar sensitivity to strong agonist conditions (e.g. high dose CVX) (Fig. 1). Patient responses varied widely, perhaps due to diverse injury conditions or treatment strategies, and partial recovery of platelet function was observed in one-third of the patients. Trauma patients also showed low $\alpha_2\beta_3$ activation, P-selectin display, and PS exposure following stimulation via flow cytometry (Fig. 2), and thromboelastography (TEG) data showed relatively fast clotting times (R,K) and rates of clot formation (angle), but initially diminished clot development (MA) and platelet aggregation (Fig. 3). The patient sample size in this study (n=16) may prevent the generalizable nature of the results and certainly warrants further investigation, but we have validated the coagulopathic phenotype of platelet dysfunction through the use of several lab and clinical techniques, which has not been reported previously to our knowledge.

A potential link between these results is the injury severity score (ISS), an anatomical quantification system to describe the extremity of a patient's injured state. The ISS accounts for multiple regions of the body, with scores ranging from 0–75 and the threshold for major trauma set at ISS=15 (24,25). Individual ISS scores for the cohort of 16 trauma patients are listed in Table 1 (median=24.5, IQR=15). This large range led us to hypothesize that the extent of platelet dysfunction may be correlated with the severity of the condition. However, we found no such relationship (Supp. Fig. S5), suggesting an added emphasis on the plasma effect observed in Figs. 4–6. The final ISS assigned to a patient is often determined at some later time point during or even after the patient's stay and does not account for the patient's previous medical history, which may explain the lack of correlation.

We also show a negative influence of plasma on platelet function. Diluting PRP with PPP to normalize platelet count has previously been shown to impair platelet aggregation (26). Further, cellular microparticles isolated from traumatic hemothorax blood, and to a lesser extent shed frozen plasma, inhibit platelet response to ADP, arachidonic acid, and collagen (27). The effect we observed was more potent with trauma patient-derived plasma, implying an amplified role of certain soluble species on cell viability. Other groups have shown elevated levels of soluble FDPs in trauma patient plasma (9,10), which we confirmed via D-dimer ELISA (Supp. Fig. S4). The role of circulating fibrin fragments on inherent and transfused platelet function is a subject of continuing study, but initial results show binding sites for fibrin on GPVI (23) and signaling defects following thrombin-mediated platelet

activation and fibrin polymerization (16). At this point it is difficult to elucidate whether these species (e.g. D-dimer) are meant to simply serve as a biomarker or if there exists an important mechanistic explanation linking elevated plasma concentrations and platelet dysfunction. Other possible hypotheses include roles of platelet-silencing species such as cAMP and cGMP, which are reported as elevated in trauma platelets and correlated with injury severity (28).

With the sustained prevalence and diversity of trauma-related injuries and mortalities, understanding the mechanisms of action as well as developing sufficient treatment strategies is becoming increasingly necessary. We highlighted the development of phenotypic profiles of platelet function to be used to guide clinicians in making informed decisions. Additionally, the observation of a plasma-mediated inhibitory effect gives rise to implications of transfusion therapies being ineffective or uninformed. Recent work has debated the appropriate volumes and ratios of blood products to administer to coagulopathic patients (29,30), but perhaps transfusions are not always optimal. In fact, platelet transfusions in trauma patients have been shown to have little effect on platelet function recovery (31) and resuscitation of hemorrhagic rats via whole blood delivery failed to restore platelet viability (32). Healthy platelets may inherit the “zombie” nature of the unresponsive but still circulating cells upon exposure to the patient’s plasma, as suggested by the *in vitro* results in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2. Trauma patient samples show impaired integrin $\alpha_{2b}\beta_3$ activation, P-selectin expression, and phosphatidylserine exposure in flow cytometry.

Fluorescence-activated cell sorting (FACS) was used to detect platelet activation following stimulation with ADP (2 μ M), convulxin (4 nM), or buffer. Fluorescent antibodies against the activated form of integrin $\alpha_{2b}\beta_3$ (A), P-selectin (B), and exposed phosphatidylserine (C) were incubated with dilute PRP (1% final concentration) prior to agonist stimulation. Conditions were tested in replicate for each donor/patient and mean fluorescent intensities (MFI) of each peak were calculated. For PS exposure, the percentage of positive cells were determined by the relative intensity of the second peak after gating the data.

Figure 3. Thromboelastography results for patient samples over time.

Five important parameters describing clot formation and strength were measured at different timepoints using a TEG[®] 6s Hemostasis System (A-E). Averaged data for trauma patients (n=14) are shown for citrated whole blood treated with RapidTEG[®] reagent (dried kaolin + tissue factor) and CaCl₂ for recalcification. The reported normal ranges (shaded) are: R=4–8 min; K=1–4 min; angle=47–74°; MA/max amplitude=55–73 mm; LY30=0–8%.

Additionally, platelet function was measured using the compatible PlateletMapping cartridge. Data was collected using heparinized whole blood and the final metric of platelet

activation was calculated as percent aggregation in response to ADP stimulation (F), where the shaded region indicates the reference range (83–100%).

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Figure 4. Healthy donor platelets lose function when reconstituted in trauma patient-derived plasma.

Washed platelets from healthy donors were prepared according to standard protocol and resuspended in platelet-poor plasma (PPP) from healthy donors or trauma patients. The platelet-plasma systems were incubated for about 10 min prior to exposure to various platelet agonists to test cell functionality. The agonists tested were 20 nM convulxin (A), 10 μ M ADP (B), 10 μ M U46619 (C), and 100 μ M SFLLRN (D). In each case, platelets in a trauma plasma setting showed ~50% decrease in calcium mobilization from platelets in a healthy plasma setting.

Figure 5. Screening several plasma samples reveals a more potent inhibitory effect in trauma samples than healthy samples.

Functionality of platelets from healthy donors (n=3–4) was screened in the presence and absence of PPP from several other healthy donors and trauma patients using the same protocol as Fig. 4. Responses to 20 nM convulxin (A) and 10 μ M ADP (B) were normalized to the control condition (no plasma). Though healthy plasma showed a down-regulation of platelet function, plasma derived from trauma patients showed a more potent inhibition in both agonist tests. Data are presented as mean $\pm$ SEM. (**p<0.01, ***p<0.001, ****p<0.0001).

Figure 6. Increasing plasma concentrations reduces the calcium fluorescent signal in both healthy and patient plasma settings.

Plasma concentration was varied from 1% to 30% and healthy washed platelets were incubated in the presence of healthy (autologous/nonautologous) plasma or trauma plasma. A control response (no plasma) was also prepared for data normalization purposes. All conditions were challenged with a potent dose of convulxin (20 nM) and data were plotted as a dose-response curve showing a negative relationship between plasma concentration and platelet response. Data are presented as mean $\pm$ SD.

Table 1.

Summary of patient demographics and clinical presentations.

Data were collected for 16 enrolled trauma patients to Penn Presbyterian Medical Center's Trauma-Induced Coagulopathy and Inflammation (TrICI) study. Patient ages were distributed over a wide range (median=31.5, IQR=32.0), and gender distribution was 14 males (87.5%) and 2 females (12.5%). Patients presented with a variety of injuries, though the mechanisms were limited to gun shot wounds, stab wounds, motor vehicle crashes, and falls. Injury severity scores (ISS) were calculated as a measure of the extent of trauma (median=24.5, IQR=15.0), where ISS>15 represents a major traumatic episode.

Patient No.	Age (y)	Gender	Mechanism of Injury (MOI)*	Injury Severity Score (ISS)**	Units of Blood Products Given	Patient Presentation Synopsis
1	014	45	M	9	43	N/A
2	019	19	M	17	2	Multiple penetrating wounds over scapula; received thoracotomy
3	020	60	M	59	13	N/A
4	025	18	M	24	11	N/A
5	027	21	M	38	28	N/A
6	028	33	M	17	7	N/A
7	036	26	M	17	96	Exsanguination, hemorrhagic shock, cardiac arrest; massive transfusion
8	038	87	F	13	0	N/A
9	044	67	M	14	0	N/A
10	055	54	M	43	4	N/A
11	068	22	M	26	19	Wounds to thigh, posterior axillary, stomach; sent to OR for thoracotomy
12	075	24	M	34	3	Wounds to right upper quadrant, liver laceration, adrenal hemorrhage
13	078	30	F	26	4	Fractures (ankle, femoral, rib) due to extrication
14	086	24	M	25	2	Wounds to left lower quadrant, sent to OR on two occasions
15	096	34	M	29	6	Wound to abdomen, sent to OR, initiated exsanguination protocol
16	102	76	M	43	13	Rib fractures, pneumothorax, liver laceration; fast taken to OR

* GSW: gun shot wound; SW: stab wound; MVC: motor vehicle crash; MCC: motorcycle crash

** ISS>15 corresponds to major trauma