

ORIGINAL ARTICLE

Plasma proteomic profile associated with platelet dysfunction after trauma

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Abstract

Background: Coagulopathic bleeding is a major cause of mortality after trauma, and platelet dysfunction contributes to this problem. The causes of platelet dysfunction are relatively unknown, but a great deal can be learned from the plasma environment about the possible pathways involved.

Objective: Describe the changes in plasma proteomic profile associated with platelet dysfunction after trauma.

Methods: Citrated blood was collected from severely injured trauma patients at the time of their arrival to the Emergency Department. Samples were collected from 110 patients, and a subset of twenty-four patients was identified by a preserved ($n = 12$) or severely impaired ($n = 12$) platelet aggregation response to five different agonists. Untargeted proteomics was performed by nanoflow liquid chromatography tandem mass spectrometry. Protein abundance levels for each patient were normalized to total protein concentration to control for hemodilution by crystalloid fluid infusion prior to blood draw.

Results: Patients with platelet dysfunction were more severely injured but otherwise demographically similar to those with retained platelet function. Of 232 proteins detected, twelve were significantly different between groups. These proteins fall into several broad categories related to platelet function, including microvascular obstruction with platelet activation, immune activation, and protease activation.

Conclusions: This observational study provides a description of the change in proteomic profile associated with platelet dysfunction after trauma and identifies twelve proteins with the most profound changes. The pathways involving these proteins are salient targets for immediate investigation to better understand platelet dysfunction after trauma and identify targets for intervention.

KEYWORDS

blood platelet disorders, hemorrhage, hemostasis, multiple trauma, proteomics

Essentials

- Platelet dysfunction after trauma is common, deleterious, and poorly understood.
- Plasma proteomics was performed in 12 trauma patients with and 12 without platelet dysfunction.
- The abundances of 12 proteins were significantly different between patient groups.
- These proteins have several known links to platelet function that are targets for future study.

1 | BACKGROUND

Trauma is a major cause of morbidity and mortality, accounting for more disability-adjusted life-years than malignancy and cardiovascular disease.¹ Hemorrhage is the second-leading cause of death after trauma,² and it is the leading cause of preventable death after injury.³

Coagulopathic bleeding is a major contributor to death from hemorrhage.^{4–6} Trauma-induced coagulopathy (TIC) is a derangement of normal hemostatic processes that was first defined by plas-matic tests of coagulation.^{4,5} The definition of TIC has since become less clear, defined sometimes by prothrombin time, sometimes by viscoelastic whole blood measurements, and sometimes by clinical judgment. However, platelet function has since been found to be an important contributor to coagulopathic bleeding after trauma.^{7,8}

The phenotype of platelet dysfunction is multifaceted. It has been found to include impaired platelet adhesion, aggregation, and contraction.^{7,9–11} Studies have found impairment of different and sometimes conflicting agonist pathways, though the degranulation mechanism appears to remain intact.¹² The platelet dysfunction phenotype may be induced by ligand alteration, surface receptor inhibition, receptor shedding, and/or cellular metabolic dysfunction.^{8,13} While much of the research in this area focuses on the platelet behaviors, a great deal can also be learned from the plasma environment after injury, which may directly alter platelet function. Studying the changes in the plasma environment using untargeted proteomics would provide unbiased insight into the presence of possible inhibitors of platelet function, changes to their major ligands, or other previously unknown pathways affecting platelet function.

Here, we present the changes in plasma proteomic profile associated with platelet dysfunction in trauma patients. We hypothesized that comparing the plasma of trauma patients with and without platelet dysfunction would reveal a proteomic signature of platelet dysfunction that could provide insights into the mechanisms underlying it. We identified 12 proteins whose levels were significantly altered and discuss the implications. These exploratory findings

should spur further study of the contribution of these proteins and their associated pathways to platelet dysfunction after injury.

2 | METHODS

2.1 | Patient population

Trauma patients presenting to Harborview Medical Center, a Level I trauma center in Seattle, WA, and meeting the criteria in Table 1 were enrolled at the time of Emergency Department (ED) arrival as part of a human subjects board-approved study. Inclusion criteria were generally meant to identify acute polytrauma patients not belonging to a vulnerable population and without dramatic exogenous alteration of the blood. The requirement for highest-level trauma team activation was used as an early surrogate for severe injury. The institutional criteria for this level of trauma team activation have been published previously.¹⁴

2.2 | Sample collection and processing

Blood was collected in 3.2% citrate tubes at the time of arrival to the ED using standard methods and existing intravenous catheters. Within 2 h of collection, samples were tested for complete blood counts and whole blood impedance aggregometry in response to each of five agonists (adenosine diphosphate, ADP; arachidonic acid, AA; thrombin receptor activating peptide, TRAP; collagen; and ristocetin). Samples were then centrifuged at 1200 G for 10 min to obtain plasma, which was then frozen at -80°C .

This was part of a larger study collecting samples on 110 patients measuring platelet function after trauma. Platelet function was measured using multiple electrode aggregation (Multiplate) immediately after blood draw.¹⁵ Platelet function was defined by aggregation response to adenosine diphosphate (ADP), arachidonic acid (AA), collagen, thrombin-receptor activating peptide

TABLE 1 Inclusion and exclusion criteria for patient enrollment

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> • Age ≥ 18 years • Highest-level trauma team activation¹³ • English-speaking (for consent) • Able to consent 	<ul style="list-style-type: none"> • ≥ 1 unit blood products transfused before screening • ≥ 2 L crystalloid infused before screening • ≥ 3 h since injury • Incarcerated • Pregnant

(TRAP), and ristocetin as the area under the aggregation curve. For this study, aggregation responses were divided into quartiles, and twelve subjects from the lowest quartile of platelet aggregation were compared to twelve subjects from the highest platelet function quartile. To ensure results were not due to laboratory error, patients in the low platelet function were also required to have clinical evidence of severe injury (injury severity score >15, blood transfusion within 24 h after ED arrival, or death). Because no preliminary data regarding plasma proteomics in trauma were available to guide a power analysis at the time of data collection, this was performed as an exploratory analysis to identify targets for future investigation. The decision to include twelve patients in each group was based on previous experience that major changes in proteomic profile can be detected with similar patient enrollments in trauma.¹⁶

2.3 | Sample preparation and protein digestion

Plasma samples were thawed when ready for mass spectrometry analysis. At this time, total protein concentration was measured by the Bradford protein assay (Bio-Rad) according to the manufacturer's instructions. Bovine serum albumin was used as the standard (Pierce™ Bovine Serum Albumin Standard Ampules, ThermoFisher Scientific).

Two isotope-labeled peptides (LSEAEFEVL*K and EAPDLVL*QR, L* = L-¹³C615 N, Anaspec) were used as internal standards (IS) to ensure samples injected for analysis reflected actual plasma concentrations before processing. Ten microliters of each plasma sample were mixed with IS and Ultra protease inhibitor (Roche). Samples were then processed with a multiple-affinity removal spin cartridge-Hu14 (Cat# 5188–6560, Agilent) according to the manufacturer's instructions to remove the 14 most abundant plasma proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3 and transthyretin). Depletion was verified by repeat total protein concentration, which indicated that 93±1% of protein was removed. Five micrograms of total protein from each depleted plasma sample were reduced with 10 mmol/L dithiothreitol for 20 min at 70°C, alkylated with 25 mmol/L iodoacetamide for 15 min at room temperature, and then digested with sequencing grade modified trypsin (Promega) (1:10 wt/wt, trypsin/protein) in 50 mmol/L Tris (pH 8) containing 5% acetonitrile and 1 mmol/L calcium chloride at 37°C overnight. Digestion was terminated by acidification with trifluoroacetic acid. The digested mixtures were concentrated and desalted with C18 extraction disk cartridges, dried under vacuum, and resuspended in 0.1% formic acid and 5% acetonitrile for proteomics analysis.

2.4 | Proteomics analysis

Tryptic peptides from each sample (100 ng/injection) were analyzed by nanoflow liquid chromatography (nanoLC) electrospray

ionization tandem mass spectrometry (nano LC-MS/MS) with a Thermo Scientific LTQ OrbitrapVelos mass spectrometer coupled to a Waters nanoAcquity Ultra Performance LC system. Peptides were separated at a flow rate of 300 nl/min on an ACQUITY UPLC M-Class HSS T3 Column (100 × 0.075 mm, 1.8 μm, Cat# 186008006, Waters), using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Peptides were eluted using a linear gradient of 5%–25% solvent B over 150 min, 25%–65% solvent B over 20 min. The LTQ OrbitrapVelos mass spectrometer was operated in data-dependent MS/MS acquisition mode with a full scan in the Orbitrap, followed by data-dependent MS/MS of the 15 most abundant precursor ions. A spray voltage was 3.0 kV, and the collision energy for MS/MS was 35%. Dynamic exclusion was used for 30 s after 3 repeats.

Mass spectrometry data were processed with Proteome Discoverer 2.4 (Thermo Scientific) software using a default setting and label free quantification work flow. MS/MS spectra were searched against the human protein database downloaded from UniProt. Search parameters allowed modified thiol residues (fixed carbamide methylation and variable methionine sulfoxide) and two incomplete cleavage sites with trypsin. The precursor tolerance was 10 ppm, and the fragment mass tolerance was 0.6 Da. Protein identifications were accepted if false discovery rate (FDR) <1.0%, at least 2 unique peptides per protein were detected, and there was a protein score >0. Protein abundance was determined by the sum of normalized peak area of precursor ions of unique and razor peptides. Average area of duplicated analysis was used for further statistical analysis.

2.5 | Gelsolin Enzyme-Linked Immunosorbent Assay (ELISA)

The protein found to be the most significantly different between groups by proteomics (gelsolin) was tested for confirmation by ELISA. Patient plasma gelsolin concentration was calculated using a human gelsolin sandwich ELISA kit (Cusabio) per manufacturer instructions. One patient each in the platelet dysfunction and control groups did not have enough remaining plasma for this test, so these data were collected on a total of 11 patients in each group.

2.6 | Statistical analysis

Because trauma patients receive differing amounts of crystalloid fluid, they likely have differing amounts of hemodilution. Controlling for this can be difficult, as patients have differing blood volumes, and crystalloid fluids are known to rapidly redistribute into the interstitium. We chose to control for hemodilution by normalizing the mass spectrometry peak intensity areas (using the standard definition^{17,18}) to the patient's corresponding total plasma protein concentration, as the best possible method from available data.

For statistical analysis, protein peak area normalized by the above methods were entered into MetaboAnalyst 4.0, a web-based software suite for metabolomics analysis that has been used for multi-omics analysis, including proteomics.¹⁹ Undetectably low values were estimated as half of the lowest detected value for each protein. Proteins with greater than half of values missing were excluded from analysis. For covariance heatmapping, data were auto-scaled (mean-centered and divided by standard deviation of each variable). For all data, continuous variables were compared by Wilcoxon rank-sum test, and dichotomous variables were compared by the chi square test. Correlation was determined by Pearson's *r* statistic. Statistical significance was considered $p < .05$. As this was an exploratory analysis, no adjustments were made for multiple comparisons. There is no consensus in the literature on the most appropriate method for handling multiple comparisons in small, exploratory omics studies. However, there is precedent for this approach.²⁰ This is deliberated further in the Discussion section below.

3 | RESULTS

3.1 | Patient information

Patient demographic, clinical, and laboratory data are summarized in Table 2. Compared to patients with high platelet function, those with low platelet function were more severely injured and trended toward receiving more blood products during entire hospital admission. As expected, they also trended toward a lower total protein concentrations, suggesting more hemodilution. Consistent with the method of group allocation, they also exhibited markedly lower platelet aggregation response to nearly all agonists tested.

3.2 | Proteomics

Two hundred thirty-two proteins were identified with the criteria listed in the Methods. A detailed list of proteins and peak areas from each analysis, as well as processed data with adjustment for hemodilution, are provided as supplemental spreadsheet files in Appendix S1 and S2. The raw data are available on Proteome Xchange with identifier PXD024370. Among the identified proteins, 12 were significantly different between platelet function groups (Figure 1). Their data are summarized in Table 3.

To begin to explore clusters of proteins that could be involved in common causal pathways, correlations between changes in protein abundance between groups are shown in Figure S1. Among the 12 proteins identified above there was a cluster of strongly co-varying proteins that included seven of these proteins: alpha-enolase, histone H4, heat shock protein 90-alpha, protein S100-A8, protein S100-A9, transketolase, and neutrophil gelatinase-associated lipocalin.

Among the 12 proteins, five decreased and seven increased in abundance in association with platelet dysfunction. The five that decreased were predominantly plasma proteins, and the seven

that increased were predominantly intracellular proteins. Two of the proteins are involved with pathways that lead to microvascular obstruction. Approximately half of the proteins are involved in inflammatory pathways, and this includes four previously identified damage-associated molecular patterns (DAMPs).

3.3 | Gelsolin ELISA

Plasma gelsolin concentration (median, IQR) was not significantly different between platelet dysfunction (17.3, 14.3–27.8 µg/ml) and control (22.3, 17.9–52.6 µg/ml) groups ($p = .200$). A simple linear regression testing for covariance between ELISA concentration and MS peak area resulted in $p = .271$. In a similar attempt to control for hemodilution as was performed with MS peak areas, these gelsolin concentrations were normalized to total protein. Normalized gelsolin concentrations were not also not significantly different between platelet dysfunction (467.8, 300.8–797.7 arbitrary units) and control (415.8, 285.1–766.7 arbitrary units) groups ($p = 0.870$).

4 | DISCUSSION

To our knowledge, this study is the first to present the changes in the plasma proteomic profile associated with platelet dysfunction after severe trauma. We identified twelve proteins whose abundance significantly change in patients with low platelet function. Interestingly, few of the identified proteins have previously described direct links to platelet function. However, several themes common to subgroups of these proteins could provide insight into the pathophysiology of platelet dysfunction after trauma to guide future study.

Two of the proteins could be related to microvascular obstruction and platelet activation. Gelsolin was the most markedly changed of the twelve proteins, showing a dramatic decrease in patients with low platelet function. It is a highly conserved actin-remodeling protein that acts intracellularly to reshape the actin cytoskeleton and, in the plasma, to scavenge actin leaked into the blood from tissue damage. After gelsolin solubilizes actin filaments, the gelsolin-actin complexes are then cleared from the plasma by an unknown mechanism.²¹ Plasma gelsolin levels are known to decline in trauma and burn patients, and lower levels are tied to poor clinical outcomes, but no link to coagulopathy has been shown in trauma patients.^{22–24} Levels also fall in several other critical illnesses, suggesting a common pathway.^{25–27} A prevailing theory is that tissue damage in critical illness leads to actin release, which leads to saturation of the actin-scavenging system and gelsolin sequestration.²⁸ Thus, low gelsolin levels could both serve as a biomarker for tissue damage and signal a decreased ability to clear further actin filaments from the blood. This decreased actin-clearing capacity could obstruct microvascular flow and worsen end-organ damage, leading to a self-perpetuating spiral of cell death. Interestingly, another one of the proteins that showed a modest decrease in this study, vitamin D-binding protein

TABLE 2 Patient demographic, clinical, and laboratory data summary

	All patients (n = 24)	High platelet function (n = 12)	Low platelet function (n = 12)	p Value
Demographic data				
Age (years)	38 (35–61)	39 (35–52)	37.5 (32–71)	.885
% Female	16.7	8.3	25	.273
Clinical data				
% Blunt mechanism	87.5	91.7	83.3	.537
% Taking aspirin	0	0	0	.999
Initial HR (bpm)	100 (77–109.5)	93.5 (70.5–106)	100.5 (86–121)	.272
Initial SBP (mmHg)	131.5 (120–141)	135 (120.5–151.5)	129.5 (104–136)	.419
ISS	20 (13.5–29.5)	13.5 (6–20)	29.5 (21.5–34.5)	.002
Crystalloid given prior to enrollment (mL)	800 (500–1500)	700 (150–1900)	950 (500–1500)	.672
% Transfused in first 24 h	20.8	8.3	33.3	.132
Total in-hospital blood products received (units)	0 (0–2.5)	0 (0–0)	0.5 (0–4)	.063
% In-hospital mortality	4.2	0	8.3	.307
Laboratory data				
Hemoglobin (mg/dl)	13.45 (12.0–14.25)	13.7 (12.95–14.4)	13.2 (11.35–13.85)	.175
Platelet count ($\times 10^9$ /L)	208 (173–262.5)	208 (177.5–264)	201.5 (168–262.5)	.644
PT (s)	14.45 (13.7–16.15)	13.0 (12.75–13.85)	13.45 (12.85–14.05)	.451
% PT ≥ 18 s	12.5	0	25	.064
Initial [lactate] (mmol/L)	2.4 (1.7–4.8)	1.5 (1.2–3.25)	3.3 (1.9–6.8)	.117
Initial [ETOH] (mg/dl)	0 (0–0)	0 (0–0)	0 (0–49.5)	.626
Total protein (mg/dl)	57.9 (48.4–60.2)	58.7 (55.7–62.9)	54.4 (36.5–59.1)	.065
Aggregometry AUC–ADP (AU)	49 (22.5–65)	59.5 (54.5–88.5)	22.5 (12.5–39)	.003
Aggregometry AUC–AA (AU)	34 (19–54)	50.5 (28.5–69)	20.5 (8–39)	.017
Aggregometry AUC–TRAP (AU)	106 (70.5–124.5)	114.5 (94.5–139.5)	74 (54–117.5)	.057
Aggregometry AUC–collagen (AU)	27.5 (12–53.5)	53.5 (30.5–62)	12 (5.5–26)	.001
Aggregometry AUC–ristocetin (AU)	28.5 (7–51)	43 (15–55.5)	8.5 (3–44)	.099
ROTEM–CT (s)	60.5 (50.5–73)	58.5 (50.5–72.5)	64.5 (51–75)	.386
ROTEM–CFT (s)	97 (80–134)	90 (79–110)	99.5 (80.5–150)	.355
ROTEM–alpha angle ($^{\circ}$)	70 (65–74)	73 (68–74.5)	70 (62–74)	.193
ROTEM–MCF (mm)	62 (56–65)	62.5 (59.5–65)	61 (51–65)	.309
ROTEM–LI30 (%)	100 (100–100)	100 (100–100)	100 (100–100)	.999
PCF (nN) at 2 min perfusion	86.5 (72.7–141.0)	98.7 (82.3–145.6)	82.9 (63.4–133.2)	.309

Continuous variables reported as median (IQR).

p values obtained by Wilcoxon rank-sum test for continuous variables and chi-square test for dichotomous variables.

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; AU, arbitrary units; AUC, area under the curve; bpm, beats per minute; CFT, clot formation time; CT, clotting time; HR, heart rate; ISS, injury severity score; LI30, lysis index after 30 min by whole blood EXTEM assay; MCF, maximum clot firmness; PCF, platelet contractile force after different durations of perfusion through previously described platelet function assay; PT, prothrombin time; ROTEM, rotational thromboelastometry whole blood EXTEM assay; SBP, systolic blood pressure; TRAP, thrombin receptor activating peptide.¹⁵

(also called “Gc protein”), is also involved in the extracellular actin scavenging system, supporting a model of actin toxicity in critical illness.²⁸ In support of this, injection of globular actin into rats leads to microvascular obstruction and microthrombus formation, and the culpable intravascular actin filaments complex with two proteins: gelsolin and vitamin D-binding protein.²⁹ Furthermore,

preincubation with supplemental vitamin D-binding protein prevents the damage caused by actin injection. The role of gelsolin in platelet function is not well understood, but a few key links have been found. First, a gelsolin knockout mouse exhibits decreased platelet shape change and activation in response to stimulation and prolonged bleeding time after tail snip, though this is likely

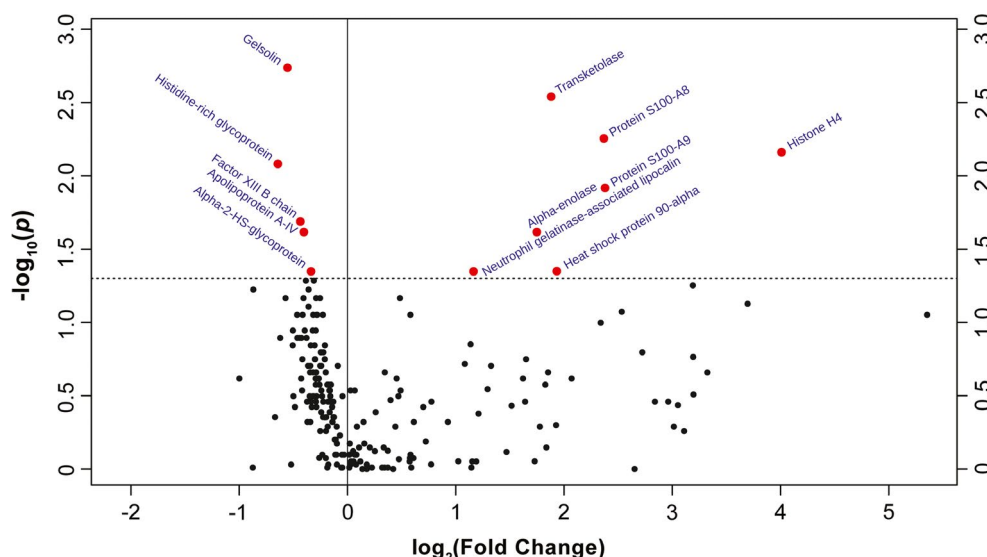


FIGURE 1 Volcano plot depicting fold-change and statistical significance of individual proteins in patients with low vs. high platelet function. Dotted horizontal line represents $p = .05$. Twelve proteins showed a statistically significant p value

due to deficiency of the cytosolic form of gelsolin that facilitates cytoskeletal remodeling.³⁰ Second, extracellular actin triggers platelet aggregation, primarily by potentiating ADP agonism.^{31,32} This suggests low gelsolin levels and accompanying high actin levels could lead to a bleeding phenotype, caused by offsite activation of platelets, as seen in disseminated intravascular coagulation. Synthesizing all of this evidence, gelsolin and possibly the greater extravascular actin-scavenging system are salient candidates for involvement in coagulopathic bleeding and possibly microvascular obstruction. These should be an immediate focus for further investigation.

Bearing some possible similarities, plasma histone H4 abundance was 16-fold higher in patients with platelet dysfunction. Histone H4, one of the major deoxyribonucleic acid-packaging proteins, resides primarily within cell nuclei but can be released upon cell death. Histone H4 released into the blood induces microvascular obstruction and platelet adhesion, activation, and aggregation, and high histone levels in the blood are associated with thrombocytopenia in a general population of critically ill patients.^{33,34} Histone levels rise in correlation with poor outcomes and coagulopathy in sepsis and in association with acute lung injury after trauma.^{35–37} Recently, histone H4 was found to coat platelets in severely injured trauma patients and convert them into procoagulant balloons.³⁸ Our findings are consistent with this mechanism and underline the importance of pursuing this pathway as a possible therapeutic target.

Another common theme among several of the identified proteins was widespread immune activation. Histidine-rich glycoprotein (HRG) was markedly decreased in association with platelet dysfunction. HRG binds a wide array of ligands and serves many roles, including modulating aspects of hemostasis. HRG inhibits fibrinolysis by inhibiting plasminogen activation.³⁹ It also

inactivates the adhesive glycoprotein thrombospondin, leading to less adhesive platelets.⁴⁰ Finally, HRG maintains circulating neutrophils in the quiescent state.⁴¹ Though not previously linked to trauma, low plasma levels have been found in multiple disease states,^{42–45} and supplementation in rodent models of sepsis and pancreatitis decreases the development of immunothrombosis and improves survival.^{43,46} The cause of decreased HRG levels are unknown, but the effect of low HRG is thought to be related to the mitigation of an overwhelming inflammatory response. Activated neutrophils have been tied to hemostasis in two ways. First, they release neutrophil elastase, a nonspecific protease that cleaves and inactivates several proteins critical to hemostasis, including platelet glycoprotein Iba,⁴⁷ platelet fibrinogen receptor $\alpha_{IIb}\beta_3$,⁴⁸ von Willebrand factor (VWF),^{49,50} fibrin/fibrinogen,^{51,52} plasminogen activator inhibitor type 1 (PAI-1),⁵³ and plasminogen.⁵⁴ Second, the reactive oxygen species released by activated neutrophils have complex effects on many hemostasis proteins, including VWF,⁵⁵ the primary VWF-cleaving protease ADAMTS13 (a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13),⁵⁶ and fibrinogen.¹⁶ Combined, these data suggest it is possible that low HRG levels permit fibrinolysis, hyper-adhesive platelets, and rampant neutrophil activation, resulting in oxidation and cleavage by elastase of numerous proteins involved in normal platelet function.

Several of the other identified proteins are involved in immune activation, though they do not have known direct links to platelet function. These include protein S100-A8 and -A9, alpha-enolase, heat shock protein 90-alpha, alpha-2-HS-glycoprotein, and neutrophil gelatinase-associated lipocalin. While these proteins do not fit into any single known pathway, they could represent widespread immune activation, which is known to impact primary hemostasis

TABLE 3 Normalized protein abundance, mean fold-change, and *p* value of the twelve proteins found to be significantly different between platelet function groups

Protein	Normalized protein abundance		Mean fold-change (Low vs. High Platelet Function Groups)	<i>p</i> value
	High platelet function (n = 12)	Low platelet function (n = 12)		
Gelsolin	1.48E + 7 (1.32E + 7–1.71E + 7)	9.70E + 6 (8.27E + 6–1.19E + 7)	0.68	.002
Transketolase	9.36E + 3 (7.67E + 3–1.76E + 4)	2.86E + 4 (1.35E + 4–4.15E + 4)	3.68	.003
Protein S100-A8	2.64E + 4 (8.29E + 3–2.87E + 4)	4.60E + 4 (2.83E + 4–6.26E + 4)	5.16	.006
Histone H4	8.47E + 3 (7.62E + 3–7.10E + 4)	5.72E + 4 (7.20E + 3–1.50E + 5)	16.10	.007
Histidine-rich glycoprotein	8.30E + 6 (7.11E + 6–9.45E + 6)	5.00E + 6 (3.50E + 6–6.04E + 6)	0.64	.008
Protein S100-A9	4.07E + 4 (2.78E + 4–5.11E + 4)	5.83E + 4 (4.42E + 4–1.17E + 5)	5.20	.012
Coagulation factor XIII B chain	1.61E + 6 (1.22E + 6–1.81E + 6)	1.09E + 6 (9.70E + 5–1.30E + 6)	0.74	.020
Apolipoprotein A-IV	3.28E + 7 (2.46E + 7–4.04E + 7)	2.30E + 7 (1.67E + 7–2.58E + 7)	0.76	.024
Alpha-enolase	4.15E + 4 (1.77E + 4–1.03E + 5)	1.02E + 5 (4.59E + 4–1.32E + 5)	3.36	.024
Heat shock protein 90-alpha	4.08E + 3 (3.10E + 3–1.25E + 4)	1.41E + 4 (6.40E + 3–2.79E + 4)	3.82	.045
Alpha-2-HS-glycoprotein	3.68E + 7 (3.06E + 7–4.26E + 7)	2.83E + 7 (2.34E + 7–3.34E + 7)	0.79	.045
Neutrophil gelatinase-associated lipocalin	1.36E + 4 (7.72E + 3–2.06E + 4)	3.44E + 4 (1.42E + 4–5.99E + 4)	2.24	.045

Normalized protein abundance reported as median (IQR).

p values obtained by Wilcoxon rank-sum test.

as mentioned above. Indeed, several of these proteins (S100-A8, S100-A9, heat shock protein 90-alpha, and histone H4) have been identified as DAMPs, intracellular molecules released after acute trauma that trigger massive immune activation.⁵⁷ Notably, the S100-A10 protein, which has been previously noted to be degraded in association with hyperfibrinolysis after trauma,⁵⁸ was no different between groups.

A third theme is that the proteins that increased in abundance were generally intracellular in origin, while those that decreased in abundance were mostly extracellular. The relative abundance of intracellular proteins is consistent with widespread tissue damage. Their association with platelet dysfunction could largely be explained through DAMP triggering of inflammation, as discussed above. The decrease in abundance of plasma proteins, even after controlling for hemodilution, could reflect the highly proteolytic environment that arises after trauma.

Apolipoprotein A-IV, which was lower with platelet dysfunction, binds platelet surface receptor $\alpha_{IIb}\beta_3$ and inhibits platelet aggregation.⁵⁹ It is unclear whether this is through direct inhibition or stabilization of the quiescent state of $\alpha_{IIb}\beta_3$, but its role in trauma requires further investigation.

Coagulation factor XIII (FXIII) B chain was lower in association with platelet dysfunction. Depletion of FXIII B chain from normal plasma leads to impaired fibrin cross-linking.⁶⁰ Although platelets contain FXIII in their alpha granules, this cytoplasmic form of FXIII does not contain the B chain. The FXIII B chain associates only with the plasma form of FXIII and acts as a carrier protein that is shed on plasma FXIII activation. There are no known direct links between the FXIII B chain and platelet function or trauma. The B chain can serve as a marker of thrombin generation, but high levels can also inhibit contact pathway activation.⁶¹ Together, low FXIII B chain levels could link platelet dysfunction with impaired fibrin cross-linking, but the causal pathways are currently unknown.

Correlation analysis revealed one cluster of tightly co-varying proteins that included seven of the proteins of interest (alpha-enolase, histone H4, heat shock protein 90-alpha, protein S100-A8, protein S100-A9, transketolase, and neutrophil gelatinase-associated lipocalin). While not directly tied to a single known pathway, nearly all of them fit into the broader category of widespread inflammatory activation. As mentioned above, four of them have been identified as trauma-induced DAMPs, which are associated with immune activation and remote organ injury, though these mechanisms are

not completely understood. This suggests that DAMP release and immune activation trigger platelet dysfunction in addition to their other known effects.

Interestingly, many of the proteins identified in this study (including gelsolin, apolipoprotein A-IV, and alpha-2-HS-glycoprotein) have shown similar alterations in trauma patients with hyperfibrinolysis, which has similar risk factors to and often coincides with platelet dysfunction.⁶² Indeed, there is a great deal of interplay between platelet function and fibrinolysis. There are likely common causes of both disorders after trauma,⁵ and poor platelet function also increases the susceptibility of a fibrin clot to external lysis.^{63,64} These overlapping results reinforce the importance of these proteins and suggest they could be involved in pathways common to both phenotypes contributing to hemostatic failure.

The gelsolin ELISA data are difficult to interpret, primarily because the power of these comparisons is unknown. The raw gelsolin concentrations had a possible weak trend toward confirming the MS data, but after normalization for hemodilution any existing trend disappeared. The gelsolin concentrations we found in these patients (platelet dysfunction and control groups both) were comparable to the lowest range of gelsolin concentrations in polytrauma patients published previously.^{22,23} They were far below the ranges of healthy controls. There are several possible reasons for this, including differences in patient populations and number of sample freeze-thaw cycles. This likely severely limits the power to detect differences by ELISA, compared to the more-sensitive modality of MS. Indeed, assuming the previously published mean of 261 µg/ml and standard deviation of 104 µg/ml, a total of 52 patients (26 in each group) would be required to detect the 0.68-fold change we found by MS with 80% power and alpha 0.05. The current sample size of 22 (11 per group) would have a power of only 43.4% to definitively say there is no difference between groups by ELISA.

Weaknesses of this study include the lack of a universal definition of platelet dysfunction after trauma. The use of combined aggregation response is probably a reasonable surrogate for global aggregatory function, but it does not directly measure other key platelet functions, such as adhesion, degranulation, and contraction. A second weakness is the use of total plasma protein concentration to control for hemodilution from crystalloid fluid administration. Hemodilution is a uniquely challenging issue for proteomic analysis in critical illness, because sicker patients tend to receive more crystalloid fluid. It is an important issue to control for, but there is no perfect method to do so. While we felt that normalizing to total protein concentration was the best method available, it introduces variability into the results, which likely reduces the overall power of this study to detect differences. Our analysis identified only 232 proteins total, which is relatively low for a proteomics study. This is a result of our decision to have a relatively high threshold for protein identification. This prioritized accurate protein identification but should be acknowledged as potentially limiting additional protein identification. Another weakness is that the mass spectrometry method used is only semi-quantitative. Although it likely accurately reflects relative concentrations, which is the most important

comparison at this stage, it does not show actual plasma concentrations of the molecules in question. Future research will likely need to test numerical concentrations for the proteins of most interest. Also, our statistical analyses did not adjust for multiple comparisons. This was intentional, as the primary goal of this pilot study is to describe with a wide view what proteins could be areas for further study. The risk of a type II error weighs more heavily against the risk of a type I error at this early stage than in later studies, especially given that the study has unknown power to detect differences in this novel area. There is not a clear consensus in the literature about the most appropriate approach to multiple comparisons in exploratory omics analyses. However, there is precedent for our approach for the reasons mentioned.²⁰ If we were to adjust for multiple comparisons, only gelsolin would remain significantly different between groups. However, the other proteins reported likely also warrant further investigation. A related limitation is the relatively low number of patients included. This pilot study was intended to identify major differences between groups, but it is likely underpowered to detect some subtler changes in the proteome. A final limitation is that this study only represent a single early snapshot in what is probably a dynamic progression of plasma proteomic changes. It is focused on the critical early phase, during which hemostasis is critically important, but subsequent changes could be meaningful, particularly to patients who subsequently become hypercoagulable.

In conclusion, this study provides a description of the change in plasma proteomic profile associated with platelet dysfunction after trauma and identifies 12 proteins whose abundance was markedly different. Themes in these proteins suggest microvascular obstruction with platelet activation, tissue damage inducing widespread inflammation, and possibly proteolysis are associated with platelet dysfunction. The possible roles of these processes and individual proteins in platelet dysfunction after injury warrant further investigation urgently.

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CONFLICT OF INTERESTS

No authors have any significant conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Study design was completed by A St. John, Y Wang, J Chen, D Chung, S Stern, N White, X Fu, and J López. Data collection, analysis, and interpretation was performed by all authors. Critical writing was performed by A St. John, Y Wang, J Chen, D Chung, N White, X Fu, and J López. All authors approved of this manuscript.

REFERENCES

1. Murray CJL, Vos T, Lozano R, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012;380:2197–2223.

2. Kauvar DS, Lefering R, Wade CE. Impact of hemorrhage on trauma outcome: an overview of epidemiology, clinical presentations, and therapeutic considerations. *J Trauma*. 2006;60:S3-S11.
3. Drake SA, Holcomb JB, Yang Y, et al. Establishing a regional trauma preventable/potentially preventable death rate. *Ann Surg*. 2020;271:375-382.
4. Cap A, Hunt BJ. The pathogenesis of traumatic coagulopathy. *Anaesthesia*. 2015;70(Suppl 1):96-101, e32-4.
5. Chang R, Cardenas JC, Wade CE, Holcomb JB. Advances in the understanding of trauma-induced coagulopathy. *Blood*. 2016;128:1043-1049.
6. Brohi K, Singh J, Heron M, Coats T. Acute traumatic coagulopathy. *J Trauma*. 2003;54:1127-1130.
7. Kutcher ME, Redick BJ, McCreery RC, et al. Characterization of platelet dysfunction after trauma. *J Trauma Acute Care Surg*. 2012;73:13-19.
8. Vulliamy P, Kornblith LZ, Kutcher ME, Cohen MJ, Brohi K, Neal MD. Alterations in platelet behavior after major trauma: adaptive or maladaptive? *Platelets*. 2020;1-10.
9. Jacoby RC, Owings JT, Holmes J, Battistella FD, Gosselin RC, Paglieroni TG. Platelet activation and function after trauma. *J Trauma*. 2001;51:639-647.
10. Solomon C, Traintinger S, Ziegler B, et al. Platelet function following trauma. A multiple electrode aggregometry study. *Thromb Haemost*. 2011;106:322-330.
11. Sillesen M, Johansson PI, Rasmussen LS, et al. Platelet activation and dysfunction in a large-animal model of traumatic brain injury and hemorrhage. *J Trauma Acute Care Surg*. 2013;74:1252-1259.
12. St John AE, Newton JC, Martin EJ, et al. Platelets retain inducible alpha granule secretion by P-selectin expression but exhibit mechanical dysfunction during trauma-induced coagulopathy. *J Thromb Haemost*. 2019;17(5):771-781.
13. John AE, White NJ. Platelets and fibrinogen: emerging complexity in trauma-induced coagulopathy. *Semin Thromb Hemost*. 2020;46:125-133.
14. St John AE, Rowhani-Rahbar A, Arbabi S, Bulger EM. Role of trauma team activation in poor outcomes of elderly patients. *J Surg Res*. 2016;203:95-102.
15. Ting LH, Fegghi S, Taparia N, et al. Contractile forces in platelet aggregates under microfluidic shear gradients reflect platelet inhibition and bleeding risk. *Nat Commun*. 2019;10:1204.
16. White NJ, Wang Y, Fu X, et al. Post-translational oxidative modification of fibrinogen is associated with coagulopathy after traumatic injury. *Free Radic Biol Med*. 2016;96:181-189.
17. Nesvizhskii AI, Vitek O, Aebersold R. Analysis and validation of proteomic data generated by tandem mass spectrometry. *Nat Methods*. 2007;4:787-797.
18. Rampler E, Egger D, Schoeny H, et al. The power of LC-MS based multionics: exploring adipogenic differentiation of human mesenchymal stem/stromal cells. *Molecules*. 2019;24(19):3615.
19. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr Protoc Bioinformatics*. 2019;68:e86.
20. Pascovici D, Handler DC, Wu JX, Haynes PA. Multiple testing corrections in quantitative proteomics: a useful but blunt tool. *Proteomics*. 2016;16:2448-2453.
21. Lind SE, Smith DB, Janmey PA, Stossel TP. Role of plasma gelsolin and the vitamin D-binding protein in clearing actin from the circulation. *J Clin Invest*. 1986;78:736-742.
22. Dahl B, Schiødt FV, Ott P, Gvozdenovic R, Yin HL, Lee WM. Plasma gelsolin is reduced in trauma patients. *Shock*. 1999;12:102-104.
23. Mounzer KC, Moncure M, Smith YR, Dinubile MJ. Relationship of admission plasma gelsolin levels to clinical outcomes in patients after major trauma. *Am J Respir Crit Care Med*. 1999;160:1673-1681.
24. Dinsdale RJ, Hazeldine J, Al Tarrah K, et al. Dysregulation of the actin scavenging system and inhibition of DNase activity following severe thermal injury. *Br J Surg*. 2020;107:391-401.
25. Huang LF, Yao YM, Li JF, et al. Reduction of plasma gelsolin levels correlates with development of multiple organ dysfunction syndrome and fatal outcome in burn patients. *PLoS One*. 2011;6:e25748.
26. Lind SE, Smith DB, Janmey PA, Stossel TP. Depression of gelsolin levels and detection of gelsolin-actin complexes in plasma of patients with acute lung injury. *Am Rev Respir Dis*. 1988;138:429-434.
27. Pan JW, He LN, Xiao F, Shen J, Zhan RY. Plasma gelsolin levels and outcomes after aneurysmal subarachnoid hemorrhage. *Crit Care*. 2013;17:R149.
28. Epstein FH, Lee WM, Galbraith RM. The extracellular actin-scavenger system and actin toxicity. *N Engl J Med*. 1992;326:1335-1341.
29. Haddad JG, Harper KD, Guoth M, Pietra GG, Sanger JW. Angiopathic consequences of saturating the plasma scavenger system for actin. *Proc Natl Acad Sci USA*. 1990;87:1381-1385.
30. Witke W, Sharpe AH, Hartwig JH, Azuma T, Stossel TP, Kwiatkowski DJ. Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell*. 1995;81:41-51.
31. Scarborough VD, Bradford HR, Ganguly P. Aggregation of platelets by muscle actin. A multivalent interaction model of platelet aggregation by ADP. *Biochem Biophys Res Comm*. 1981;100:1314-1319.
32. Vasconcellos CA, Lind SE. Coordinated inhibition of actin-induced platelet aggregation by plasma gelsolin and vitamin D-binding protein. *Blood*. 1993;82:3648-3657.
33. Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA*. 2010;107:15880-15885.
34. Alhamdi Y, Abrams ST, Lane S, Wang G, Toh CH. Histone-associated thrombocytopenia in patients who are critically ill. *J Am Med Assoc*. 2016;315:817-819.
35. Zeerleder S, Zwart B, Willemin WA, et al. Elevated nucleosome levels in systemic inflammation and sepsis. *Crit Care Med*. 2003;31:1947-1951.
36. McDonald B, Davis RP, Kim SJ, et al. Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood*. 2017;129:1357-1367.
37. Abrams ST, Zhang N, Manson J, et al. Circulating histones are mediators of trauma-associated lung injury. *Am J Respir Crit Care Med*. 2013;187:160-169.
38. Vulliamy P, Gillespie S, Armstrong PC, Allan HE, Warner TD, Brohi K. Histone H4 induces platelet ballooning and microparticle release during trauma hemorrhage. *Proc Natl Acad Sci USA*. 2019;116:17444-17449.
39. Blank M, Shoenfeld Y. Histidine-rich glycoprotein modulation of immune/autoimmune, vascular, and coagulation systems. *Clin Rev Allergy Immunol*. 2008;34:307-312.
40. Leung LL, Nachman RL, Harpel PC. Complex formation of platelet thrombospondin with histidine-rich glycoprotein. *J Clin Invest*. 1984;73:5-12.
41. Nishibori M, Wake H, Morimatsu H. Histidine-rich glycoprotein as an excellent biomarker for sepsis and beyond. *Crit Care*. 2018;22:209.
42. Kuroda K, Wake H, Mori S, Hinotsu S, Nishibori M, Morimatsu H. Decrease in histidine-rich glycoprotein as a novel biomarker to predict sepsis among systemic inflammatory response Syndrome. *Crit Care Med*. 2018;46:570-576.
43. Terao K, Wake H, Adachi N, et al. Histidine-rich glycoprotein suppresses hyperinflammatory responses of lung in a severe acute pancreatitis mouse model. *Pancreas*. 2018;47:1156-1164.
44. Matsumoto A, Nakamura T, Shinomiya A, et al. Histidine-rich glycoprotein could be an early predictor of vasospasm after aneurysmal subarachnoid hemorrhage. *Acta Med Okayama*. 2019;73:29-39.
45. Winiarska A, Zareba L, Krolczyk G, Czyzewicz G, Zabczyk M, Undas A. Decreased levels of histidine-rich glycoprotein in advanced lung cancer: association with prothrombotic alterations. *Dis Markers*. 2019;2019:8170759.

46. Wake H, Mori S, Liu K, et al. Histidine-rich glycoprotein prevents septic lethality through regulation of immunothrombosis and inflammation. *EBioMedicine*. 2016;9:180-194.
47. Beer JH, Buchi L, Steiner B. Glycocalicin: a new assay—the normal plasma levels and its potential usefulness in selected diseases. *Blood*. 1994;83:691-702.
48. Kornecki E, Ehrlich YH, De Mars DD, Lenox RH. Exposure of fibrinogen receptors in human platelets by surface proteolysis with elastase. *J Clin Investig*. 1986;77:750-756.
49. Bonnefoy A, Legrand C. Proteolysis of subendothelial adhesive glycoproteins (fibronectin, thrombospondin, and von Willebrand factor) by plasmin, leukocyte cathepsin G, and elastase. *Thromb Res*. 2000;98:323-332.
50. Jelenska M, Bykowska W, Kopec M, Vigh Z, Scharrer I, Breddin K. Effects of human elastase on von Willebrand factor in highly purified factor VIII concentrate and in cryoprecipitate. *Thromb Res*. 1990;59:295-307.
51. Bach-Gansmo ET, Godal HC, Skjongsberg OH. Degradation of fibrinogen and cross-linked fibrin by human neutrophil elastase generates D-like fragments detected by ELISA but not latex D-dimer test. *Thromb Res*. 1998;92:125-134.
52. Hayakawa M, Sawamura A, Gando S, et al. Disseminated intravascular coagulation at an early phase of trauma is associated with consumption coagulopathy and excessive fibrinolysis both by plasmin and neutrophil elastase. *Surgery*. 2011;149:221-230.
53. Wu K, Urano T, Ihara H, et al. The cleavage and inactivation of plasminogen activator inhibitor type 1 by neutrophil elastase: the evaluation of its physiologic relevance in fibrinolysis. *Blood*. 1995;86:1056-1061.
54. Barrett CD, Moore HB, Banerjee A, Silliman CC, Moore EE, Yaffe MB. Human neutrophil elastase mediates fibrinolysis shutdown through competitive degradation of plasminogen and generation of angiotatin. *J Trauma Acute Care Surg*. 2017;83:1053-1061.
55. Chen J, Fu X, Wang Y, et al. Oxidative modification of von Willebrand factor by neutrophil oxidants inhibits its cleavage by ADAMTS13. *Blood*. 2010;115:706-712.
56. Wang Y, Chen J, Ling M, Lopez JA, Chung DW, Fu X. Hypochlorous acid generated by neutrophils inactivates ADAMTS13: an oxidative mechanism for regulating ADAMTS13 proteolytic activity during inflammation. *J Biol Chem*. 2015;290:1422-1431.
57. Vourc'h M, Roquilly A, Asehnoune K. Trauma-induced damage-associated molecular patterns-mediated remote organ injury and immunosuppression in the acutely ill patient. *Front Immunol*. 2018;9:1330.
58. Gall LS, Vulliamy P, Gillespie S, et al. The S100A10 pathway mediates an occult hyperfibrinolytic subtype in trauma patients. *Ann Surg*. 2019;269:1184-1191.
59. Qu J, Ko C-W, Tso P, Bhargava A. Apolipoprotein A-IV: a multifunctional protein involved in protection against atherosclerosis and diabetes. *Cells*. 2019;8:319.
60. Souri M, Osaki T, Ichinose A. The non-catalytic B subunit of coagulation factor XIII accelerates fibrin cross-linking. *J Biol Chem*. 2015;290:12027-12039.
61. Halkier T, Magnusson S. Contact activation of blood coagulation is inhibited by plasma factor XIII b-chain. *Thromb Res*. 1988;51:313-324.
62. Banerjee A, Silliman CC, Moore EE, et al. Systemic hyperfibrinolysis after trauma: a pilot study of targeted proteomic analysis of superposed mechanisms in patient plasma. *J Trauma Acute Care Surg*. 2018;84:929-938.
63. Tutwiler V, Peshkova AD, Le Minh G, et al. Blood clot contraction differentially modulates internal and external fibrinolysis. *J Thrombosis Haemost*. 2019;17:361-370.
64. Moore HB, Moore EE, Chapman MP, et al. Viscoelastic measurements of platelet function, not fibrinogen function, predicts sensitivity to tissue-type plasminogen activator in trauma patients. *J Thrombosis Haemost*. 2015;13:1878-1887.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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