HHS Public Access

Author manuscript

J Trauma Acute Care Surg. Author manuscript; available in PMC 2019 June 01.

Published in final edited form as:

J Trauma Acute Care Surg. 2018 June ; 84(6): 929–938. doi:10.1097/TA.0000000000001878.

Systemic Hyperfibrinolysis after Trauma: A Pilot study of Targeted Proteomic Analysis of Superposed Mechanisms in Patient Plasma

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Abstract

Background—Viscoelastic measurements of hemostasis indicate that 20% of seriously injured patients exhibit systemic hyperfibrinolysis, with increased early mortality. These patients have normal clot formation with rapid clot lysis. Targeted proteomics was applied to quantify plasma proteins from hyperfibrinolytic (HF) patients to elucidate potential pathophysiology.

Methods—Blood samples were collected in the field or at Emergency Department arrival and thrombelastography (TEG) was used to characterize *in vitro* clot formation under native and tissue plasminogen activator (tPA)-stimulated conditions. Ten samples were taken from injured patients exhibiting normal lysis time at 30 min (Ly30), "eufibrinolytic" (EF), 10 from HF patients, defined

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The authors have no conflict of interest with the submitted work.

Author Contributions

AB and CCS wrote the manuscript and had major inputs into data analyses and interpretation. EEM, AS, AD'A, EP, helped write the manuscript and were key in data analyses. MD completed all of the QConCATs and wrote portions of the manuscript. MK performed all ELISA data and constructed the tables and Figure 3. KJ added valuable statistical advice and helped to write the manuscript. MPC, EG, HBM, BEH, PE helped to accrue data and aided in data analyses and manuscript preparation. JC and AG were in charge of all sample accrual, supervised the running of all TEGs, and maintained the subject databases and integrity of the data. KH supervised all mass spectrometry studies and ensured that the QConCAT was performed properly and helped in data analysis and writing the manuscript.

as tPA-stimulated TEG Ly30 >50%, and 10 from healthy controls. Trauma patient samples were analyzed by targeted proteomics and ELISA assays for specific coagulation proteins.

Results—HF patients exhibited increased plasminogen activation. Thirty-three proteins from the HF patients were significantly decreased compared to healthy controls and EF patients; 17 were coagulation proteins with anti-protease consumption ($p<0.005$). The other 16 decreased proteins indicate activation of the alternate complement pathway, depletion of carrier proteins, and 4 glycoproteins. CXC7 was elevated in all injured patients versus healthy controls ($p<0.005$), and 35 proteins were unchanged across all groups ($p>0.1$ and fold change of concentrations of 0.75–1.3).

Conclusion—HF patients had significant decreases in specific proteins and support mechanisms known in trauma-induced hyperfibrinolysis and also unexpected decreases in coagulation factors, **factors II, X, and XIII**, without changes in clot formation (SP, R times or angle). Decreased clot stability in HF patients was corroborated with tPA-stimulated TEGs.

Level of Evidence—III prognostic.

Keywords

Coagulation; Targeted Proteomics; Fibrinolysis; Traumatic Injury

Background

Systemic hyperfibrinolysis following traumatic injury with hemorrhagic shock occurs in up to 20% of critically injured patients and is associated with a mortality of >50%.[1–6] Previous descriptions of trauma-induced coagulopathy (TIC) relied on standard measures of coagulation in fresh plasma, e.g. prothrombin times/International normalized ratios (PT/INR).[7–10] A number of coagulation proteins has been assayed to depict this hyperfibrinolytic phenotype with high sensitivity but poor specificity, i.e. minimal capacity to discriminate across many heterogeneous patient samples.[10–14] Although delayed clotting may occur due to an apparent decrease in soluble clotting factor levels, this etiology appears to be attributable to a dilutive mechanism from major hemorrhage and crystalloid resuscitation, rather than consumption.[3,8,10] Changes in resuscitation practice over the past two decades have significantly ameliorated iatrogenic dilution as a driver of TIC, and attention has shifted to hyperfibrinolysis.

Severe trauma distorts physiological clot remodeling, towards one of two extremes: systemic hyperfibrinolysis (HF) or fibrinolysis shutdown, both of which are associated with increased mortality.[5,15–17] The CRASH 2 trial underlines the importance of clinical strategies for inhibiting plasmin-mediated fibrinolysis acutely following injury, and this and other studies suggest the therapeutic utility of the lysine analog tranexamic acid (TXA) in a subset of severely hyperfibrinolytic patients.[5,18–20] While the hypothesis that TIC is solely due to diminished clotting factors has been displaced and the key role of deregulated fibrinolysis in TIC recognized, an unbiased survey of the hyperfibrinolytic plasma proteome is lacking.

Thrombelastography (TEG) evaluates not only clot formation times with different initiators but also the kinetics of clot formation, clot strength, and clot remodeling/lysis in whole blood.[5,6,21–24] Time course studies establish that many injured patients with systemic

hyperfibrinolysis have normal or even rapid clot formation times (R times) and low maximum amplitude (MA, an indicator of clot strength).[1,17,18,25,26] While HF presents in many patients as increased lysis measured at 30 minutes after MA (Ly30), HF can also be rapidly detected by observing marked increases in Ly30 following the addition of exogenous tissue plasminogen activator (tPA) to the TEG assay.[17,18,23,24,27] The tPA-challenged TEG assay allows the rapid stratification of trauma patients into HF, eufibrinolytic (EF:physiologic), and fibrinolysis shutdown phenotypes and is a better predictor of massive transfusion, a hallmark of systemic hyperfibrinolysis.[17,24,27,28] We hypothesize that examination of the plasma proteome of injured patients reveals differences is specific proteins, which further define the fibrinolytic phenotype and provide mechanistic insight into the pathogenesis of trauma-induced coagulopathy (TIC). Application of a controlled mass spectroscopy approach of 142 specific proteins was completed and allowed for quantification of these plasma proteins, which include serpins, coagulation factors, and other proteins known to affect hemostasis, either clotting or fibrinolysis.[29,30] Such data may provide a better scientific basis for individualized, goal-directed resuscitation of the critically injured.

Methods

Study Population

Consecutive adult trauma patients (n=130) meeting criteria for the highest level of activation at our Level I trauma center (Denver Health Medical Center) from April 2014 to April 2016 were assigned to the Trauma Activation Protocol approved by the Combined Multi-Institutional Review Board with a waiver of consent. The criteria are patients >18 years of age and traumatic injury with any of the following: (a) blunt trauma with systolic blood pressure SBP <90 mmHg (b) mechanically unstable pelvic injury (open or obvious by physical exam) (c) penetrating neck/torso injuries with (SBP) <90 mmHg (d) gunshot wounds to the neck/torso or stab wounds to the neck/torso that require endotracheal intubation. Patients who did not have blood drawn within 60 minutes of ED arrival, received blood products, being treated with anticoagulants, or transferred from another facility, were excluded. No pre-hospital blood products were administered prior to arrival. Citrated and heparinized whole blood and plasma samples were obtained upon arrival, and 5 different TEG assays were completed: rapid-TEG (rTEG: re-calcified immediately prior to loading into the TEG cup with tissue factor and kaolin), citrated functional fibrinogen (CFF: platelet-blocked, reptilase-initiated + Factor XIII in heparin), CFF + TXA, citrated native (CNTEG, re-calcified native TEG), and CNTEG stimulated with tPA [75 ng/ml]. Fresh plasma samples isolated from whole blood by an initial centrifugation at 5,000*g* for 7 min followed by a second spin at 12,500*g* to remove platelets and acellular debris were immediately frozen at −80°C. These samples were used for targeted proteomic analysis and ELISA measurements. The groups were stratified by the Ly30 obtained from tPA (75 ng/ml)-stimulated CNTEG traces: ten consecutive injured patients with HF, defined as Ly30 ≥50% (10 samples), were paired with 10 injured EF patients: injured patients with EF defined as tPA-stimulated CNTEG 20%≥Ly30≥5% over the same time frame as the HF patients, and 10 healthy control subjects.

Plasma depletion of albumin and IgG

Albumin and IgG, were removed from the plasma samples using serum protein immunodepletion resins (Proteome Purify2, R&D Systems, Inc., Minneapolis, MN) as published.[29,30] Because of the intentional removal of albumin and immunoglobulins these proteins are obviated from further analysis.

Targeted proteomics

Recombinant isotopically labeled QConCAT proteins, containing a chimeric concatamer of peptides labeled at the lysine and arginine residues ($^{13}C_6$ isotopologues) were mixed with the albumin- and IgG-depleted plasma at 200 or 100 fmol per injection as published.[29,30] The QConCAT palette of targeted proteins includes 142 proteins made up of coagulation factors, serpins, carrier proteins, known to affect hemostasis.[29,30]

Enzyme-linked immunosorbent assays (ELISAs)

ELISA assays were performed in duplicate, with dilutions to ensure proper quantification, per the manufacturer's instructions. ELISA's were completed for thrombin (MyBiosource.com, San Diego, CA), antithrombin (AT), thrombin:antithrombin (TAT) complexes, plasminogen (Plg) (AssayPro, St. Charles, MO), α_2-antiplasmin, plasmin: α_2-antiplasmin (PAP) complexes, tPA, tPA:PAI-1 complexes and PAI-1 (Molecular Innovations, Novi, MI), and thrombin-activated fibrinolysis inhibitor (TAFI) (Sekisui Diagnostics, Stamford, CT).

Statistical analyses

The data are reported as the median ± interquartile ranges for all patient demographics, coagulation assays, and targeted proteomics and the mean ± the standard error of the mean for the ELISA data. Because the data was not normally distributed, statistical differences among the 3 patient groups were compared using a non-parametric Kruskal-Wallis test followed by the Dwass, Steel, Critchlow-Fligner multiple comparison procedure with statistical differences at $p<0.005$ for proteins and $p<0.05$ for other clinical tests. Z means testing at 1.25 times the standard deviation was employed to determine the proteins that were unchanged among groups. For the normally distributed ELISA data, as determined by the Shapiro-Wilk test for normal distribution, statistical differences ($p<0.05$) were determined by an independent analysis of variance (ANOVA) followed by a Bonferroni test for multiple comparisons.

Results

Patient Demographics

Patients with HF were the most severely injured cohort and compared to EF patients and had higher injury severity scores (ISS, NISS) and Glasgow coma scales (GCS) (Table 1). The HF patients showed no difference in age or BMI compared to the EF patients. Moreover, HF patients evidenced a lower plasma pH with higher base deficits versus the EF patient group, although there was no difference in initial field fluid administration or in time to blood sample collection after injury: 19/20 samples were acquired within 1 hour (Table 1). The

longest interval to sample collection was 3 hours 25 minutes in a stabbed female, who had vital signs in the field and survived without receiving any pre-hospital fluids. Seventy percent of the HF patients died within 12 hours of injury, all required massive transfusions, and emergency thoracotomy were required in 50% (Table 1). The citrated r-TEGs were employed for clinical decisions as published because: 1) tissue factor- and kaolin-activated TEGs provide faster results and 2) citrate prevents sample clotting because some of these patients are paradoxically hypercoagulable soon after injury.[6,28]

TEG Measurement of HF Patients

TEG assays were conducted concomitantly in whole blood samples of all injured patients upon admission. These data included measurements from A) CNTEGs, B) tPA-CNTEGs with 75 ng/ml tPA (tPA-CNTEGs), and C) rTEGs (Table 2), along with CFF-TEGs, which assess platelet independent clotting in whole blood initiated with tissue factor, and CFF-TEG with tranexamic acid (CFF+TXA), to inhibit plasmin activity (Supplementary Data, Table 1.). The HF plasma samples selected for QConCAT proteomic analyses all showed high Ly30 (≥50%) in routinely obtained tPA-CNTEG assays compared to samples from EF patients 5%≥Ly30≤20% and healthy controls (Table 2) ($p<0.001$). In addition, for all TEG experiments the initial clot formation parameters Split Time (SP) and R were not significantly different between whole blood samples from HF patients vs. samples from EF patients and healthy controls ($p>0.05$). However, both the angle and the MA, which are dependent upon fibrinogen and platelets, from HF patients were significantly decreased versus EF patients and healthy controls ($p<0.01$ for both) (Table 2).[6,28]

Increases in Ly30 with tPA for HF patients are 40.4±9.4% to 80.0±3.0% from CNTEG to tPA-CNTEG ($p<0.001$), with smaller changes in EF patients (0.9±0.2% to 7.9±3.0%) vs. healthy controls (3.0±1.0% to 9.5±1.4%) similar to previous data.[17,27,31] Importantly, half (5/10) of the HF cohort exhibited lysis at the cutoff of Ly30≥50% in the CNTEG and are also close to the line of identity suggesting a minimal response to the tPA challenge (Supplementary Fig. 1). The lack of tPA response appears to occur if the Ly30 is already >70%. Also, the HF patients had a higher Ly30 in CFF-TEGs (Supplementary Fig. 2, panel A, 51.2±11.3%) compared to the EF patients (8.32±8.32%) and the healthy controls (0.23±0.1%). Furthermore, the addition of TXA to the CFF-TEGs nullified the Ly30 (the HF and EF patients were 0.0±0.0%, and the controls 0.1±0.1%) in these platelet-independent clots (Supplementary Fig. 2, panel B), confirming overactive plasminolysis as the likely dominant mechanism. Although increased, the rTEG Ly30 did indicate significant increases in Ly30 in the HF patients versus either the EF patients or healthy controls, which is most likely due to the small sample size, although the Ly30 cutoff was ≥3.0% for all HF patients. These patients had Ly30 ≥50% on the tPA-stimulated CN-TEGs further reinforcing the likely requirement for tPA-CNTEGs to define the HF group.

Coagulation Assays and Coagulation Proteins

The HF patients had significantly increased PT/INR's and activated partial thromboplastin times (aPTT) versus healthy controls ($p=0.001$ and $p=0.0016$) and the lower fibrinogen concentration demonstrated significance ($p=0.0094$) (Table 1). The EF patients had PT/INR and aPTT values that were were significantly decreased ($p=0.0069$ and $p=0.0033$,

respectively) vs. controls (Table 1). In comparison to the EF patients the HF group also had significantly lower plasma pH (p=0.0045), increased PT/INR and aPTT (p=0.0022 and p=0.0025, respectively). The HF patient group also received increased number of RBC units and plasma versus the EF patients (both p=0.0017).

ELISA measurement of coagulation proteins and related serpins revealed that in comparison to normal controls the HF patients demonstrated similar thrombin activity with significantly decreased anti-thrombin concentrations ($p<0.05$) and significantly increased TAT complexes ($p<0.05$). As compared to EF patients, the concentrations of anti-thrombin and TAT complexes from HF patients were not different (Fig. 1, panel A). Conversely, plasminogen concentrations in HF patients was significantly less than either the EF patients or the healthy controls with a concomitant increase in PAP complexes with plasminogen concentrations not showing statistical differences across the three groups (Fig. 1, panel C). TAFI concentrations were also not different across the three groups (Fig. 2). Moreover, previous data from these same patient groups indicate that the HF patients had significantly increased tPA activity versus healthy controls and EF patients ($p<0.005$) (Fig. 1 panel Bi). The HF patients also had decreased but not statistically significant PAI-1 activity (Fig. 1, panel Bii) vs. both healthy controls and EF patients, but there was a significant increase ($p<0.005$) tPA:PAI-1 complexes in the EF patients and HF patients compared to healthy controls (Fig. 1, panel Biii).

Mass spectroscopy Measurement of Plasma Proteins

Of the 142 proteins analyzed by the targeted mass spectrometry approach with heavy labeled internal standards (QConCAT), 11 were measured against two reporter proteotypic peptides with excellent agreement between the quantification of both parent ions and transition fingerprints: correlation >0.95. The concentrations of 35 plasma proteins did not change amongst the three groups as defined by $p>0.1$ and fold change of (0.75–1.3), which, included two distinct polypeptides from von Willebrand factor (Supplementary Table 2). These proteins consist of the most abundant soluble plasma proteins including: α_2-macroglobulin (A2M), all fibrinogen chains, haptoglobin, apo-lipoprotein E, etc. Structural proteins including fibronectin, vimentin, and filamin were unchanged among groups. Coagulation factors: Factors V and VIII, serpins: A1, E1, and G1 and complement Factor C9 all remained unchanged among all three groups as well as the antiprotease α_2-macroglobulin.

Importantly, intracellular biomarker proteins from circulating blood cells were not increased compared to normal plasma. Specifically, myeloperoxidase (MPO), matrix metalloprotease-2 (MMP2), MMP8, MMP9, and neutrophil elastase (ELANE) from neutrophils (PMNs) and other leukocytes, and platelet factor 4 (PLF4) and platelet glycoprotein 5 (GP5) from platelets were not changed vs. the healthy controls, despite severe injuries. Surprisingly, CXCL7, a platelet granule chemokine, was elevated in all injured patients (HF and EF) versus healthy controls ($p<0.001$) and there were no other statistically different proteins between the EF patients and the healthy controls ($p>0.005$).[32] Seventy-one proteins were not statistically different amongst the three groups and did not make the

statistical cutoff of $p<0.005$ nor the cutoffs for “no change”: $p>0.1$ and fold change 0.75–1.3 <2 (Supplementary Table 2).

There were a few rare, dramatic increases in plasma proteins. Thus, dramatic changes >two-fold of the mean were documented for fatty acid binding protein-1 (FABP1) and glyceraldehyde 3-phopsphate dehydrogenase (GAPDH) in a few patients; however, most of the injured subjects evidenced similar levels as compared to the healthy controls ($p\geq0.01$). Isolated increases in intracellular proteins may reflect the mechanism of injury because the patient who was crushed in an escalator had a 10-fold increase in muscle myoglobin vs. all other groups.

Coagulation proteins

Thirty-three of the 142 measured proteins were significantly decreased as compared to EF patients with physiologic fibrinolysis and the healthy controls ($p<0.005$) (Table 3). Fourteen of these 33 plasma proteins were coagulation-related enzymes, including: prothrombin (factor II), thrombin, a serine-protease, and factor XIIIB. The depletion of prothrombin structural fragments (F2a, F2b) in hyperfibrinolytic patients does not appear to affect clot formation (ACT or R, in any TEG experiment, Table 2) or in the ELISA measure of active thrombin, which may be expected in a decrease of only 50%. Three distinct protein epitopes implicate the contact pathway: factor XII, plasma kallikrein (both serine-proteases), together with their co-factor Kinninogen-1 (KNG1), a cystatin, decreased by 50%.[33] The probe sequence for KNG1 (starting at aa 67) is within the first cystatin domain of the KNG1 heavy chain and unaffected by possible cleavage by factor XII or kallikrein (aa 379 and 389).[34] The plasma ‘anticoagulants’, proteins C and S, were also significantly decreased as was fibrinogen in the HF group which was significantly decreased compared to the healthy controls (Table 1).

Three fragments characterized the plasminolytic aspect of hyperfibrinolysis. One fragment of plasminogen (another short-lived serine-protease, aa 181–191), along with 2 epitopes of its dedicated serpinF2 (α_2-antiplasmin) were all concomitantly reduced ($p<0.005$). These data coincide with both decreased plasminogen and increased PAP complexes in the HF group by ELISA (Fig. 1, Panel B i & iii). However, fibrinogen was depleted, and the TAFI concentrations were unchanged across groups. In addition specific serpins were decreased, including: serpin F2 (α_2-antiplasmin), serpin A4 (kallistatin), serpin D1 (Heparin Cofactor II), and the broadly acting inter α-trypsin inhibitor component, ITIH2 compared to EF patients and healthy controls.

Complement

QConCAT probes of 3 proteins confirm the activation of the complement pathway after severe trauma. These include, depletion of the complement zymogens, C3 (53%, $p=0.0015$) and C5 (56%, $p<0.0006$). Notably the suicide inhibitor of C5 activation CFH, is also decreased (61% compared to EF samples, $p= 0.0043$). These results imply that acute injury activates the alternate pathway since neither C2, C4, or C9 reached significance.[35]

Proteins involved with stress-induced oligomerization

Serum amyloid-P (component APCS) is an oligomerizing protein that was significantly decreased in HF patients. However, the better known inducible pentraxin, C-reactive protein, which was assessed at two sites (CRPa, CRPb), was unchanged, except dramatically in one patient with a documented infection prior to injury, in whom both fragments were elevated >10-fold higher along with significant increases in both lipopolysaccharide binding protein (LBP) and all three fibrinogen chains in comparison to the other injured patients and healthy controls ($p<0.001$). Supporting traumatic stress denaturation, clusterin (CLU) and hemopexin were also depleted. Lastly, of the apo-lipoproteins constituting HDL, only APOA 2,4, was depleted.

Carrier proteins

Both retinol-binding protein-4 (RBP4) and vitamin D binding protein (GC) were decreased in HF patients vs. EF patients and the healthy controls ($p=0.0008$). Both the Fe-binding proteins serotransferrin (TF) and hemopexin (HPX) were also significantly decreased in HF patients as was ceruloplasmin (CP).

Glycoproteins

The abundant cystatin domain protein α_2-Heremans Schmid (HS)-glycoprotein, fetuin A, was significantly decreased in the HF group compared to the EF patients and the healthy controls, while another minor cystatin, histidine-rich glycoprotein was not. Moreover, glycosylphosphatidyl-inositol specific phospholipase D, a plasma phospholipase D, cleaving inositol anchored proteoglycans was also decreased in the HF group.[36] Zinc α_2-glycoprotein-1 is an incompletely understood protein that may be an adipokine, which contains the major histocompatibility complex and immunoglobulin domains was also decreased as was glycosylphosphatidyl-inositol specific phospholipase D.[37]

Discussion

The reported data from this pilot study has demonstrated that injured HF patients were more seriously injured, had a decreased pH, and increased mortality, NISS, PT/INR and PTT, and required more transfusion support in the first 6 hours with both RBCs and plasma. The HF patients also had increased plasmin activity as documented by significantly increased Ly30 on CNTEG, the further augmentation of Ly30 in the tPA-stimulated lysis, and the concomitant increases in tPA and PAP complexes (600-fold) with decreases in plasminogen, PAI-1, and fibrinogen. Importantly, all patients were bleeding when the samples were obtained and were stratified only by the Ly30 on the tPA-CNTEGs: $Ly30\geq50\%$ for the HF group and $5\%\leq Ly30\leq20\%$ for the EF group. These patients' presentation and treatment were over a similar time frame with supportive care and surgical interventions completed by the identical teams. The angle, MA, a measure of clot strength, and fibrinogen were significantly decreased in the HF patients, particularly in those TEG assays that involve thrombin activation (rTEG, CNTEG,) but less likely to be significant in platelet-independent assays, CFF-TEG. Thirty-three proteins were significantly decreased in the HF patient group, versus both the EF patients and the normal controls, while 106 did not demonstrate a statistical difference ($p>0.005$) with 35 of these demonstrating no change ($p>0.1$, fold

change 0.75–1.3). Other TEG parameters are supportive of this hyperfibrinolytic phenotype, especially the CFF-TEG and tranexamic acid reversed the lysis on TEGs from the HF patients. This HF patient group experienced significantly increased mortality of 70% and consisted of patients with high ISS, hemorrhagic shock, and required massive transfusions. These data are supported by a retrospective study of fibrinolytic phenotype in 2,540 trauma patients with 18% of severely injured patients (ISS>15) who appeared hyperfibrinolytic (Ly30≥3%) and suffered a death rate of 44%, which was exacerbated by shock, irrespective of the mechanism of injury.[3–5,31,38]

Previous work has detailed that the overwhelming increases in tPA, not degradation of PAI-1, is responsible for the observed hyperfibrinolysis in injured patients.[4,17] The reported data are similar with significantly increased tPA activity and tPA:PAI-1 complexes with decreased, but not significantly, levels of PAI-1 in HF patients versus healthy controls and EF patients. Moreover, the HF patients had significant amounts of plasmin activity, documented by both the increased amounts of plasmin:α_2-antiplasmin complexes with the significantly decreased amounts of plasminogen versus both control and EF patients. Thus, plasmin has been directly implicated, which is downstream of tPA and is directly responsible for the hyperfibrinolytic phenotype described.

The reported data is focused on HF patients, which differs from other reports on trauma-induced coagulopathy (TIC).[8,9,21,25,26] TIC has been postulated to represent a sub-type of disseminated intravascular coagulation (DIC), which should result in decreased platelet count, which did not occur.[15,21] The reported data are consistent with the clinical series from Copenhagen, which indicated that TIC was not similar to DIC.[14,39] In addition, TIC has been described as a dilutional coagulopathy secondary to overzealous administration of crystalloid without proper reconstitution of hemostatic potential.[3,8,10] Importantly, 19/20 samples analyzed were collected within 1 hour of injury with comparable saline volumes infused, and 35 proteins were not different between the injured patient groups would argue against crystalloid dilution as a mechanism for the observed systemic hyperfibrinolysis subset of TIC.

Decreases in specific coagulation proteins, Factors II, X, and XIII, as well as TAFI point towards appropriate intervention by slowing/stopping systemic hyperfibrinolysis with an antifibrinolytic, tranexamic acid, followed by plasma, which is the best source of factors II and X and TAFI.[40,41] Factor XIII has an *in vivo* half-life of ~12 days and thus, there are significant amounts of it in plasma, although cryoprecipitate is the best source.[40,41] One must be cautious for intervention with tranexamic acid after the first three hours post-injury correlates with increased adverse events and even mortality.[42–45]

Systemic increases in activated protein C (APC) have also been postulated to be a mechanism for TIC. If this is the case, then one would expect that the SP- and R-times on CNTEG would be increased with concomitant decreases in fibrinogen, factor V, and factor VIII levels/activities.[19] In contrast, the reported data does not demonstrate increased SP-/R-times. The thrombin activity is not different in HF patients vs. EF patients and healthy controls, and the fibrinogen concentrations are unchanged (all three chains), although both factor V and factor VIII remain in the normal range. In addition, APC-induced systemic

hyperfibrinolysis has been reported to be due to thrombin binding to thrombomodulin via activation of protein C and consumption of PAI-1 with TAFI inhibition being an important mechanism for “fibrinolysis derepression” with TAFI-induced activation of protein C resulting in APC binding to thrombin-thrombomodulin.[2,19] The reported data demonstrate a modest increase in TAFI in HF patients, which was not significantly different from the EF patients or the healthy controls, and such maintenance of normal TAFI concentrations in the HF patients requires more data to better define role of APC in injury-induced hyperfibrinolysis.[2,19]

HF patients, as defined by TEG measurements, had the highest plasma levels of tPA with a concomitant diminished anti-protease defense, or alternatively, near complete conversion of plasminogen to active plasmin. However, EF patients and healthy controls also showed modest response to tPA in the tPA-stimulated TEGs (vertical increase <20%), which may reflect lower levels of tPA or may be due to insufficient anti-protease tPA “buffering”. The HF patients also had significantly prolonged PT/INR and aPTT, which may be due to multiple factor diminutions and/or interference with the clotting cascade.

The proteomic signature of the HF group is dominated by decreases in 1) coagulation proteins, 2) the complement system, specifically anaphylatoxins, 3) proteins involved in stress-induced oligomerization, 4) carrier proteins, and 5) glycoproteins. The decrease in coagulation proteins: factors II, V, XIII and fibrinogen, attests that both anti-protease regulated clot formation and plasminolysis are ongoing. The clearance of serpins, and activation of the contact pathway: factor XII, Kallikrein B1, and Kininogen-1 (F12, KLKB1 KNG) with and significant decrease of both protein S (ProS1) fragments are notable.

HF patients also have decreased plasma concentrations of proteins involved with stress-induced oligimerization. The depletion of such self-associating soluble zymogens that form large oligomers, may lead to unexpected consequences including immunodeficiency.[46–48] In this regard serum amyloid P is a pentraxin, self-associates in 5 and 10 units, provides a mechanism for recognizing DAMPs and PAMPs and accompanies amyloid deposits.[49] Clusterin (CLU), hemopexin (HPX), and α_2-macroglobulin (A2M), also form a class of chaperones oligomerizing with mis-folded proteins in plasma, which often arise during stress.[50] However, CLU can stabilize up to 10 equivalents of certain mis-folded plasma proteins, especially at pH ≤7.1.[51,52]

Traumatic injuries have not been previously linked to decreased plasma concentration of carrier proteins, and the significantly decreased concentrations of retinol binding protein-4 (RBP4), ceruloplasmin (CP), vitamin D binding protein (GC), serotransferrin (TF), and hemopexin (HPX) were unexpected. In plasma, the RBP-retinol complex interacts with transthyretin (TTR), which prevents its loss by filtration through the kidney glomeruli.[53] TTR mis-folding leading to amyloids is well known.[54] The deficiency of GC has not been linked to trauma before, and may be involved in coagulation.[55,56] Similar to RBP4 and TTR, both TF and CP contain beta sheets amenable to common amyloid formation.[57] In contrast HPX (like CLU), could stabilize certain mis-folded plasma proteins.

The presented data have a number of limitations. First, viscoelastic measurements of hemostasis and the fibrinolytic system are low shear assays and the effects of factor XIII depletion and other proteins, including fibrinogen, may not be as "functionally represented" as coagulation *in vivo*. Second, the employed targeted mass spectrometry-based proteomics approach relies heavily on the presence of unique prototypic peptides derived from the concomitant tryptic digestion of the proteins in the sample and the spiked QConCAT standards. Therefore, the approach is limited to the measurement of such peptides and cannot measure the activity of serine-proteases and other enzymes that cut at different residues than arginine or lysine. Future generations of QConCAT peptides could be designed to address this issue by including sequences that can be targeted by proteases in the coagulation and other cascades. Differential clearance of pro-domains and other cleaved domains and the presence of endopeptidases could also confound the results by changing the peptide sequences from those monitored in the QConCAT. With the present set of internal standards, the levels and activation status of tPA and PAI-1 could not be accurately determined. Lastly, plasma samples are filtered prior to mass spectroscopy analysis via QConCAT and there may be some proteins that are non-specifically retained by these columns. This non-specific retention of proteins by these columns is currently being investigated.

In conclusion, HF patients exhibited significant decreases in specific proteins and buffering mechanisms, which were expected in TIC, as well as unexpected decreases in Factors II, X, XII and XIII. Notably, these changes are well correlated to both hyperfibrinolysis with decreased clot strength (MA) with little impact on clot initiation, both the SP and R-times. Thus, the hyperfibrinolytic component of TIC appears mechanistically distinct from derangements of clot formation related to soluble factor dilution/consumption. The unique set of proteome alterations in this subset of severely HF patients may better explain the mechanistic underpinnings of the onset of systemic hyperfibrinolysis after severe injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants P50 GM049222, NIGMS, NIH, T32 GM008315, NIGMS, NIH, and UM1-HL120877, NHLBI, NIH and the Department of Surgery and the Colorado Clinical and Translational Sciences Institute, School of Medicine, University of Colorado Denver, and Bonfils Blood Center.

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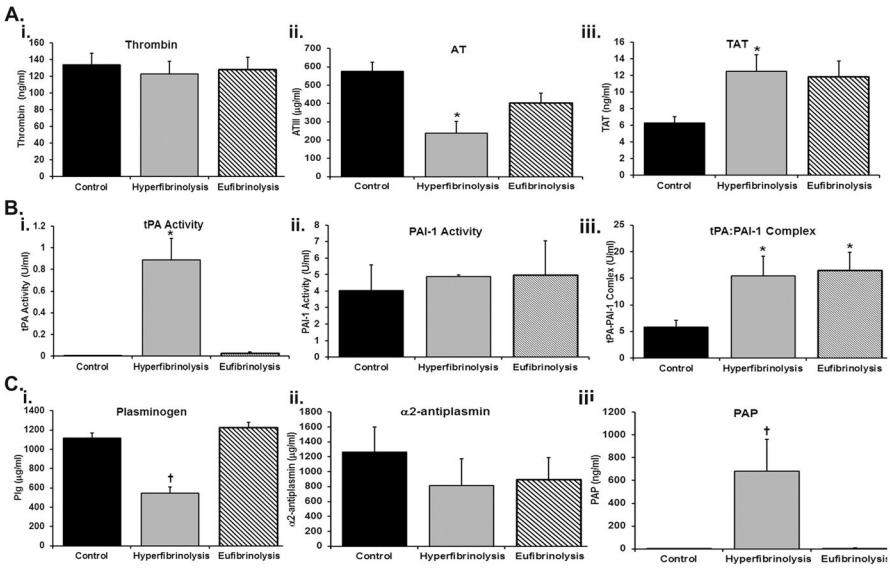

Figure 1.

Quantification of coagulation factors, serpins, and serine protease:inhibitor complexes in injured patietns with HF and EF vs. healthy controls. The figure illustrates the measured protein activity or concentrations by ELISA. Panel A, from left to right, consists of thrombin activity (i), anti-thrombin (ii), thrombin:anti-thrombin complexes (TAT) (iii). Panel B depicts from left to right the concentration of tissue plasminogen activator (tPA) (i), plasminogen activator inhibitor (PAI-1) (ii) and tPA:PAI-1 complexes (iii). Panel C shows from left to right: plasminogen (i), α_2-antiplasmin (ii), and the plasmin:α_2-antiplasmin (PAP) complexes (iii). All data are expressed as the means ± the standard error of the means. *=$p<0.05$ versus the healthy controls and †=$p<0.05$ versus both EF patients and the healthy controls. Significance was measured by an independent analysis of variance followed by Bonferroni's test for multiple comparisons.

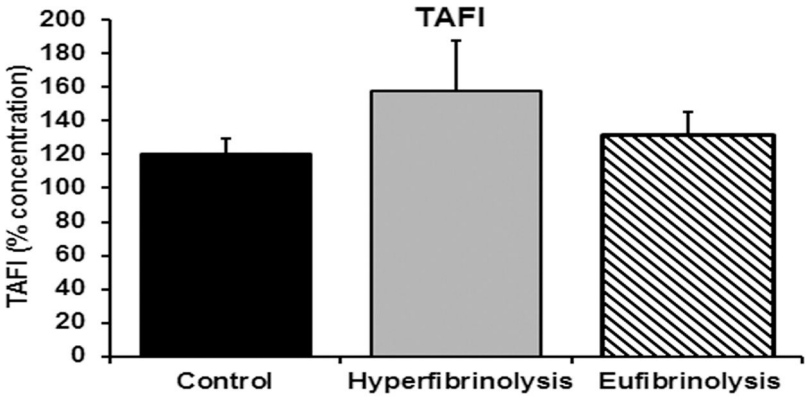

Figure 2.

Thrombin-activated fibrinolysis inhibitor (TAFI) in injured patients with HF, EF and healthy controls. TAFI (% concentration) is illustrated for healthy controls (control) and injured patients with hyperfibrinolysis and eufibrinolysis. All data are expressed as the means ± the standard error of the means. *=$p<0.05$ versus the healthy controls and †=$p<0.05$ versus both EF patients and the healthy controls. Significance was measured by an independent analysis of variance followed by Bonferroni's test for multiple comparisons.

Table 1

Patient demographics, clinical characteristics, and laboratory values.

Variables		Controls (C) n=10						
		Median	Lower quartile	Upper quartile	p-value			
					C vs HF	C vs EF		
Total Hematocrit (%)		46.9	45.1	47.7	0.0147	0.0176		
INR		1.0	1.0	1.1	0.001	0.0069		
PTT		29.3	28.5	29.9	0.0016	0.0033		
Fibrinogen		273	242.8	292	0.0094	No Data		
		Hyperfibrinolysis (HF) n=10			Eufibrinolysis (EF) n=10			p-value * HF vs EF
		Median	Lower quartile	Upper quartile	Median	Lower quartile	Upper Quartile	
Age (years)		32.3	24.6	43.4	36.4	23.4	59.6	0.8242
Men		80%			90%			1.00
Blunt mechanism		40%			20%			0.6285
NISS		46.5	38	51.8	17	12	23.8	0.0074
Max AIS	**Head/Neck**	0	0	3.5	0	0	0	0.2696
	Chest	3.5	0	5	1.5	0	3	0.0340
	Abdomen/Pelvis	2.0	0	3.5	1.0	0	3.3	0.5070
	Extremities	1.0	0.8	2.3	1.0	0	2.3	0.8740
Time from injury (min)		42.0	26.0	58.8	35.0	28.0	42.8	0.5418
1st Measurement (ED)	**SBP (mmHg)**	40.0	0.0	121.5	108.0	100.0	128.0	0.1509
	GCS	3.0	3.0	3.0	15.0	12.25	15	0.0073
	pH	6.9	6.8	7.0	7.3	7.3	7.3	0.0045
	Base Excess (mEq/L)	−19.5	−11	−13.3	−5.0	−7.0	−2.0	0.0128
	Hematocrit (%)	35.9	30.7	40.5	40.3	38.9	44.0	0.1509
	INR	1.8	1.4	2.0	1.1	1.1	1.2	0.0022
	PTT	66.1	48.7	98.9	27.5	25.3	28.2	0.0025
	Fibrinogen	107	87.5	108.5	No data	No data	No data	
Crystalloid infused (ml)		450	62.5	500	400	200	875	0.8582
Units/6 hrs	**Red Blood Cells**	17.5	3.8	26.3	0	0	0	0.0017

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Variables		Controls (C) n=10						
		Median	Lower quartile		Upper quartile		p-value	
							C vs HF	C vs EF
	Plasma (FFP)	10	2.0	12.0	0	0	0	0.0017
	Platelets	1.5	0.0	2.8	0	0	0	0.3143
	Cryoprecipitate	0.5	0	1.8	0	0	0	0.3143
Death /24 hrs.		70%			0			0.0031

The data are presented as the median ± the interquartile range.

*Statistical differences were determined by Wilcoxon's, Kruskal-Wallis, or Fisher's Tests;

NISS: New Injury Severity Score; Max AIS: Maximum Abbreviated Injury Scale score; ED: emergency department (arrival): C = healthy controls. Massive transfusion = 10 RBC units transfused in 24 hours. A unit of apheresis platelets = 3×10^{11} platelets.

Table 2

Thrombelastography of injured patients with systemic hyperfibrinolysis versus injured patients with eufibrinolysis and healthy controls

TEG		Control (C)			C vs. HF	Hyperfibrinolysis (HF)			HF vs. EF	Eufibrinolysis (EF)		
		Med	25th %	75th %	P-Value	Median	25th %	75th %	P-Value	Med	25th %	75th %
CNTEG	SP	11.4	10.8	12.5	0.1221	8.5	6.0	9.9	0.7750	7.6	6.8	8.6
	R	13.2	12.2	13.6	0.1221	9.9	6.9	10.7	0.6595	8.5	7.8	9.4
	Angle	52.9	42.4	54.4	0.5378	44.0	39.5	50.6	0.0337	60.9	55.1	65.6
	MA	57.5	54.5	60.5	0.0054	38.2	28.1	45.5	0.0008	64.2	62.5	66.3
	LY30	1.8	1.1	3.2	0.0023	41.1	11.0	69.3	0.0004	0.8	0.7	0.9
CNTEG +tPA (75 ng /ml)	SP	9.1	0.9	10.2	0.8560	7.3	5.6	11.6	0.7748	6.9	5.8	7.2
	R	12.4	11.3	13.3	0.3049	8.3	6.6	13.5	0.8173	7.8	6.8	8.4
	Angle	44.3	27.5	48.3	0.8352	48.2	23.1	50.2	0.0603	60.7	57.7	62.0
	MA	50.5	48.0	56.5	0.0035	20.3	14.0	32.0	0.0006	60.0	57.5	63.0
	LY30	8.8	5.9	10.3	0.0007	80.0	72.0	89.7	0.0005	7.6	7.1	8.8
r-TEG	SP	0.5	0.5	0.5	0.0381	0.7	0.6	0.8	0.6172	0.6	0.6	0.7
	R	0.7	0.7	0.7	0.0379	0.8	0.8	1.5	0.6401	0.8	0.7	1.0
	Angle	70.6	67.9	72.4	0.0071	58.6	49.4	65.5	0.0055	71.6	67.2	77.1
	MA	60.0	57.5	62.5	0.0020	45.3	31.0	52.0	0.0007	63.5	61.5	67.5
	LY30	2.1	1.3	3.8	0.0569	46.1	4.1	70.1	0.2195	1.5	1.1	2.6

Med = median; CN = Citrated Native; r = rapid TEG; SP = Split Time; R = time to beginning of clot formation MA=Maximum Amplitude; Ly30 = lysis measured at 30 minutes after MA.

Table 3

Plasma proteins that are decreased in HF patients as compared to EF patients and healthy controls

Gene	Common Name	Hyperfibrinolysis			Eufibrinolysis			HF vs EF
		Median	Q1	Q3	Median	Q1	Q3	P value
APCS	Serum amyloid P-component	0.22	0.15	0.27	0.46	0.45	0.55	0.0005
F2a	Prothrombin	0.57	0.34	0.78	1.18	1.05	1.20	0.0005
F2b	Prothrombin	0.49	0.28	0.68	0.96	0.85	1.01	0.0005
GC	Vitamin D-binding Protein	1.43	0.88	1.68	2.38	2.12	2.56	0.0005
AZGP1	Alpha-2-Glycoprotein 1	1.23	0.65	1.33	1.84	1.72	1.98	0.0006
C5	Complement 5	0.46	0.34	0.61	0.83	0.72	0.91	0.0006
KLKB1	Kallikrein B1 (plasma)	0.29	0.22	0.40	0.56	0.51	0.61	0.0006
ITIH2	Inter-α-Trypsin Inhibitor Heavy Chain 2	1.09	0.53	1.31	1.77	1.68	1.90	0.0008
RBP4	Retinol Binding Protein 4	0.94	0.57	1.07	1.73	1.51	2.20	0.0008
SERPINF2a	Alpha 2-Antiplasmin	0.54	0.39	0.92	1.41	1.27	1.48	0.0008
APOA4	Apolipoprotein A4	1.92	1.17	2.86	3.29	3.20	3.76	0.0011
TF	Transferrin	17.67	12.29	22.16	31.52	26.98	35.98	0.0011
APOA2	Apolipoprotein A2	17.04	10.65	19.54	25.92	23.17	30.84	0.0015
C3	Complement 3	3.59	2.04	4.23	6.01	5.46	6.81	0.0015
PROS1a	Protein S alpha	0.08	0.04	0.11	0.16	0.13	0.16	0.0015
SERPINA4	Kallikrein Inhibitor	0.19	0.11	0.22	0.36	0.30	0.40	0.0015
SERPINF2b	Alpha 2-Antiplasmin	0.95	0.50	1.33	1.77	1.57	1.89	0.0015
AHSG	Alpha 2-HS Glycoprotein	2.74	2.14	3.66	5.91	5.42	6.52	0.0019
F12	Coagulation Factor XII	0.09	0.06	0.16	0.28	0.24	0.33	0.0019
ITIH1	Inter-α-Trypsin Inhibitor Heavy Chain 1	1.21	0.57	1.37	1.83	1.61	2.02	0.0019
CP	Ceruloplasmin	1.66	1.23	2.11	2.80	2.28	3.53	0.0025
PROS1b	Protein S	0.11	0.07	0.15	0.20	0.18	0.22	0.0025
F10	Coagulation Factor X	0.20	0.09	0.23	0.33	0.27	0.39	0.0033
F13Bb	Factor XIII B Chain	0.17	0.15	0.23	0.33	0.27	0.41	0.0033
GPLD1	Glycosylphosphatidyl inositol specific Phospholipase D1	0.12	0.07	0.15	0.22	0.18	0.26	0.0033
GSN	Gelsolin	0.47	0.41	0.79	1.19	1.02	1.38	0.0033

Gene	Common Name	Hyperfibrinolysis			Eufibrinolysis			HF vs EF
		Median	Q1	Q3	Median	Q1	Q3	P value
PLGb	Plasminogen	0.95	0.73	1.25	1.79	1.59	1.97	0.0033
PROCb	Protein C	0.18	0.13	0.24	0.33	0.29	0.36	0.0033
SERPIND1	Heparin Cofactor 2	0.72	0.32	0.86	1.15	1.10	1.36	0.0033
CFH	Complement Factor H	1.04	0.76	1.22	1.81	1.62	2.02	0.0043
CLU	Clusterin	0.84	0.43	1.04	1.49	1.36	1.67	0.0043
HPX	Hemopexin	7.05	4.59	8.94	11.39	11.04	12.32	0.0043
KNG1	Kininogen-1	0.97	0.74	1.30	1.82	1.64	2.04	0.0043

The data are expressed as the median ± interquartiles. All 33 proteins were decreased versus identical QConCAT values from healthy controls as well as the EF patients ($p<0.005$).