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ORIGINAL ARTICLE

Early hemostatic responses to trauma identified with hierarchical clustering analysis

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Summary. *Background:* Trauma-induced coagulopathy is a complex multifactorial hemostatic response that is poorly understood. *Objectives:* To identify distinct hemostatic responses to trauma and identify key components of the hemostatic system that vary between responses. *Patients/Methods:* A cross-sectional observational study of adult trauma patients at an urban level I trauma center emergency department was performed. Hierarchical clustering analysis was used to identify distinct clusters of similar subjects according to vital signs, injury/shock severity, and comprehensive assessment of coagulation, clot formation, platelet function, and thrombin generation. *Results:* Among 84 total trauma patients included in the model, three distinct trauma clusters were identified. Cluster 1 ($N = 57$) showed platelet activation, preserved peak thrombin generation, plasma coagulation dysfunction, a moderately decreased fibrinogen concentration and normal clot formation relative to healthy controls. Cluster 2 ($N = 18$) showed platelet activation, preserved peak thrombin generation, and a preserved fibrinogen concentration with normal clot formation. Cluster 3 ($N = 9$) was the most severely injured and shocked, and showed a strong inflammatory and bleeding phenotype. Platelet dysfunction, thrombin inhibition, plasma coagulation dysfunction and a decreased fibrinogen concentration were present in this cluster. Fibrinolytic activation was present in all clusters, but was particularly increased in cluster 3. Trauma clusters were most noticeably different in their relative fibrinogen concentration, peak thrombin generation, and platelet-induced clot contraction. *Conclusions:* Hierarchical clustering analysis identified three distinct hemostatic responses to trauma. Further insights into the underlying hemostatic mechanisms responsible for these responses are needed.

Keywords: coagulation; fibrinogen; hemostasis; platelets; thrombin; trauma.

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Introduction

Trauma-induced coagulopathy (TIC) is an important and incompletely understood multifactorial response to trauma that is associated with increased bleeding, greater transfusion requirements, and increased mortality [1]. Brohi *et al*. [2] first described spontaneous anticoagulation according to an increase in the International Normalized Ratio (INR) in the plasma of emergency department (ED) trauma patients. Further work supported an anticoagulant mechanism resulting from a systemic increase in the level of thrombomodulin, which, when bound to thrombin, activated protein C [3]. Activated protein C (APC) can inhibit factor V and FVIII, thus reducing thrombin generation. Fibrinolysis is also enhanced by simultaneous inhibition of plasminogen activator inhibitor-1 (PAI-1) and increased plasma tissue-type plasminogen activator (t-PA) levels [3,4]. Others have stratified trauma patients according to disseminated intravascular coagulopathy (DIC) score criteria, noting the presence of an activated, rather than anticoagulated, hemostatic state leading to DIC with a hyperfibrinolytic phenotype [5]. There is also emerging evidence for a

prominent role for platelet dysfunction in the pathomechanism of TIC. Even mildly impaired platelet aggregation is strongly associated with poor outcomes in trauma patients [6]. In addition, inflammation may play an important role in modulating the acute coagulation response to trauma and blood loss. Levels of the cytokines interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) are elevated almost immediately after severe injury and/or blood loss in trauma and surgical patients [7]. An increased IL-6 level, in particular, is associated with massive transfusion, the development of multiorgan failure, and mortality [8]. IL-6 can also directly activate fibrinogen gene promoters, thus linking its presence in plasma directly to subsequent coagulation responses [9]. Endothelial activation is also a prominent component of TIC, and may initiate coagulopathy after endothelial glycocalyx shedding, allowing for direct interaction between activated inflammatory cells and the endothelial surface [10,11]. However, there is limited understanding of how various components of the hemostatic and inflammatory systems and injury/patient characteristics interact to produce overall hemostatic phenotypes after trauma.

Much of our current understanding of coagulopathy after trauma comes from studies that grouped subjects according to injury severity, shock severity, and/or single estimates of coagulopathy, such as INR, viscoelastic clot strength, or platelet aggregation. These approaches have yielded significant knowledge regarding individual components of the hemostatic response to trauma. However, the information gained from these investigations is limited, owing to selection bias, *a priori* assumptions about outcome measures, and the chosen method of coagulopathy classification. In addition, the generally accepted multifactorial nature of coagulopathy limits the ability of single outcome measurements to adequately identify the syndrome of traumatic coagulopathy. Alternatively, data mining techniques such as hierarchical clustering analysis (HCA) can adequately identify distinct groups or phenotypes within larger cohorts by grouping similar variables and characteristics without requiring *a priori* assumptions. HCA is a multivariate technique of grouping together rows in a dataset that share similar values, and whose values are close to each other relative to those of other clusters. It is a process that starts with each point in its own cluster; next, the two clusters that are closest together are combined into a single cluster, until there is only one cluster containing all of the points. This type of clustering permits the detection of subgroups that would otherwise be disguised, owing to high variation or *a priori* assumptions [12]. HCA has been used successfully as a data mining tool to identify blood plasma lipidomics profiles [13] and to identify complex metabolic states in critically injured patients [14]. The strategy of using HCA to group patients by combining their clinical profiles with broadly measured hemostatic parameters may be useful to identify both distinct hemostatic responses and the relationships governing these responses. Such information can be used to improve our understanding of the pathophysiology of TIC and to guide further studies.

The primary aim of this study was to use HCA to identify distinct hemostatic responses within a general trauma patient cohort. We hypothesized that hierarchical clustering would identify multiple distinct hemostatic responses. We also aimed to characterize the key differences between clusters, and interpret how patient characteristics and individual components of the hemostatic system interact to produce an overall hemostatic response to trauma. To this end, we applied HCA to a prospective cohort of adult ED polytrauma patients who presented to a US urban level I trauma center, and identified key differences in hemostatic parameters between clusters.

Methods

This was a cross-sectional study of trauma patients presenting to a US level I trauma center ED. Informed consent was obtained from all subjects according to an institutional human subjects review board-approved protocol consistent with the Declaration of Helsinki. A group of healthy non-injured volunteers who were not taking anticoagulants were recruited from the local population as a control group for comparison. Patients were excluded if they were < 18 years of age, incarcerated, pregnant, suicidal, or known to be taking anticoagulant medications, including aspirin, clopidogrel, or warfarin. To avoid confounding influences of blood transfusion or massive non-survivable injury with a perimortem state, patients were excluded if they received any blood products prior to study sample blood draw, or were not expected to live for 3 days, owing to obvious massive non-survivable injury. The coagulation response in such perimortem patients is often dominated by massive hyperfibrinolysis, which would have added bias and skewed our data unnecessarily [15]. We also sought a mixed cohort of severely injured trauma patients who would be more likely to vary in their individual hemostatic responses. Therefore, we excluded patients who were not expected to be admitted to the inpatient trauma service, owing to isolated or minor injury. Trauma patients were identified in the ED, and blood was collected into standard citrated (1 : 9 ratio of citrate to blood), EDTA and sodium–heparin vacutainers prior to the patients receiving any blood products. Samples were collected on arrival at the ED prior to subjects receiving any blood products, and blood was sampled again 8 h later for repeat plasma coagulation and thromboelastography (TEG) measurements. Samples were either processed immediately for viscoelastic clot formation, platelet adhesion and platelet aggregation determination in whole blood, fixed for flow cytometry analysis, or centrifuged at 5000 × *g* for 15 min to produce platelet-poor plasma and frozen at − 80 °C for later analysis. Clinical data, including vital signs, transfusion requirements, inju-

ries, clinical laboratory results, and outcomes, were collected from the medical record for each patient. Injury severity scores were obtained for each patient from the hospital trauma databank.

Laboratory methods

Plasma coagulation Platelet-poor plasma was assayed for INR, activated partial thromboplastin time (APTT) and fibrinogen concentration with the START-4 coagulation analyzer, according to the manufacturer's instructions (Diagnostica Stago, Parsippany, NJ, USA).

Platelet function Adhesion of platelets under high flow conditions was determined from time to occlusion of a small orifice by use of the PFA-100 (Dade International, Miami, FL, USA) with exposure to collagen/ADP. This device measures time to occlusion of a small aperture as whole blood is drawn through it under high-flow conditions, and has been shown to respond to changes in primary platelet adhesion mediated by the glycoprotein (GP) Ib–IX–V surface membrane complex in addition to von Willebrand factor activity, and GPIIb/IIIa activation [16,17]. Platelet aggregation was also determined from impedance in whole blood (Chronolog Series 500 aggregometer; Chrono-Log Corp., Havertown, PA, USA) in response to ADP (10 μmol L^{-1}) to evaluate platelet aggregation. Flow cytometry (BD Accuri C6; Accuri, Ann Arbor, MI, USA) was used to measure platelet activation via the GP P-selectin, because it is rapidly translocated to the platelet surface on stimulation. The P-selectin content on the platelet surface was detected with the CD62-P mAb conjugated with phycoerythrin (mouse anti-human: BD Pharmingen, San Jose, CA, USA). We also measured GPIIb/IIIa surface integrin transition to its high-affinity state by using the mAb against high-affinity glycoprotein IIb/IIIa platelet surface integrin (PAC-1) conjugated with fluorescein isothiocyanate (BD Biosciences, San Jose, CA, USA). These assays provide an estimate of the platelet activation state.

Viscoelastic measurements of whole blood clot formation TEG (TEG 5000; Haemonetics, Niles, IL, USA) was used to measure whole blood viscoelastic clot formation. The TEG 5000 reports time to onset of clot formation (R), which positively correlates with thrombin generation, the time needed to reach a predetermined level of clot stiffness (K) and the angle (α-angle), which is the clot formation rate and correlates with the fibrin generation rate, the maximal clot amplitude (MA) or stiffness, and the percentage of clot breakdown resulting from fibrinolysis in the first 30 min after MA (LY30%). The TEG with Platelet Mapping Assay (TEG-PM; Haemonetics) was also used to determine the platelet contribution to clot amplitude. Platelets were selectively activated with ADP, and the amplitude difference between platelet-specific activation and maximal thrombin-induced clot formation during TEG measurement was calculated and expressed as percentage inhibition from MA.

Fibrinolytic activation and fibrinolysis We determined clot lysis from TEG LY30% and fibrinolytic activation from D-dimer measured by ELISA (Technozym D-Dimer; Technoclone, Vienna, Austria). D-dimer is a plasmin-specific cleavage product of fibrin.

Thrombin generation Thrombin generation profiles for each subject were generated by the use of calibrated automated thrombography with a thrombin-specific fluorogenic substrate (CAT; Thrombinoscope BV, Mastricht, The Netherlands). Platelet-poor plasma was first activated with low-level tissue factor (5 pmol) to activate thrombin generation. The elapsed time from activation to onset of thrombin generation (thrombin lag time), maximal thrombin concentration (C_{max}) and area under the thrombin generation curve (endogenous thrombin potential [ETP]) were recorded and included in the final analysis. Thrombin–antithrombin complexes were also measured in plasma by ELISA (TAT micro; Siemens Healthcare Diagnostics, Marburg, Germany).

Inflammation The cytokines IL-6 (Human IL-6 ELISA Kit II; BD Biosciences) and TNF-α (Human TNF ELISA Kit II; BD Biosciences) were measured in plasma by ELISA, as estimates of the inflammatory response. Cell counts, including leukocyte count as an estimate of inflammation, and hemoglobin concentration were measured by automated cell counting in the research laboratory (ABX-60; Horiba, Irvine, CA, USA).

Statistical methods

Descriptive statistics (median and interquartile range [IQR]) were used to summarize demographics and outcome measurements, owing to significantly skewed data distributions. Outcome variables included IL-6, TNF-α, TEG parameters, categorical descriptions of injury severity, hemostatic transfusion thresholds, and blood products received. Clusters and control groups were then compared by use of the non-parametric Kruskal–Wallis test. Steel–Dwass adjustment was performed to adjust for multiple comparisons for *post hoc* comparisons. Chi-square analysis with likelihood ratio testing was used to compare nominal outcomes between clusters. Matched-pairs analysis was used to measure the mean difference and 95% confidence interval of the difference between the ED arrival sample and the 8-h sample for plasma and TEG measurements.

Variables were selected for inclusion in the clustering analysis if they were measured in ≥ 90% of subjects and they represented an important component of clot formation (e.g. extrinsic and intrinsic pathways, fibrinogen,

platelet activation and function, fibrinolysis, and inflammation). Stringent HCA variable selection was used, limiting the variables used to build the model to direct laboratory measurements. We chose to not use any clinical variables or subject characteristics (ISS, vital signs, resuscitation fluid volumes, etc.) in the model. These clinical and treatment variables have multiple colinear effects on coagulopathy that have not yet been clearly defined, making them poor contributors to independent cluster selection. We also excluded TEG parameters (R, K, angle, MA, and LY30%), because these measurements are derived from one overall clot formation curve, and are therefore strongly collinear, making them less useful for clustering. Final variables included in the model were: INR, APTT, fibrinogen concentration, thrombin lag time, ETP, C_{max}, platelet count, PFA-100 collagen/ADP closure time, ADP-activated platelet aggregation determined by impedance, ADP-activated TEG-PM percentage inhibition, PAC-1 (%), P-selectin (%), D-dimer, and leukocyte count (white blood cells [WBCs]).

The purpose of HCA is to classify data of previously unknown structure into discrete groups in an unbiased fashion. The procedure starts by considering each observation, or subject, as a single cluster, and applying the model-specific selection criteria for merging of adjacent clusters for each variable assigned to the model. Model-based HCA then proceeds by successively merging pairs of clusters until all subjects are clustered together [18].

Ward's minimum variance method was compared with average linkage, complete linkage and centroid linkage models. Ward's minimum variance method uses classification likelihood, based on the sum-of-squares, when comparing variables to assign subjects to clusters according to their similarity to one another. The distance between two clusters is the ANOVA sum of squares between the two clusters added up over all the variables. Average linkage uses the average distance between pairs of observations to join clusters, and is slightly biased towards producing clusters with the same variance. Complete linkage uses the maximum distance between a subject in one cluster

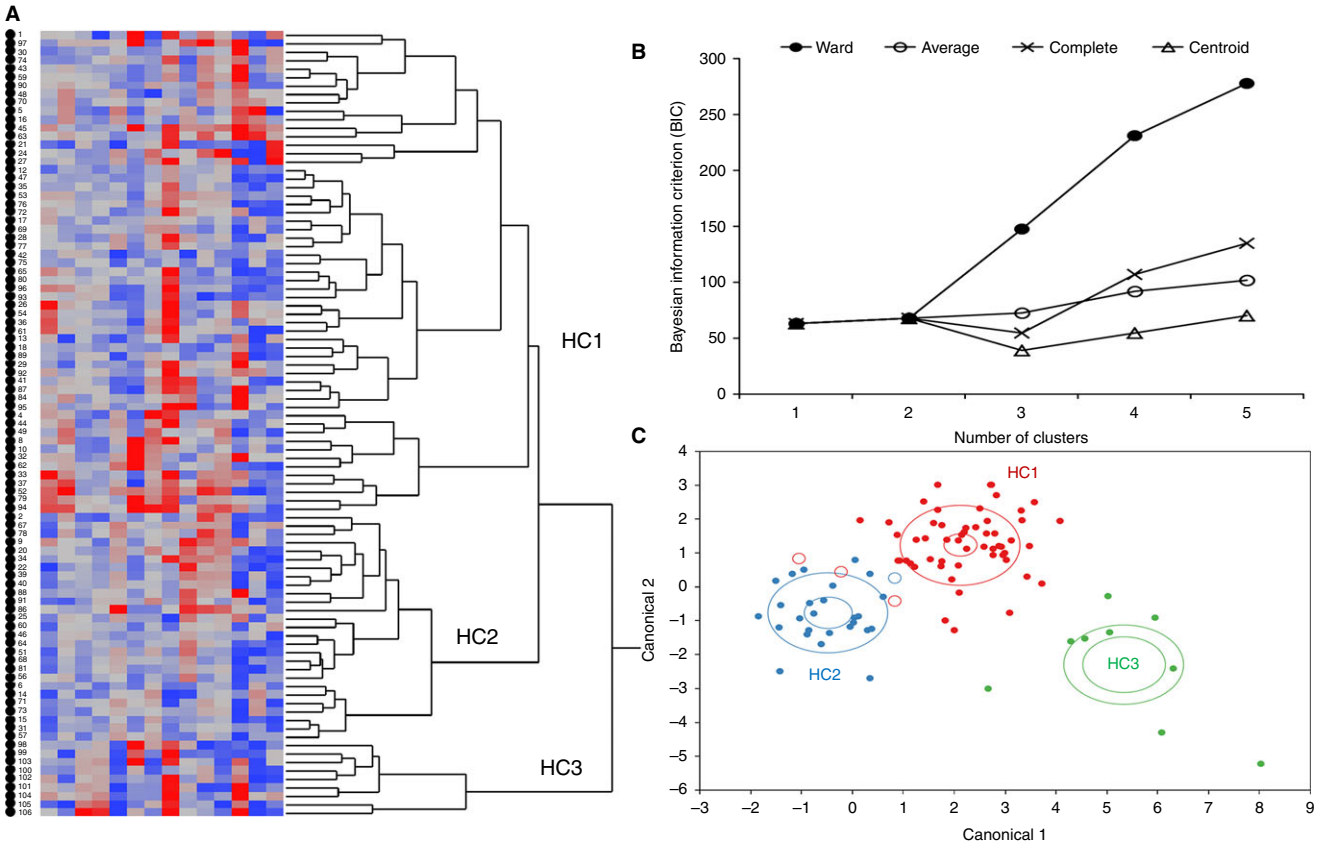

Fig. 1. Selection of appropriate model for hierarchical analysis. (A) Heat map and dendrogram of hierarchical clustering analysis, showing the left-to-right clustering of individual subjects into separate clusters based on the similar values for each test variable. The results of analysis with a three-cluster (HC1–HC3) Ward method are shown. (B) Selection of the best hierarchical clustering model by use of the Bayesian information criterion (BIC) plotted against number of clusters. The Ward method produced the highest BIC with the least number of clusters, and was selected over other models. (C) Discriminant analysis canonical plot of the three-cluster Ward model. Good discrimination between clusters was possible, with misclassification of only four subjects (open circles). Cluster 1 (red) and cluster 2 (blue) were more similar, whereas cluster 3 (green) was clearly discriminated. Larger ellipses identify the area in which 50% of the subjects are found for each cluster. Smaller ellipses identify the 95% confidence interval for the center point of each cluster.

Table 1 Demographics, injury profiles, and mortality

	Healthy controls	Injured subjects		
		Cluster 1	Cluster 2	Cluster 3
Age (years), median (IQR)	41 (29–46)	30 (24–50)	52 (30–58)	36 (21–62)
Mean body mass index (IQR)	22 (20–26)	25 (23–28)	28 (24–32)	26 (20–29)
Prehospital transport time (min) (IQR)	NA	42 (21–59)	34 (21–60)	30 (19–38)
N	10	57	18	9
Male gender, *n* (% of total)	6 (60)	49 (86)	16 (89)	5 (55)
Injury category, *n* (%)				
Penetrating	NA	15 (26)	5 (28)	2 (22)
Blunt	NA	40 (70)	13 (72)	7 (78)
Both	NA	2 (4)	0	0
Cause of injury, *n* (%)				
Stab wound	NA	0	1 (6)	0
Gunshot wound	NA	14 (25)	4 (22)	2 (22)
Motor vehicle crash	NA	31 (54)	11 (61)	5 (56)
Pedestrian struck by motor vehicle	NA	7 (12)	2 (11)	2 (22)
Fall	NA	5 (9)	0	0
Traumatic brain injury	NA	17 (30)	2 (11)	5 (55)
72-h mortality	NA	2 (3.5)	1 (5.6)	4 (44)

IQR, interquartile range; NA, not applicable.

and a subject in the other clusters to link clusters, and tends to produce clusters with roughly equal diameters that are susceptible to the influence of outliers. The centroid method uses the squared Euclidean distance between subject means to link clusters. The centroid method is more robust to the influence of outliers. A procedure outlined by Fraley and Raftery, based on identifying the model with the highest Bayesian information criterion with the least number of clusters for each proposed model, was used to select the best-performing model, which was then used for HCA [19]. After model selection and clustering, discriminant analysis was used to confirm that the selected model demonstrated an acceptable degree of separation between clusters, with minimal misclassifications. All statistical analyses were performed with JMP statistical software (version 9.0.0: SAS Institute, Cary, NC, USA). The overall level of significance for all statistical tests was $P < 0.05$.

Results

One hundred and sixty-three trauma subjects were screened for enrollment. Forty-nine subjects were immediately excluded because they either declined to participate ($N = 16$), had known anticoagulant use ($N = 5$), had received blood products prior to blood sampling ($N = 2$), or for other reasons ($N = 26$). Additional subjects were excluded prior to HCA because they had incomplete data ($N = 6$), or were not expected to survive for at least 72 h ($N = 9$). A total of 99 trauma subjects met the clinical

Table 2 Initial emergency department vital signs, injury severity and 8-h fluid resuscitation for injured subjects

	Cluster 1		Cluster 2		Cluster 3	
Vital signs	Median	IQR	Median	IQR	Median	IQR
Systolic blood pressure (mmHg)	120	111–142	146	117–165	93	76–124
Diastolic blood pressure (mmHg)	78	64–90	80	62–100	58	48–91
Respiratory rate (breaths min^{-1})	18	14–24	18	15–22	20	16–29
Pulse (beats min^{-1})	100	80–117	95	76–108	101	77–144
Anatomic injury severity						
Injury Severity Scale	19	14–27	14	10–29	41*,†	28–49
Glasgow Coma Scale	15	3–15	15	14–15	3†	3–11
Shock severity						
Lactate (mmol L^{-1})	4.0	2.8–5.3	2.9	1.9–4.8	2.1	1.2–7.1
Base deficit (meq L^{-1})	2.8	6.0 to 1.0	0.9	2.7 to 1.1	8.1*,†	16.2 to 5.0
Hemoglobin concentration (g dL^{-1})	12.5	10.8–13.7	12.1	10.8–13.7	9.3*	6.2–12.0
Resuscitation fluid volume (mL)						
Crystalloid at time of sampling	1400	500–2000	500	0–2000	2000	1350–2300
Crystalloid at 8 h	1169	486–2599	346	56–2000	3240†	2195–7075
Blood products at 8 h (mL)						
Packed red blood cells	0	0–465	0	0–310	2325*,†	310–6433
Fresh frozen plasma	0	0–0	0	0–0	1350*,†	169–3251
Platelet concentrate	0	0–0	0	0–0	100*,†	0–350
Cryoprecipitate	0	0–0	0	0–0	215*,†	38–633

IQR, interquartile range. Non-parametric multiple comparisons with Steel–Dwass all-pairs adjustment for individual differences. *Difference versus cluster 1, $P < 0.05$. †Difference versus cluster 2, $P < 0.05$.

inclusion criteria and were considered for HCA analysis. They were compared with 10 healthy uninjured volunteer control subjects, who were enrolled separately.

The Ward three-cluster model was chosen as the best-performing model for HCA of this dataset (Fig. 1B). A total of 99 subjects and 14 variables were entered into the model for clustering. The total number of missing values was 28 of 1386 (1.7%). Owing to the missing values, 84 of 99 (85%) subjects entered into the model could be clustered. The variable with the most missing values was TEG-PM percentage inhibition, with seven of 99 (7%) of values missing. The resulting HCA showed three distinct clusters. According to the dendrogram, cluster 1 ($N = 57$) and cluster 2 ($N = 18$) were in closer proximity to each other than to cluster 3 ($N = 9$), which was located further away and was more distinct. The dendrogram and heat map generating the three clusters are shown in Fig. 1. Smaller subclusters present in the dendrogram were not evaluated, owing to small sample numbers.

Demographics, injury patterns, shock severity and mortality for each cluster are shown in Table 1. Note that no subjects received hemostatic agents, including tranexamic acid, prior to the ED or 8-h blood samples being obtained. Overall mortality measured 72 h after hospital admission for those included in clustering was seven of 84 (8%); of these, one subject died before 8 h, and this subject was assigned to cluster 2. Subjects were mostly middle-aged males with normal to overweight body composition. For each cluster, blunt injury from motor vehicle crashes was the primary mechanism of injury, followed by penetrating injury from gunshot wounds. The prevalence of traumatic brain injury was also increased in cluster 3.

Vital signs, shock severity and fluid resuscitation characteristics are given in Table 2 and Fig. 2. Cluster 3 showed significantly abnormal vital signs, greater injury, and greater shock. Cluster 3 also clearly required more crystalloid and blood products over the first 8 h of hospital treatment. Significantly more subjects met fibrinogen replacement criteria in cluster 1 than in cluster 2 (Fig. 2B).

Hemostatic and inflammatory parameters are shown in Table 3. Again, cluster 3 showed the strongest bleeding phenotype, with significantly different plasma coagulation pathway test results, fibrinogen concentration, thrombin generation, and platelet dysfunction, and increased levels of inflammatory markers. Interestingly, cluster 1 differed from healthy controls and cluster 2 by a slight prolongation of the INR, a moderate decrease in fibrinogen concentration, strongly inhibited platelet-induced clot contraction, and an increased WBC count.

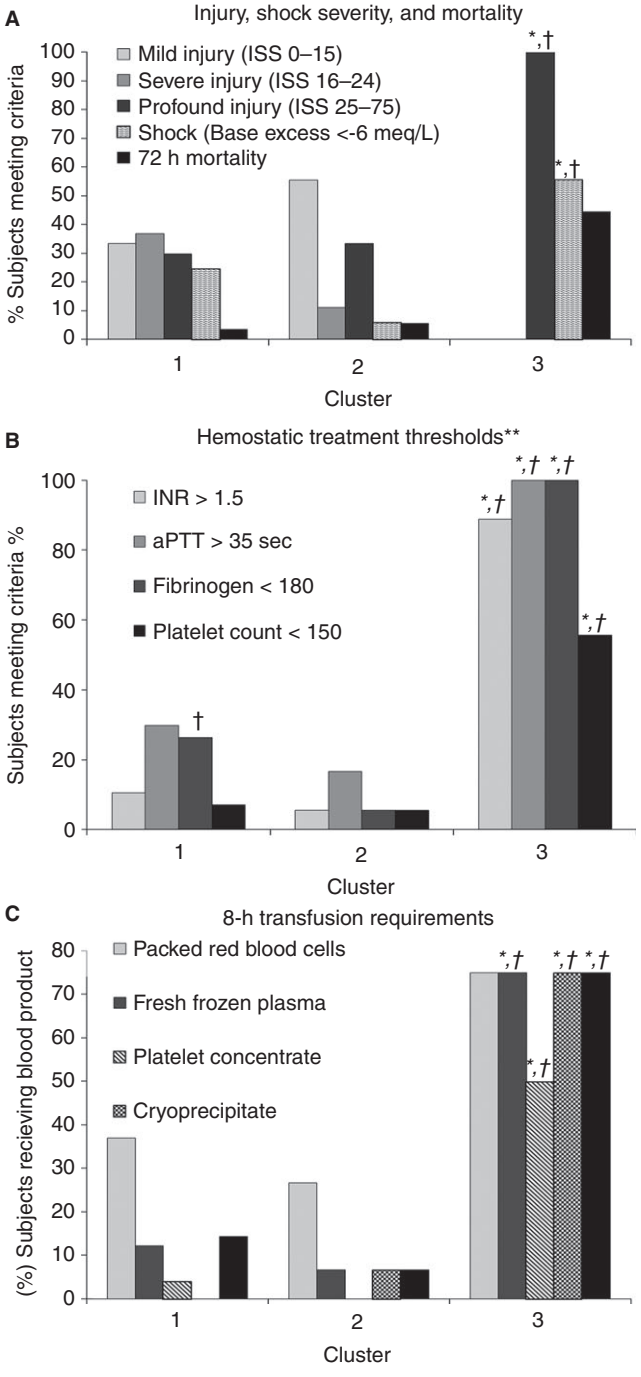

Fig. 2. Characterization of subjects in each cluster. (A) Histogram of nominal categories for injury severity, shock severity and mortality for each cluster. There was significantly more profound injury and hemorrhagic shock in cluster 3 than in clusters 1 and 2. Seventy-two-hour mortality was also greatest in cluster 3, but statistical analysis of a comparison with other clusters was not possible, owing to the low number of non-survivors in the other clusters. (B) Histogram of the prevalence of standard hemostatic transfusion thresholds present in each cluster. **Criteria from [20]. (C) Histogram of prevalence of subjects receiving blood products within the first 8 h of hospitalization by cluster ($N = 68$). Subjects were counted if they received any amount of specified blood product. Hemostatic transfusion was defined as the receipt of any amount of fresh frozen plasma, platelet concentrate, or cryoprecipitate. Chi-square likelihood ratio: *$P < 0.05$ versus cluster 1; †$P < 0.05$ versus cluster 2. APTT, activated partial thromboplastin time; INR, International Normalized Ratio.

Table 3 Clustered hemostatic and inflammatory variables measured on arrival in the emergency department

		Healthy controls		Cluster 1		Cluster 2		Cluster 3	
Category	Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Plasma coagulation	INR	1.0	0.9–1.2	1.2*	1.1–1.4	1.1	1.1–1.3	2.0*,†,‡	1.8–2.7
	APTT (s)	34.8	31.0–35.9	32.0	29.6–35.7	31.0	28.7–34.2	53.0*,†,‡	48.3–77.3
	Fibrinogen concentration (mg dL^{-1})	337.1	307.7–379.4	220.4*	175.6–322.1	285	216.8–359.3	113.2*,†,‡	100.0–129.8
Thrombin generation	Thrombin lag time (min)	2.0	1.6–3.1	2.1	1.7–2.6	3.7*,†	2.5–4.0	2.3‡	1.8–2.7
	Endogenous thrombin potential (AUC)	2147.7	1828.3–2281.3	1889.3	1609.2–2317.8	2050.2	1837.8–2342.0	1712.3	1260.7–2002.0
	Maximal thrombin concentration (nmol L^{-1})	382.6	335.7–426.0	369.8	322.5–414.3	381.2	332.4–436.3	203.7*,†,‡	186.2–235.6
Platelet function	Platelet count (10^9 L^{-1})	217	184–263	247	184–310	236	195–250	149*,†,‡	91–192
	PFA-100-COLL/ADP (s)	90	68.3–93.5	63	52.5–83.0	63	52.8–75.3	129‡	67.5–263.0
	Aggregation-ADP (Ω)	10.0	9.4–12.1	11.0	8.0–13.8	10.0	8.4–11.4	5.5†	3.3–9.0
	TEGPM-% inhibition-ADP	52.7	25.4–58.1	92.7*	74.6–98.4	46.6†	30.1–65.0	99.2*,†,‡	94.9–99.9
	PAC-1 (%)	11.0	4.6–28.9	4.9	1.4–12.3	5.0	1.2–14.4	0.5†	0.1–2.1
	P-selectin (%)	2.7	0.7–3.6	3.5	1.9–8.9	1.5	1.0–3.3	3.4	0.9–8.0
Fibrinolytic activation	D-dimer (ng mL^{-1})	73.8	41.3–178.9	2349.0*	630.0–4201.3	628.9*	206.4–2469.1	3551.1*	1817.9–5000.0
Inflammation	WBCs (10^9 L^{-1})	6.7	5.7–7.4	13.9*	11.2–18.7	8.2†	5.4–10.7	13.0*	10.2–17.6
	IL-6 (pg mL^{-1})	NA		58.4	13.6–160.7	25.9	7.3–65.9	179.8‡	54.5–210.2
	TNF-α (pg mL^{-1})	NA		4.5	3.4–6.3	5.7	3.3–10.3	4.4	2.9–17.5

Aggregation-ADP, impedance platelet aggregometry with ADP activation; APTT, activated partial thromboplastin time; AUC, area under the curve; IL-6, interleukin-6; INR, International Normalized Ratio; IQR, interquartile range; NA; PAC-1, high-affinity glycoprotein IIb/IIIa platelet surface receptor; PFA-100-COLL/ADP, aperture closure time with collagen and ADP activation; TEGPM-% Inhibition-ADP, Thrombelastography Platelet Mapping Assay percentage inhibition after ADP activation; TNF-α, tumor necrosis factor-α; WBC, white blood cell. Non-parametric multiple comparisons with Steel Dwass all-pairs adjustment for individual differences. *Difference versus control, $P < 0.05$. †Difference versus cluster 1, $P < 0.05$. ‡Difference versus cluster 2, $P < 0.05$. NA, not applicable.

Table 4 Plasma coagulation and thrombelastography measurements at emergency department (ED) arrival and after 8 h of hospitalization for each cluster

		Cluster 1		Cluster 2		Cluster 3	
Plasma coagulation	Measurement time	Median	IQR	Median	IQR	Median	IQR
INR	ED arrival	1.2	1.1–1.4	1.1	1.1–1.3	2.0*,†	1.8–2.7
	8 h	1.2	1.1–1.3	1.1	1.1–1.3	1.3	1.2–1.4
APTT (s)	ED arrival	32.0	29.6–35.7	31.0	28.7–34.2	53.0*,†	48.3–77.3
	8 h	34.0	30.3–37.0	34.5	30.4–38.3	38.6	33.1–45.1
Fibrinogen (mg dL^{-1})	ED arrival	220	175.6–322.1	285	216.8–359.3	113.2*,†	100.0–129.8
	8 h	238.9	206.8–307.2	265.0	241.7–361.8	209.5	152.1–321.0
TEG clot formation							
R (min)	ED arrival	4.3	3.3–5.1	4.2	3.4–4.8	4.6	4.2–6.0
	8 h	4.8	4.2–5.7	4.8	3.1–5.8	4.6	3.9–5.2
K (min)	ED arrival	1.4	1.2–1.6	1.2	1.1–1.6	2.2*,†	1.6–3.1
	8 h	1.4	1.2–1.8	1.3	1.1–1.8	1.9	1.2–2.4
ANG (°)	ED arrival	70.2	66.2–72.9	71.2	64.9–74.3	61.7*,†	52.2–67.5
	8 h	69.7	63.6–71.6	70.0	63.5–73.6	65.1	58.8–71.6
MA (mm)	ED arrival	61.7	57.7–65.6	61.9	60.2–68.0	51.5*,†	45.2–60.8
	8 h	61.7	58.2–64.8	64.3	60.0–65.6	57.2	53.6–64.0
LY30%	ED arrival	0.3	0.0–1.4	0.8	0.3–2.5	0.0	0.0–1.6
	8 h	0.1	0.0–0.8	0.4	0.0–1.6	0*,†	0.0–0.0

ANG, clotting angle; APTT, activated partial thrombplastin time; INR, International Normalized Ratio; IQR, interquartile range; *K*, clot formation time; LY30%, percentage clot lysis at 30 min; MA, maximal clot amplitude; *R*, clot onset time; TEG, thromboelastography. Non-parametric multiple comparisons with Steel–Dwass all-pairs adjustment for individual differences. *Difference versus cluster 1, $P < 0.05$. †Difference versus cluster 2, $P < 0.05$.

Plasma coagulation and TEG parameters measured at ED arrival and again after 8 h of hospitalization are shown in Table 4, and the interval changes for each parameter over 8 h of hospitalization are shown in Fig. 3. At ED arrival, cluster 3 showed impaired clot formation, as demonstrated by a significantly prolonged *K*, a decreased angle and decreased MA as compared with other clusters. Note that there were no differences in clot onset time (*R*), even though extrinsic and intrinsic plasma pathway tests showed significant prolongation. At 8 h, the greatest changes were normalization of the INR and APTT, an increased fibrinogen concentration and decreased clot lysis with concurrent normalization of other TEG parameters in cluster 3. No measured parameters changed significantly over 8 h in cluster 1 or cluster 2 (Fig. 3).

Discussion

Application of HCA to this selected trauma cohort revealed three distinct hemostatic responses to injury. Our results agree with previous characterizations of TIC showing that overt coagulopathy was most often present in those patients with severe anatomic injury and tissue hypoperfusion resulting from hemorrhagic shock [2]. These patients also required more blood transfusions and were the most likely to die. It is clear that the most severely injured and shocked patients (cluster 3) showed a profoundly decompensated and impaired hemostatic and proinflammatory response that required significant hemostatic therapy. However, trauma patients have traditionally been stratified into only coagulopathic or non-coagulopathic groups. Only more recently have investigators identified a third acute hypercoagulable or activated hemostatic state as also existing in ED trauma patients. Branco *et al.* found that coagulation activation was associated with less blood product transfusion and low mortality, and was therefore protective during the acute phase of trauma treatment [21]. Similarly, we also identified a small cluster of subjects (cluster 2) with an overall activated hemostatic response that required minimal hemostatic therapy during the acute phase of trauma treatment (i.e. the first 8 h of hospitalization). Increasingly, it appears that an acutely activated hemostatic response may be optimal to reduce blood loss, and that any impairment of this activated state may signal an increased risk of bleeding. This concept is supported by evidence that even a minor prolongation of the INR (> 1.2) into what most providers accept as being the 'low normal' range is associated with increased mortality in trauma patients [22].

The majority of subjects in this cohort (58%, cluster 1) showed a distinct compensated initial hemostatic state with viscoelastic clot formation that was not significantly abnormal. This largest subgroup represents a newly recognized and important group of trauma patients, and its presence helps to bridge the previously identified prohemostatic and bleeding responses to trauma. The hemostatic components that were most different between cluster 1 and cluster 2 were fibrinogen concentration and the relative platelet activation state. Hagemo *et al.* showed that 229 mg dL^{-1} fibrinogen was the threshold

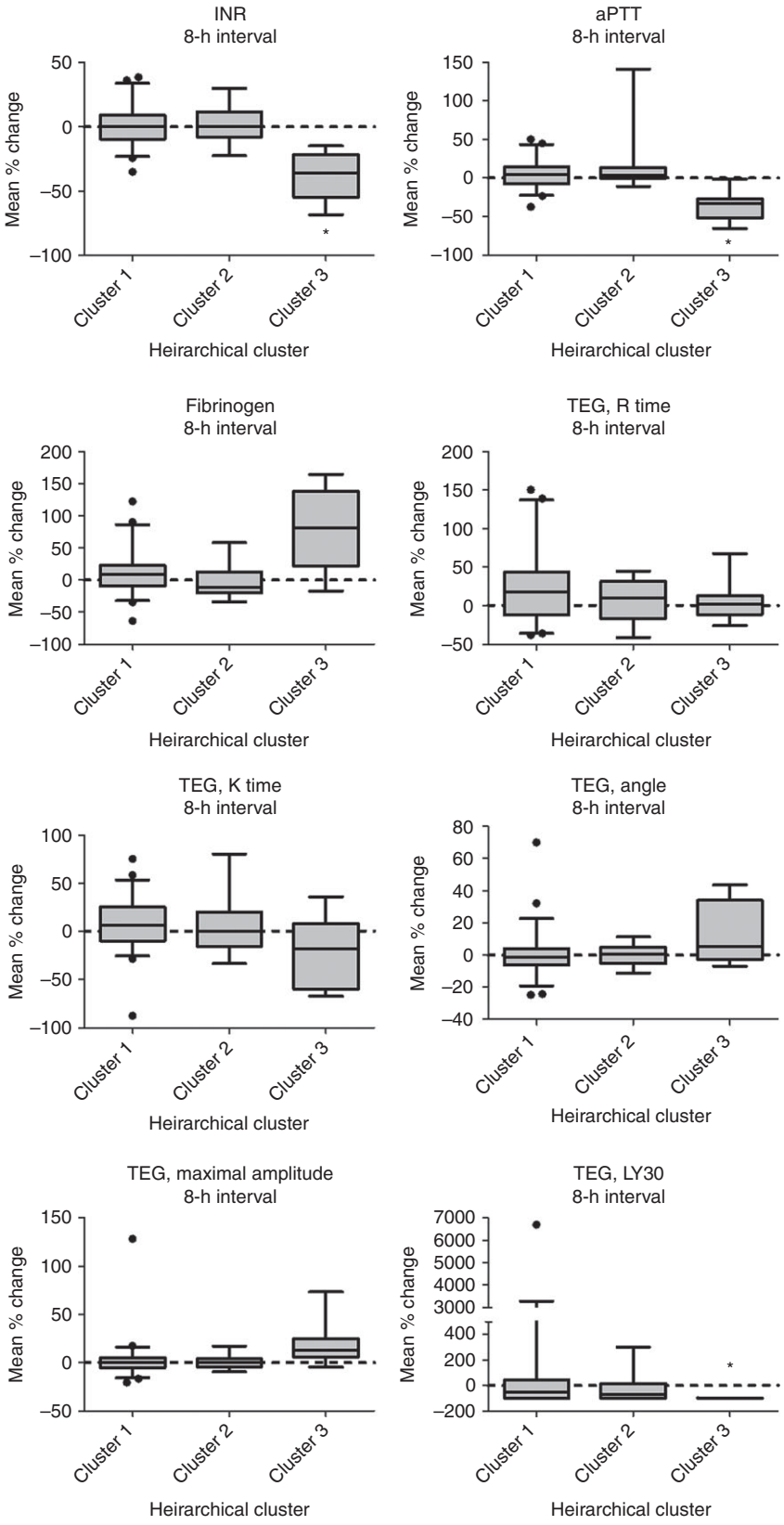

Fig. 3. Box and whisker plots of average percentage changes in coagulation and thrombelastography (TEG) measurements when measured at emergency department arrival and again after 8 h of hospitalization. Whiskers represent the 5th and 95th percentiles. Plots that do not include zero represent statistically significant changes at $\alpha = 0.05$. APTT, activated partial thromboplastin time; INR, International Normalized Ratio; *K*, clot formation time; LY30%, percentage clot lysis at 30 min; *R*, clot onset time.

for mortality in a recent multicenter observational study [23]. The fibrinogen concentration in cluster 1 (median 220, IQR 175.6–322.1) mirrored this critical threshold, theoretically placing this cluster at risk for increased mortality. D-dimer levels were also elevated, suggesting that significant fibrinolytic activation and ADP-specific clot contraction was also severely impaired. However, overall clot formation was not impaired. Perhaps subtle changes in clot formation were obscured in kaolin-activated TEG. However, according to the cell-based model of hemostasis, small increases in plasma thrombin can activate platelets prior to the more dramatic 'thrombin burst' at the platelet surface [24]. Therefore, it is logical that preserved maximal plasma thrombin generation after tissue factor activation was seen concurrently with preserved platelet adhesiveness and aggregation in clusters 1 and 2. Platelet adhesiveness under high shear (PFA-100 collagen/ADP closure time) was enhanced in these clusters, even though there was no concurrent increase in PAC-1 or P-selectin binding, suggesting that either GPI–IX–V-dependent adhesion or von Willebrand factor may have been activated to increase adhesiveness. Preserved platelet reactivity appears to be critical, because platelet aggregation defects, even within the low normal range, are strongly associated with mortality in trauma patients [6]. A similar trend towards shortened high-shear closure times with the PFA-100 was found by Jacoby *et al*. when comparing trauma patients with healthy controls, indicating a degree of platelet activation after injury [25]. The preserved maximal thrombin generation in plasma in cluster 1 may have supported adequate platelet adhesion/aggregation and clot formation in spite of fibrinolysis with moderate fibrinogen depletion.

All trauma clusters in this cohort showed significant fibrinolytic activation by D-dimer as compared with normal healthy controls. D-dimer levels were lowest in cluster 2, and were relatively increased nearly four-fold in cluster 1 and six-fold in cluster 3. These differences were not statistically significant, probably because of the limited sample size. However, this trend does lend support to different degrees of fibrinolytic regulation after trauma. Moore *et al*. recently obtained similar results, in which three distinct clot lysis responses were seen in trauma patients, one of which was fibrinolytic shutdown [26]. Using D-dimer, we found that lysis was reduced, but not abolished, in cluster 2 as compared with healthy controls. Interestingly, ADP-activated TEG-PM was also normal in this cluster, suggesting that adequate platelet-induced clot contraction may also contribute to fibrinolytic resistance after trauma. Nevertheless, fibrinolytic activation was present and associated with coagulopathy, possibly because of either the presence of fibrin formed at injured tissue, an increased t-PA concentration resulting from endothelial release, or PAI-1 inhibition by APC [3–5].

Limitations

This study is limited in several ways. The primary limitation is its small sample size. This was primarily because we limited the study to severely injured trauma patients who could yet be saved. Thus, the exclusion of massively injured subjects who were not expected to live regardless of treatment may have minimized the effects of fibrinolysis in this cohort, and other cohorts may not cluster similarly. We can also only provide associations between measured parameters, and cannot infer causality from this study. The use of kaolin-activated TEG may also have obscured subtle changes in TEG *R*, thus affecting our interpretation of the results. We also did not measure components of the protein C pathway or endothelial activation and/or dysfunction in this study. Understanding these components is critical for understanding the biochemical response to trauma and bleeding after trauma, and they should be incorporated in future HCA efforts. Another limitation is retained colinearity between the variables selected for HCA. Specifically, multiple tests of the platelet ADP receptor activation response may have added bias to the clustering results. Clustering may also have been affected by the exclusion of subjects who were not expected to live for 72 h. This exclusion criterion introduces a degree of survival bias that should be considered when these results are generalized. This is the initial attempt at HCA in an undifferentiated trauma cohort that provides new insights into the various hemostatic responses to trauma. Therefore, our results require further confirmation in larger trauma cohorts. In addition, further specific analyses of the behavior of the individual hemostatic and inflammatory components measured in this study are required and are underway.

Conclusion

HCA identified three types of distinct hemostatic response in ED trauma patients. Further investigations into hemostatic mechanisms after trauma are warranted.

Addendum

N. J. White is responsible for study concept and design, data analysis/interpretation, and primary manuscript writing. D. Contaifer and N. J. White performed HCA, and contributed to data collection, analysis, and manuscript writing. E. J. Martin contributed to data collection, data interpretation, and manuscript review. J. C. Newton, B. M. Mohammed, and J. L. Bostic contributed to data collection, critical data interpretation, and manuscript review. G. M. Brophy, B. D. Spiess, and A. E. Pusateri contributed to study concept and design, data interpretation, and critical manuscript review. K. R. Ward and D. F. Brophy were responsible for study management, critical writing and review of the manuscript, and final approval of the published version.

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Disclosure of Conflict of Interests

N. J. White reports receiving grants from iTrauma Care Inc., the Life Science Discovery Fund, the Coulter Foundation, and KITECH; and personal fees from Vidacare and CSL Behring, outside the submitted work. In addition, N. J. White has a patent from the University of Washington licensed to Stasys Medical Corp. for a platelet diagnostic device, and a patent from the University of Washington for a hemostatic biopolymer. The other authors state that they have no conflict of interest.

References

1 Jenkins DH, Rappold JF, Badloe JF, Berséus O, Blackbourne L, Brohi KH, Butler FK, Cap AP, Cohen MJ, Davenport R, DePasquale M, Doughty H, Glassberg E, Hervig T, Hooper TJ, Kozar R, Maegele M, Moore EE, Murdock A, Ness PM. Trauma hemostasis and oxygenation research position paper on remote damage control resuscitation: definitions, current practice, and knowledge gaps. *Shock* 2014; **41**: 3–12.

2 Brohi K, Singh J, Heron M, Coats T. Acute traumatic coagulopathy. *J Trauma Acute Care Surg* 2003; **54**: 1127–30.

3 Brohi K, Cohen MJ, Ganter MT, Matthay MA, Mackersie RC, Pittet J. Acute traumatic coagulopathy: initiated by hypoperfusion: modulated through the protein C pathway? *Ann Surg* 2007; **245**: 812–18.

4 Cardenas JC, Matijevic N, Baer LA, Holcomb JB, Cotton BA, Wade CE. Elevated tissue plasminogen activator and reduced plasminogen activator inhibitor promote hyperfibrinolysis in trauma patients. *Shock* 2014; **41**: 514–21.

5 Oshiro A, Yanagida Y, Gando S, Henzan N, Takahashi I. Makise H Hemostasis during the early stages of trauma: comparison with disseminated intravascular coagulation. *Crit Care* 2014; **3**: R61-70.

6 Kutcher ME, Redick BJ, McCreery RC, Crane IM, Greenberg MD, Cachola LM, Nelson MF, Cohen MJ. Characterization of platelet dysfunction after trauma. *J Trauma Acute Care Surg* 2012; **73**: 13–19.

7 Roumen RM, Hendriks T, van der Ven-Jongekrijg J, Nieuwenhuijzen GAP, Sauerwein RW, van der Meer JW, Goris RJA. Cytokine patterns in patients after major surgery, hemorrhagic shock, and severe blunt trauma. *Ann Surg* 1993; **6**: 769–76.

8 Bogner V, Keil L, Kanz KG, Kirchhoff C, Leidel BA, Mutschler W, Biberthaler P. Very early posttraumatic serum alterations are significantly associated to initial massive RBC substitution, injury severity, multiple organ failure and adverse clinical outcome in multiple injured patients. *Eur J Med Res* 2009; **14**: 284–91.

9 Hu CH, Harris JE, Davie EW, Chung DW. Characterization of the 5′-flanking region of the gene for the alpha chain of human fibrinogen. *J Biol Chem* 1995; **270**: 28342–9.

10 Johansson PI, Sørensen AM, Perner A, Welling KL, Wanscher M, Larsen CF, Ostrowski SR. High sCD40L levels early after trauma are associated with enhanced shock, sympathoadrenal activation, tissue and endothelial damage, coagulopathy and mortality. *J Thromb Haemost* 2012; **10**: 207–16.

11 Haywood-Watson RJ, Holcomb JB, Gonzalez EA, Peng Z, Pati S, Park PW, Wang W, Zaske AM, Menge T, Kozar RA. Modulation of syndecan-1 shedding after hemorrhagic shock and resuscitation. *PLoS One* 2011; **6**: e23530.

12 Berkhin P. *A Survey of Clustering Data Mining Techniques*. Berlin, Heidelberg: Grouping Multidimensional Data – Springer, 2006: 25–71.

13 Draisma HH, Reijmers TH, Meulman JJ, van der Greef J, Hankemeier T, Boomsma DI. Hierarchical clustering analysis of blood plasma lipidomics profiles from mono- and dizygotic twin families. *Eur J Hum Genet* 2013; **21**: 95–101.

14 Cohen MJ1, Grossman AD, Morabito D, Knudson MM, Butte AJ, Manley GT. Identification of complex metabolic states in critically injured patients using bioinformatic cluster analysis. *Crit Care* 2010; **14**: R10–21.

15 Schöchl H, Frietsch T, Pavelka M, Jámbor C. Hyperfibrinolysis after major trauma: differential diagnosis of lysis patterns and prognostic value of thromboelastometry. *J Trauma Acute Care Surg* 2007; **67**: 125–31.

16 Mammen EF, Comp PC, Gosselin R, Greenberg C, Hoots WK, Kessler CM, Larkin EC, Liles D, Nugent DJ. PFA-100 system: a new method for assessment of platelet dysfunction. *Semin Thromb Hemost* 1998; **24**: 195–202.

17 Di Paola J, Federici AB, Mannucci PM, Canciani MT, Kritzik M, Kunicki TJ, Nugent D. Low platelet alpha2beta1 levels in type I von Willebrand disease correlate with impaired platelet function in a high shear stress system. *Blood* 1999; **93**: 3578–82.

18 Milligan GW. Clustering validation: results and implications for applied analyses. Clustering and Classification 1996; 341–75.

19 Fraley C, Raftery AE. Model-based clustering, discriminant analysis, and density estimation. *J Am Stat Assoc* 2002; **97**: 611–31.

20 Holcomb JB, Minei KM, Scerbo ML, Radwan ZA, Wade CE, Kozar RA, Gill BS, Albarado R, McNutt MK, Khan S, Adams PR, McCarthy JJ, Cotton BA. Admission rapid thrombelastography can replace conventional coagulation tests in the emergency department: experience with 1974 consecutive trauma patients. *Ann Surg* 2012; **56**: 476–86.

21 Branco BC, Inaba K, Ives C, Okoye O, Shulman I, David JS, Schöchl H, Rhee P, Demetriades D. Thromboelastogram evaluation of the impact of hypercoagulability in trauma patients. *Shock* 2014; **41**: 200–7.

22 Davenport R, Manson J, De'Ath H, Platton S, Coates A, Allard S, Hart D, Pearse R, Pasi KJ, MacCallum P, Stanworth S, Brohi K. Functional definition and characterization of acute traumatic coagulopathy. *Crit Care Med* 2011; **39**: 2652–8.

23 Hagemo JS, Stanworth S, Juffermans NP, Brohi K, Cohen M, Johansson PI, Røislien J, Eken T, Næss PA, Gaarder C. Prevalence, predictors and outcome of hypofibrinogenaemia in trauma: a multicentre observational study. *Crit Care* 2014; **18**: R52–60.

24 Hoffman M, Monroe DM 3rd. A cell-based model of hemostasis. *Thromb Haemost* 2001; **85**: 958–65.

25 Jacoby RC, Owings JT, Holmes J, Battistella FD, Gosselin RC, Paglieroni TG. Platelet activation and function after trauma. *J Trauma* 2001; **51**: 639–47.

26 Moore HB, Moore EE, Gonzalez E, Chapman MP, Chin TL, Silliman CC, Banerjee A, Sauaia A. Hyperfibrinolysis, physiologic fibrinolysis, and fibrinolysis shutdown: the spectrum of postinjury fibrinolysis and relevance to antifibrinolytic therapy. *J Trauma Acute Care Surg* 2014; **77**: 811–17.