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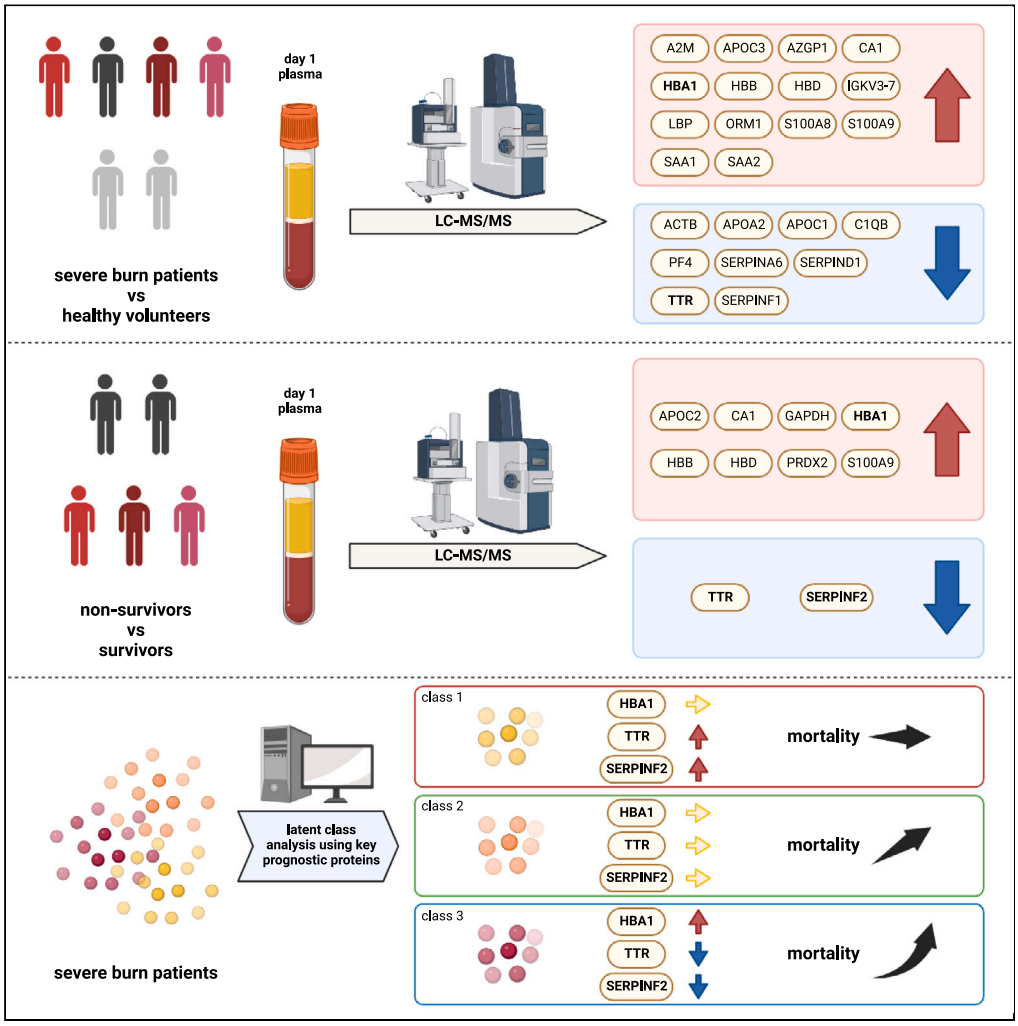

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Highlights

Plasma mass spectrometry revealed ten proteins associated with burn prognosis

The ten proteins are involved in metabolic processes and toxin response

HBA1, TTR, and SERPINF2 showed AUCs > 0.8 for prediction of 28-day mortality

Molecular pathotypes based on HBA1, TTR, and SERPINF2 correlated with outcomes

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Combination of HBA1, TTR, and SERPINF2 in plasma defines phenotype correlated with severe burn outcome

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SUMMARY

Recent advancements in proteomics allow for the concurrent identification and quantification of multiple proteins. This study aimed to identify proteins associated with severe burn pathology and establish a clinically useful molecular pathology classification. In a retrospective observational study, blood samples were collected from severe burn patients. Proteins were measured using mass spectrometry, and prognosis-related proteins were extracted by comparing survivors and non-survivors. Enrichment and ROC analyses evaluated the extracted proteins, followed by latent class analysis. Measurements were performed on 83 burn patients. In the non-survivor group, ten proteins significantly changing on the day of injury were associated with metabolic processes and toxin responses. ROC analysis identified HBA1, TTR, and SERPINF2 with AUCs > 0.8 as predictors of 28-day mortality. Latent class analysis classified three molecular pathotypes, and plasma mass spectrometry revealed ten proteins associated with severe burn prognosis. Molecular pathotypes based on HBA1, TTR, and SERPINF2 significantly correlated with outcomes.

INTRODUCTION

Severe burns are one of the most common injuries requiring intensive care even today. They often cause severe circulatory, immune, metabolic, and coagulation system dysregulation. Acute burn shock due to decreased intravascular volume from increased capillary permeability and myocardial depression requires fluid resuscitation, which in turn leads to oedema formation and other complications.[1] Cellular immunity is reduced immediately after injury, reaches a minimum level within 4–7 days, and is thought to be the main cause of the easy progression to systemic infection in the early phase of the injury.[2] Metabolic dysregulation, such as elevated resting energy expenditure, persists from immediately after burn injury until approximately 3 years later.[3] A coagulopathy in extensive burns differs in character from that in other traumatic injuries and is more dynamic over time.[4] Thus, many clinicians and researchers have struggled with the treatment of burns. Despite medical advances, deaths from severe burns are still frequent.[5] Further pathophysiological studies are needed to improve mortality rates, and new approaches are needed that have never been undertaken before.

Recent developments in mass spectrometry and quantification techniques have improved the technology of proteome analysis, enabling the identification and quantification of hundreds to thousands of proteins at a time, thus providing researchers with comprehensive biomolecular information.[6–11] In reports of proteomics in sepsis, analysis of filters used for blood purification found elevated levels of CA1 and LRG1 in the blood, which had not been previously reported.[9] Other reports have newly identified glycopeptides specific to non-survivors of sepsis and found that NGAL and vascular cell adhesion molecule-1 (VCAM-1), previously reported biomarkers of sepsis, are downregulated according to their expression levels.[10] In burns, an analysis of extracellular vesicles in plasma showed that SAA1 and C-reactive protein correlate with total body surface area (TBSA) and length of hospitalization and may be potential markers for estimating the course of the injury.[11] The proteomic study of burns is still an emerging field of research, and there is much to be learned about the pathophysiology of burns through such study. While there have been some proteomic studies on burns, their findings have been limited, and the pathophysiology of burns has not been fully elucidated through these studies.

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Table 1. Patient characteristics

Characteristic	Burn patients (n = 83)		Volunteers (n = 10)		p Value
Age (years)	61	(43–78)	40	(29–54)	<0.001[a]
Sex, n (%)					0.497
Male	58	(70)	6	(60)	
Female	25	(30)	4	(40)	
Height (cm)	162	(155–170)			
Weight (kg)	60	(49–69)			
Body mass index (kg/m^2)	23	(20–25)			
28-day mortality, n (%)	15	(18)			
TBSA (%)	35	(28–48)			
Burn index	22	(17–36)			
PBI	89	(67–103)			
Baux score	100	(81–115)			
Revised Baux score	104	(88–120)			
Flame/scald/chemical burn	62/18/3				
Inhalation injury, n (%)	30	(36)			
APACHE II score	14	(8–21)			
SOFA score on the date of injury	3	(1–7)			
Time from burn injury to 1st examination (h)	4	(2–12)			

Data are shown as group number or median (interquartile range). TBSA: Total Body Surface Area; PBI: Prognostic Burn Index; APACHE: Acute Physiology and Chronic Health Evaluation; SOFA: Sequential Organ Failure Assessment.

[a]Significance between burn patients and volunteers was assessed using Welch's *t*-test or Fisher's exact test.

Therefore, the purposes of the present study were first, to identify proteins associated with the pathogenesis of severe burns that previously have not received attention by using mass spectrometry to comprehensively measure many proteins in blood at once, and second, to investigate novel molecular pathologies of clinical usefulness based on the identified proteins.

RESULTS

Characteristics of the patients and healthy volunteers

This study included 83 burn patients and ten volunteers. The median age of the patients was 61 years, and their median burn extent was 35%. Fifteen died within 28 days of injury. There was a significant difference in age between the patients and volunteers. Other patient information is described in Table 1.

Principal component analysis of the patients and healthy volunteers

There were 642 proteins detected in this study. Principal component analysis (PCA) was performed on 146 proteins, excluding those that showed no variation at all (mean absolute deviation = 0) between all samples from the burn patients and healthy controls. The healthy subjects were represented as a single cluster, whereas the distribution of the burn group was scattered, with some cases distributed near the healthy subjects (Figure 1A).

Volcano plot analysis of the patients and healthy volunteers

A volcano plot analysis was performed to visualize the differences in expression of each protein. Fourteen proteins (red dots) were significantly more highly expressed in the burn group compared to the healthy subjects, and nine proteins (blue dots) were less highly expressed (Figure 1B and Table S1). We conducted correlation analyses of age and each protein within the volunteer group to investigate the impact of age on protein expression. Except for APOA2, none of the proteins correlated significantly with age (Table S2).

Enrichment analysis of the patients and healthy volunteers

To determine which biological processes are affected, we performed enrichment analysis of the significant proteins based on biological processes in Gene Ontology (GO) and Kyoto Encyclopedia of Genes and

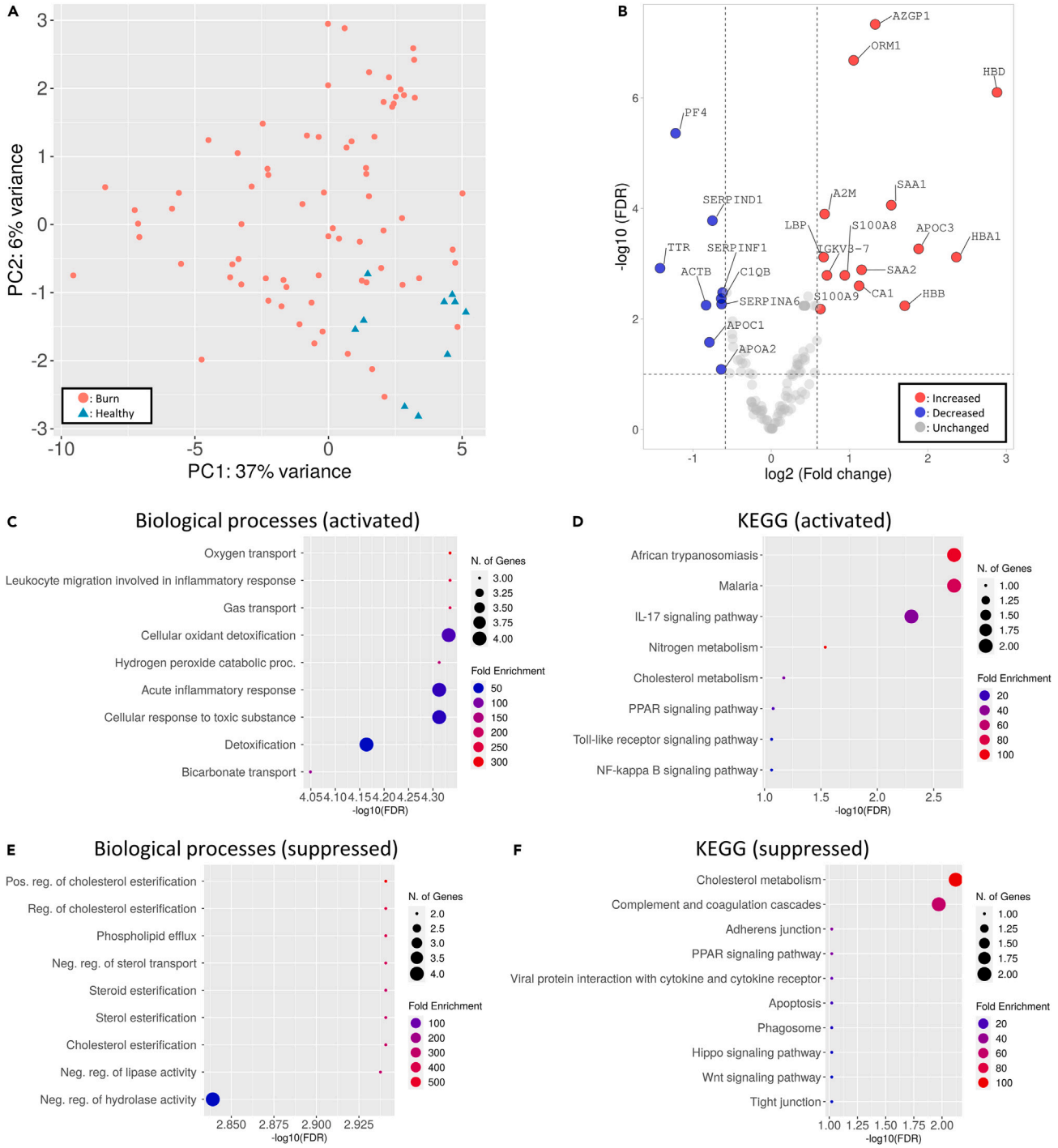

Figure 1. Comparison of burn patients with healthy volunteers

(A) Red dots are burn patients and blue triangles are volunteers.

(B) Red dots indicate proteins that were more highly expressed in the burn patients compared to the healthy volunteers, and blue dots indicate proteins that were less highly expressed. The names of proteins with significantly different expression levels are noted (false discovery rate [FDR] <0.1, fold changes >|1.5|).

(C–F) The results of enrichment analysis based on data of biological processes and KEGG are shown. FDR is calculated based on the nominal p value from the hypergeometric test. Fold Enrichment is defined as the percentage of genes belonging to a pathway divided by the corresponding percentage in the background. The size of the dots indicates the number of genes in the pathway. (C and D) The analysis is based on significantly up-regulated proteins. (E and F) The analysis is based on significantly down-regulated proteins. The analyses were based on the results of the day 1 samples. Burn patients (n = 83), volunteers (n = 10).

Table 2. Patient characteristics (survivors vs. non-survivors)

Characteristic	Survivors (n = 68)		Non-survivors (n = 15)		p Value
Age (years)	56	(38–71)	80	(78–82)	<0.001[a]
Sex, n (%)					0.134
Male	50	(74)	8	(53)	
Female	18	(26)	7	(47)	
Height (cm)	165	(157–170)	155	(149–156)	<0.001[a]
Weight (kg)	63	(52–70)	49	(44–57)	0.001[a]
Body mass index (kg/m^2)	23	(20–25)	20	(19–25)	0.284
TBSA (%)	33	(26–45)	41	(30–66)	0.131
Burn index	21	(15–31)	28	(22–66)	0.070
PBI	83	(65–96)	108	(102–146)	<0.001[a]
Baux score	94	(80–106)	119	(114–146)	<0.001[a]
Revised Baux score	99	(81–114)	134	(115–157)	<0.001[a]
Flame/scald/chemical burn	49/16/3		13/2/0		0.727
Inhalation injury, n (%)	22	(32)	8	(53)	0.146
APACHE II score	12	(7–19)	20	(17–29)	<0.001[a]
SOFA score on the date of injury	3	(1–5)	7	(2–9)	0.009[a]
Time from burn injury to 1st examination (h)	5	(3–14)	2	(2–5)	0.005[a]

Data are shown as group number or median (interquartile range).TBSA: Total Body Surface Area; PBI: Prognostic Burn Index; APACHE: Acute Physiology and Chronic Health Evaluation; SOFA: Sequential Organ Failure Assessment.

[a]Significance between Survivors and Non-survivors was assessed using Welch's *t*-test or Fisher's exact test.

Genomes (KEGG). In the pathway based on the biological processes, transport processes such as oxygen and inflammatory processes are activated, and processes related to cholesterol esterification are inactivated, resulting in suppression of cholesterol metabolism (Figures 1C–1F).

Characteristics of the non-survivors and survivors

Patients in the non-survivor group were significantly older than those in the survivor group. There was no significant difference in TBSA, but the Acute Physiology and Chronic Health Evaluation (APACHE) II and Sequential Organ Failure Assessment (SOFA) scores on the date of injury were significantly higher in the non-survivor group (Table 2). To minimize the impact of age differences on the outcomes, we imposed an age restriction of over 70 years of age (Table S3).

PCA of the non-survivors and survivors

The analysis was performed using 148 proteins, excluding proteins with mean absolute deviation = 0 within the specimens from the burn group only. PCA showed that the distributions were different between the non-survivor and survivor groups, although there were some areas of intersection between the two groups (Figure 2A). For age greater than 70 years, just under half of the patients with a poor prognosis had a split distribution, whereas the rest were mixed with the distribution of the survivor group (Figure S1A).

Volcano plot analysis of the non-survivors and survivors

Eight proteins were significantly up-regulated (red dots) and two proteins were down-regulated (blue dots) in the non-survivor group (Figure 2B and Table S4). The proteins found to be significant in the patient group over 70 years of age showed a high degree of similarity and overlap (Figure S1B and Table S5). However, SERPINF2 showed no difference.

Enrichment analysis of the non-survivors and survivors

Enrichment analysis based on biological processes in GO and KEGG showed that multiple response processes to metabolism and toxins were activated in the non-survivor group (Figures 2C and 2D). Analysis of the deactivation process could not be performed due to the small number of proteins that were significantly reduced.

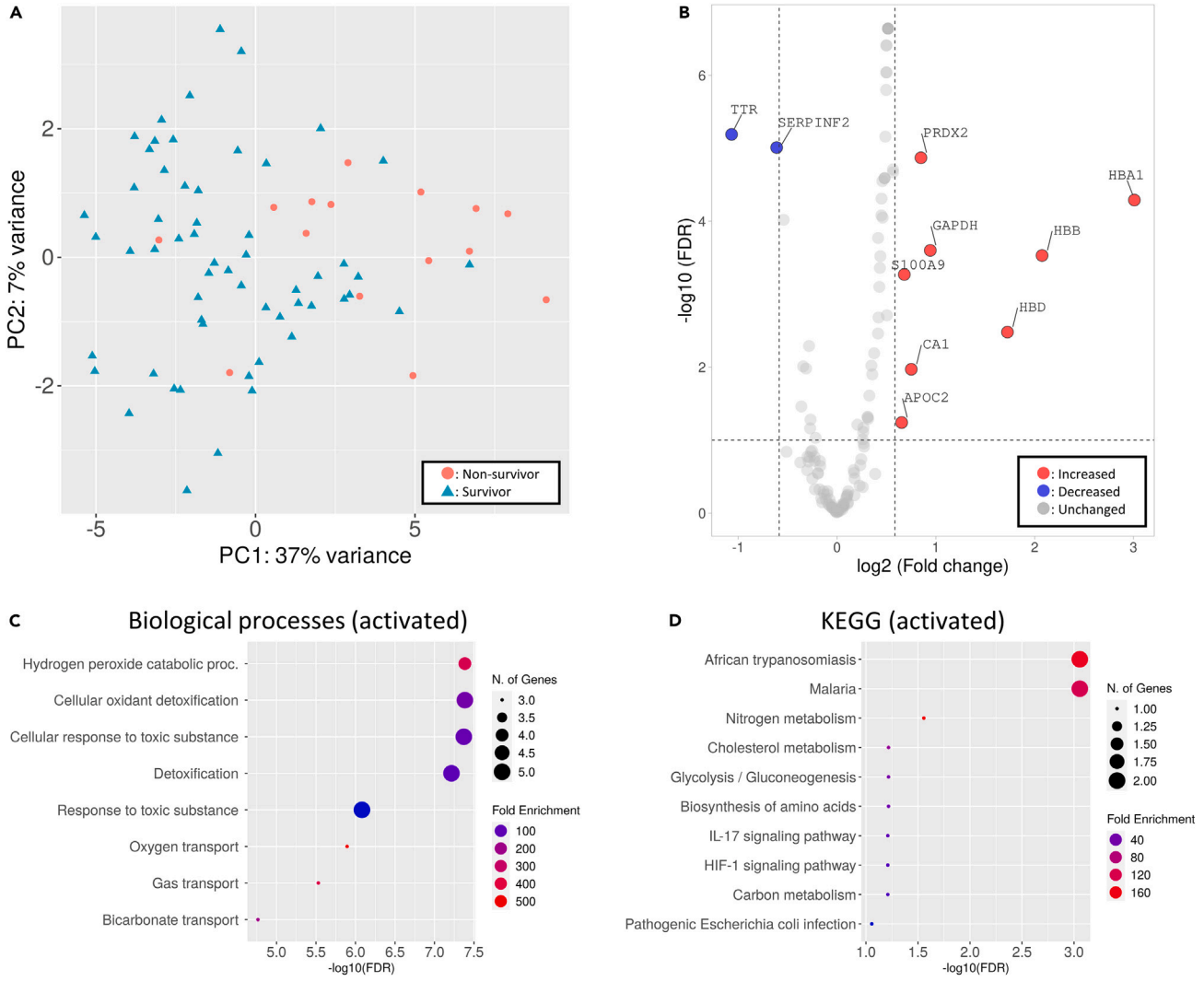

Figure 2. Comparison of non-survivors with survivors

(A) Red dots are non-survivors and blue triangles are survivors.

(B) Red dots indicate proteins that were more highly expressed in the non-survivor group compared to the survivor group, and blue dots indicate proteins that were less highly expressed. The names of proteins with significantly different expression levels are noted (false discovery rate < 0.1, fold changes > |1.5|).

(C and D) The results of enrichment analysis based on data such as biological processes and KEGG are shown. The analysis is based on significantly up-regulated proteins. Analysis of down-regulated proteins was not possible due to the small number of proteins. The analyses were based on the results of the day 1 samples. Survivors (n = 68), non-survivors (n = 15).

Time course of each protein in the non-survivors and survivors

The time course of the ten proteins that differed significantly between the non-survivor and survivor groups on the date of injury was analyzed. Among the proteins that were significantly higher in the non-survivor group on the day of injury, none were still significantly higher seven days later. Among the proteins significantly lower on the day of injury, only TTR was still significantly lower seven days later (Figure 3A). Two additional proteins, SAA1, which has been reported previously, and AZGP1, which is the most distant from the reference point on the volcano plot between burns and healthy volunteers, were added for sub-analysis (Figure S2A). SAA1 was significantly higher in the survival group on day 1, whereas AZGP1 did not differ between the survivor and non-survivor groups.

Relation to other clinical data

The relation between these proteins and the patients' basic data, blood count and biochemistry values, coagulation capacity, and various cytokines is shown using Spearman's correlation coefficient (Figure 3B).

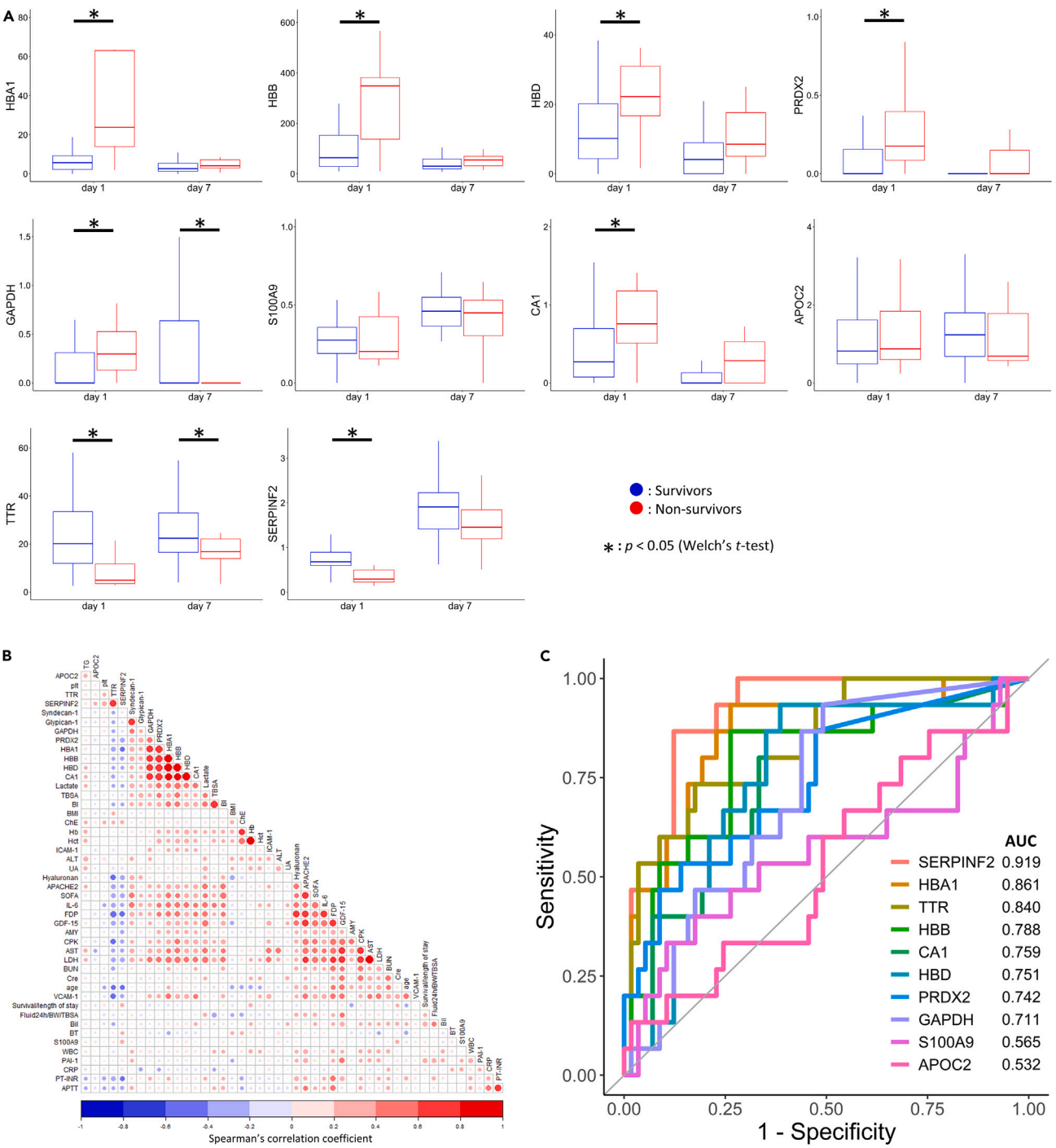

Figure 3. Characteristics of each protein

(A) Time trends of each protein between the survivor and non-survivor groups are shown. Asterisks indicate a statistical difference between survivors and non-survivors (Welch's *t*-test, $p < 0.05$). All vertical axis values for each protein are exponentially modified protein abundance index values.

(B) Correlation chart between each protein and clinical information using Spearman's correlation coefficient.

(C) ROC curve analysis using ten proteins that differed significantly between the survivor and non-survivor groups. AUCs were calculated to assess the prognostic predictive ability of each value on the day of injury. Survivors (n = 68), non-survivors (n = 15). plt, platelet count; Fluid24h/BW/TBSA, Volume of infusion administered within 24 h of injury/body weight/percentage of total body surface area; Bil, total bilirubin; APACHE2, Acute Physiologic Assessment and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment score; IL-6, interleukin-6; FDP, fibrinogen/fibrin degradation products; GDF-15, growth differentiation factor-15; AMY, amylase; CPK, creatinine phosphokinase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; BUN,

Figure 3. *Continued*

blood urea nitrogen; Cre, creatinine; VCAM-1, vascular cell adhesion molecule-1; BT, body temperature; WBC, white blood cell count; PAI-1, plasminogen activator inhibitor-1; CRP, C-reactive protein; PT-INR, prothrombin time-international normalized ratio; APTT, activated partial thromboplastin time; BI, burn index; BMI, body mass index; ChE, cholinesterase; Hb, hemoglobin; Hct, hematocrit; ICAM-1, intracellular adhesion molecule-1; ALT, alanine aminotransferase; UA, uric acid; ROC, receiver operating characteristics; AUC, area under the curve.

HBA1 and HBB showed a remarkable positive correlation with TBSA, APACHE II and SOFA scores, aspartate aminotransferase (AST), fibrinogen/fibrin degradation products (FDP), lactate, creatinine phosphokinase (CPK), lactate dehydrogenase (LDH), and interleukin-6 (IL-6). TTR was strongly negatively correlated with FDP, CPK, VCAM-1, and hyaluronan, whereas SERPINF2 was strongly negatively correlated with age, prothrombin time-international normalized ratio, and FDP. SAA1 was negatively correlated with lactate, and AZGP1 was negatively correlated with hemoglobin subunits (Figure S2B).

Prognostic value of each protein

The prognostic value of blood proteins on the day of injury was investigated with receiver operating characteristics (ROC) analysis (Figure 3C). The area under the curve (AUC) was high for HBA1, TTR, and SERPINF2 at 0.861, 0.840, and 0.919, respectively, but those of SAA1 and AZGP1 were not high (Figure S2C). Additionally, the results were compared and combined with existing indices that are commonly used to predict outcomes (Figure S3). The prognostic burn index, Baux score, and revised Baux score each showed an AUC greater than 0.9. When each protein was integrated with each score, the AUC increased in almost all scenarios, with the exception of the combination of SERPINF2 and SOFA score, which resulted in a lower AUC than that for SERPINF2 alone.

Phenotyping in the burn patient

Phenotyping of burn patients was performed using three proteins that were significantly different between the non-survivor and survivor groups and had a high AUC (> 0.8). The Bayesian information criterion (BIC) for the latent class analysis (LCA) model increased continuously with the number of classes, with changes in the BIC decreasing when the number of classes was ≥ 3 (Figure 4A). We determined the optimal number of classes in this study to be 3. The discriminative power of each variable in the LCA was then expressed to check the contribution of each variable when classifying them (Figure 4B). HBA1 was overwhelmingly the most discriminating, followed by TTR. HBA1 was highly expressed in class 3, whereas TTR and SERPINF2 were less highly expressed in class 3 and more highly expressed in class 1 (Figure 4C). PCA revealed that class 3 had a different distribution compared to the other two (Figure 4D). Survival analysis showed that class 3 had a drastically reduced survival rate compared to the other class groups (Figure 4E). Table 3 shows the number of patients and basal characteristics of each class. TBSA and burn index values did not differ between classes. Age increased gradually with each class. There were significant differences in the APACHE II and SOFA scores on the date of injury. Injury data values for the three proteins used for phenotyping are shown for each class (Figure 4F). Class 3 that with the highest mortality, had significantly higher HBA1 and lower TTR and SERPINF2 values. Sub-analysis performed with the addition of SAA1 and AZGP1 resulted in four classifications (Figure S4 and Table S6). AZGP1 did not contribute to the classification (Figure S4B). SAA1 was highly but sparsely expressed in class 1, and AZGP1 was gradually down-regulated from class 1 to class 4 (Figures S4C and S4F). There was no 28-day mortality in class 1, but the mortality rate in class 4 was high (Figure S4E).

Mediation analysis

Mediation analysis was conducted to investigate the combined influence of age, TBSA, and three specific proteins (HBA1, TTR, and SERPINF2) on the outcome. In conducting our mediation analysis, we assumed a simple model, as shown in Figure S5A. The effect of age on 28-day mortality was partially mediated by HBA1. As Figure S5B illustrates, the regression coefficients between age and 28-day mortality and between HBA1 and 28-day mortality were significant. The indirect effect was (0.534)*(0.003) = 0.001. We assessed the significance of the indirect effect through the implementation of bootstrapping procedures. Unstandardized indirect effects were calculated for each of the 1000 bootstrapped samples. The bootstrapped unstandardized indirect effect was 0.001 and was statistically significant ($p < 0.05$). Similarly for TTR and SERPINF2, the effect of age was partially mediated (Figures S5D and S5F). The effect of TBSA on outcome was statistically completely mediated by HBA1. However, no evidence of the involvement of TTR and SERPINF2 on the impact of TBSA was found in the current model (Figures S5E and S5F).

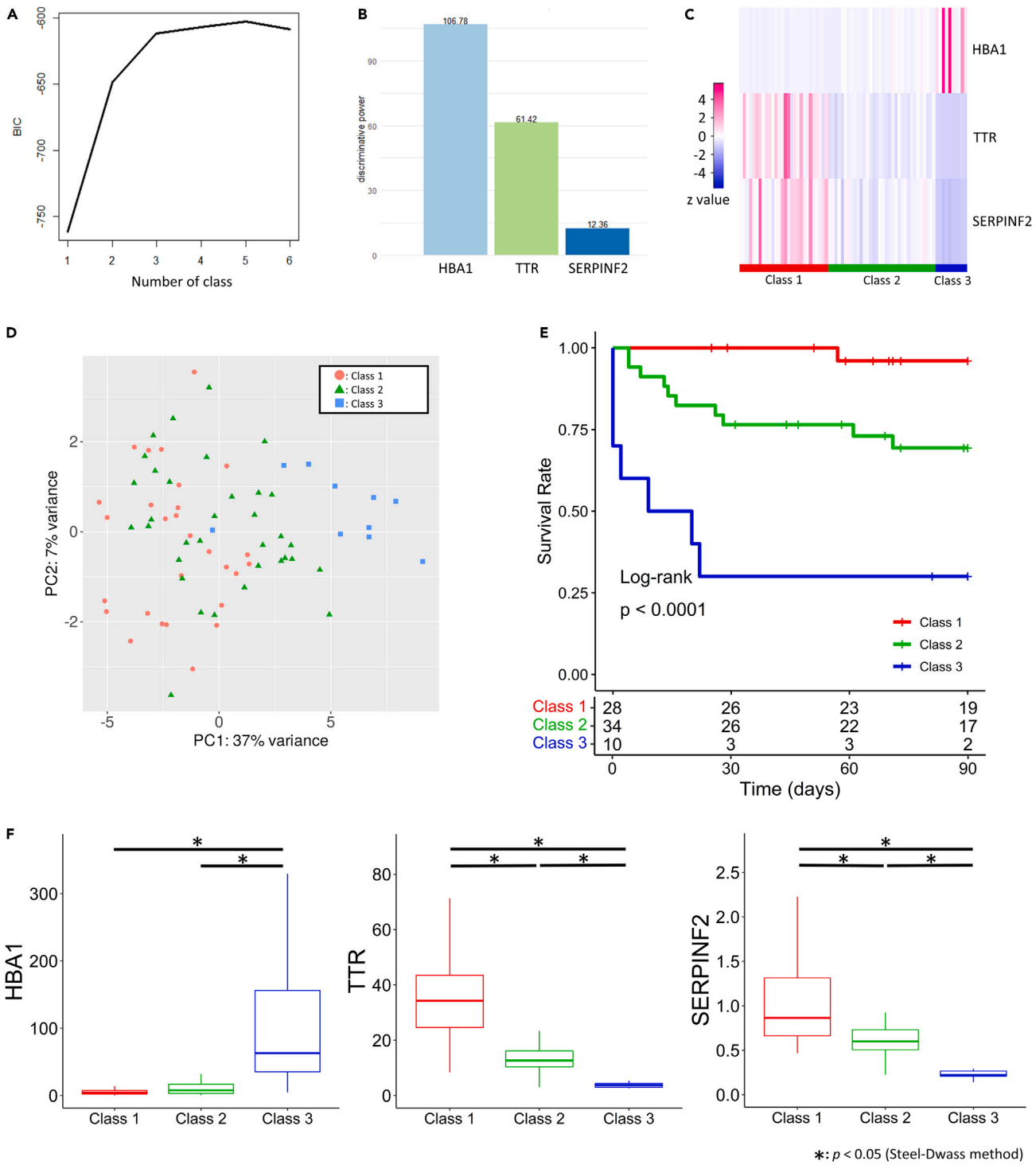

Figure 4. Phenotyping in the burn patient

(A) BIC calculation results. There is almost no change when the number of classes is 3 or more.

(B) Graph showing the contribution of the three proteins with the highest prognostic ability after classifying. HBA1 has a major influence on classifying.

(C) Heatmap showing the bias in expression of the three proteins among cases within the three classes. Blue indicates lowest *Z* score and red indicates highest *Z* score. Actual measurements are converted to Z-scores.

(D) Scatterplot showing PCA by class. Class 3 is the most independently distributed.

(E) Kaplan-Meier plots by class. Significant differences were observed, with class 3 having by far the lowest survival rate.

Figure 4. *Continued*
(F) Differences between each class by protein on day 1. Asterisks indicate a statistical difference (Steel-Dwass method, $p < 0.05$). All vertical axis values for each protein are exponentially modified protein abundance index values. The analyses were based on the results of the day 1 samples. Class 1 (n = 28), Class 2 (n = 34), Class 3 (n = 10).

DISCUSSION

We performed an exhaustive mass spectrometry analysis of plasma to determine differences between healthy subjects and patients with severe burn and between survivors and non-survivors among the patients with severe burn. The results revealed significant changes in ten proteins between the survivor and non-survivor groups, with HBA1 and TTR and SERPINF2 being particularly important in the molecular pathogenesis of prognosis.

Comparison between the patients with severe burns and the healthy subjects revealed that some patients have profiles that are almost identical to those of the healthy subjects. The outcomes imply a reduction in cholesterol esterification in burn patients, albeit direct quantification was not feasible. Burns result in fatty liver and liver dysfunction, which increases mortality; however, the mechanism behind the occurrence of these conditions is not exactly clear.[12] When esterification is suppressed, free cholesterol accumulates, which leads to liver inflammation.[13] Further research and analysis of the key proteins identified in this study may help control liver dysfunction, which may lead to improved mortality.

Hemoglobin, which is composed of alpha and beta subunits (HBA and HBB), is a protein in red blood cells that binds and transports oxygen. When red blood cells collapse, hemoglobin is released to the extracellular space. Free hemoglobin has been shown to exacerbate inflammatory responses and activate innate immunity via Toll-like receptor 4 present in innate immune cells.[14] The high expression of HBA and HBB in serum of patients with ovarian cancer and that of HBA in serum of those with nonalcoholic liver disease have been reported in studies using mass spectrometry, similar to the present study.[15,16] Not only HBA1 but also other hemoglobin subunits were significantly different in the present study, and the correlation chart shows that these hemoglobin subunits have a strong correlation with AST and LDH, which may indicate the intensity of hemolysis. When hemolysis occurs, hemoglobin is released from within the red blood cell to the outside to become free hemoglobin. Studies in animal burn models have reported that free hemoglobin in plasma is associated with burn area and depth, and free hemoglobin impairs Kupffer cell function.[17–19] Measuring hemoglobin subunits or free hemoglobin may be useful in determining the true severity of a patient's injury at an earlier phase.

TTR, also known as prealbumin, is a tetrameric plasma protein with a molecular weight of 56 kDa that is normally synthesized in the liver and choroid plexus of the brain and secreted into the bloodstream and cerebrospinal fluid.[20] Plasma mass spectrometry in coronary artery disease revealed that TTR is downregulated compared to that in healthy individuals and that TTR shares interactions with the apolipoprotein family and also plays an important role in cholesterol transport mechanisms.[21] In sepsis, TTR has been found to be initially higher in the non-survivor group but gradually declines.[22] However, in urogenital sepsis, TTR within 24 h was lower in the shock group.[23] TTR has been found to be associated with mortality and protein energy malnutrition in ICU admissions, in that TTR in blood reflects protein catabolic losses in critically ill patients.[24] TTR reduction increases the generation of reactive oxygen species and induces apoptosis, which is associated with acute kidney injury.[25] Regarding the association between burns and TTR, initial and persistently low values of TTR were associated with mortality, regardless of the superiority of nutritional support.[26,27] TTR has long been used to understand changes in nutritional status, but recent developments in proteomics have shown associations in areas other than nutrition, such as those with mortality in various diseases. In the present study, mass spectrometry results in burn patients showed an association with mortality, similar to a previous report that did not use mass spectrometry. Vascular endothelium is known to be important in the pathogenesis and treatment strategies of burns.[28] It is interesting that TTR correlated inversely with VCAM-1, hyaluronan, and age in the present study because these factors are closely related to vascular endothelium. Further studies are needed to investigate whether TTR can be used for the regulation of vascular endothelium.

SERPINF2 is known as alpha-2-antiplasmin and has been reported to be one of the early diagnostic biomarkers in serum mass spectrometry in high-grade ovarian cancer.[29] Patients who develop venous thrombosis after lower extremity trauma have been reported to have elevated pre-trauma SERPINF2 in plasma mass spectrometry.[30] Mass spectrometry analysis of serum-derived exosomal components in rectal cancer showed that patients with high expression of SERPINF2 respond poorly to neoadjuvant radiation therapy.[31] Serum mass spectrometry reports of purpura fulminans showed significantly decreased expression of SERPINF2, suggesting its

Table 3. Patient characteristics by class (latent class analysis)

Characteristic	Class 1 (N = 28)		Class 2 (N = 34)		Class 3 (N = 10)		p Value
Age (years)	51	(38–69)	69	(45–79)	79	(69–81)	0.035[a]
Sex, n (%)							0.937
Male	21	(75)	24	(71)	7	(70)	
Female	7	(25)	10	(29)	3	(30)	
Height (cm)	165	(159–169)	160	(155–170)	156	(149–166)	0.348
Weight (kg)	65	(57–73)	56	(45–65)	55	(48–63)	0.021[a]
Body mass index (kg/m^2)	23	(22–26)	21	(19–24)	23	(20–24)	0.024[a]
TBSA (%)	33	(25–48)	36	(30–42)	34	(30–96)	0.331
Burn index	19	(15–29)	22	(18–31)	26	(18–96)	0.134
PBI	78	(57–95)	88	(71–105)	102	(94–168)	0.013[a]
Baux score	87	(71–106)	102	(83–115)	111	(102–168)	0.031[a]
Revised Baux score	94	(79–108)	104	(98–118)	119	(102–185)	0.031[a]
Flame/scald/chemical burn	19/8/1		26/6/2		9/1/0		0.670
Inhalation injury, n (%)	7	(25)	12	(35)	4	(40)	0.613
APACHE II score	10	(4–16)	15	(11–20)	25	(9–34)	0.021[a]
SOFA score on the date of injury	1	(0–3)	4	(2–6)	8	(2–11)	0.011[a]
Time from burn injury to 1st examination (h)	6	(3–13)	4	(3–10)	3	(2–5)	0.037[a]
28-day mortality, n (%)	0	(0)	8	(24)	7	(70)	<0.001[a]

Data are shown as group number or median (interquartile range).TBSA: Total Body Surface Area; PBI: Prognostic Burn Index; APACHE: Acute Physiology and Chronic Health Evaluation; SOFA: Sequential Organ Failure Assessment.

[a]Significance between classes was assessed using Welch's *t*-test or Fisher's exact test.

association with uncontrolled fibrinolysis and thrombosis.[32] With regard to burns, a pediatric report showed that alpha-2-antiplasmin is decreased after burns and contributes to an increase in total proteolytic activity.[33] In the present study, we newly showed that the degree of decrease in SERPINF2 is related to mortality and correlated with measures of clinical severity such as the SOFA score. A decrease in alpha-2-antiplasmin indicates an imbalance in the fibrinolytic system, and the development of therapeutic strategies to control the fibrinolytic system in the acute phase of burn injury may be a future challenge.

Regarding the prognostication of burn outcomes, it is worth noting that the Baux score, revised Baux score, and prognostic burn index are widely recognized and utilized indices.[34–36] The high prognostic value of these indices is further augmented by all three proteins reported in this study. In this regard, we posit that the present proteins may also hold value as biomarkers.

In recent years, there have been an increasing number of reports leading to the elucidation of new pathological conditions and the creation of phenotypic classifications through comprehensive protein analysis. Shu et al. are using mass spectrometry to distinguish the severity and clarify new pathogenesis of COVID-19.[37] Our laboratory used exhaustive protein analysis to discover a new phenotypic classification for COVID-19 survival.[38] The phenotyping results in the present study showed significant differences in mortality rates, even though there were no significant differences in TBSA or burn index by class. The APACHE II and SOFA scores differed among these classes, suggesting that several proteins identified in this study may be key to the mechanism of the differences in the true severity of burns, which cannot be determined solely by the apparent extent or depth of the burns.

In summary, comprehensive mass spectrometry of plasma revealed that ten proteins are associated with prognosis in severe burns. The clinical phenotypes based on HBA1, TTR, and SERPINF2 were significantly associated with patient prognosis.

Limitations of the study

This study has several limitations. First, it is a single-center retrospective study. The timing of specimen collection is not standardized, with day 1 data defined as that collected within 24 h of injury. Burns are

injuries with dynamic pathological changes in the early phase of injury, and proteins in the blood are also changing, which is assumed to have affected the results of this measurement. Second, as patients transferred from other hospitals were included, treatment by previous physicians, mainly the infusion of extracellular fluid, may have affected the results. Adjusting for this factor is difficult because burns are often treated first at the nearest hospital because of the importance of initial care. Third, the age difference between the burn and volunteer groups and between the survivor and non-survivor groups is the most important limitation of this study. It continues to be reported that older age is associated with worse outcome.[39] To examine the association between age and the proteins, we performed a restricted reanalysis of patients and a mediation analysis. Interventions that attempt to lessen the direct impact of age on outcomes are difficult. However, our current analysis indicates that the effect of aging on outcome is mediated by a specific protein, suggesting a potential target for future interventions aimed at mitigating this indirect effect. A thorough assessment of this protein's utility and elucidation of its underlying mechanism necessitate a collaboration involving a considerable cohort of patients from various institutions. Fourth, unknown confounding factors may be influencing the results. Finally, this study is based on measurements in a single cohort, and the results have not been validated in other cohorts.

QUANTIFICATION AND STATISTICAL ANALYSIS

PCA was performed by using the web portal for integrated differential expression and pathway analysis (iDEP.96; http://bioinformatics.sdstate.edu/idep, accessed on 25 April 2022).[40] We performed volcano plot analysis based on the limma voom algorithm[50,51] to detect proteins differentially expressed between burn patients and healthy volunteers or between the non-survivor and survivor groups using the web portal (VolcaNoseR; https://goedhart.shinyapps.io/VolcaNoseR/, accessed on 25 April 2022).[41] In each comparison, significance was defined as a false discovery rate <0.1 with fold change >|1.5|. Subsequently, GO and KEGG enrichment analysis was performed using the web portal (ShinyGO 0.76; http://bioinformatics.sdstate.edu/go/, accessed on 25 April 2022).[42] Welch's *t*-test was used to compare each protein between the non-survivor and survivor groups. The Steel-Dwass method was used to compare proteins between classes. We created ROC curves and compared the AUC to determine the prognostic value of each protein at the date of injury. The association of each protein with other clinical data was evaluated using Spearman's correlation coefficient.

The appropriate number of classes was determined, and class assignment for each patient was performed using LCA, which calculates the BIC, and high values are more appropriate for the number of classes. For each combination of patient and class, LCA calculates a posterior probability that represents the likelihood that the patient belongs to that class. The posterior probability ranges from 0 to 1. The higher the posterior probability for a given patient and a given class, the higher the likelihood that this patient belongs to this class. Patients are assigned to the class with the highest posterior probability. We performed LCA using the VarSelLCM package in R. The heatmap of the expression level of each protein was used to confirm the bias within each class. Mortality rates are expressed as Kaplan-Meier curves and compared between classes by the log rank test.

Mediation analysis was performed for age, TBSA, and each protein.[52] We assessed the significance of the indirect effect through the implementation of bootstrapping procedures. Unstandardized indirect effects were calculated for each of the 1000 bootstrapped samples.

A value of $p < 0.05$ was considered to indicate statistical significance. Statistical analyses were performed with R Statistical Software (version 4.1.1; R Foundation for Statistical Computing, Vienna, Austria) and JMP Pro 16.2.0 (SAS Institute Inc., Cary, NC, USA).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107271.

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AUTHOR CONTRIBUTIONS

All authors participated in the study design, data acquisition, and interpretation. S.O. performed the statistical analysis and drafted the manuscript. F.S. performed protein mass spectrometry analysis and wrote the relevant parts of the manuscript. H.Matsumoto, T.E., H.Matsuura, A.O., D.O., H.O., and J.O. supervised the study and revised the manuscript. All authors read and agreed with the contents of the final manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
C18 tip	GL Sciences Inc.	https://www.glsciences.com/product/spe_columns/tip_columns/01124.html
Deposited data		
Proteomic data	This work	http://www.ebi.ac.uk/pride Project accession: PXD043065
Software and algorithms		
Bruker Data Analysis software	Bruker	https://www.bruker.com/en/products-and-solutions/mass-spectrometry/ms-software.html
MASCOT Server ver. 2.7	Matrix Science Inc.	https://www.matrixscience.com/search_form_select.html
Scaffold	Proteome Software, Inc.	https://www.proteomesoftware.com/products/scaffold-5
iDEP.96	Ge et al.[40]	http://bioinformatics.sdstate.edu/idep
VolcaNoseR	Goedhart and Luijsterburg[41]	https://goedhart.shinyapps.io/VolcaNoseR/
ShinyGO 0.76	Ge et al.[42]	http://bioinformatics.sdstate.edu/go/
R Statistical Software ver.4.1.1	R Foundation for Statistical Computing	https://www.r-project.org/
JMP Pro 16.2.0	SAS Institute Inc.	https://support.sas.com/downloads/browse.htm?fil=&cat=536
Other		
timsTOF Pro mass spectrometer	Bruker	https://www.bruker.com/content/bruker/int/ko/products-and-solutions/mass-spectrometry/timstof/timstof-pro.html?q=%7B%7Bspellcheck%7D%7D
Nano Elute nanoLC system	Bruker	https://www.bruker.com/en/products-and-solutions/mass-spectrometry/lc-ms/nanoelute-2.html
Bruker TEN column	Bruker	https://store.bruker.com/products/pepsep-ten-columns

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. H.Matsumoto (h-matsumoto@hp-emerg.med.osaka-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD043065.[43,44]
- This study did not generate new code. Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

- Shinya Onishi and Hisatake Matsumoto have access to all data used in this study and are responsible for the integrity and accuracy of the data and analysis. The datasets used and/or analyzed during the current study are available from these authors on reasonable request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study design

In this retrospective, observational study, specimens were collected at a single institution, the Center for Trauma and Emergency Medicine, Chukyo Hospital, Japan Organization for Regional Health Care Functions (Naka-ku, Nagoya, Japan), and analyses were performed at the Department of Traumatology and Acute Critical Medicine, Osaka University Graduate School of Medicine (Suita, Osaka, Japan). Between October 2014 and December 2019, patients who participated in studies aimed at investigating the pathogenesis of severe burns, including this study, were retrospectively included. We received written informed consent for inclusion from the patients themselves, if possible, and from their family members if not. This study was conducted in accordance with the Declaration of Helsinki and was approved by our Ethics Committee (approval number: 2014015).

Patients and volunteers

Patients were included if they were at least 16 years of age and TBSA >20%. Patients who developed cardiopulmonary arrest before arrival at the hospital, patients transported from other hospitals more than 24 h after the injury, and patients who did not consent to participate in the study were excluded. For the treatment of burn patients, we use a mostly uniform treatment plan based on the Advanced Burn Life Support Course Provider Manual, with slight modifications depending on each patient's condition.[45] Furthermore, blood samples were collected and used from healthy adult volunteers after we received informed consent for their inclusion. No restrictions on sex were placed on the inclusion of the patients or volunteers. There were no restrictions on race or ethnicity, but all patients and volunteers who were included were Japanese.

Data collection

We collected the following clinical information from the patient's electronic medical record: age, sex, body mass index, outcome, TBSA, burn index, PBI, Baux score, revised Baux score, causes of the burns, presence of inhalation injury, and time from injury to the first blood sample. Outcome was defined as alive or dead at 28 days after the injury. We calculated the APACHE II score and SOFA score at each sample collection.[46,47] Blood samples were collected immediately after the patient's arrival at our hospital and seven to ten days thereafter. We collected blood samples once from each healthy volunteer. Blood samples were collected in a tube with anticoagulant (ethylenediaminetetraacetic acid) and immediately centrifuged at 3500 rpm for 5 min to separate plasma. Separated plasma was stored at −30°C until testing. The storage time of each sample until mass spectrometry ranged from 1.5 to 6.7 years.

METHOD DETAILS

Mass spectrometry methods

The method is the same as in other studies previously conducted at our institution.[48] The plasma protein was precipitated by adding methanol and chloroform and dissolved into PTS solution. The protein solution was reduced with dithiothreitol, followed by alkylation with iodoacetamide, digestion by trypsin, and purification with a C18 tip (GL Sciences Inc., Tokyo, Japan). The trypsinised and purified solution was subjected to liquid chromatography with tandem mass spectrometry (LC-MS/MS) with a Bruker TEN column (Bruker, Billerica, MA, USA) on a Nano Elute nanoLC system coupled with a timsTOF Pro mass spectrometer (Bruker). The column temperature was set to 50.0°C. The mobile phase consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Peptides were eluted by the gradient setting of 2–35%B for 18 min at a flow rate of 500 nL/min. The mass scanning range was set to 300–2000 *m/z*, and the ion mobility revolution mode was set to custom with a range of 0.85–1.30 Vs/cm^2. The ion spray voltage was set to 1.6 kV in positive ion mode. MS/MS spectra were acquired by automatic switching between MS and MS/MS modes. Bruker Data Analysis software was used for the processing of the mass spectrometry data. Peptides were identified by a database search using the MASCOT Server (ver. 2.7, Matrix Science Inc., Boston, MA, USA). Precursor mass tolerance was set to 15 ppm, and fragment mass tolerance was set to 0.05 Da. Carbamidomethylation of cysteine was set as a static modification, and oxidation of methionine, acetyl of protein N-term, and deamination of N, Q were set as variable modifications. The Swiss-Prot database was used for the Mascot search, and the taxonomy was limited to *Homo*

sapiens. The search results were summarised using Scaffold (Proteome Software, Inc., Portland, OR, USA), quantified using the exponentially modified protein abundance index,[49] and exported in the CSV format for further analysis. All samples for LC-MS/MS were prepared at the same time and measured consecutively with one blank measurement inserted in between to reduce batch effects. Due to the large number of samples, measurements were taken continuously for several days. After DECOY proteins and immunoglobulins were removed, 642 proteins were used. Each statistical analysis was then performed after excluding proteins with a mean absolute deviation = 0 within each cohort.