BJA

British Journal of Anaesthesia, 121 (5): 1065–1074 (2018)

doi: 10.1016/j.bja.2018.05.072

Advance Access Publication Date: 26 July 2018

Critical Care

CRITICAL CARE

Proteolysis in septic shock patients: plasma peptidomic patterns are associated with mortality

J. Bauzá-Martinez[1,#], F. Aletti[2,#], B. B. Pinto[3], V. Ribas[4], M. A. Odena[1], R. Díaz[1], E. Romay[5], R. Ferrer[6], E. B. Kistler[7], G. Tedeschi[8,9], G. W. Schmid-Schönbein[2], A. Herpain[10], K. Bendjelid[3,*] and E. de Oliveira[1,*]

[1]Proteomics Platform, Barcelona Science Park, Barcelona, Spain, [2]Department of Bioengineering, University of California San Diego, La Jolla, CA, USA, [3]Geneva University Hospital, Geneva, Switzerland, [4]Eurecat, Technology Centre of Catalonia, Barcelona, Spain, [5]University Hospital Mútua Terrassa, Barcelona, Spain, [6]Intensive Care Department, Vall d'Hebron University Hospital, Barcelona, Spain, [7]Department of Anesthesiology and Critical Care, VA San Diego Healthcare System, San Diego, CA, USA, [8]Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Milan, Italy, [9]Fondazione Filarete Milano, Milan, Italy and [10]Université Libre de Bruxelles, Brussels, Belgium

*Corresponding authors. E-mails: Karim.Bendjelid@hcuge.ch, eoliveira@pcb.ub.es

[#] These authors contributed equally to this study.

Abstract

Background: Uncontrolled proteolysis contributes to cell injury and organ dysfunction in animal models of circulatory shock. We investigated in humans the relationship between septic shock, proteolysis, and outcome.

Methods: Intensive care patients with septic shock (n=29) or sepsis (n=6) and non-hospitalised subjects (n=9) were recruited as part of the prospective observational trial '*ShockOmics*' (ClinicalTrials.gov Identifier NCT02141607). A mass spectrometry-based approach was used to analyse the plasma peptidomes and the origin of circulating peptides from proteolysis in the enrolled subjects.

Results: Evidence of systemic proteolysis was indicated by a larger number of circulating peptides in septic shock patients, compared with septic patients and non-hospitalised healthy subjects. The peptide count and abundance in the septic shock patients were greater in patients who died (n=6) than in survivors (n=23), suggesting an association between magnitude of proteolysis and outcome. *In silico* analysis of the peptide sequences and of the sites of cleavage on the proteins of origin indicated a predominant role for serine proteases, such as chymotrypsin, and matrix metalloproteases in causing the observed proteolytic degradation.

Conclusions: Systemic proteolysis is a novel fundamental pathological mechanism in septic shock. Plasma peptidomics is proposed as a new tool to monitor clinical trajectory in septic shock patients.

Clinical trial registration: NCT02141607.

Keywords: in-hospital mortality; mass spectrometry; peptidomics; proteolysis; septic shock

Editorial decision: 13 June 2018; **Accepted:** 13 June 2018

Editor's key points

- Recent data have suggested that systemic proteolysis occurs in sepsis but its clinical relevance is uncertain.
- In this observational study, circulating concentrations of peptide were increased in patients with septic shock compared with patients with sepsis and healthy controls.
- There was an association between increased peptide concentrations and mortality in patients with septic shock.
- Further translational and clinical research into the possible role of proteolysis in sepsis is needed.

Sepsis is a life-threatening condition caused by a dysregulated host response to infection.[1] Septic shock is the most severe form of sepsis, characterised by circulatory failure and tissue injury, and associated with high mortality.[2]

Despite longstanding efforts to develop new treatments for septic shock, interventions are still based on haemodynamic support, antibiotics and anti-infective drugs, and source control (e.g. surgery). To date, no therapy addresses the root cause of septic shock, largely because of an incomplete understanding of the cascade leading to multiorgan dysfunction.

Uncontrolled proteolysis has recently been proposed as a fundamental pathological mechanism in shock, possibly mediated by digestive enzymes.[3–9] Enteral blockade of pancreatic proteases in animals mitigates organ injury and improves outcome.[3–9] I.V. serine protease blockade has also shown some efficacy in severe sepsis.[10] Further, we recently reported the occurrence of systemwide proteolysis and the appearance of a large number of circulating peptides in a rat model of shock.[8]

The objectives of this study are: (i) to investigate proteolysis in septic shock patients compared with septic patients without shock and healthy donors by assessment of plasma peptide concentrations from mass spectrometry-based peptidomics, and by *in silico* analysis of proteolytic activity; and (ii) to determine the association between proteolysis and in-hospital mortality in septic shock.

Methods

Study design and participants

This study is part of the prospective observational trial '*ShockOmics*' (ClinicalTrials.gov Identifier NCT02141607)[11] and was approved by: the Geneva Regional Research Ethics Committee (study number 14-041); the Ethical committee of Hôpital Erasme-Université Libre De Bruxelles (study number P2014/171), and the Mutua Terrassa Hospital Institutional Review Board (study number EO/1407).

Adult (>18 yr old) patients admitted between October 2014 and March 2016 to the ICU of Geneva University Hospitals, Geneva, Switzerland (38-bed, mixed) and Erasme University Hospital, Brussels, Belgium (36-bed, mixed) with septic shock[12] diagnosis, Sequential Organ Failure Assessment (SOFA) score $\geq$6, arterial lactate $\geq$2 mmol L^{-1} and a documented source of infection were screened for enrolment. Patients at high risk of death within the first 24 h after admission, systemic immunosuppression, haematological diseases, metastatic cancer, pre-existing dialysis, decompensated cirrhosis, or who received more than four units of red blood cells or any fresh frozen plasma were excluded.[11] Given the stringency of the exclusion criteria, the thresholds on SOFA and lactate were chosen to include severe enough septic shock patients, while ensuring adequate patient enrolment and recruitment rate.

A convenience sampled cohort of patients with sepsis (infection with inflammatory response and one organ dysfunction),[11,12] but not in shock, was included as control. Healthy donors were enrolled at Mutua Terrassa Hospital, Terrassa, Spain for an additional non-sepsis control. Informed consent was obtained from patients or proxies. Patients in the ICU were managed according to international guidelines.[13] Patient characteristics, organ function, and haemodynamic data were prospectively collected into a custom-made electronic case report form. In-hospital mortality was assessed by consultation of the local death registry, or by telephone call to the patient or proxies.

Sample collection

Blood (total volume 18 ml for the multiomics analyses of the '*ShockOmics*' project)[11] was collected from either the arterial or venous line in septic shock and sepsis patients; venous blood was drawn from healthy donors. For peptidomics, plasma (0.5 ml) was isolated within 30 min of sample collection in K_2-EDTA tubes (BD Biosciences, San Jose, CA, USA) by centrifugation (twice at 1200g for 10 min) to pellet cellular elements. COmplete Protease Inhibitor Cocktail (Roche Applied Sciences, Mannheim, Germany) was added and samples were stored at −80°C until in-batch analyses. Plasma samples collected from the septic shock patients at three time points were analysed: within 16 h of ICU admission (T_1); at 48 h (T_2) after ICU admission; and before discharge from the ICU or on Day 7 at latest (T_3). Only one sample was collected from the sepsis patients (at T_1) and healthy donors.

Liquid chromatography–mass spectrometry analysis

Residual protease activity in the plasma samples was tested by a fluorometric assay (Supplementary material S.1). Peptides were extracted by filtration (Supplementary material S.2), separated by reverse phase liquid chromatography, and analysed by mass spectrometry (Supplementary material S.3) in the Orbitrap (Thermo Fisher Scientific, Bremen, Germany).

Peptide and protein identification: qualitative approach

Proteins and peptides were identified (Supplementary material S.4) using Mascot search engine (v2.3.01, Matrix Science, Boston, MA, USA) against the SwissProt Human (SPH) database (v160127). Venn diagrams of peptide counts were generated using R packages.[14]

Peptide and protein quantification: label-free approach

Raw data were processed with Progenesis QI for Proteomics (Non-Linear Dynamics, Waters, Milford, MA, USA). Pool samples were used as alignment reference. A total of 219 825 mass spectra (z>1 and Rank<5) were considered for database search. Mascot search engine was used against SPH (Supplementary material S.4).

Table 1 Clinical parameters. *Mean (SD) and †*n* (%). Blood samples were drawn at three different time points. T_1: within 16 h of ICU admission; T_2: at 48 h after ICU admission; T_3: before discharge from the ICU or on Day 7 at latest. SS, septic shock

	SS non-Survivors (*n*=6)	SS survivors (*n*=23)	Sepsis (*n*=6)	Healthy controls (*n*=9)
Age (yr)*	62–93	19–85	41–90	31–51
Sex (female)†	2 (33.3%)	7 (30.4%)	2 (33.3)	4 (44.4%)
BMI (kg m^{-2})*	25.5 (0.93)	27.3 (6.20)	21.4 (6.43)	–
SOFA*				
T_1	13.2 (1.94)	11.6 (2.79)	5.33 (3.01)	–
T_2	10.5 (3.51)	7.65 (2.62)	6.67 (3.98)	–
T_3	6.50 (3.62)	5.50 (3.25)	–	–
APACHE II* (T_1)	27.2 (5.85)	23.4 (7.08)	13.0 (3.16)	–
Temperature (°C)*				
T_1	37.9 (1.3)	37.5 (1.3)	37.0 (0.7)	–
T_2	37.5 (1.1)	37.6 (0.8)	36.6 (0.4)	–
T_3	37.3 (2.0)	37.6 (0.9)	–	–
Lactate (mmol L^{-1})*				
T_1	6.25 (2.94)	4.22 (2.20)	3.23 (2.79)	–
T_2	2.77 (0.90)	1.52 (0.64)	0.75 (0.07)	–
T_3	1.56 (0.51)	1.16 (0.56)	–	–
Heart rate (beats min^{-1})*				
T_1	109 (19)	107 (26)	95 (21)	–
T_2	92 (25)	92 (23)	74 (32)	–
T_3	82 (24)	104 (19)	–	–
MAP (mm Hg)*				
T_1	59 (7)	59 (6)	80 (17)	–
T_2	67 (6)	67 (9)	68 (18)	–
T_3	73 (15)	76 (13)	–	–
Patients on vasopressor†				
T_1	6 (100)	22 (95.7)	0 (0.0)	–
T_2	4 (66.7)	12 (52.2)	0 (0.0)	–
T_3	2 (33.3)	3 (21.4)	0 (0.0)	–
Patients on mechanical ventilation†				
T_1	6 (100)	18 (78.3)	0 (0.0)	–
T_2	6 (100)	13 (56.5)	0 (0.0)	–
T_3	3 (50.0)	6 (42.9)	0 (0.0)	–
Renal replacement therapy†				
T_1	0 (0.0)	1 (4.35)	0 (0.0)	–
T_2	0 (0.0)	0 (0.0)	0 (0.0)	–
T_3	0 (0.0)	0 (0.0)	0 (0.0)	–
Source of infection†				
Abdominal	1 (16.7)	8 (34.8)	1 (16.7)	–
Respiratory	5 (83.3)	7 (30.4)	3 (50.0)	–
Urinary tract	0 (0.0)	8 (34.8)	2 (33.3)	–
Mortality†				
ICU	5 (83.3)	0 (0.0)	0 (0.0)	–
Hospital	6 (100)	0 (0.0)	0 (0.0)	–

Both the total number (i.e. the sum of peptides found in the subjects belonging to a group) and the number of exclusive peptides identified (i.e. the sum of peptides found exclusively in such group) in the septic shock, sepsis, and heathy donor groups were compared.

The level of proteolysis is related to the intensity of the peptide signal detected by the mass spectrometry, which is proportional to the peptide abundance in a sample, as calculated by Progenesis. The abundance of a cleaved protein is the cumulative abundance of all peptides derived uniquely from cleavage of this protein. Then, the median abundance of each protein in a patient group is computed, and the total protein abundance is obtained from the sum of the medians of all the cleaved proteins in the patient group. This serves as an estimate of proteolysis: an incremental change in the abundance of cleaved proteins can be interpreted as an incremental change in proteolysis.

In silico protease effector estimation

In order to estimate the proteases responsible for protein cleavage, we split the peptide dataset into two: protein fragments with median abundance ratio higher or exclusive to the healthy group (357 peptides) and protein fragments with median higher or exclusive to the septic shock group (500 peptides). We extracted the C-terminus amino acid of every identified peptide, obtaining $P1_{C\text{-term}}$ amino acids and $P1_{N\text{-term}}$ amino acids (immediately before each peptide N-terminus position) by matching the peptides to the SPH database using a variant of a Perl Script.[15] The analysis of proteases responsible for both C- and N-terminus cleavage focused specifically on matrix metalloproteases (MMPs), for their role in shock pathophysiology,[16] and on serine proteases, which could have a dual role of cleaving proteins and activating pro-MMPs.

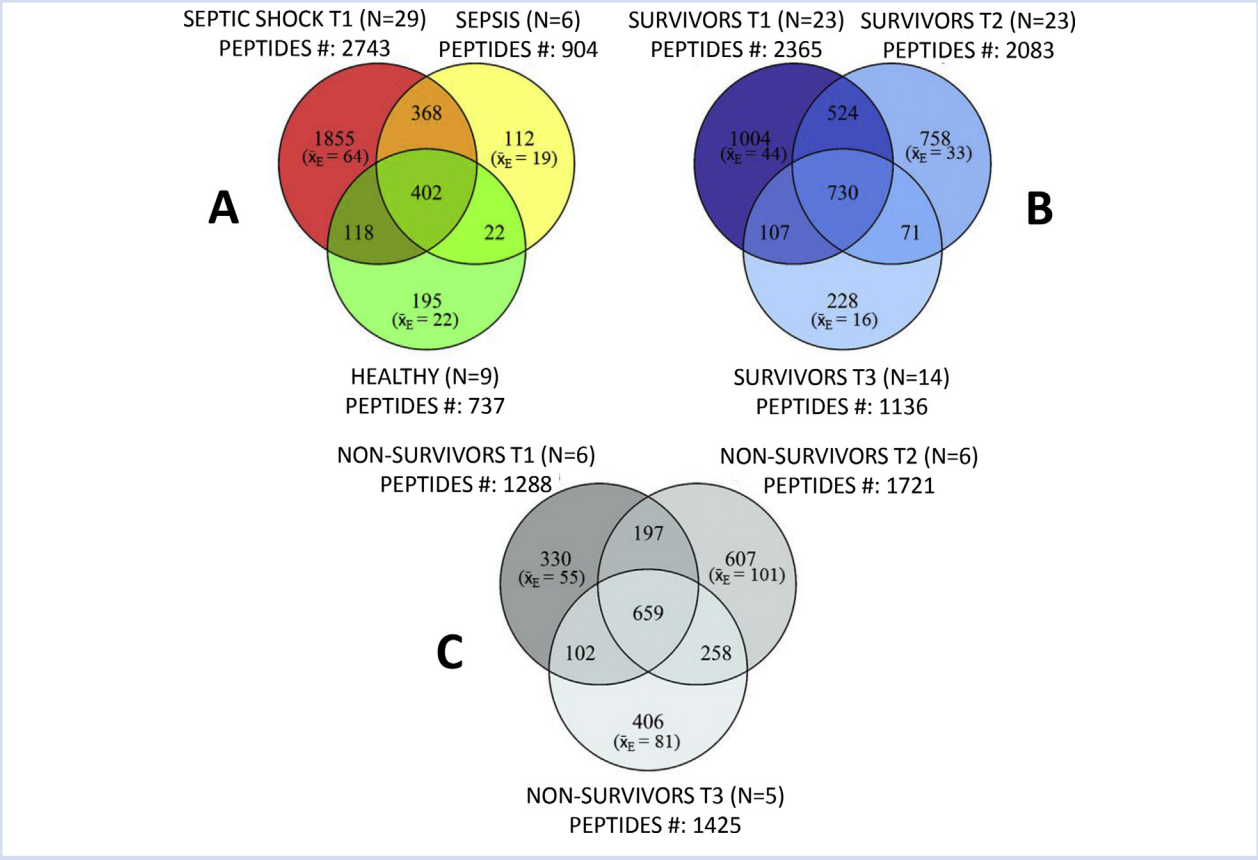

Fig 1. Venn diagrams of: (A) peptides generated by proteolysis in the septic shock group (n=29), in the sepsis group (n=6), and in the healthy controls (n=9) at T_1; (B) peptides generated by proteolysis in the survivor subgroup (n=23) of the septic shock patients at the three different time points T_1, T_2, and T_3; (C) peptides generated by proteolysis in the non-survivors subgroup (n=6) of the septic shock patients at the three different time points T_1, T_2, and T_3. Numbers in brackets indicate the number of peptides exclusively observed in each group divided by the number of patients in each group (exclusive peptides/n). Blood samples were drawn at three different time points. T_1: within 16 h of ICU admission; T_2: at 48 h after ICU admission; T_3: before discharge from the ICU or on Day 7 at latest (T_3).

Statistical analysis

For group homogeneity, calculated P-values correspond to Student's t-test and χ^2 test for numerical and categorical data, respectively.

In order to assess the spread of the data of cleaved proteins and derived peptides, data variance was tested by one-way analysis of variance (ANOVA) after $log2$-transformation of the dataset. Proteins showing significant differences (P<0.05) between groups by the ANOVA test were evaluated for normality through the Shapiro-Wilk test. For non-normally distributed protein abundances, comparisons of the mean abundances between groups were evaluated by Wilcoxon (Mann–Whitney) test; for normally-distributed protein abundances, comparisons were evaluated by Student's t-test (P<0.05). Statistical analyses were performed using R packages.[14]

Results

Study participants

Out of 529 screened patients, 29 patients with septic shock (of whom six died during the hospital stay) and six septic controls were enrolled in the study. Healthy blood donors (n=9) served as additional controls (Table 1). At T_1, patients with septic shock not surviving to hospital discharge were older than survivors (P=0.034), with higher acute physiology and chronic health evaluation II (APACHEII) (P=0.00091) and SOFA (P=0.0005) scores than septic patients not in shock. No significant difference in the SOFA score was found between septic shock survivors and non-survivors at either T_1 or T_2. At T_2, lactate was higher (P=0.017) in the non-survivors. No patient in the sepsis group not in shock died during the hospital stay.

Proteolysis in septic shock

Both the total (2743) and exclusive (1855) peptide population counts in the septic shock group at T_1 were higher than in the sepsis and healthy groups (Fig. 1A). The mean value of exclusive peptides per patient was computed to account for the different group sizes. Septic shock patients had approximately a three-fold larger peptide population than the sepsis control and healthy donor groups.

Septic shock peptidome: correlation with outcome and source of infection

The mean values of the peptides exclusive for each group (Fig. 1B and C) were lower for survivors than non-survivors at

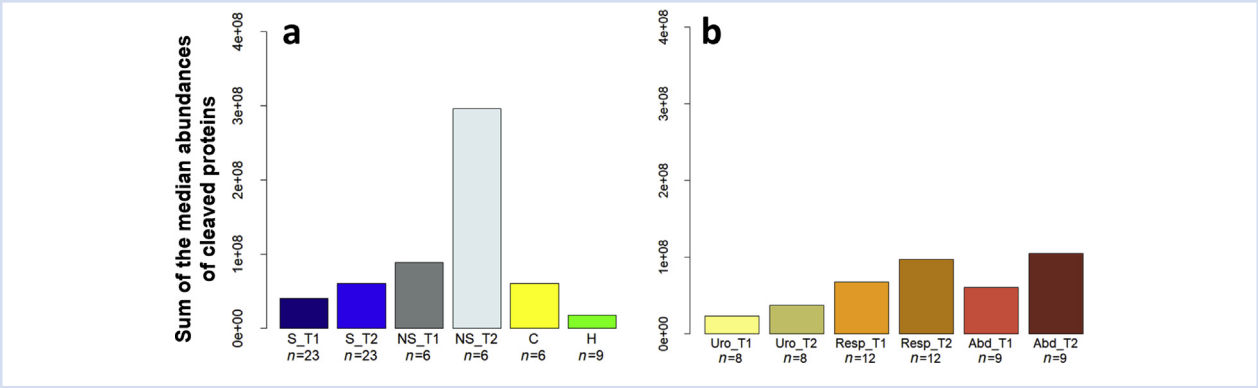

Fig 2. Proteolysis levels at the time points T_1 and T_2 expressed as total abundance (see Peptide and protein quantification: label-free approach in the Methods section) of the proteins cleaved in different patient groups, classified according to: (a) survival (S=survivor septic shock; NS=non-survivor septic shock); and (b) source of infection (Uro=urosepsis; Resp=respiratory septic shock; Abd=abdominal septic shock). C=septic control; H=healthy donor. T_1: within 16 h of ICU admission; T_2: at 48 h after ICU admission.

all times. In addition, survivors showed a decrease from T_1 to T_2, while the non-survivor group showed a large increase at T_2, the time when the highest peptide count was reached in non-survivors.

To investigate the relationship between proteolysis and outcome, we compared the peptidomes of survivors *vs* non-survivors at T_1 and T_2. This quantitative analysis identified 218 proteins as the source for 913 peptides detected in the plasma samples. At both time points, the proteolysis levels, estimated from the abundance of the cleaved proteins, were increased in the non-survivor group (Fig. 2a) *vs* survivors.

Further, when comparing the sources of infection, the magnitude of proteolysis was higher in respiratory-initiated and abdominal-initiated septic shock compared with urosepsis (Fig. 2b). In the urinary and abdominal septic shock subgroups, the numbers of total, exclusive peptides and mean of exclusive peptides decreased from T_1 to T_3 (Table 2). The respiratory subgroup displayed a different behaviour. Both survivors and non-survivors in this subgroup presented a higher number of peptides at T_2 than at T_1, although the increase was larger in the non-survivors group, which displayed the highest number of peptides at T_2. Except for the respiratory non-survivors, the number of peptides at T_3 was smaller than at T_1 in all groups.

No protein from the urosepsis group was characterised by significantly increased proteolysis either at T_1 or T_2, while we found five proteins subject to significantly increased proteolysis in the abdominal septic shock group at T_1, and 10 proteins with significantly increased proteolysis in the group that had a respiratory-initiated septic shock at T_2 (Supplementary Table S2).

Group differences in proteolysis levels were assessed by computing the difference between protein abundance in the two groups at T_2 (Fig. 3), the time point at which the two groups of survivors and non-survivors clearly separated. At T_1 there was no appreciable difference between the two groups. At T_2, six proteins showed significantly increased proteolysis in non-survivors (Fig. 3a–f), and one protein in survivors (Fig. 3g).

Effector proteases estimation

In total, 644 and 929 $P1_{N/C\text{-term}}$ amino acids for the healthy and septic shock groups, respectively, were classified according to the protease-specific cleavage annotated in databases[17] and the literature.[7,18] Despite comparable protease cleavage distributions, the contribution of chymotrypsin-like enzymes to proteolysis was larger in shock (42% of the circulating peptides) than in the healthy controls (37%) (Fig. 4).

Discussion

This study demonstrates that plasma from septic shock patients displayed approximately a three-fold increment in total peptide count compared with healthy individuals, as well as 40% higher peptide abundance, indicating elevated proteolysis above physiological levels in septic shock. The protein fragments resulted mostly from the proteolytic action of serine proteases, such as chymotrypsin-like enzymes, and MMPs, in

Table 2 Source of infection and number of circulating peptides. Blood samples were drawn at three different time points. T_1: within 16 h of ICU admission; T_2: at 48 h after ICU admission; T_3: before discharge from the ICU or on Day 7 at latest (T_3)

Source of infection	*n*	Total no. peptides	Exclusive peptides	Exclusive peptides/*n*
Urinary tract: survivors				
T_1	8	1016	352	44
T_2	8	885	241	30
T_3	3	512	85	28
Abdominal: survivors				
T_1	8	1429	601	75
T_2	8	1119	318	40
T_3	6	785	182	30
Respiratory: survivors				
T_1	7	1442	522	75
T_2	7	1556	663	95
T_3	5	704	140	28
Respiratory: non-survivors				
T_1	5	1265	344	69
T_2	5	1689	634	127
T_3	4	1252	343	86

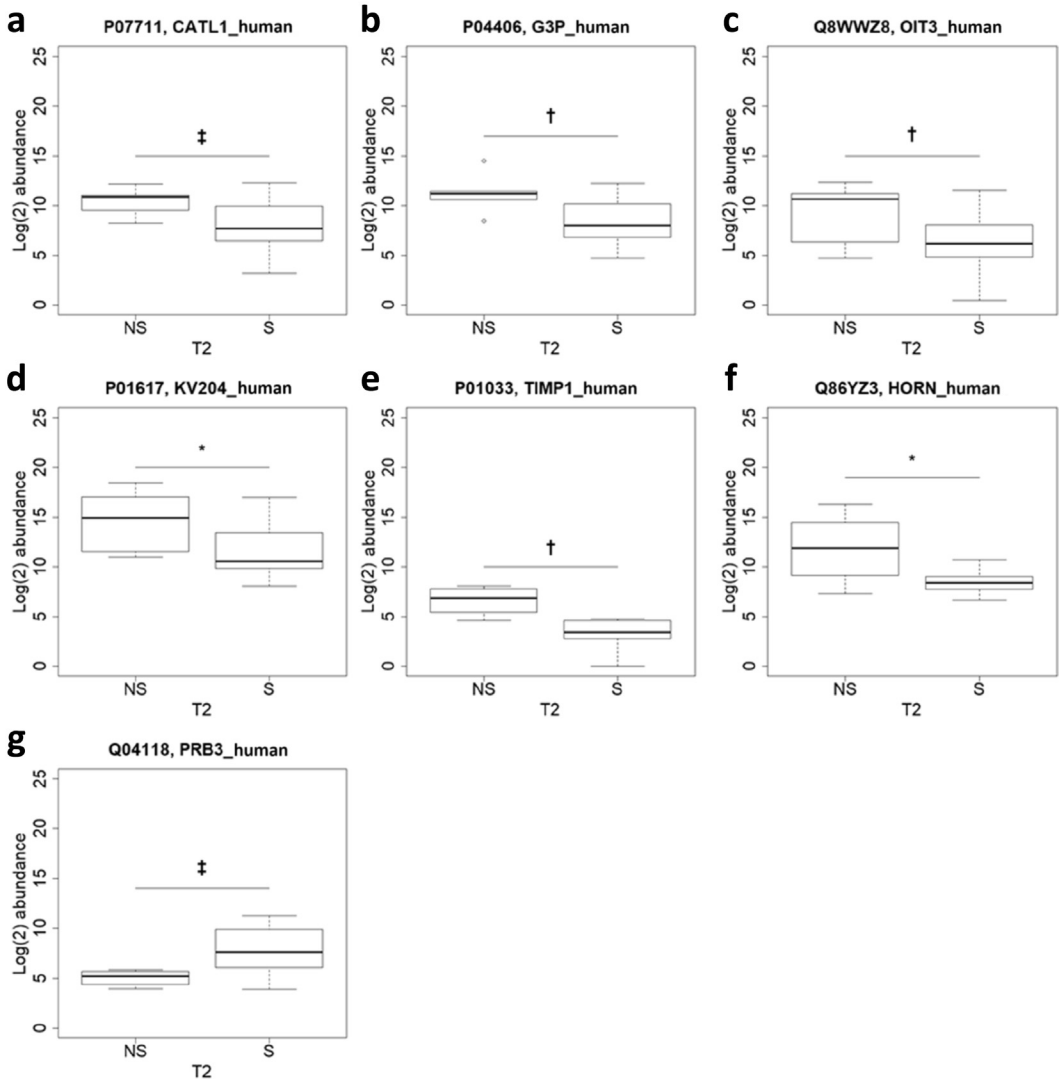

Fig 3. Boxplots representing the difference between survivors and non-survivors in protein abundance used to estimate proteolysis at T_2. Central marks in the boxplot represent median Log_2 abundance, the edges represent 1st and 3rd quartiles and outliers are defined as those observations being greater than 1.5 times the inter-quartile range (whiskers) and are plotted as a blank circle. *P-value<0.05 for Wilcoxon (Mann–Whitney) test; †P-value<0.05, and ‡P-value<0.01 for Student's *t*-test. S: survivors; NS: non-survivors; T_2: at 48 h after ICU admission. (a) CATL1: cathepsin L1; (b) G3P: glyceraldehyde-3-phosphate dehydrogenase; (c) OIT3: oncoprotein-induced transcript 3 protein; (d) KV204: immunoglobulin kappa variable 2D-28; (e) TIMP1: tissue inhibitor of metalloproteases 1; (f) HORN: hornerin; (g) PRB3: basic salivary proline-rich protein 3.

accordance with previous evidence.[7,19–22] The distribution of protease effectors responsible for proteolytic cleavage in shock was similar to the one in healthy controls: the largest number of circulating peptides were derived by proteolytic cleavage because of chymotrypsin-like enzymes in both groups, while elastase-like/MMPs, and MMPs and trypsin-like enzymes contributed to a similar extent to proteolysis in both groups, with a slight reduction of the contribution of MMPs and trypsin-like enzymes and a slight increase of elastase-like/MMPs. These results: (i) hint at a pathological shift in the concentration or activity of circulating enzymes or in the balance between endogenous protease inhibitors (the reduced presence of which in shock could also explain the increased proteolysis) and active proteases; (ii) confirm in humans the concept, previously proposed in experimental models of shock, that increased enzymatic activity and diffuse proteolysis are associated with mortality;[3–6,21,22] and (iii) are consistent with previous reports on the effectiveness of anti-protease treatments in acutely ill humans.[23] Therefore, this body of evidence points to a novel pathological mechanism with evident therapeutic implications in the complex framework of septic shock.

The comparison between non-survivors and survivors yielded a significantly larger total number of circulating

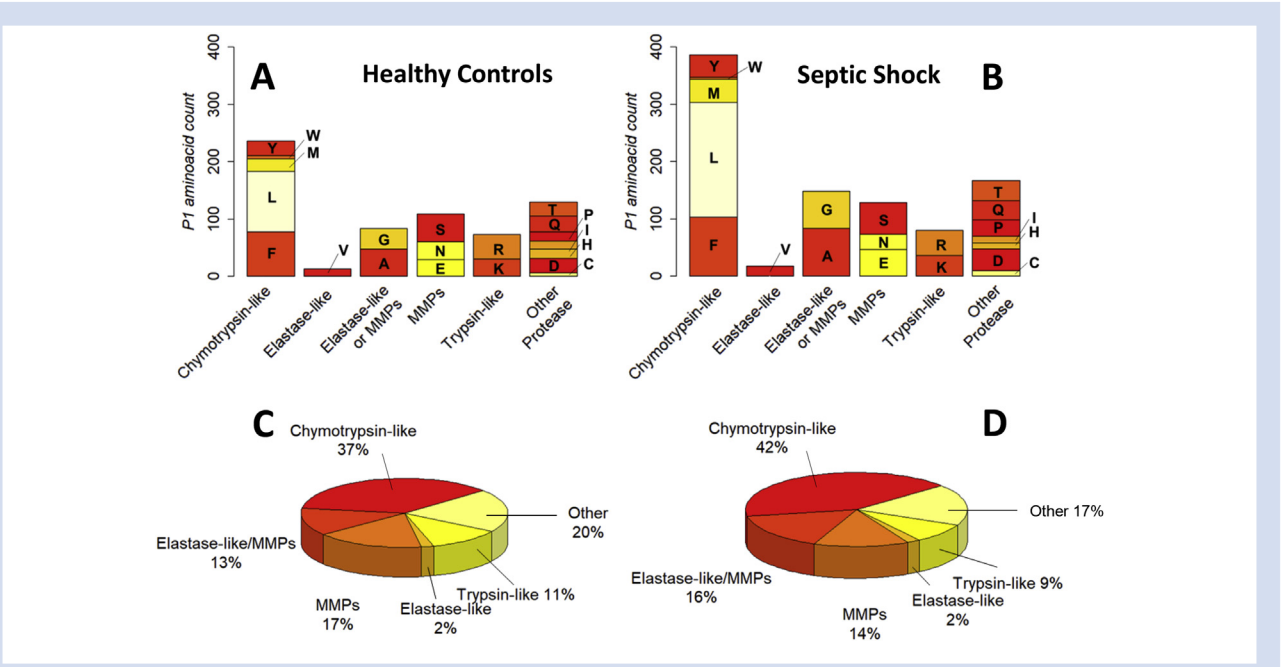

Fig 4. Proteases acting in the proteolytic process according to the peptide datasets observed in the healthy and septic shock groups. (A) Number (664) of P1 amino acids obtained from the 357 peptide sequences more abundant or present only in the healthy group; bar plots represent the amount of different amino acids observed at the P1 cleavage position, including N and C-term; each letter code represents an amino acid and each column accounts for all the cleaving sites of every enzyme listed on the x axis. (B) Number (929) of P1 amino acids obtained from 500 peptide sequences more abundant or present only in the septic shock group (at T_1 and T2); bar plots represent the amount of different amino acids observed at the P1 cleavage position, including N and C-term; each letter code represents an amino acid and each column accounts for all the cleaving sites of every enzyme listed on the *x* axis. The percentage of cleavage associated to *in silico* predicted effector proteases is shown in (C) for the healthy group and in (D) for the septic shock group. T_1: within 16 h of ICU admission; T_2: at 48 h after ICU admission. MMPs, matrix metalloproteases.

peptides in the non-survivor group at admission to the ICU, which increased further at 48 h after ICU admission (Figs 1 and 2). This result is even more striking in light of the low number of subjects in the non-survivor group and reinforces the notion that protein cleavage is increased in lethal septic shock.

Several important proteins were differentially cleaved between non-survivors and survivors. Specifically, at T_2, six proteins were more proteolysed in non-survivors, suggesting the pathologic disruption of important biological processes (Table 3). Most of these proteins are physiologically expressed in multiple tissues, and they are involved in the regulation of several functions known to be altered in shock, such as immune response, complement activation, coagulation, toll-like receptor signalling, etc. Of interest in the context of our findings, several proteins are involved in the regulation of proteolytic activity, especially by acting as endopeptidase inhibitors. For instance, the increased cleavage of glyceraldehyde-3-phosphate dehydrogenase (Fig. 3b) may explain the reduction of aspartic-type endopeptidase inhibitor activity, and the increased cleavage of tissue inhibitor of metalloproteases 1 (Fig. 3e), a potent MMP inhibitor, may reflect a higher release of MMP[24] into plasma of non-surviving individuals. The cleavage of inhibitors (inter-alpha-trypsin inhibitors, alpha-1-antichymotrypsin, alpha-1-antitrypsin, alpha-2-antiplasmin), even in the absence of significant differences in the proteolytic levels between subgroups of patients, supports the evidence of an imbalance between the pathologically increased proteolytic activity and the physiological endogenous inhibitory potential, which should trigger the testing of proteases as new therapeutic targets in septic shock.

A fragment of the activation peptide of cathepsin L1 (residues 75–89) also had increased abundance in non-survivors (Fig. 3a), along with immunoglobulin kappa variable 2D-28, hornerin, and the liver-specific[25] oncoprotein-induced transcript 3 protein.

Only one protein was more proteolysed in the survivors group (Fig. 3g) (i.e. basic salivary proline-rich protein 3, a salivary gland secreted protein involved in a 'first barrier' immune response to infection, given its role in the saliva as receptor for the Gram-negative *Fusobacterium nucleatum*). The identification of this protein is based on the qSLNEDVSQEESPSVISGKPEGR peptide sequence (residues 17–39 of the protein), which corresponds to the protein N-terminus after signal peptide release, detected with modified N-terminal glutamine (q) to pyroglutamate.[26]

We also found that 10 proteins were only proteolysed in survivors at T_1 or T_2, while in contrast, no protein was exclusively proteolysed in non-survivors. The proteolysis of these 10 proteins (Supplementary Table S1) may have beneficial roles in the progression of shock and be part of enhanced recovery in survivors. Among these proteins, a 29-amino acid peptide fragment of lipopolysaccharide-binding protein (P18428) was found in 57% of survivors at T_1. The identification of this protein is based on the ANPGLVARITDKGLQYAAQEGLLALQSEL peptide. As a protein that

Table 3 Significantly differently cleaved proteins between survivors and non-survivors. Protein name; origin (where the protein is physiologically expressed); general molecular function; biological processes or pathways in which the protein is involved

Protein	Origin	Function	Biological processes
Cathepsin L1	Lysosome	Cysteine- and serine-type endopeptidase activity; collagen, elastin, alpha-1 protease inhibitor binding	Intracellular proteolysis; adaptive immune response; toll-like receptor signalling pathway
Oncoprotein-induced transcript 3 protein	Liver	Hepatocellular function	Urate homeostasis
Immunoglobulin kappa-variable 2D-28	Membrane-bound or secreted	Antigen binding; serine-type endopeptidase activity	Immune response (humoral immunity); immune response regulation; complement activation pathway
Tissue inhibitor of metalloproteases 1	Multiple tissues	Irreversible inactivation of specific MMPs, regulation of extracellular matrix composition	Involvement in the regulation of multiple processes (e.g. wound healing, response to cytokines, response to hormones, etc.)
Hornerin	Cytoplasm	Calcium ion binding	Innate immune system
Glyceraldehyde-3-phosphate dehydrogenase	Intracellular	Aspartic-type endopeptidase inhibitor activity, etc.	Glycolysis; nuclear functions
Basic salivary proline-rich protein 3	Saliva	Receptor for the Gram-negative *Fusobacterium nucleatum*	Immune response

facilitates binding of lipopolysaccharide (LPS) to recognition receptors (e.g. CD14, TLR4),[27] P18428 degradation may signal a reduced response to Gram-negative endotoxin-mediated signalling and its absence in non-survivors could indicate complete degradation of the protein into fragments below the peptide range in the current analysis. The significance of this protein in survival remains to be investigated with independent measurements of LPS concentrations in patients.

Peptidomic profiles were different according to the source of infection. Uroseptic shock typically has better prognosis than shock derived from pulmonary or abdominal origins.[28] Consistently, proteolysis was more limited in the uroseptic patients, further confirming the association between severity of septic shock, outcome, and extent of proteolysis. Reduced proteolysis in these patients could be related to a favourable response to the early therapeutic interventions which, when successful, limit hypoperfusion and protect the tissues, including the gut.[29] Furthermore, in hyperdynamic states such as urosepsis, blood flow to heart, gut, and kidney is better preserved.[30]

In contrast, septic shock of pulmonary origin accounted for five of the six deaths and it was the most common source of infection, similar to previous observations.[28] Comparison of proteolysis in abdominal and respiratory infection showed that five proteins were significantly more cleaved in the abdominal group at T_1, while 12 proteins were significantly more cleaved in the respiratory-initiated shock patients (Supplementary Table S2). Despite the same overall level of proteolysis at T_1, these differences could indicate a broader impact of proteolysis on physiologic and repair processes in the respiratory group and therefore explain the association between outcome and proteolysis. These observations indicate that, much like in urosepsis, the patterns of proteolysis in the pulmonary and abdominal groups were consistent with their clinical severity.

The present study acknowledges some limitations. First, non-survivors were on average older than survivors and of healthy donors. Still, the data on circulating peptides detected in the whole septic shock group (survivors and non-survivors together), as well as in both the abdominal and respiratory septic shock subgroups at T_1 do not point to a positive correlation between age and levels of proteolysis. This is consistent with previously reported evidence of the negative correlation between ageing and proteasome activity,[31] and of the decreasing trypsin and MMP-9 activity with age[32] (with possible pathological implications), which is also accompanied by increased alpha-1-antitrypsin concentration. Second, the number of enrolled patients was not very large. However, this work can serve as a guide for future studies designed to validate the peptidomic approach and its significance in larger and more diverse cohorts.

Conclusions

We propose a novel peptidomics-based analysis to test proteolysis in septic shock patients as a fundamental pathological mechanism contributing to outcome. Our data suggest an enhanced proteolytic activity with an association between proteolysis and mortality.

Even though the current analysis was limited to plasma only, the main findings of our study confirm previous animal reports. We observed that the increased proteolysis is the result of the activity of serine proteases and MMPs, and could also be explained by the systematic cleavage of endogenous protease inhibitors. Thus, a patient's peptidomic profile could serve as a useful tool for interpreting the trajectory and outcome of patients in septic shock and to design new therapeutic treatments aimed to target proteases and support protease inhibition.

Authors' contributions

Draft of the first manuscript: J.B.M., F.A.

Study design: F.A., K.B.
Data analysis: J.B.M., F.A., V.R.
Data discussion and interpretation: J.B.M., F.A., B.B.P., V.R., E.B.K., G.W.S.S., K.B., E.D.O.
Preparation of figures and table: J.B.M.
Patient recruitment: B.B.P., K.B., A.H.
Blood sample and data collection: B.B.P., E.R.M., R.F., A.H.
Statistical analysis: V.R., J.B.M.
Healthy donor recruitment: E.R.M., R.F.
Mass-spectrometry experiments: J.B.M., M.A.O., R.D.
Definition of methodology for peptidomics analysis: G.T., E.D.O.
Manuscript approval: E.R.M., G.T.
Preparation of the initial and of the final manuscript: F.A.
Manuscript revision and approval: J.B.M., B.B.P., V.R., M.A.O., R.D., R.F., E.B.K., G.W.S.S., A.H., K.B., E.D.O.

Acknowledgements

The authors acknowledge the European Union-funded '*ShockOmics*' consortium (Principal Investigator: Professor Giuseppe Baselli, Politecnico di Milano), and the Spanish Proteomics Network.

Declaration of interest

F.A. has a pending patent application (PCT/US2017/028583). G.W.S.S. owns shares in InflammaGen, a company by Leading Bioscience Inc., San Diego, and has a pending patent application (PCT/US2017/028583). The other authors declare no conflict of interest.

Funding

The 7th Framework Program of the European Union under the '*ShockOmics*' grant #602706; the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development and Career Development Award (CDA2) 1IK2BX001277-01A1; the Instituto de Salud Carlos III ProteoRed-ISCIII.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bja.2018.05.072.

References

1. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016; **315**: 801–10
2. Seymour CW, Liu VX, Iwashyna TJ, et al. Assessment of clinical criteria for sepsis. *JAMA* 2016; **315**: 762
3. Fitzal F, Delano FA, Young C, Rosario HS, Schmid-Schönbein GW. Pancreatic protease inhibition during shock attenuates cell activation and peripheral inflammation. *J Vasc Res* 2002; **39**: 320–9
4. Fitzal F, DeLano FA, Young C, Rosario HS, Junger WG, Schmid-Schönbein GW. Pancreatic enzymes sustain systemic inflammation after an initial endotoxin challenge. *Surgery* 2003; **134**: 446–56
5. Chang M, Alsaigh T, Kistler EB, Schmid-Schönbein GW. Breakdown of mucin as barrier to digestive enzymes in the ischemic rat small intestine. *PLoS One* 2012; **7**: 1–12
6. DeLano FA, Hoyt DB, Schmid-Schonbein GW. Pancreatic digestive enzyme blockade in the intestine increases survival after experimental shock. *Sci Transl Med* 2013; **5**: 169ra11
7. Aletti F, Maffioli E, Negri A, et al. Peptidomic analysis of rat plasma: proteolysis in hemorrhagic shock. *Shock* 2015; **45**: 540–54
8. Santamaria MH, Aletti F, Li JB, et al. Enteral tranexamic acid attenuates vasopressor resistance and changes in α1-adrenergic receptor expression in hemorrhagic shock. *J Trauma Acute Care Surg* 2017; **83**: 263–70
9. DeLano FA, Schmid-Schönbein GW. Pancreatic digestive enzyme blockade in the small intestine prevents insulin resistance in hemorrhagic shock. *Shock* 2014; **41**: 55–61
10. Karnad DR, Bhadade R, Verma PK, et al. Intravenous administration of ulinastatin (human urinary trypsin inhibitor) in severe sepsis: a multicenter randomized controlled study. *Intensive Care Med* 2014; **40**: 830–8
11. Aletti F, Conti C, Ferrario M, et al. ShockOmics: multiscale approach to the identification of molecular biomarkers in acute heart failure induced by shock. *Scand J Trauma Resusc Emerg Med* 2016; **24**: 9
12. Levy MM1, Fink MP, Marshall JC, et al. SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Intensive Care Med* 2001; **2003**(29): 530–8
13. Dellinger RP, Levy MM, Rhodes A, et al. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock. *Crit Care Med* 2012; **2013**(41): 580–637
14. R Development Core Team R. R: a language and environment for statistical computing. *R Found Stat Comput* 2011; **1**: 409
15. Secher A, Kelstrup CD, Conde-Frieboes KW, et al. Analytic framework for peptidomics applied to large-scale neuropeptide identification. *Nat Commun* 2016; **7**: 11436
16. Vanlaere I, Libert C. Matrix metalloproteinases as drug targets in infections caused by gram-negative bacteria and in septic shock. *Clin Microbiol Rev* 2009; **22**: 224–39
17. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* 2003; **31**: 3784–8
18. Turk BE, Huang LL, Piro ET, Cantley LC. Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. *Nat Biotechnol* 2001; **19**: 661–7
19. Schmid-Schönbein GW, Penn A, Kistler E. The autodigestion hypothesis in shock and multi-organ failure: degrading protease activity. *Bol Soc Port Hemorreol Microcirc* 2011; **26**: 6–15
20. Kistler EB, Alsaigh T, Chang M, Schmid-Schönbein GW. Impaired small bowel barrier integrity in the presence of lumenal pancreatic digestive enzymes. *Shock* 2012; **38**: 262–7
21. Schmid-Schönbein GW, Chang M. The autodigestion hypothesis for shock and multi-organ failure. *Ann Biomed Eng* 2014; **42**: 405–14
22. Altshuler AE, Kistler EB, Schmid-Schönbein GW. Autodigestion: proteolytic degradation and multiple organ failure in shock. *Shock* 2016; **45**: 483–9
23. Lee YT, Wei J, Chuang YC, et al. Successful treatment with continuous enteral protease inhibitor in a patient with severe septic shock. *Transplant Proc* 2012; **44**: 817–9
24. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. *Biochim Biophys Acta* 2010; **1803**: 55–71

25. Xu ZG, Du JJ, Zhang X, et al. A novel liver-specific zona pellucida domain containing protein that is expressed rarely in hepatocellular carcinoma. *Hepatology* 2003; **38**: 735–44
26. Vitorino R, Alves R, Barros A, et al. Finding new post-translational modifications in salivary proline-rich proteins. *Proteomics* 2010; **10**: 3732–42
27. Ryu JK, Kim SJ, Rah SH, et al. Reconstruction of LPS transfer cascade reveals structural determinants within LBP, CD14, and TLR4-MD2 for efficient LPS recognition and transfer. *Immunity* 2017; **46**: 38–50
28. Leligdowicz A, Dodek PM, Norena M, Wong H, Kumar A, Kumar A. Association between source of infection and hospital mortality in patients who have septic shock. *Am J Respir Crit Care Med* 2014; **189**: 1204–13
29. Sertaridou E, Papaioannou V, Kolios G, Pneumatikos I. Gut failure in critical care: old school versus new school. *Ann Gastroenterol* 2015; **28**: 307–20
30. Giantomasso D Di, May CN, Bellomo R. Vital organ blood flow during hyperdynamic sepsis. *Chest* 2003; **124**: 1053–9
31. Saez I, Vilchez D. The mechanistic links between proteasome activity, aging and age-related diseases. *Curr Genomics* 2014; **15**: 38–51
32. Paczek L, Michalska W, Bartlomiejczyk I. Trypsin, elastase, plasmin and MMP-9 activity in the serum during the human ageing process. *Age Ageing* 2008; **37**: 318–23

Handling editor: J.P. Thompson