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Cell-free DNA increase over first 48 hours in emergency intensive care unit predicts fatal outcome in patients with shock

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Abstract

Objective: To investigate whether circulating cell-free (cf)-DNA levels are a useful biomarker for survival in patients with shock in the emergency intensive care unit (EICU).

Methods: This prospective observational study enrolled patients admitted to the EICU diagnosed with shock. Blood cf-DNA levels were analysed on admission, and after 24 and 48 h. As a measure of circulating cf-DNA, copy number of the β-globin gene in plasma was assessed using quantitative real-time polymerase chain reaction.

Results: Circulating cf-DNA levels were higher at hospital admission and after 24 h in EICU patients with shock who died than in those who recovered. Change in cf-DNA levels over the first 48 h in critical care was independently associated with 28-day mortality. The critical cut-off value for cf-DNA change over 48 h in predicting 28-day mortality was +16.12% (sensitivity 68.9%, specificity 89.7%).

Conclusions: Increased circulating cf-DNA levels in EICU patients with shock are associated with risk of death and measuring cf-DNA change over 48 h improves risk prediction. The present study suggests that cf-DNA may serve as a viable plasma biomarker of mortality risk in EICU patients with shock.

Keywords

Shock, cell-free DNA, cell-free DNA change, mortality, prognosis

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Introduction

One-third of patients admitted to the intensive care unit (ICU) are in shock[1] – a life-threatening complicated state of acute circulatory failure,[2] associated with a high mortality rate.[3] In critically ill patients, procalcitonin and C-reactive protein biomarkers are used to identify infections.[4] Intensive care scoring systems, such as the Acute Physiology and Chronic Health Evaluation II (APACHE II), have been studied as clinical tools for assessing illness severity and predicting outcome of ICU patients.[5–7] Despite the improvement in prognosis afforded by these tools,[5,7] there is a need to develop biomarkers that can help clinicians further improve risk prediction for patients with shock in the emergency (EICU), as only a few biomarkers for assessing the severity of shock are validated for routine use in clinical practice.[3]

Repeated risk assessment and close clinical monitoring of patients with shock in the EICU are vital for improving the chances of patient survival. Pulse Index Continuous Cardiac Output device parameters have been used to assess the effectiveness of treatment and to guide further treatment for patients with shock.[8,9] Assessment of serum lactate concentrations early in the diagnostic process is also recommended, to evaluate the severity of the patient's condition, to observe the effectiveness of treatment and for predicting mortality risk due to shock.[3,10] Circulating cell-free (cf)-DNA may be a useful biomarker of mortality risk in critically ill patients,[11,12] and has been proposed as a novel diagnostic and prognostic biomarker for various conditions including sepsis,[11,13] stroke,[14] myocardial infarction,[15] cancer[16] and trauma.[17]

The mechanisms of shock are complicated, involving any of four pathophysiologic subtypes (hypovolemic, cardiogenic, distributive and obstructive), and result in circulatory failure.[18] The subsequent inadequate oxygen utilization by cells results in cellular dysfunction and eventually cell necrosis, and is general in all patients with shock.[18] Degeneration of the cell nucleus has been shown to release cf-DNA into the circulation.[19] Healthy subjects have low levels of cf-DNA in plasma,[20] which originates from phagocytes removing dead cell debris.[21] As cf-DNA levels have been proposed as biomarkers of risk in critically ill patients,[11,12] the present authors hypothesized that circulating cf-DNA levels would be associated with risk of mortality in patients with shock admitted to the EICU, and may be used to improve risk prediction. Thus, the objective of the present study was to investigate whether measurement of circulating cf-DNA levels could be a useful biomarker of mortality risk in patients with shock in the EICU.

Patients and methods

Study population

This prospective cohort study was conducted in the EICU of the First Affiliated Hospital of Anhui Medical University, Hefei, China between June 2012 and April 2015. All patients aged ≥ 18 years, meeting the criteria for shock on admission to the EICU, diagnosed according to the definition of the European Society of Intensive Care Medicine consensus conference, 2014,[3] were enrolled. Exclusion criteria included patients or patient's families who refused consent for participation, patients with shock following a surgical procedure, patients with terminal malignant tumours, elderly patients (>80 years), and those with autoimmune diseases. Age- and sex-matched controls were recruited from the Physical Examination Centre, First Affiliated Hospital of Anhui Medical University between June 2012 and August 2012. All patients in the EICU received supportive treatment based on individual need. An electronic chart was used to record demographic data, prognosis, length of EICU and hospital stay, clinical diagnosis, Karnofsky scores,[22] skin condition

(degree of cutaneous perfusion), co-morbidities, vital signs, APACHE II score at EICU admission, any organ dysfunction, details of any mechanical ventilation and/or continuous renal replacement therapy, use of vasopressors, enteral nutrition and laboratory results (including lactate levels). All study population data were retrieved from medical records held at the First Affiliated Hospital of Anhui Medical University. The primary study endpoint was mortality at 28 days.

This study was approved by the Medical Research Ethics Board of the First Affiliated Hospital of Anhui Medical University and written informed consent was provided by each patient or their legal proxy.

Blood sampling

On admission to the EICU and after 24 and 48 h, 5 ml of whole blood was obtained from each patient into EDTA-containing tubes and kept on ice prior to transfer to the Molecular Biology Department, Central Laboratory of the First Affiliated Hospital of Anhui Medical University. Blood samples were then centrifuged at 16 000 *g* for 10 min at 4°C, plasma was aspirated carefully to avoid residual cells and then immediately stored at −80°C to avoid DNA fragmentation.[23] The same process was conducted for control blood samples.

DNA was extracted using QIAamp® DNA Blood Mini Kits (QIAGEN GmbH, Hilden, Germany) and eluted in a final volume of 200 µl using QIAamp® DNA Blood Mini Kit buffer (pH 7), according to the manufacturer's 'blood and body fluid' protocol. The DNA samples were stored at –80°C prior to use in quantitative real-time polymerase chain reaction (PCR) assays.

Quantification of circulating cf-DNA

Plasma cf-DNA was measured using a SYBR Green dye-based quantitative real-time PCR assay for the human β-globin gene,[24] a housekeeping gene.[25] The forward primer sequence was 5′-GCA CCT GAC TCC TGA GGA GAA-3′ and the reverse primer sequence was 5′-CAC CAA CTT CAT CCA CGT TCA-3′.[26] Each 20 µl reaction mix contained 2 µl DNA, 10 µl SYBR Premix Ex Taq™ II (TaKaRa, Dalian, China), 0.8 µl forward primer (10 µmol/l), 0.8 µl reverse primer (10 µmol/l), 0.4 µl ROX reference dye II (TaKaRa, Dalian and China) and 6 µl distilled water. Human genomic DNA (Roche Diagnostics GmbH, Penzberg, Germany) of known concentration 100 µl/500 µl (2×10^8 pg/ml) was serially diluted (1:10) to prepare a standard curve against which the patient cf-DNA could be compared. The serially diluted DNA concentrations spanned 10^8 pg/ml to 10^3 pg/ml. Standard curve and no DNA template control samples were included in each quantitative PCR reaction. The PCR temperatures used to detect cf-DNA were an initial 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s and elongation at 58°C for 30 s, slowly warming to a final elongation of 95°C for 5 s (performed using an Agilent Technologies Inc. thermocycler, Palo Alto, CA, USA). The size of the PCR product was 95 bp.[21] A conversion factor of 6.6 pg of DNA per diploid cell was used. Results are expressed as genome equivalents (GE) per ml (1 GE = 6.6 pg DNA).

Dissociation curve analysis showed a single melting temperature of approximately 83.5°C, indicating one specific product was generated with the standard template (Figure 1).

Statistical analyses

Continuous variables were analysed using Student's *t*-test and Mann–Whitney *U*-test, and are presented either as mean ± SD or median (interquartile range [IQR], 25th to 75th percentiles). Categorical variables were analysed as percentages using χ^2-test or

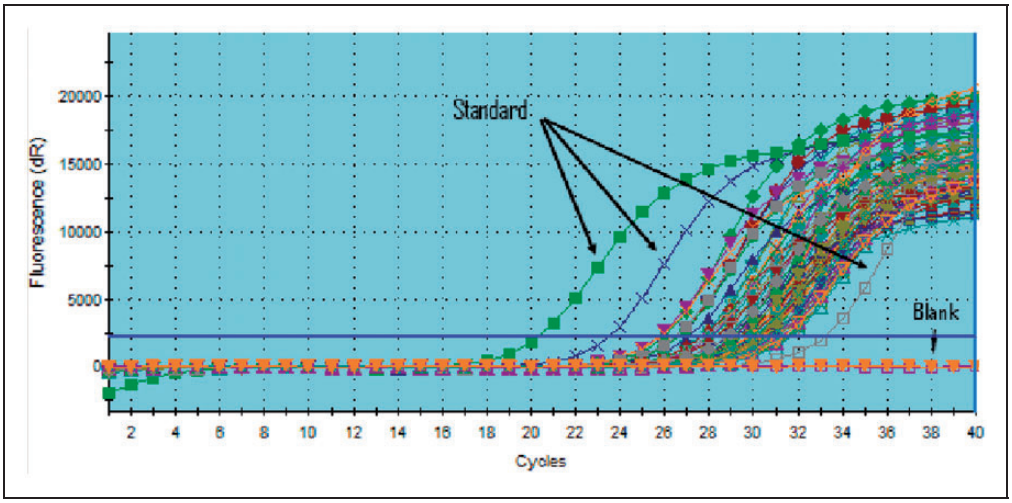

Figure 1. Amplification plot of part of the human β-globin gene (95 bp), showing standard curves of serially diluted known DNA concentrations from 10^8 pg/ml to 10^3 pg/ml, DNA free 'blank' control, and DNA samples from emergency intensive care unit patients with shock. Amplification curves from patient samples were within the concentration range of the known DNA standard curves.

Fisher's exact test. Receiver operating characteristic (ROC)[27] curves were drawn to evaluate the discriminative power of lactate levels 24 h following EICU admission, APACHE II scores at admission, cf-DNA levels at admission, cf-DNA levels 24 h following admission and the change in cf-DNA levels over 48 h in EICU to predict 28-day mortality. The area under the ROC curve (AUC) was calculated for each ROC curve, and reflects the value of the predictive variable, as follows: AUC = 0.5, the variable has no predictive value; AUC = 1.0, the variable is fully predictive of the outcome; AUC between 0.5 and 0.7, the variable has some predictive value; AUC between 0.7 and 0.9, the variable has moderate predictive value; and AUC > 0.9, the variable is predictive. The Youden index[28] was defined as the sum of the highest value of sensitivity and specificity (sensitivity + specificity-100%) or the ROC-curve point at which both sensitivity and specificity are maximal. As cf-DNA levels were not normally distributed, continuous levels were dichotomized at the cut-off value for maximal sensitivity and specificity. Univariate analysis using forward variable selection was used to identify which variables were independently associated with 28-day mortality. All significant variables were then tested in a multivariable logistic regression model to investigate which variables were independently associated with 28-day mortality.

All statistical analyses were performed using the SPSS® software, version 22.0 (SPSS; IBM Corp. Armonk, NY, USA). A P-value < 0.05 was considered statistically significant in all tests.

Results

Patient characteristics

Out of 173 patients with shock, 169 were included in this study, 4 patients were excluded due to missing plasma samples (Table 1). In addition, 140 plasma samples were collected 24 h after EICU admission, and 124 plasma samples after 48 h.

Table 1. Characteristics of 169 patients ≥ 18 years of age admitted to the emergency intensive care unit with shock.

Characteristic	All patients $n = 169$	28-Day survivors $n = 88$	28-Day non-survivors $n = 81$	Statistical significance
Age, years	61.0 ± 20.0	58.6 ± 19.4	63.5 ± 20.0	NS
Sex, male/female	98/71	49/39	49/32	NS
Type of shock				$P < 0.001$
Hypovolemic	25 (14.8%)	14 (56.0%)	11 (44.0%)	
Cardiogenic	41 (24.3%)	18 (43.9 %)	23 (56.1%)	
Obstructive	4 (2.4%)	2 (50.0%)	2 (50.0%)	
Distributive	99 (58.6%)	54 (54.5%)	45 (45.5%)	
EICU length of stay, days	5.0 (3.0–10.5)	6.0 (4.0–11.8)	4.0 (2.0–15.0)	$P < 0.001$
Total hospital stay, days	12.0 (5.0–23.0)	19.0 (11.0–31.8)	6.0 (3.0–15.0)	$P = 0.004$
Lactate level 24 h after admission, mmol/l	1.80 (1.13–3.69)	1.28 (0.93–2.65)	2.38 (1.56–5.62)	$P < 0.001$
APACHE II score on admission	22.0 ± 7.6	19.3 ± 7.1	24.9 ± 7.2	$P < 0.001$
cf-DNA on admission, pg/ml	2.86×10^5 (8.75×10^4–9.68×10^5)	1.20×10^5 (4.59×10^4–3.12×10^5)	8.02×10^5 (2.71×10^5–1.98×10^6)	$P < 0.001$
cf-DNA after 24 h in EICU, pg/ml	2.85×10^5 (5.16×10^4–7.20×10^5)	9.06×10^4 (3.38×10^4–3.19×10^5)	5.23×10^5 (2.94×10^5–2.05×10^6)	$P < 0.001$
cf-DNA after 48 h in EICU, pg/ml	2.24×10^5 (3.34×10^4–1.70×10^6)	3.98×10^4 (2.08×10^4–1.24×10^5)	1.90×10^6 (5.08×10^5–5.12×10^6)	$P < 0.001$
Change in cf-DNA over first 48 h in EICU, %	−37.5 (−33.92 to +42.33)	−26.06 (−2.56 to −50.96)	+ 37.93[c] (−1.07 to +137.88)	$P < 0.001$

"c" means that the cf-DNA concentrations increased 37.93% on the basis of admission.

Data presented as *n* (%) patient prevalence, median (interquartile range), mean ± SD, or mean (range).

NS, no statistically significantly difference ($P > 0.05$; Mann-Whitney *U*-test).

EICU, emergency intensive care unit; APACHE II, Acute Physiology and Chronic Health Evaluation; cf-DNA, cell-free DNA.

In total, 81 of 169 patients (47.9%) died within 28 days of EICU admission. In the entire patient cohort, more patients presented with distributive shock than other subtypes of shock. There were statistically significant differences in total hospital length of stay ($P = 0.004$), and EICU length of stay ($P < 0.001$) between 28-day survivors and non-survivors of shock. Patients who died within 28 days had higher mean APACHE II scores on admission and higher median 24 h lactate values compared with patients who survived (both $P < 0.001$). The median cf-DNA concentration in patient plasma was significantly higher in non-survivors than in survivors on admission to the EICU, after 24 h and after 48 h in the EICU (all $P < 0.001$). The median change in cf-DNA levels during the first 48 h in EICU was statistically different between patients who died and those who survived beyond 28 days (median −26.06% [IQR −2.56% – −50.96%] versus +37.93% [IQR −1.07% – +137.88%]; $P < 0.001$).

Out of a total of 30 healthy individuals (one sample taken from each patient during an examination at the Physical Examination Centre, First Affiliated Hospital of Anhui Medical University), the median cf-DNA concentration was 1.68×10^4 pg/ml (IQR 8.44×10^3 pg/ml – 1.30×10^5 pg/ml), and was significantly lower than cf-DNA levels in EICU patients with shock ($P < 0.001$).

Variables predictive of 28-day mortality

Predictions of EICU 28-day mortality were made with ROC curve analyses (Figure 2 and Table 2). In patients with shock, cf-DNA levels at admission and 24 h after admission, as well as change in cf-DNA levels over the initial 48 h in EICU, were found to be significant predictors of death; lactate levels at 24 h and APACHE II scores

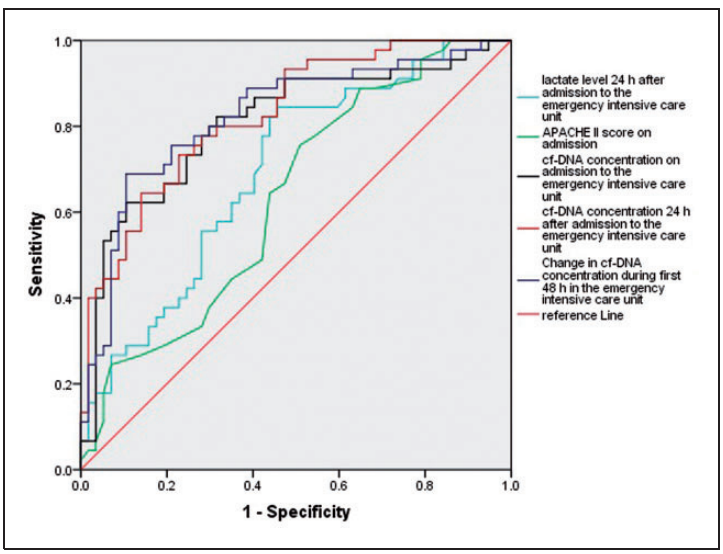

Figure 2. Receiver operating characteristic curves comparing the power to predict 28-day mortality between lactate levels 24 h after admission to the emergency intensive care unit (EICU), APACHE II score on admission, cell-free (cf)-DNA concentration on admission to the EICU, after 24 h in the EICU, and after 48 h in the EICU, and the change in cf-DNA concentration over the first 48 h of EICU admission in 169 patients ≥ 18 years of age admitted to the emergency intensive care unit with shock.

Table 2. Receiver operating characteristic curve analysis to determine which variables best predict 28-day mortality in 169 patients ≥ 18 years of age admitted to the emergency intensive care unit with shock.

Variable	AUC (95% CI)	Statistical significance	Standard error	Best cut-off value	Sensitivity	Specificity
Lactate levels 24 h after EICU admission, mmol/l	0.689 (0.587, 0.790)	$P = 0.001$	0.052	1.285	84.4%	53.4%
APACHE II score on EICU admission	0.637 (0.530, 0.743)	$P = 0.018$	0.054	19.5	75.6%	50.0%
cf-DNA on EICU admission, pg/ml	0.808 (0.719, 0.896)	$P < 0.001$	0.045	635119.5	62.2%	89.7%
cf-DNA after 24 h in EICU, pg/ml	0.830 (0.753, 0.907)	$P < 0.001$	0.039	325779.5	73.3%	77.6%
cf-DNA change over 48 h, %	0.825 (0.741, 0.909)	$P < 0.001$	0.043	+16.12	68.9%	89.7%

EICU, emergency intensive care unit; AUC, area under the curve; APACHE II, Acute Physiology and Chronic Health Evaluation; cf-DNA, cell-free DNA.

at admission had lower predictive values for 28-day mortality. The AUC of the ROC curve analysis for the cf-DNA value at admission for predicting death was 0.808 (95% confidence interval [CI] 0.719, 0.896; $P < 0.001$). A cf-DNA value at admission of 6.35×10^5 pg/ml predicted 28-day mortality with a sensitivity of 82.2% and a specificity of 68.4%. A cf-DNA value 24 h after admission of 3.26×10^5 pg/ml was also predictive of death during the 28-day follow-up, with a sensitivity of 73.3%, a specificity of 77.6 and an AUC of 0.830 (95% CI 0.753, 0.907; $P < 0.001$). The AUC in the ROC curve analysis for the cf-DNA change over the first 48 h in EICU for predicting death was 0.825 (95% CI 0.741, 0.09; $P < 0.001$). If the cf-DNA concentration increased by 16.12% or more during the first 48 h of EICU admission, the patient would likely die within 28 days. The sensitivity of this result was 68.9 and the specificity 89.7.

Multivariate logistic regression analysis

When all measured variables: 24 h lactate levels, APACHE II scores on admission, cf-DNA levels at admission, cf-DNA levels after 24 h in EICU and cf-DNA change over 48 h, were analysed together in a multivariate logistic regression analysis, only cf-DNA change over 48 h was significant as predictive of 28-day mortality (Table 3; odds ratio 4.232, 95% CI 1.547, 11.579; $P = 0.005$).

Discussion

Accurate and convenient predictive tools for repeated risk assessment and close monitoring of critically ill patients are vitally important to a patient's chances of survival.[29] Predictive biomarkers that can be objectively and rapidly measured and respond to clinical recovery could add relevant, reliable, real-time information for the care of patients with shock. Studies have shown that critically ill patients who died had higher circulating cf-DNA levels compared with surviving patients,[11,13,27,30] and circulating cf-DNA has been shown to independently predict patients with severe sepsis and septic shock in the ICU.[31] Measurements of cf-DNA levels in ICU patients with severe sepsis was shown to be predictive of patient mortality, with a very high AUC of 0.97 and high levels of sensitivity (87%) and

Table 3. Multivariate logistic regression analysis to determine which variables are predictive of 28-day mortality in 169 patients ≥ 18 years of age admitted to the emergency intensive care unit with shock.

Variable	OR (95% CI)	Statistical significance
Lactate levels 24 h after EICU admission	–	NS
APACHE II score on EICU admission	–	NS
cf-DNA on EICU admission	–	NS
cf-DNA after 24 h in the EICU	–	NS
cf-DNA change over 48 h	4.232 (1.547, 11.579)	$P = 0.005$

OR, odds ratio; CI, confidence interval; EICU, emergency intensive care unit; APACHE II, Acute Physiology and Chronic Health Evaluation; cf-DNA; cell-free DNA.
NS, not statistically significant ($P > 0.05$).

specificity (93%).[13] In addition, cf-DNA has been shown to have a high prognostic value to predict mortality in febrile patients.[26] In the current study, plasma cf-DNA concentration at admission and 24 h after admission, cf-DNA change over the first 48 h of EICU admission, and lactate levels and APACHE II scores at admission, were measured in critically ill EICU patients with shock, and were found to have predictive significance for 28-day mortality.

The present study showed that median circulating cf-DNA concentrations in EICU patients with shock were significantly higher compared with healthy controls. Median circulating cf-DNA concentrations on EICU admission and after 24 h in the EICU were significantly higher in patients with shock whose outcome was fatal within 28 days compared with those who survived. The main finding of the present study was that change in cf-DNA concentration over the first 48 h of critical care treatment was an accurate mortality predictor in intensively-treated patients with shock. This finding was independent of the initial severity assessment, including cf-DNA levels on admission, lactate levels and APACHE II scores. Other variables, including 24 h lactate levels, APACHE II scores, cf-DNA concentration on admission and cf-DNA concentration 24 h after admission were more dependent on the underlying diseases or other confounders, and when accounted for in multivariate logistic regression analysis, they were not found to be predictive of 28-day mortality, despite the results of the initial analysis.

The present data suggest that high circulating cf-DNA concentrations in patients with shock are dynamic changes reflecting the change of the patients' condition. Close monitoring of plasma cf-DNA levels of patients with shock in the first 48 h of EICU care would provide information to help decisions about whether the patient could be released from EICU care to a general ward or whether they should continue to receive treatment escalation. The patient population of the present study was stratified according to whether the cf-DNA concentration change over 48 h was >16.12%, as a change of this size may help identify individuals at high risk of mortality and who would benefit by continuing to receive intensive treatment. Patients with a cf-DNA concentration change over 48 h ≤ 16.12% were found to be at reduced mortality risk and, therefore, would be good early EICU discharge candidates, freeing beds to accept other critically ill patients. Accurate stratification of EICU patients with shock is a prerequisite for therapeutic options to lighten the financial burden of patients, improve patient satisfaction and reduce the expense of medical treatment.[29]

Accurate risk assessment and close clinical monitoring of disease severity would provide accurate clinical course prediction, and assists in setting realistic expectations regarding the illness.

Previous studies have used circulating cf-DNA as a biomarker to investigate patients with circulation issues,[11–13,26,31–36] however, these studies only explored the connection between cf-DNA levels and patient outcomes, and obtained an optimal cf-DNA concentration level. The present study is the first to research and demonstrate a significant association between cf-DNA concentration change over 48 h and outcome among EICU patients with shock.

The present work has several limitations: First, the mechanism for removing cf-DNA *in vivo* is unclear,[37] although investigations using experimental models suggest that the liver is a major site for metabolizing plasma nucleic acids;[38] Secondly, levels of plasma nucleic acids may be influenced by patient age and underlying diseases, and mechanical ventilation and vasoactive agents increase the circulating level of DNA;[33] Thirdly, the present study enrolled only 4 patients with obstructive shock, which precluded exploration of cf-DNA levels between each shock sub-group; Fourthly, confounders, such as the duration of shock before EICU entry, were difficult to control for and the study sample was too small to adjust for multiple confounders. The present study is also limited by only using a single centre for sample collection. The present results, therefore, need additional prospective validation in a multicentre study with a larger patient population.

In summary, circulating cf-DNA concentrations in EICU patients with shock were significantly higher than in healthy controls. Circulating cf-DNA concentrations at EICU admission and after 24 h were significantly higher in patients with shock who died than in those who survived at least 28 days. Lactate levels at 24 h and APACHE II scores at EICU admission had lower predictive values for 28-day mortality than cf-DNA concentrations. The most accurate predictor of fatal outcome for a patient in the EICU with shock was cf-DNA change over the first 48 h of critical care. The cut-off value for cf-DNA change over 48 h found in the present study may help physicians in deciding which patients with shock should continue with escalating treatment and which can be discharged from the EICU.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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