ORIGINAL ARTICLE

Sustained high serum caspase-3 concentrations and mortality in septic patients

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Abstract Caspase-3 is the main executor of the apoptotic process. Higher serum caspase-3 concentrations in non-survivor compared to survivor septic patients have been found. The objectives of this work (with the increase of sample size to 308 patients, and the determination of serum caspase-3 concentrations also on days 4 and 8 of diagnosis of severe sepsis) were to know whether an association between serum caspase-3 concentrationss during the first week, degree of apoptosis, sepsis severity, and sepsis mortality exists. We collected serum samples of 308 patients with severe sepsis from eight intensive care units on days 1, 4 and 8 to measure concentrations of caspase-3 and caspase-cleaved cytokeratin (CCCK)-18 (to assess degree of apoptosis). End point was 30-day mortality. We found higher serum concentrations of caspase-3 and CCCK-18 in non-survivors compared to survivors on days 1 (p < 0.001), 4 (p < 0.001), and 8 (p < 0.001). We found an association between serum caspase-3 concentrations on days 1, 4 and 8 of severe sepsis diagnosis and serum CCCK-18 concentrations (p < 0.001), SOFA (p < 0.001), serum acid lactic concentrations

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(p < 0.001), and 30-day sepsis mortality (p < 0.001). The new findings of this work were that an association between serum caspase-3 concentrations during the first week, apoptosis degree, sepsis severity, and sepsis mortality exists.

Introduction

Severe sepsis leads to burden of health resources and many deaths annually [1, 2]. Cell death by apoptosis occurs during sepsis. Apoptosis is initiated mainly through two pathways, by death receptors (or extrinsic pathways) or by mitochondrial contribution (or intrinsic pathways). In both pathways, caspase-3 is activated and leads to cell death [3–6].

An increase of caspase-3 activity in different body sites in septic animal models has been found [7–18]. Besides, higher caspase-3 activity has been found in lymphocytes of septic patients compared to healthy controls [19–21], in spleen samples of septic patients (post mortem obtained) comparted to non-septic patients (obtained from emergent splenectomy due to bleeding) [22], and in plasma of septic patients compared to non-septic patients [23]. In addition, another study found higher serum caspase-3 levels over 72 h in septic patients with decreased lactate clearance, and higher mortality rate in those patients with lower lactate clearance [24]. In addition, we previously determined serum caspase-3 concentrations in 216 severe septic patients at the time of diagnosis [25], and an association between serum caspase-3 concentrations and 30-day mortality was found. The objectives of this work (with the increase of sample size to 308 patients, and the determination of serum caspase-3 concentrations also on days 4 and 8 of diagnosis of severe sepsis) were to know whether an association between serum caspase-3 concentrations during the first week, degree of apoptosis, sepsis severity, and sepsis mortality exists. If those associations were found, then serum caspase-3 concentration determination could be proposed to estimate the prognosis of those patients, and could open interest for research about agents that modulate caspase-3 activation in those patients.

Methods

This was a multicenter study carried out with 308 severe septic patients of intensive care units from eight Spanish hospitals: H General de La Palma (La Palma), HU Insular (Las Palmas de Gran Canaria), H Quirón (Santa Cruz de Tenerife), HU de Valencia (Valencia), HU de Canarias (La Laguna), H San Jorge (Huesca), HU Dr. Negrín (Las Palmas de Gran Canaria), and HU Nuestra Señora de Candelaria (Santa Cruz de Tenerife). Ethic review boards from each hospital approved the project. Patients or their legal guardians signed the informed consent.

We included patients with severe sepsis on the basis of the International Sepsis Definitions Conference [26]. We excluded patients with human immunodeficiency virus (HIV), white blood cell count <1000/μL, solid or hematological tumor, steroid, immunosuppressive or radiation therapy, pregnancy, lactation or age < 18 years.

Previously, we determined other circulating biomarkers in some of those patients [27–31]. In a previous work, we analyzed serum caspase-3 concentrations in 216 severe septic patients at the time of diagnosis [25]. In this work, we determined serum caspase-3 concentrations also on days 4 and 8 of diagnosis of severe sepsis in 308 patients.

The following variables were collected: age, acute physiology and chronic health evaluation II (APACHE II) score [32], activated partial thromboplastin time (aPTT), bloodstream infection, bilirubin, caspase-3, caspase-cleaved cytokeratin (CCCK)-18, chronic renal failure (defined as glomerular filtration rate < 60 ml/min/1.73 m^2), chronic obstructive pulmonary disease (COPD), diabetes mellitus, creatinine, empiric antimicrobial treatment, international normalized ratio (INR), lactic acid, ischemic heart disease, leukocytes, microorganism responsible, pressure of arterial oxygen/fraction inspired of oxygen (PaO$_2$/FIO$_2$), platelets, sepsis-related organ failure assessment [SOFA] score [33], site of infection, and sex. We considered mortality at 30 days as the end point.

Serum blood samples at days 1, 4 and 8 were taken to measure concentrations of caspase-3 and CCCK-18 serum levels, and were stored at −80 °C until the determination moment in the Laboratory Department of the Hospital Universitario de Canarias (La Laguna, Tenerife, Spain). Enzyme-linked immunosorbent assay (ELISA) assays were used to measure caspase-3 with Human Caspase 3 Elisa BlueGene Biotech® kit (Shanghai, China) and CCCK-18 with M30 Apoptosense® ELISA, PEVIVA AB kit (Bromma, Sweden). For caspase-3, the intra- and inter-assay coefficients of variation (CV) were <5.6% and <7.9%, respectively, and the detection limit was 0.1 ng/mL. For CCCK-18, the intra- and inter-assay CV were <10%, and the detection limit was 25 U/L. Serum concentrations of CCCK-18 levels were determined to assess degree of apoptosis [27, 34–36].

We used frequencies (and percentages) and chi-square test to report and compare categorical variables, and medians (and interquartile ranges) and Mann-Whitney U test to report and compare continuous variables. Receiver operating characteristic (ROC) analyses with serum concentrations of caspase-3 at days 1, 4 and 8, and survival at 30 days were carried out; and we used the Youden J index to select the prognostic cut-off value for each day. Kaplan-Meier 30-day survival curves using cut-off values of serum caspase-3 concentrations (selected according to Youden J index) of 0.22 ng/mL on day 1, 0.16 ng/mL on day 4, and 0.14 ng/mL on day 8 were carried out. Logistic regression analyses were carried out to determine whether an association between 30-day mortality and serum concentrations of caspase-3 on days 1, 4 and 8 exists.

Table 1 Baseline clinical and biochemical characteristics of survivor and non-survivor patients

Characteristic	Survivors (N = 206)	Non-survivors (N = 102)	P-value
Age, median years (p 25–75)	60 (47–69)	65 (55–74)	0.003
APACHE-II score, median (p 25–75)	19 (15–23)	24 (19–29)	<0.001
aPTT, median seconds (p 25–75)	32 (28–39)	36 (29–45)	0.01
Bilirubin (mg/dl), median (p 25–75)	0.88 (0.50–1.40)	0.97 (0.50–2.33)	0.22
Bloodstream infection, n (%)	28 (13.6)	16 (15.7)	0.61
Caspase-3, median ng/mL (p 25–75)	0.12 (0.10–0.23)	0.41 (0.23–0.49)	<0.001
CCCK-18, median u/L (p 25–75)	299 (242–398)	474 (326–694)	<0.001
Chronic renal failure, n (%)	12 (5.8)	13 (12.7)	0.046
COPD, n (%)	28 (13.6)	11 (10.8)	0.59
Creatinine (mg/dl), median (p 25–75)	1.30 (0.80–2.10)	1.66 (1.00–2.80)	0.01
Diabetes Mellitus, n (%)	51 (24.8)	40 (39.2)	0.01
Empiric antimicrobial treatment adequate			0.64
Unknown due to negative cultures, n (%)	105 (51.0)	55 (53.9)	
Unknown due to diagnosis by antigenuria, n (%)	15 (7.3)	4 (3.9)	
Adequate, n (%)	82 (39.8)	40 (39.2)	
Inadequate, n (%)	4 (1.9)	3 (2.9)	
INR, median (p 25–75)	1.25 (1.10–1.50)	1.42 (1.15–1.91)	0.004
Ischemic heart disease, n (%)	20 (9.7)	11 (10.8)	0.84
Microorganism responsible			
Unknown, n (%)	105 (51.0)	55 (53.9)	0.63
Gram-positive, n (%)	52 (25.2)	24 (23.5)	0.78
Gram-negative, n (%)	49 (23.8)	23 (22.5)	0.89
Fungi, n (%)	4 (1.9)	4 (3.9)	0.45
Anaerobe, n (%)	2 (1.0)	1 (1.0)	0.99
Lactic acid, median mmol/L (p 25–75)	2.00 (1.10–3.50)	3.40 (1.60–6.00)	<0.001
Leukocytes, median ×10^3/mm^3 (p 25–75)	14.2 (9.2–18.9)	15.0 (7.1–20.6)	0.96
Pa0$_2$/FI0$_2$ ratio, median (p 25–75)	180 (123–271)	170 (104–240)	0.17
Platelets, median × 10^3/mm^3 (p 25–75)	197 (130–270)	129 (61–227)	<0.001
Sex			0.52
Female, n (%)	67 (32.5)	37 (36.3)	
Male, n (%)	139 (67.5)	65 (63.7)	
Site of infection			0.95
Respiratory, n (%)	119 (57.8)	57 (55.9)	
Abdominal, n (%)	55 (26.7)	28 (27.5)	
Urinary, n (%)	12 (5.8)	5 (4.9)	
Skin, n (%)	9 (4.4)	5 (4.9)	
Endocarditis, n (%)	6 (2.9)	5 (4.9)	
Arthritis, n (%)	1 (0.5)	1 (1.0)	
CNS, n (%)	4 (1.9)	1 (1.0)	
Sodium, median mEq/L (p 25–75)	138 (134–142)	137 (133–142)	0.35
SOFA score, median (p 25–75)	9 (7–11)	11 (9–14)	<0.001
Source of sepsis, n (%)			0.59
Community, n (%)	160 (77.7)	78 (76.5)	
Nosocomial extra-UCI, n (%)	23 (11.2)	15 (14.7)	
Nosocomial intra-UCI, n (%)	23 (11.2)	9 (8.8)	

APACHE II acute physiology and chronic health evaluation, *aPTT* activated partial thromboplastin time, *CCCK* caspase-cleaved cytokeratin, *COPD* chronic obstructive pulmonary disease, *INR* international normalized ratio, *PaO$_2$*/FIO$_2$ pressure of arterial oxygen/fraction inspired oxygen, *CNS* central nervous system, *SOFA* sepsis-related organ failure assessment score

We used Spearman's rank coefficient to test the correlation between serum concentrations of caspase-3, CCCK-18, and acid lactic, and SOFA score at days 1, 4 and 8. *P*-values < 0.05 were considered statistically significant, and Bonferroni correction was applied in multiple comparisons. We carried out statistical analyses by NCSS 2000 (Kaysville, UT, USA) and SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Demographic and clinical characteristics in non-survivor (*n* = 102) and survivor patients (*n* = 206) are shown and compared in Table 1. Statistically significant differences between non-survivor and survivor patients were not found on PaO_2/FIO_2 ratio, leukocytes, bilirubin, sex, ischemic heart disease, COPD, bloodstream infection, site of infection, and microorganism responsible for sepsis. However, we found that non-survivors in comparison to survivors had higher rate of diabetes mellitus and chronic renal failure, and lower platelet count. In addition, non-survivors had higher lactic acid, age, INR, aPTT, SOFA score, creatinine, APACHE-II score, serum caspase-3 concentrations, and serum CCCK-18 concentrations. Besides, non-survivors compared to survivors had higher serum concentrations of caspase 3 (Fig. 1) on day 1 (p < 0.001), day 4 (p < 0.001), and day 8 (p < 0.001).

Correlations between serum concentrations of caspase 3, CCCK-18 and acid lactic, and SOFA score during the first

Table 2 Correlations between serum levels of caspase-3, CCCK-18 and lactic acid, and SOFA score during the first week of severe sepsis

Measured variable	Day 1	Day 4	Day 8
CCCK-18 (u/L)	$r = 0.37$	$r = 0.25$	$r = 0.30$
	P < 0.001	P < 0.001	P < 0.001
Acid lactic (mmol/L)	$r = 0.26$	r = 0.25	$r = 0.22$
	P < 0.001	P < 0.001	P < 0.001
SOFA score	$r = 0.20$	$r = 0.21$	r = 0.30
	P < 0.001	P < 0.001	P < 0.001

CCCK caspase-cleaved cytokeratin, *SOFA* sepsis-related organ failure assessment score

P-values < 0.005 are statistically significant after Bonferroni correction

week are shown in Table 2. We found a statistically significant positive association of serum concentrations of caspase 3 with serum concentrations of CCCK-18 (p < 0.001) and acid lactic (p < 0.001), and with SOFA score (p < 0.001) during the first week.

ROC analyses of serum concentrations of caspase-3 on days 1, 4 and 8 as well as 30-day mortality prediction are shown in Table 3 and Fig. 2. We found that serum concentrations of caspase-3 on day 1 (p < 0.001), day 4 (p < 0.001), and day 8 (p < 0.001) predict 30-day mortality.

Logistic regression analyses are shown in Table 4. We found an association between 30-day mortality and serum concentrations of caspase-3 on days 1 (p < 0.001), 4 (p < 0.001) and 8 (p < 0.001) controlling for SOFA and age.

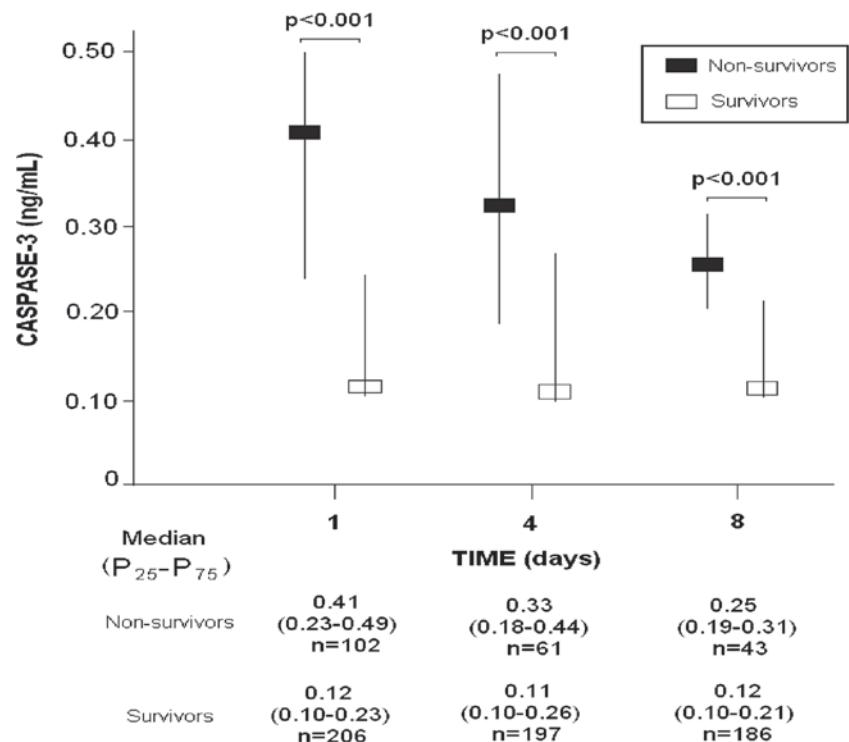**Fig. 1** Serum caspase-3 concentrations on days 1, 4 and 8 in non-survivor and survivor patients

Table 3 Receiver operation characteristic analysis using serum concentrations of caspase-3 at day 1, 4 and 8 as predictor of mortality at 30 days

Measure	Day 1	Day 4	Day 8
Cut-off of serum caspase-3 levels	>0.22 ng/mL	>0.16 ng/mL	>0.14 ng/mL
Sensitivity (95% CI)	78 (69–86)	85 (74–93)	93 (81–98)
Specificity (95% CI)	74 (68–80)	62 (55–69)	61 (53–68)
Positive likelihood ratio (95% CI)	3.1 (2.4–3.9)	2.3 (1.8–2.8)	2.4 (1.9–2.9)
Negative likelihood ratio (95% CI)	0.3 (0.2–0.4)	0.2 (0.1–0.4)	0.2 (0.1–0.3)
Positive predicted value (95% CI)	60 (51–69)	41 (33–50)	35 (27–45)
Negative predicted value (95% CI)	87 (82–92)	93 (87–97)	97 (93–99)

CI confidence intervals

Kaplan-Meier 30-day survival curves with cut-off values of serum concentrations of caspase-3 at days 1, 4 and 8 are shown in Fig. 3. We found that patients with higher serum concentrations of caspase-3 on days 1 ($p < 0.001$), 4 ($p < 0.001$) and 8 ($p < 0.001$) had higher risk of death.

Discussion

The new findings of this work were that an association between serum caspase-3 concentrations during the first week, apoptosis degree, sepsis severity, and sepsis mortality exists.

Previously, higher caspase-3 activity in lymphocytes of septic patients compared to healthy controls [19–21], in spleens of septic patients compared to non-septic patients [22], in plasma of septic patients compared to non-septic patients [23], and in serum of septic patients with decreased lactate clearance [24] have been found. In addition, we previously found higher serum caspase-3 concentrations at time of severe sepsis diagnosis in 30-day non-survivors than in survivor patients, and an association between serum caspase-3 concentrations at severe sepsis diagnosis and 30-day mortality

[25]. Thus, new findings of this work were that 30-day non-survivors had higher serum caspase-3 concentrations during the first week than survivor patients, and that there is an association between serum caspase-3 concentrations during the first week and 30-day mortality.

We found in our previous study that serum caspase-3 levels at severe sepsis diagnosis could be used as 30-day sepsis mortality prediction biomarker [25]. Thus, another new finding of this study was that serum caspase-3 levels on days 4 and 8 of severe sepsis diagnosis also could be used as a 30-day sepsis mortality prediction biomarker according to the results of receiver operating characteristic analysis.

In our previous study, we found that an association between serum concentrations of caspase-3 (as the main executor of apoptosis) and CCCK-18 levels (as biomarker of degree of apoptosis) at the moment of severe sepsis diagnosis exists [25]. Then, another interesting and new finding of this work is the association between serum concentrations of caspase-3 and CCCK-18 during the first week.

Another new finding of this work was that an association between serum concentrations of caspase-3 during the first week and sepsis severity (assess by serum concentration of acid lactic and SOFA score) exists.
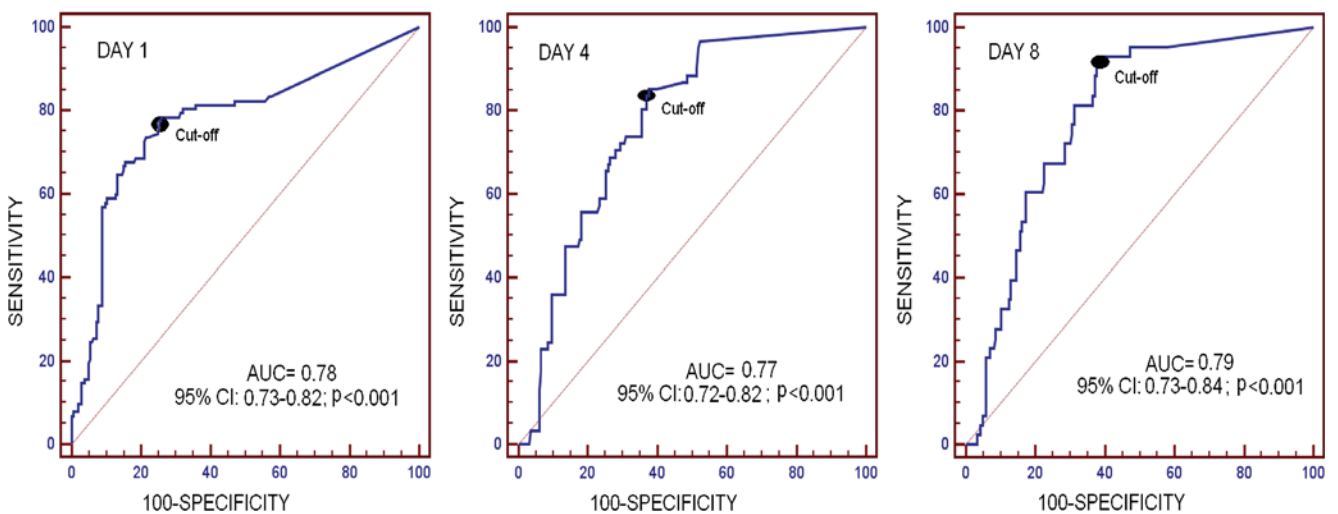

Fig. 2 Receiver operation characteristic curves of serum caspase-3 levels on days 1, 4 and 8 to predict 30-day mortality

Table 4 Multiple logistic regression analyses to predict mortality at 30 days

Measure	Odds ratio	95% confidence interval	p-value
Model: Mortality estimated at day 1			
Caspase-3 levels > 0.22 ng/mL on day 1	9.420	5.229–16.972	<0.001
SOFA at day 1	1.171	1.079–1.270	<0.001
Age (years)	1.022	1.001–1.043	0.04
Model: Mortality estimated at day 4			
Caspase-3 levels > 0.16 ng/mL on day 4	8.274	3.615–18.940	<0.001
SOFA at day 4	1.187	1.092–1.290	<0.001
Age (years)	1.019	0.994–1.045	0.14
Model: Mortality estimated at day 8			
Caspase-3 levels > 0.14 ng/mL on day 8	15.661	4.519–54.276	<0.001
SOFA at day 8	1.189	1.084–1.304	<0.001
Age (years)	1.025	0.996–1.056	0.09

SOFA sepsis-related organ failure assessment

There are mainly two different pathways of cell death by apoptosis: the mitochondrial (or intrinsic) pathway and the death receptor (or extrinsic) pathway [3–6]. The intrinsic pathway could be initiated by reactive oxygen species or by cytokines as interleukin (IL)-1 and IL-6, and could be slowed by IL-10 (through to the activation of Bcl-2 anti-apoptotic family); and afterwards, cytochrome c is released from mitochondria and caspase 3 is activated. The extrinsic pathway is initiated when the TNF ligand superfamily (TNFSF) binds to the TNF membrane receptors superfamily (TNFRSF), such as FasL (Apo-1 L or TNFSF-6 or CD95L) binding to Fas (Apo-1 or TNFRSF-6 or CD95) or TNF-related apoptosis-inducing ligand (TNFSF-10 or TRAIL) binding to its different receptors (TNFRSF-10A to D or TRAILR1 to 4); afterwards, a death signal is generated to cleave pro-caspase-8 generating caspase-8 that activates caspase-3. After the activation of caspase-3, by either of two pathways, the cell death is initiated. Cytokeratin-18 protein, that is present in most parenchymal and epithelial cells, could cleave during apoptosis by caspases

and its CCCK-18 fragments appear afterward in the blood [37, 38]. Thus, we think that the association that we found in our work between serum concentrations of caspase-3 during the first week and sepsis mortality could reflect that patients with higher caspase-3 (main apoptosis executor) have higher degree of apoptosis (with higher serum CCCK-18 concentrations), higher sepsis severity (with higher SOFA score and higher serum acid lactic concentrations) and sepsis mortality.

In an animal model with septic rats, the caspase inhibitors administration have reduced apoptosis and mortality rates [7–9]; thus, all those findings could stimulate interest for research about agents that modulate caspase-3 activation in those patients.

Certain limitations exist in our work, for example, we have not determined other molecules related to apoptosis activation, such as Fas, FasL, and Bcl-2. Besides, we have not determined the degree of apoptosis by other methods, such as annexin-V to assess membrane alterations or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to assess DNA fragmentation.
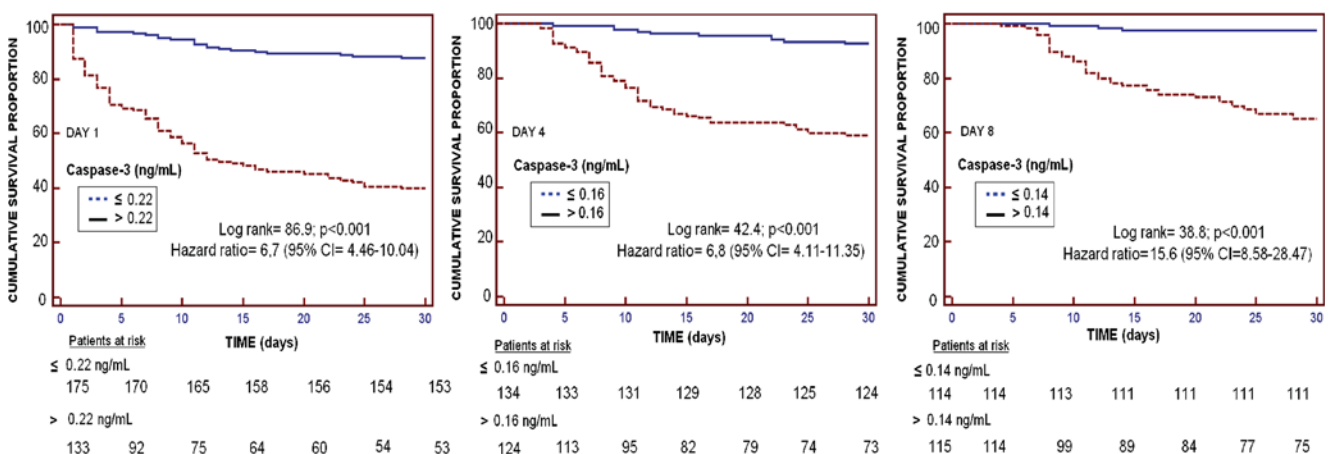

Fig. 3 Kaplan-Meier 30-day survival curves of serum caspase-3 concentrations on days 1, 4 and 8

Conclusions

The new findings of this work were that an association be-tween serum caspase-3 concentrations during the first week, apoptosis degree, sepsis severity, and sepsis mortality exists. Thus, serum caspase-3 concentration determination could be proposed to estimate the prognosis of those patients, and could open the interest for research about agents that modulate caspase-3 activation in those patients.

Author contributions LLo conceived, designed and coordinated the study, participated in acquisition and interpretation of data, and drafted the manuscript.

MMM, ROL, JF, JSV, LLa, CD, SP participated in acquisition of data. APC and AFGR participated in determination of serum concentrations.

AJ participated in the interpretation of data.

All authors revised the manuscript critically for important intellectual content and made the final approval of the version to be published.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The study was approved by the research Ethics Committee of each hospital: H General de La Palma (La Palma), HU Insular (Las Palmas de Gran Canaria), H Quirón (Santa Cruz de Tenerife), HU de Valencia (Valencia), HU de Canarias (La Laguna), H San Jorge (Huesca), HU Dr. Negrín (Las Palmas de Gran Canaria), and HU Nuestra Señora de Candelaria (Santa Cruz de Tenerife).

Informed consent Patients or family members signed the informed consent to participate in the study.

Abbreviations *APACHE*, Acute physiology and chronic health evaluation; *aPTT*, Activated partial thromboplastin time; *COPD*, Chronic obstructive pulmonary disease; *FIO$_2$*, Fraction inspired oxygen; *INR*, International normalized ratio; *ICU*, Intensive care unit; *PaO$_2$*, Pressure of arterial oxygen; *TNF*, Tumor necrosis factor; *SOFA*, Sepsis-related organ failure assessment score

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