

ORIGINAL RESEARCH



Blood concentrations of proapoptotic sFas and antiapoptotic Bcl2 and COVID-19 patient mortality

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ABSTRACT

Background: There are no data on circulating concentrations of sFas (proapoptotic protein of extrinsic pathway) and Bcl2 (antiapoptotic protein of intrinsic pathway) in COVID-19 patients. Thus, our objective study was to determine whether an association exists between serum concentrations of sFas and Bcl2 and COVID-19 patient mortality.

Methods: This observational and prospective study of COVID-19 patients was performed in eight Intensive Care Units (ICU) from Canary Islands (Spain). Serum levels of sFas and Bcl2 at ICU admission were determined. Mortality at 30 days was the end-point study.

Results: Surviving patients ($n = 42$) compared to non-surviving ($n = 11$) had lower APACHE-II ($p < 0.001$), lower SOFA ($p = 0.004$), lower serum sFas levels ($p = 0.001$) and higher serum Bcl2 levels ($p < 0.001$). Logistic regression showed an association between high serum sFas levels and mortality after controlling for APACHE-II (OR = 1.004; 95% CI = 1.101–1.007; $p = 0.01$) or SOFA (OR = 1.003; 95% CI = 1.101–1.106; $p = 0.004$), and between low serum Bcl2 levels and mortality after controlling for APACHE-II (OR = 0.927; 95% CI = 0.873–0.984; $p = 0.01$) or SOFA (OR = 0.949; 95% CI = 0.913–0.987; $p = 0.01$).

Conclusions: Thus, to the best of our knowledge, this is the first study reporting blood levels of sFas and Bcl2 in COVID-19 patients and its association with mortality.

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KEYWORDS

sFas; BCL2; COVID-19; patients; mortality

1. Introduction

Coronavirus disease 2019 (COVID-19), the emerging health threat in the world detected for the first time in December 2019 in Wuhan (China), is produced by the severe acute respiratory syndrome coronavirus 2 (SARSCoV-2). To January 7 of 2021, approximately 87,857,553 cases and 1,895,431 deaths (2.16%) from COVID-19 have been reported in the world [1]. Several prognosis factors have been reported as arterial hypertension, age, cardiovascular diseases, smoking, chronic obstructive pulmonary disease (COPD), diabetes mellitus, kidney dysfunction, cerebrovascular diseases, cardiac injury, coagulation alterations, the development of acute respiratory distress syndrome (ARDS), liver dysfunction, interleukin-6, genetic, red blood cell distribution width, and nitrates [2–9].

Cell death by apoptosis, which is produced by a programmed and actively way, occurs in different physiological processes (tissue remodeling and morphogenesis) and in different diseases as infectious diseases [10]. There are two

main apoptosis pathways, the intrinsic (also names as mitochondrial pathway) and the extrinsic (also named as death receptor pathway). The activation of extrinsic pathway occurs when a tumor necrosis factor ligand superfamily (TNFSF) binding with its tumor necrosis factor membrane receptor superfamily (TNFRSF). The main receptors and ligands of the extrinsic pathway are the Fas receptor and its Fas ligand, and the TNF-related apoptosis-inducing ligand receptors (TRAILR1–4) and its TNF-related apoptosis-inducing ligand (TRAIL). When the TNFSF bindings with its TNFRSF is produced, a death signal leads to activation of pro-caspase-8 in caspase-8 (initiator caspase). Afterward, the activated caspase-8 produces the activation of caspase-3 (executor caspase) producing the cell death by apoptosis [10]. The activation of intrinsic pathway can occur through different stressors (oxidative stress, proinflammatory cytokines, excitotoxicity deficits of growth factors, and genetic mutations) that open mitochondrial transition pores (MTP). Then, cytochrome c exits from mitochondria to cytosol and binds with procaspase-9 and

Article highlights

- Non-surviving showed higher serum sFas levels and lower serum Bcl2 levels
- There is an association between serum levels of sFas and Bcl2 and mortality
- Serum levels of sFas and Bcl2 could be used as mortality biomarker

apoptotic protease activating factor (Apaf)-1 forming apoptosomes. These apoptosomes activate caspase-9 (initiator caspase). Afterward, the caspase-9 activated produces the activation of caspase-3 and 7 (executor caspases) producing the cell death death by apoptotic [10]. There are a family of anti-apoptotic B-cell lymphoma-2 (Bcl-2) proteins that block MTP formation and cytochrome c release from mitochondria to cytosol (Bcl-2, Bcl-XL, Bclw) and a family of pro-apoptotic Bcl-2 proteins that promote MTP formation and cytochrome c release (such as Bax, Bak, Bad, Bim, Bid) [10].

There are scarce data published about apoptosis and COVID-19 patients [11–17]. Higher apoptosis of lymphocytes [11,12] and higher expression of Fas in lymphocytes [13,14] of COVID-19 patients than in control subjects have been found, and this is associated with lymphopenia of COVID-19 patients. In addition, higher blood concentrations of caspase-8 [15], caspase-3 [16], sFasL [16] and caspase-cleaved cytokeratin 18 [17] in COVID-19 patients than in control subjects have been found. However, we have not found data about the association between apoptosis and mortality of COVID-19 patients. We had previously found a higher mortality in septic patients with high serum sFas concentrations [18] and low serum Bcl2 concentrations [19]. Thus, the objective of this study was to analyze whether there is an association between serum concentrations of sFas and Bcl2 and mortality of COVID-19 patients.

2. Methods

2.1. Design and subjects

The patients of this observational and prospective study were recruited during April 2020 in eight intensive care units from six hospitals from Canary Islands (Spain). The study (code CHUC-2020-26) was approved by the Ethics Committee of all hospitals. In the specific context in which the Spain Government forbid patient visits during the recruitment patient study time, the requirement of written informed consent was waived.

We included patients with laboratory confirmation of SARS-CoV-2 by real-time fluorescence reverse transcription-polymerase chain reaction (RT-PCR) from nasopharyngeal swab sample or from bronchial aspirate.

Previously, we have determined HLA genetic polymorphisms [4], serum nitrates [7], and red blood cell distribution width in some of the patients [9]. The objective of our study

was to assess apoptosis by determining the serum concentrations of sFas and Bcl2.

2.2. Determination of serum concentrations of sFas and Bcl2

Serum samples at ICU admission were collected and stored at -80°C until to blood determination time. We used the Human Fas ELISA Kit (Elabscience, Houston, Texas, USA) for the determination of serum sFas concentrations, with an assay detection limit of 19 pg/mL and coefficient of variation of intra-assay and inter-assay lower than 6%. We used the Human Bcl-2 ELISA Kit (Elabscience) for the determination of serum Bcl-2 concentrations, with an assay detection limit of 0.10 ng/mL, and coefficient of variation of intra-assay and inter-assay lower than 6%.

2.3. Variables recorded

We recorded demographic and clinical data at ICU admission as sex, age, body max index, chronic renal failure, diabetes mellitus, COPD, ischemic heart disease, smoking, arterial hypertension, steroid agents, human immunodeficiency virus (HIV), solid tumor, hematological tumor, ARDS [15], Acute Physiology and Chronic Health Evaluation (APACHE)-II score [16] Sepsis-related Organ Failure Assessment [SOFA] score [17], and chest radiography findings. Also, we recorded laboratory data at ICU admission as lactic acid, creatinine, sodium, protein, glucose, creatine kinase, albumin, bilirubin, alanine transaminase, alkaline phosphatase, aspartate transaminase, lactate dehydrogenase, gamma-glutamyl transpeptidase, interleukin-6, ferritin, procalcitonin, C-reactive protein, hemoglobin, hematocrit, white blood cell, lymphocytes, basophils, neutrophils, monocytes, eosinophils, D-dimer, platelets, fibrinogen, activated partial thromboplastin time (aPTT), international normalized ratio (INR), pressure of arterial oxygen (PaO_2), and fraction inspired of oxygen (FIO_2). Besides, we recorded data regarding ICU treatment as tocilizumab, hydroxychloroquine, steroid agents, interferon, lopinavir/ritonavir, and respiratory support. In addition, survival at 30 days was the end point of our study.

2.4. Statistical Methods

Continuous and categorical variables were reported and compared between patient groups using medians (percentile 25–75), frequencies (percentages), Mann–Whitney *U* test and chi-square test and. We used receiver operating characteristic analysis to determine the capability of serum concentrations of sFas and Bcl2 for mortality prediction. Kaplan–Meier 30-day survival curves using serum concentrations of sFas higher and lower than 846.5 ng/mL and serum concentrations of Bcl2 higher and lower than 7.6 ng/mL (cutoffs selected by Youden J index) were performed. We used logistic regression to explore the association between serum concentrations of sFas and Bcl2 and 30-day mortality after controlling by SOFA or APACHE-II. As only 11 patients deceased in our series, we

reported fourth regression models including two predictor variables in each regression model. SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and p -values < 0.05 were used for the statistical analysis and to establish significant differences.

3. Results

Surviving patients ($n = 42$) compared to non-surviving ($n = 11$) had lower APACHE-II ($p < 0.001$) and lower SOFA ($p = 0.004$) (Table 1). Besides, surviving compared to non-surviving patients had lower serum sFas concentration ($p = 0.001$), higher Bcl2 concentration ($p < 0.001$) and higher platelet count ($p = 0.02$) (Table 2).

Logistic regression analyses showed an association between serum sFas levels and 30-day mortality after controlling for APACHE-II (OR = 1.004; 95% CI = 1.101–1.007; $p = 0.01$) or SOFA (OR = 1.003; 95% CI = 1.101–1.106; $p = 0.004$). And also an association between serum Bcl2 levels and 30-day mortality after controlling for APACHE-II (OR = 0.927; 95%

Table 1. Demographic data, clinical data, and treatment of non-surviving and surviving patients.

	Survivors ($n = 42$)	Non-survivors ($n = 11$)	p -Value
Gender female, n (%)	27 (64.3)	7 (63.6)	0.99
COPD, n (%)	5 (11.9)	3 (27.3)	0.34
Smoking, n (%)	2 (4.8)	2 (18.2)	0.19
Chronic renal failure, n (%)	1 (2.4)	0	0.99
Arterial hypertension, n (%)	16 (38.1)	6 (54.5)	0.49
Ischemic heart disease, n (%)	1 (2.4)	1 (9.1)	0.38
Diabetes mellitus, n (%)	12 (28.6)	3 (27.3)	0.99
Solid tumor, n (%)	1 (2.4)	0	0.99
Hematological tumor, n (%)	2 (4.8)	0	0.99
Steroid agents, n (%)	1 (2.4)	2 (18.2)	0.11
Human immunodeficiency virus, n (%)	1 (2.4)	0	0.99
Chest radiography findings, n (%)			0.95
– Consolidation only	5 (11.9)	1 (9.1)	
– Ground glass opacity plus consolidation	21 (50.0)	6 (54.5)	
– Ground glass opacity only	16 (38.1)	4 (36.4)	
ARDS, n (%)	36 (85.7)	9 (81.8)	0.67
Age (years), median (p 25–75)	65 (51–70)	70 (59–75)	0.10
Body max index (kg/m^2), median (p 25–75)	28.1 (24.8–32.4)	27.1 (23.0–30.2)	0.29
APACHE-II score, median (p 25–75)	12 (7–15)	18 (16–20)	< 0.001
SOFA score, median (p 25–75)	5 (3–7)	8 (5–9)	0.004
Respiratory support, n (%)			0.30
– Conventional oxygen therapy	4 (9.5)	0	
– High-flow nasal cannula	4 (9.5)	0	
– noninvasive mechanical ventilation	3 (7.1)	0	
– Invasive mechanical ventilation	31 (73.8)	11 (100)	
Tocilizumab, n (%)	15 (35.7)	6 (54.5)	0.31
Lopinavir/Ritonavir, n (%)	39 (92.9)	10 (90.9)	0.99
Interferon beta-1b, n (%)	26 (61.9)	7 (63.6)	0.99
Hydroxychloroquine, n (%)	39 (92.9)	11 (100)	0.99
Steroid agents, n (%)	31 (73.8)	9 (81.8)	0.71

COPD, chronic obstructive pulmonary disease; APACHE, Acute Physiology and Chronic Health Evaluation; SOFA, Sepsis-related Organ Failure Assessment; ARDS, acute respiratory distress syndrome.

Table 2. Laboratory data at ICU admission of non-surviving and surviving patients.

	Survivors ($n = 42$)	Non-survivors ($n = 11$)	p -Value
Serum sFas levels (ng/mL), median (p 25–75)	223 (109–475)	1000 (314–1000)	0.001
Serum Bcl-2 levels (ng/mL), median (p 25–75)	83.8 (18.8–121.3)	6.4 (5.0–31.2)	< 0.001
Glucose (g/dL), median (p 25–75)	168 (122–208)	160 (135–271)	0.46
Lactic acid (mmol/L), median (p 25–75)	1.33 (1.09–1.80)	1.60 (1.30–2.20)	0.11
Sodium (mEq/L), median (p 25–75)	138 (134–141)	140 (135–144)	0.20
Creatine kinase (U/L), median (p 25–75)	152 (43–286)	200 (50–1467)	0.51
Protein (g/L), median (p 25–75)	6.4 (5.8–7.1)	6.0 (5.6–7.0)	0.60
Albumin (g/L), median (p 25–75)	3.0 (2.6–3.5)	3.0 (2.3–3.7)	0.94
Creatinine (mg/dl), median (p 25–75)	0.87 (0.68–1.03)	1.07 (0.72–1.73)	0.23
Total bilirubin (mg/dl), median (p 25–75)	0.62 (0.48–1.20)	0.59 (0.35–1.23)	0.65
Alanine transaminase (U/L), median (p 25–75)	38 (27–75)	34 (14–48)	0.14
Aspartate transaminase (U/L), median (p 25–75)	37 (29–77)	40 (19–123)	0.79
Lactate dehydrogenase (U/L), median (p 25–75)	353 (284–463)	418 (263–556)	0.58
Gamma-glutamyl transpeptidase (U/L), median (p 25–75)	61 (39–132)	84 (33–447)	0.91
Alkaline phosphatase (U/L), median (p 25–75)	58 (50–73)	67 (41–96)	0.99
Ferritin (ng/ml), median (p 25–75)	1039 (653–1817)	1383 (859–2761)	0.50
Procalcitonin (ng/ml), median (p 25–75)	0.17 (0.08–0.48)	0.58 (0.06–0.76)	0.49
Interleukin-6 (pg/ml), median (p 25–75)	50 (6–179)	61 (24–140)	0.77
C-reactive protein (mg/gl), median (p 25–75)	20 (10–76)	24 (18–67)	0.34
Hemoglobin (g/dL), median (p 25–75)	12.8 (11.7–14.4)	12.8 (11.0–15.0)	0.95
White blood cell, median $\times 10^3/\text{mm}^3$ (p 25–75)	7.7 (6.0–11.6)	7.9 (5.3–13.1)	0.95
Neutrophils, median $\times 10^3/\text{mm}^3$ (p 25–75)	7.2 (4.9–10.2)	7.4 (4.3–10.4)	0.90
Lymphocytes, median $\times 10^3/\text{mm}^3$ (p 25–75)	0.66 (0.50–0.90)	0.54 (0.40–1.28)	0.44
Eosinophils, median $\times 10^3/\text{mm}^3$ (p 25–75)	0.00 (0.00–0.02)	0.02 (0.00–0.02)	0.45
Monocytes, median $\times 10^3/\text{mm}^3$ (p 25–75)	0.37 (0.23–0.58)	0.46 (0.18–0.58)	0.66
Basophils, median $\times 10^3/\text{mm}^3$ (p 25–75)	0.01 (0.00–0.03)	0.01 (0.01–0.03)	0.69
D-dimer (ng/mL), median (p 25–75)	1102 (744–2202)	3516 (682–21,480)	0.21
Fibrinogen (mg/dl), median (p 25–75)	711 (506–829)	699 (600–910)	0.49
Platelets, median $\times 10^3/\text{mm}^3$ (p 25–75)	246 (173–383)	158 (108–278)	0.02
aPTT (s), median (p 25–75)	27 (25–32)	30 (23–36)	0.52
INR, median (p 25–75)	1.17 (1.06–1.36)	1.18 (1.02–1.32)	0.83
PaO ₂ /FIO ₂ ratio, median (p 25–75)	133 (103–201)	111 (100–140)	0.30

aPTT, activated partial thromboplastin time; INR, international normalized ratio; PaO₂, pressure of arterial oxygen; FIO₂, fraction inspired of oxygen.

Table 3. Multiple logistic regression analyses to predict mortality at 30 days.

	Odds ratio	95% Confidence interval	p-Value
Model 1			
APACHE-II (points)	1.349	1.071–1.700	0.011
Serum sFas levels (ng/mL)	1.004	1.101–1.007	0.010
Model 2			
APACHE-II (points)	1.720	1.112–2.661	0.015
Serum Bcl2 levels (ng/mL)	0.927	0.873–0.984	0.013
Model 3			
SOFA score (points)	1.665	1.119–2.478	0.012
Serum sFas levels (ng/mL)	1.003	1.001–1.006	0.004
Model 2			
SOFA score (points)	1.915	1.192–3.075	0.007
Serum Bcl2 levels (ng/mL)	0.949	0.913–0.987	0.010

APACHE, Acute Physiology and Chronic Health Evaluation; SOFA, sepsis-related Organ Failure Assessment.

CI = 0.873–0.984; $p = 0.01$) or SOFA (OR = 0.949; 95% CI = 0.913–0.987; $p = 0.01$) (Table 3).

We found that the area under curve (AUC) for mortality prediction by serum sFas levels was 82% (95% CI = 69–96%; $p < 0.001$) (Figure 1), by serum Bcl2 levels was 87% (95% CI = 75–98%; $p < 0.001$) (Figure 2), by APACHE II was 85% (95% CI = 75–96%; $p < 0.001$), and by SOFA was 80% (95% CI = 66–94%; $p < 0.001$). No significant differences were found in the AUC between serum sFas levels and SOFA ($p = 0.92$), serum Fas levels and APACHE-II ($p = 0.58$), serum Bcl2 levels and SOFA ($p = 0.59$), serum Bcl2 levels and APACHE-II ($p = 0.93$), and between serum levels of sFas and Bcl2 ($p = 0.62$).

Serum sFas levels cutoff of 846.5 ng/mL for mortality prediction had specificity 88% (74%–96%), sensitivity 73% (39–

94%), negative likelihood ratio 0.3 (0.1–0.8), negative predictive value 93% (82–97%), positive likelihood ratio 6.1 (2.5–15.0), and positive predictive value 62% (39–80%). Serum Bcl2 levels cutoff of 7.6 ng/mL for mortality prediction had specificity 93% (81%–98%), sensitivity 73% (39%–94%), negative likelihood ratio 0.3 (0.1–0.8), negative predictive value 93% (83–97%), positive likelihood ratio 10.2 (3.2–32.1), and positive predictive value 73% (46–89%).

Kaplan–Meier analysis showed higher mortality rate in patients with serum sFas levels >846.5 ng/mL (hazard ratio = 37.0; 95% CI = 8.0–170.3; $p < 0.001$) (Figure 3) and with serum Bcl2 levels <7.6 ng/mL (hazard ratio = 122.9; 95% CI = 22.5–671.5; $p < 0.001$) (Figure 4).

4. Discussion

To the best of our knowledge, our study is the first one describing blood levels of sFas and Bcl2 in patients with COVID-19. The main new findings of our study were the association of high serum sFas concentrations and low Bcl2 concentrations with mortality of COVID-19 patients. Another interesting finding in our study was the similar mortality prediction by serum levels of sFas and Bcl2 than by SOFA and APACHE-II, and those findings could help in the prognosis estimation of those patients to clinicians.

Recently, higher Fas expression on CD4 + T and CD8 + T cells has been reported in COVID-19 patients than in healthy controls [13,14]; however, the association between serum sFas concentrations and COVID-19 mortality is a new finding of our study. We have no found data in respect to Bcl2 in COVID-19 patients; thus, the association between serum Bcl2

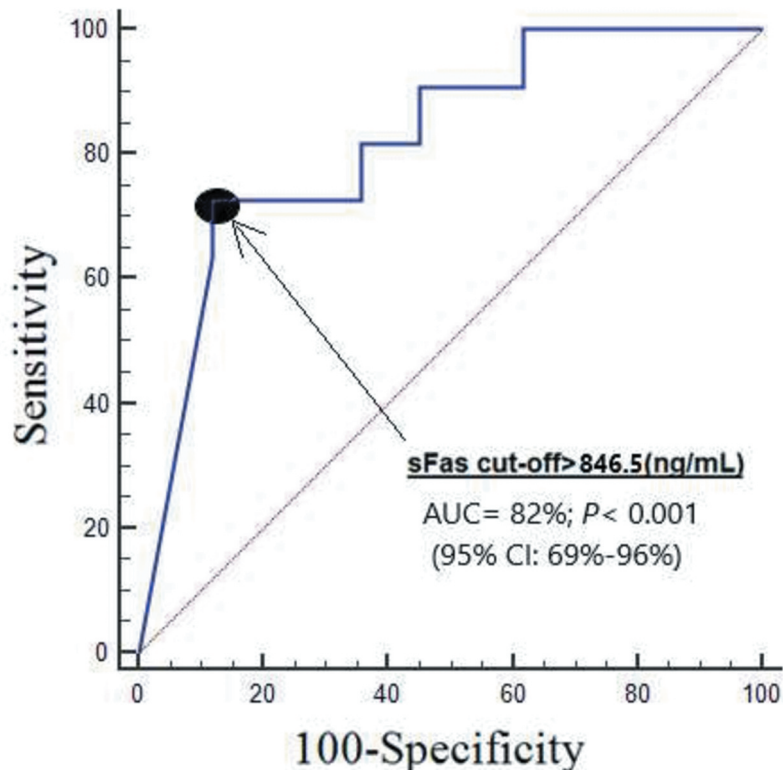


Figure 1. Receiver operating characteristic analysis using serum sFas concentration for prediction of mortality at 30 days.

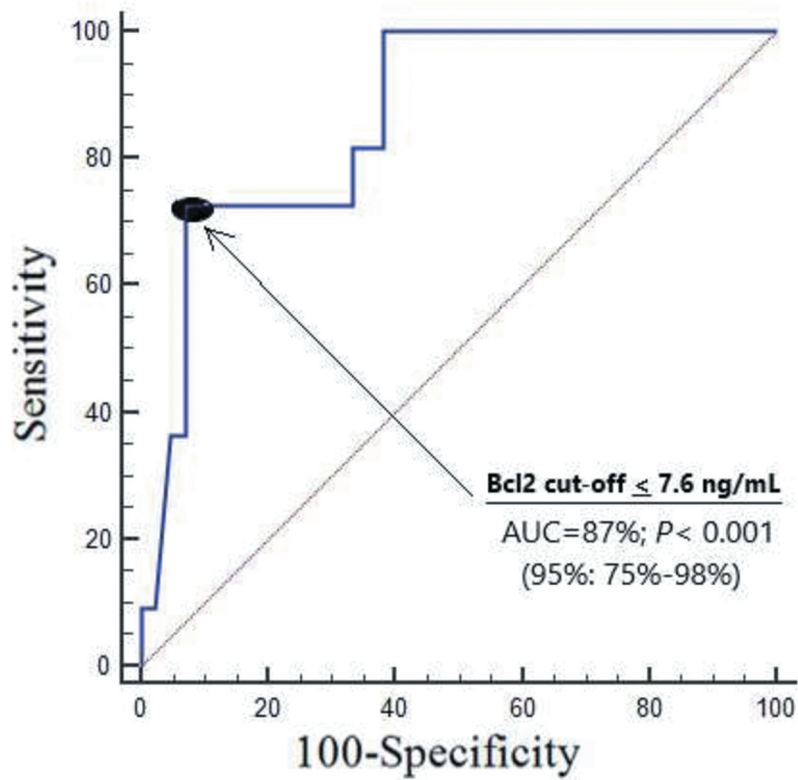


Figure 2. Receiver operating characteristic analysis using serum Bcl2 concentration for prediction of mortality at 30 days.

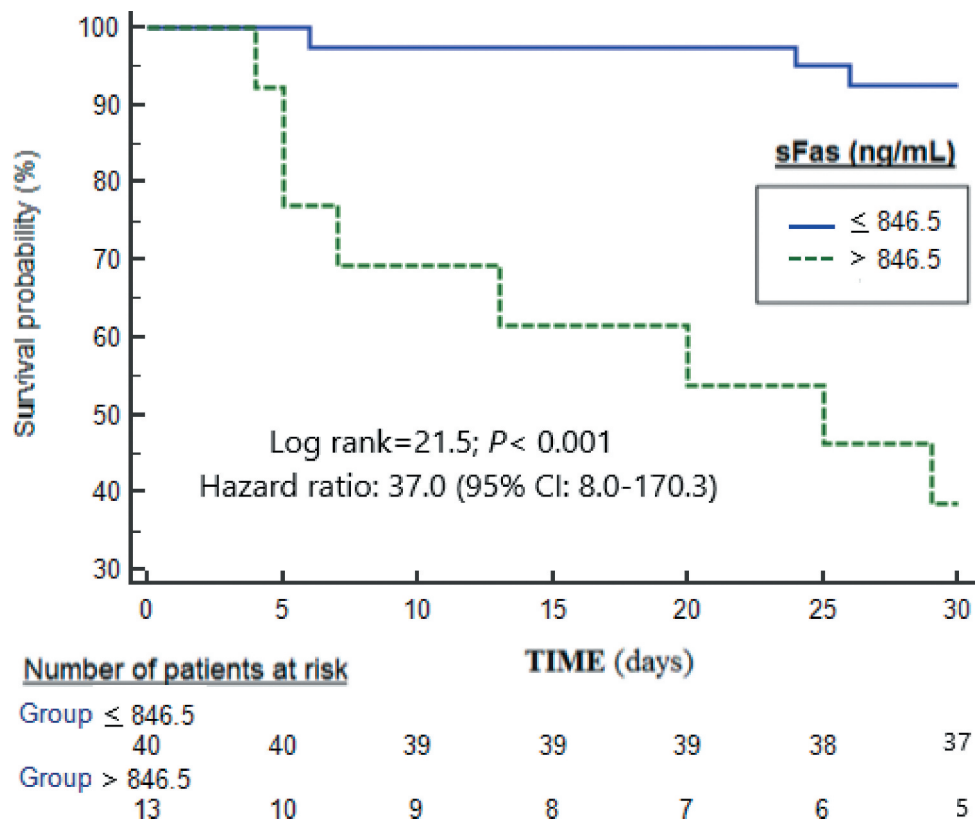


Figure 3. Survival curves at 30 days using serum Fas concentrations lower or equal vs higher than 846.5 ng/mL.

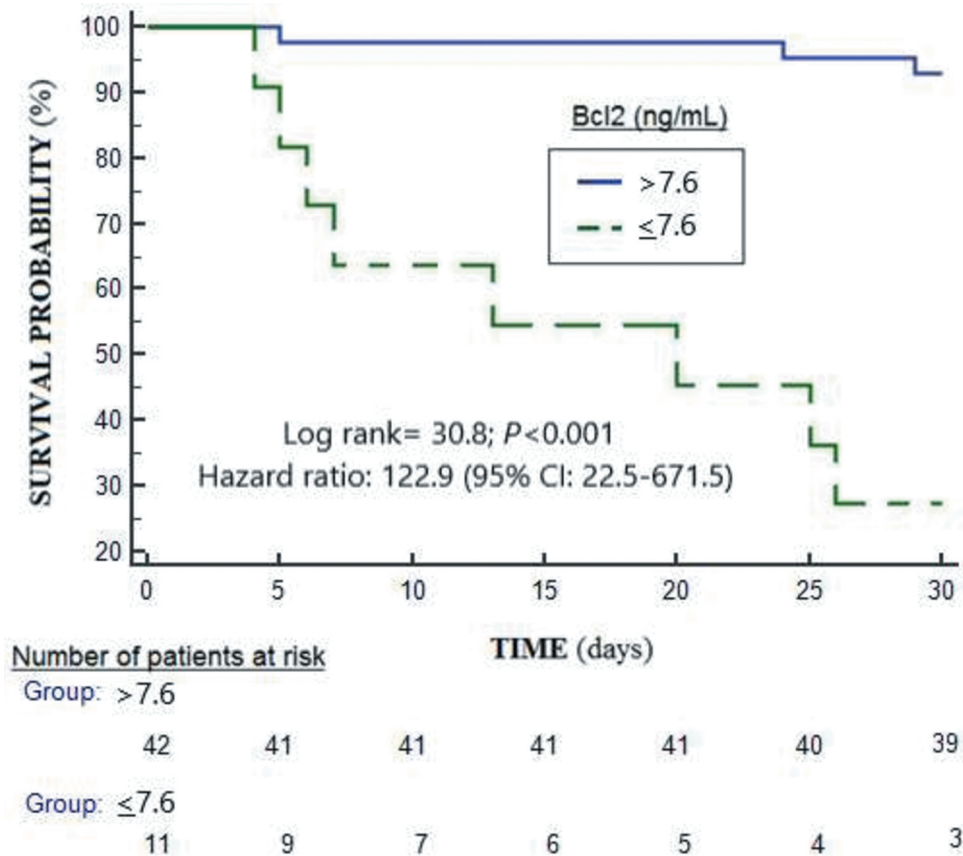


Figure 4. Survival curves at 30 days using serum Bcl2 concentrations lower or equal vs higher than 7.6 ng/mL.

concentrations and COVID-19 mortality is another new finding of our study.

Fas, one of the main death receptors of apoptosis extrinsic pathway, activates apoptosis when binding with its ligand. Then, a death signal is generated that will activate caspase-8 and then will activate caspase-3 leading to cellular damage by extrinsic apoptosis [10]. Bcl-2, one member of the anti-apoptotic Bcl-2 family, blocks MTP formation that will avoid the release of cytochrome c from mitochondria to the cytosol. Then, the formation of the apoptosis (between cytochrome c, Apaf-1 and procaspase-9) is blocked and therefore also the activation of caspase-9 is blocked. Thus, the activation of executor caspases (caspase-3 and -7) is decreased [10]. We think, that those higher serum sFas concentrations and lower blood Bcl-2 concentrations that we found in non-surviving patients compared to surviving patients contribute to higher apoptosis activation by the extrinsic pathway (due to high serum concentrations of proapoptotic sFas protein) and by the intrinsic pathway (due to low serum concentrations of antiapoptotic Bcl2 protein), which contribute in a higher activation of effector caspases, in a higher cellular damage by apoptosis, and finally in the death of patients.

In septic animals, the use of different antiapoptotic agents has reduced apoptosis [23–27]. The use of small interfering RNA (siRNA) against Fas has reduced apoptosis and increased survival [23–26], and the use of adrenomedullin has increased Bcl-2 expression and decreased apoptosis [27].

We recognize some limitations of our study. First, no serum levels of sFas and Bcl2 were determined in healthy subjects and in patients with other respiratory diseases; however, our objective study was to analyze whether serum concentrations of sFas and Bcl2 could help in the prognosis estimation of COVID-19 and not whether serum concentrations of sFas and Bcl2 could help in the diagnosis of COVID-19. Second, no assays to assess cellular damage by apoptosis were performed as Annexin V or terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL). Third, we performed regression analysis with only two variables due to the low number of deceased patients in our series. Fourth, another limitation was the reduced sample size of our study that could contribute in the absence of differences between survivor and non-survivor patients in other variables that have been found to be associated with the prognosis of COVID-19 patients in different meta-analyses [28,29].

However, we think that our study could have some strengths. First, the association between serum levels of sFas and Bcl2 and mortality is present in all regression models (controlling for SOFA or APACHE-II). Second, those high serum sFas concentrations and low serum Bcl2 concentrations in non-survivor patients are in line with the poor prognosis found in septic patient patients [18,19]. We think all those findings could motivate the research on apoptosis in COVID-19 patients.

5. Conclusions

To the best of our knowledge, this is the first study reporting blood levels of sFas and Bcl2 in COVID-19 patients and its association with mortality. However, further study should be done to confirm the results of our study due to its limited sample size.

Abbreviations

APACHE II = Acute Physiology and Chronic Health Evaluation
 aPTT = activated partial thromboplastin time
 ARDS = acute respiratory distress syndrome
 COPD = chronic obstructive pulmonary disease
 FIO₂ = fraction inspired oxygen
 GCS = Glasgow Coma Scale
 INR = international normalized ratio
 NTproBNP = N-terminal prohormone of brain natriuretic peptide
 PaO₂ = pressure of arterial oxygen
 SOFA = Sepsis-related Organ Failure Assessment

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Authors' contributions

L Lorente conceived, designed and coordinated the study, participated in acquisition and interpretation of data, and drafted the manuscript. MM Martín, AF González-Rivero, A Pérez-Cejas, M Argueso, A Perez, L Ramos, JS Violán, JA Marcos y Ramos and N Ojeda participated in acquisition of data. A Jiménez participated in the interpretation of data. All authors revised the manuscript critically for important intellectual content, made the final approval of the version to be published, and were agreed to be accountable for all aspects of the work.

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