Bradykinin and Galectin-3 in Survived and Died Patients With Covid-19 Pneumonia: An Increasingly Promising Biochemical Target

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Abstract

Still, there are no curative or preventive strategies for COVID-19, noting the importance of understanding the pathogenesis of COVID-19 infections for the rational development of pharmacological protocols.

Considering the proinflammatory, oxidative and other roles of BK and Galectin-3, it is more than relevant to investigate the clinical repercussion of that molecules in patients who survived and died after COVID-19 confirmed pneumonia. This was prospective cross-sectional study which included 47 adult patients with confirmed SARS-CoV-2 infection and with criteria for hospital treatment who are admitted to tertiary Clinical Center “Bezanijska kosa” during June of 2021. Our study strongly supports the bradykinin storm hypothesis which underlying in many of COVID-19 fatal outcomes. In that sense, future therapeutical strategies must be focused on reducing a bradykinin serum concentration in COVID-19 patients. “Bradykinin storm” and oxidative stress in patients with fatal outcome are probably responsible for the overactive inflammatory response in COVID-19 patients and resulting symptoms. Understanding the mechanisms by which the two storms, bradykinin and cytokine, affect the body, separate or together, is crucial to mitigating the pandemic’s effect, saving lives, and producing effective treatments that we presently lack.

Introduction

The coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 viral infection has resulted in more than 31 million infected people and more than one million of deaths over world (1). SARS-CoV-2 is characterized by four main structural proteins that are important for infectivity and replication. These proteins include the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. The specific structure is one of the reasons for the various clinical manifestations of these infectious disease (2). The spectrum of COVID-19 disease ranges from asymptomatic to critical including mortality (2). The pathology of COVID-19 usually occurs as moderate or severe respiratory syndrome, in 81% case was classified as mild and in 14% as severe diseases (2). In some cases, other clinical features are reported, such as coagulation disturbance, multiple organ failure, shock and death (3).

Pathophysiology process of SARS-CoV-2 infection is based on disturbance of laboratory markers and decreasing of respiratory function, but also with venous and arterial thrombosis (4). The main enzyme which is used by virus is angiotensin converting enzyme-2 (ACE-2) and its receptor, which is dominant present in blood vessels, lungs, heart, kidneys and other tissues (5).

Bradykinin (BK) is a hypotensive cytokine that is mentioned in previous studies as a potential contributing factor in pathophysiology of COVID-19 disease (7). “Bradykinin storm” is an imbalance of bradykinin caused by dysregulation of kallikrein-kinin system (KKS). From its discovery from snake venom, bradykinin explained many physio-pathological phenomenon in inflammatory conditions. BK is a regulator of tissues blood flow and vasomotor activity and it is additional element of renin-angiotensin

system (RAS). On the other hand, in very high concentrations, BK has a prominent role in the inflammatory and oxidative process (7).

Galectin – 3 a beta-galactoside-binding lectin is described as a marker of lung disease and important contributor of COVID-19 disease (8). Its role is on the immune system response, such as modulating immune cells lifecycle, angiogenesis and reparative injury in lungs. During SARS-CoV-2 viral infection, galectin-3 could make easing viral entrance into immune cells of host and enhance cytokine production and release (9, 10). Galectin-3, also known as Mac-2, L29, CBP35, and etaBP, is a secreted lectin that acts in anti-microbial immunity by pathogen opsonization, macrophage recruitment, and the activation of mast cells and neutrophils. It can also contribute to chronic inflammation and fibrosis (8–10).

On the other hand, very important is host response, based on immune response and antibody releasing (11, 12). Infection with SARS-CoV-2 has been also shown to cause hypoxaemia. These changes lead to accumulation of oxygen free radicals, changes in intracellular pH, accumulation of lactic acid, electrolyte changes and further cellular damage (13–17). We know that different virus employs different mechanisms to induce redox imbalance and oxidative stress (17). In COVID-19 disease and respiratory disease underlying, production of Reactive oxygen Species (ROS) are expected and all patients with pulmonary disease are affected with chronic oxidative stress (18). Pulmonary alveolar macrophages produce ROS usually but in small amount, but during their higher activity it is expected to producing more than usually. Also, immune cells represent a huge source of ROS, where by activating the nitric oxidase – 2 starts producing the ROS in viral infections. Well, viral pneumonia caused by SARS-CoV-2 induce overactivation of immune response in the lungs and this process is accompanied by oxidative stress (19, 20).

Connection between oxidative stress and inflammatory response during COVID-19 disease is a result of activity of endothelial cells and immune response at the same time. The authors suggested that regulation the endothelial function could be a way for preventing the cytokine storm (20, 21).

Still, there are no curative or preventive strategies for COVID-19, noting the importance of understanding the pathogenesis of COVID-19 infections for the rational development of pharmacological protocols.

Considering the proinflammatory, oxidative and other roles of BK and Galectin-3, it is more than relevant to investigate the clinical repercussion of that molecules in patients who survived and died after COVID-19 confirmed pneumonia. Several studies revealed that the hyperinflammatory response induced by SARS-CoV-2 infection and acute pneumonia is a major factor risk for severe form of disease and death. Still, there is no exactly information does and how cytokine bradykinin and peptide galectin-3 are involved in the bad prognosis of COVID-19 pneumonia and fatal outcome. These finding could be a promising therapeutical target and independent predictor of patient's survival.

Patients And Methods

3.1. Ethical concerns

This study was approved from the Local Institutional Committee Clinical Center “Bezanijska kosa” in Belgrade Serbia (reference number 6609). All procedures were done in accordance with the Declaration of Helsinki (revision 2013) and with Good Clinical Practice. From all individual participants was obtained written and informed consent before inclusion in study.

3.2. Design of study

This was prospective cross-sectional study which included 47 adult patients with confirmed SARS-CoV-2 infection and with criteria for hospital treatment who are admitted to tertiary Clinical Center “Bezanijska kosa” during June of 2021. The main inclusion criteria were old above 18 years; confirmed COVID pneumonia; voluntary participation in study. All patients were managed with supportive care and specific pharmacological protocols created by the hospital’s COVID-19 management guidelines committee in accordance with the Ministry of Health Republic of Serbia.

3.3. Diagnosis of COVID-19 pneumonia

From all participants was used a sample by swabbing nose and throat for confirmation of SARS-CoV-2 viral infection by reverse transcriptase polymerase chain reaction (RT-PCR). COVID-19 pneumonia was defined as an adult with fever or suspected respiratory infection plus one of the following signs: respiratory rate > 30 breaths/min, severe respiratory distress, SpO_2 < 90% at room air according to the definition of WHO (22).

3.4. Laboratory data collection at admission

From all patients at admission were collected demographic data, comorbidities and laboratory parameters. Laboratory parameters included complete hemogram, neutrophile-lymphocyte ratio, blood sugar, serum ferritin, coagulation status and D dimer, serum Lactate dehydrogenase and troponin, renal function test, liver function test, electrolyte balance. Treatment details are also collected such as use of steroids, anticoagulants, high-flow nasal cannula and noninvasive ventilation.

3.5. Systemic Oxidative stress markers

From plasma samples of COVID-19 patients we measured following biomarkers of oxidative stress: superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), nitrites (NO_2^-), index of lipid peroxidation measured as TBARS (thiobarbituric acid reactive substances), and activity enzymes: catalase (CAT), superoxide-dismutase (SOD) and reduced glutathione (GSH). All mentioned biochemical parameters of oxidative stress were determined spectrophotometrically (*Shimadzu UV-1800 spectrophotometer, Japan, manufacturer number 00182*).

O_2^- concentrations were determined according to Auclair, by using the NTB (Nitro Blue Tetrazolium) reagent in TRIS buffer (assay mixture) with the sample, while the measurement was performed at a wavelength of 530 nm (23).

The determination of H_2O_2 was based on the oxidation of phenol red by H_2O_2 which is catalysed by horseradish peroxidase. The level of H_2O_2 was then measured at 610 nm of wavelength (24).

Index of lipid peroxidation in the blood samples was estimated via measuring of TBARS using 1% thiobarbituric acid (TBA) in 0.05M sodium hydroxide (NaOH) incubated with the sample at 100 C for 15 min and then measured at 530 nm (25).

Nitrites level was measured in order to indirectly asses nitric oxide level. Nitrites were quantified by method according to Green using the Griess-reagent (26). The sample was precipitated with 30% sulfo-salicylic acid, vortexed for 30 min, and centrifuged at 30009g. Equal volumes of the supernatant and Griess's reagent, containing 1% sulfanil-amide in 5% phosphoric acid/0.1% napthalene ethylenediamine-dihydrochloride, were added and incubated for 10 min in the dark and measured at 543 nmol/l.

Determination of antioxidant enzyme CAT was carried out according to Aebi`s method (27). CAT buffer, prepared lysate sample, and 10 mM H_2O_2 were used for CAT determination. The activity of CAT was measured spectrophotometrically at 360 nm of wave length and was expressed in nmol/ml plasma.

SOD activity was evaluated by using epinephrine method according to Beutler (28). The sample was first mixed with carbonate buffer, and afterwards epinephrine was added to the mixture. SOD activity was measured at 470 nm of wave length and was expressed as U/ml plasma.

The level of GSH was determined according to Beutler (29). The method involves the reaction of GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid. The level of GSH was measured spectrophotometrically at 420 nm of wave length and was expressed in nmol/ml plasma.

3.6. Enzyme-linked immunosorbent assay for bradykinin and galectin-3 (ELISA)

The serum bradykinin level was measured using a bradykinin ELISA kit (ab136936, Abcam) following the manufacturer's instructions. All samples were performed in duplicate. The optical density (OD) is measured at a wavelength of 450 nm on a microtiter plate reader (UT-2100C, MRC, UK, manufacturer number 452104038IEX).

Ninety-six-well microplates were pre-coated with capture polyclonal antibody (goat) to human galectin-3 (Human galectin-3 ELISA solid Phase Sandwich Elisa, R&D system, Inc) and washed three times with wash buffer 1% Tween 20 (Sigma–Aldrich, St. Louis, MO) in PBS. Samples (50 ll) were added in duplicate to each well, which contained 50 ll of sample diluent. Detection antibody diluted in Reagent Diluent was then added to each well and incubated at room temperature (RT) for 2 h on a microplate shaker set at 200 rpm. After a period of washings and incubation, reaction was stopped by stop solution. The absorbance of each sample was determined at 450 nm in a microtiter plate reader (UT-2100C, MRC, UK, manufacturer number 452104038IEX). A standard curve ranging from 0.156 to 10 ng/ml of galectin-3 was generated for each ELISA.

3.7. Secondary outcomes of patients with COVID-19 pneumonia

These data were collected retrospectively by trained investigators from the hospital's electronic medical records, using an electronic data capture tool. We evaluated time from first symptoms to admission, duration in ICU, duration of mechanical and invasive ventilation. In relation to these data, we evaluated data of demographics, comorbidities, severity of disease and laboratorial test results at admission.

3.8. Statistical analysis

Patient characteristics were summarized using the standard descriptive statistics: means for continuous variables and count/percent for categorical variables. The correlation analyses assessed the association of the cytokines and oxidative biomarkers. Laboratory test was evaluated by using the Mann–Whitney U test as appropriate, while the patient characteristics are tested by using Chi square Test. Additionally, Cox regression models was used to test the association of the cytokine values with patient demographics and comorbidities.. The Cox proportional hazards model was used to estimate the hazard of death adjusting for the covariates (for example, patient demographics, comorbidities and laboratory test results), which were determined by the backward elimination method. Point estimates (HRs), along with the corresponding 95% CIs, predicted survival probabilities and cumulative incidence curves, were provided. The analyses were performed using two-sided tests and the SPSS version 26.0 for Macintosh.

Results

Between August and September of 2021 were recruited 47 patients with confirmed COVID-19 pneumonia of which 31,2% were with fatal outcome. Mean age of survived patients 46.50 years and died patients was 72.53 (Table 1.). Among all groups male gender was predominately distributed and present in high percent.

Table 1
Demographics characteristics and comorbidity of COVID-19 patients at admission. Statistical significance was confirmed by Mann-Whitney U test or Chi Square Test.

Variables	Survived [n = 32]	Died [n = 15]	Statistical significance
Age [years], mean [SD]	46.50 ± 13.84	72.53 ± 9.95	**p < 0.05***
Gender, n [%] (M/F)	F 31.3% M 68.7%	F 26.7% M 73.3%	**p < 0.05***
Hypertension, n [%]	Yes 40.6%	Yes 93.3%	**p < 0.05***
Diabetes, n [%]	Yes 15.6%	Yes 20.0%	**p < 0.05***
Morbid Obesity, n [%]	Yes 0%	Yes 6.67%	**p < 0.05***
Chronic obstructive pulmonary disease, n [%]	Yes 0%	Yes 0%	p > 0.05
Asthma, n [%]	Yes 0%	Yes 0%	p > 0.05
Coronary disease, n [%]	Yes 0%	Yes 6.67%	**p < 0.05***
Cardiomyopathy, n [%]	Yes 0%	Yes 6.67%	**p < 0.05***
Chronic kidney disease, n [%]	Yes 0%	Yes 6.67%	**p < 0.05***

Regarding the presence of comorbidities in survived and died patients, we observed that the higher percent of patients with hypertension (93.3%), diabetes (20.0%) and obesity (6.67%) was in patients who died in comparison with patients who survived. Other comorbidities, such as coronary disease, cardiomyopathy and chronic kidney disease were more frequently in patients who died, while the chronic obstructive pulmonary disease and asthma were not present neither in groups (Table 1).

Laboratory blood test was significantly altered in survived and died group of patients (Table 2). In died patients we observed that the levels of serum creatinine, uremic acid, direct bilirubin, AST, LDH, CK, hsTnT, chloride ions and C-reactive protein were significantly higher, while the serum concentration of urea was significantly decreased in survived patients in comparison with died patients at admission (Table 2).

Table 2
Laboratory blood test (liver and renal function, inflammatory markers) of COVID-19 patients at admission. Statistical significance was confirmed by Mann-Whitney U test.

Variables	Survived [n = 32]	Died [n = 15]	Statistical significance
Urea [mmol/l], mean [SD]	46.50 ± 13.84	10.55 ± 4.44	**p < 0.05***
Creatinine [mg/dl], mean [SD]	90.08 ± 15.21	124.46 ± 57.22	**p < 0.05***
Uremic acid [mg/dl], mean [SD]	271.92 ± 88.17	336.54 ± 158.26	**p < 0.05***
Glucose [mmol/l], mean [SD]	7.21 ± 3.01	8.06 ± 4.53	p > 0.05
Direct Bilirubin [µmol/l], mean [SD]	2.24 ± 0.68	4.23 ± 2.87	**p < 0.05***
Total Bilirubin [µmol/l], mean [SD]	6.47 ± 3.33	8.83 ± 6.16	p > 0.05
AST [U/l], mean [SD]	26.67 ± 13.67	53.00 ± 42.68	**p < 0.05***
ALT [U/l], mean [SD]	31.50 ± 26.86	31.77 ± 25.01	p > 0.05
ALP [U/l], mean [SD]	58.31 ± 13.20	61.38 ± 28.92	p > 0.05
LDH [U/l], mean [SD]	351.92 ± 74.89	734.77 ± 278.73	**p < 0.05***
CK [U/l], mean [SD]	125.21 ± 97.17	382.15 ± 365.28	**p < 0.05***
hsTnT [ng/ml], mean [SD]	6.96 ± 3.52	59.92 ± 12.23	**p < 0.05***
gamaGT [U/l], mean [SD]	104.08 ± 62.76	104.92 ± 22.12	p > 0.05
K [mmol/l], mean [SD]	4.33 ± 0.40	4.25 ± 0.61	p > 0.05
Na [mmol/l], mean [SD]	141.21 ± 1.47	140.23 ± 7.87	p > 0.05
Ca [mmol/l], mean [SD]	2.25 ± 0.18	2.08 ± 0.14	p > 0.05
P [mmol/l], mean [SD]	1.05 ± 0.21	1.09 ± 0.27	p > 0.05
Manganese [mmol/l], mean [SD]	0.84 ± 0.11	0.89 ± 0.18	p > 0.05
CL [mmol/l], mean [SD]	2.25 ± 0.18	100.52 ± 6.99	**p < 0.05***
Total Proteins [g/l], mean [SD]	67.75 ± 6.43	69.92 ± 6.59	p > 0.05
Albumin [g/l], mean [SD]	42.50 ± 4.87	35.54 ± 4.77	p > 0.05
CRP [mg/l], mean [SD]	23.58 ± 33.08	167.64 ± 19.93	**p < 0.05***

Regarding the total blood count and coagulation status, we observed significant differences between survived and died group of patients (Table 3). Statistically significant were higher count of neutrophiles, concentration of D-dimer and activity of fibrinogen in died patients. On the other hand, lower levels of hemoglobin, platelets, lymphocytes, monocytes, activity of Factor IX and Factor XII in died patients (Table 3).

Table 3
Total blood count and Coagulation status of COVID-19 patients at admission. Statistical significance was confirmed by Mann-Whitney U test.

Variables	Survived [n = 32]	Died [n = 15]	Statistical significance
Leu [x10^9/l], mean [SD]	4.81 ± 1.30	5.52 ± 3.58	p > 0.05
Er [x10^{12}/l], mean [SD]	4.67 ± 0.67	3.92 ± 0.77	p > 0.05
HGB [g/l], mean [SD]	136.50 ± 14.86	115.31 ± 23.12	**p < 0.05***
HCT [l/l], mean [SD]	0.41 ± 0.04	0.38 ± 0.11	p > 0.05
MCV [fl], mean [SD]	86.72 ± 3.41	88.75 ± 4.41	p > 0.05
TR [x10^9/l], mean [SD]	209.38 ± 87.27	168.54 ± 69.23	**p < 0.05***
Neu [x103/µl], mean [SD]	2.85 ± 0.95	4.29 ± 3.63	**p < 0.001****
Lym [x103/µl], mean [SD]	1.33 ± 0.56	0.60 ± 0.35	**p < 0.001****
Mon [x103/µl], mean [SD]	0.37 ± 0.23	0.14 ± 0.11	**p < 0.05***
INR, mean [SD]	0.94 ± 0.06	1.12 ± 0.47	p > 0.05
PT, mean [SD]	109.18 ± 8.85	93.58 ± 25.32	p > 0.05
aPTT, [s], mean [SD]	26.33 ± 4.18	35.14 ± 14.73	p > 0.05
D-dimer [ng/ml], mean [SD]	355.50 ± 372.70	1460.23 ± 1135.17	**p < 0.001****
Factor II [ng/ml], mean [SD]	113.52 ± 17.00	93.31 ± 24.44	p > 0.05
Factor V [ng/ml], mean [SD]	121.80 ± 21.04	124.06 ± 18.37	p > 0.05
Factor VII [mg/ml], mean [SD]	119.88 ± 30.36	96.24 ± 29.31	p > 0.05
Factor VIII [ng/ml], mean [SD]	101.31 ± 36.23	113.01 ± 33.86	p > 0.05
Factor IX [ng/ml], mean [SD]	120.00 ± 20.19	98.65 ± 33.68	**p < 0.05***
Factor X [ng/ml], mean [SD]	111.60 ± 19.11	97.35 ± 33.17	p > 0.05
Factor XI [ng/ml], mean [SD]	102.69 ± 31.19	91.57 ± 29.76	p > 0.05
Factor XII [ng/ml], mean [SD]	114.20 ± 31.22	77.92 ± 22.66	**p < 0.05***
AT III [g/dl], mean [SD]	97.12 ± 12.22	81.02 ± 14.22	p > 0.05
Fibrinogen [g/l], mean [SD]	3.95 ± 1.06	5.09 ± 1.43	**p < 0.05***

Secondary outcomes in survived and died patients were also different (Table 4). Duration of hospital treatment was significantly different, so died patients were longer in hospital treatment in comparison with survived patients with confirmed COVID-19 pneumonia (Table 4). Also, CT score, duration on

respirator mechanical ventilation as well as duration in ICU was different. Interestingly, duration from first symptoms to the admission was very similar in survived and died patients (Table 4).

Table 4
Secondary Outcomes of COVID-19 patients. Statistical significance was confirmed by Chi Square Test.

Secondary Outcomes	Survived [n = 32]	Died [n = 15]	Statistical significance
Duration from first symptoms to admission (days)	7.36 ± 4.15	6.31 ± 3.88	p > 0.05
Duration of hospital treatment (days)	7.46 ± 5.53	11.54 ± 4.27	**p < 0.05***
CT score	5.10 ± 3.23	20.30 ± 4.14	**p < 0.05***
Duration on respirator (days)	0.00	5.00 ± 3.81	**p < 0.05***
Duration of mechanical ventilation (days)	0.00	5.00 ± 3.81	**p < 0.05***
Duration of N mechanical ventilation (days)	0.00	4.23 ± 2.95	**p < 0.05***
Duration in ICU (days)	0.00	8.54 ± 2.99	**p < 0.05***

Redox status and “bradykinin storm” in patients with COVID-19 pneumonia

On the Figs. 1 and 2 are shown the mean concentrations of oxidative stress markers, activities of antioxidant enzymes and concentrations of bradykinin and galectin-3 in survived and died patients with confirmed COVID-19 pneumonia at admission.

We observed significantly lower levels of nitric oxide and activity of superoxide dismutase in died group of patients, and higher levels of superoxide anion radical and index of lipid peroxidation in died patients in comparison with survived patients (Figs. 1–3).

On the other hand, bradykinin and galectin-3 concentrations were significantly higher in died patients with previously confirmed COVID-19 pneumonia in comparison with survived (Fig. 4).

Correlation analysis among all COVID-19 patients confirmed statistically significant correlation between some markers of oxidative stress and cytokine bradykinin and peptide galectine-3 (Table 5). Serum bradykinin was in positive weak correlation with levels of plasma hydrogen peroxide, and in inverse weak correlation with activity of superoxide dismutase. Also, galectin-3 correlate with index of lipid peroxidation in similar manner (Table 5).

Table 5
Correlation analysis between cytokines and parameters of redox balance in hospitalized patients with confirmed SARS-CoV-2 infection.

Variables		NO^-	H_2O_2	O_2-	TBARS	SOD	CAT	GSH
Bradykinin	Pearson Correlation Coeff.	-0.007	**0.332***	0.259	0.282	**-0.289***	-0.122	0.142
	p value	0.963	**0.023**	0.079	0.055	**0.049**	0.416	0.341
Galectin-3	Pearson Correlation Coeff.	0.075	0.166	0.193	**0.322***	-0.049	-0.224	0.279
	p value	0.623	0.277	0.204	**0.031**	0.749	0.140	0.063

Since the bradykinin showed good linear association with levels of oxidative stress, we evaluated potential cut-off values of serum bradykinin which could be a borderline between positive and fatal outcome in patients with confirmed COVID-19 pneumonia. In the Figs. 10 to 16 are presented cut-off values of bradykinin in form of the red line. We observed that the levels of serum bradykinin from 200000 to 280000 pg/ml represent a significant borderline between distribution of survived and died patients. These marked values could be a significant diagnostic and prognostic sign for changing the therapy protocols and preventing fatal outcome (Figs. 5–8).

In the Cox regression analysis we observed that age above 50 years, duration of stay in hospital more than 4 days, bradykinin levels above 220000 pg/ml, elevated D-dimer, creatinine and CRP, the presence of comorbidities (hypertension and diabetes) could be an independent predictor of mortality in patients with COVID-19 pneumonia (Table 6).

Table 6
COX regression analysis of predictors of factors associated with mortality in patients with COVID-19 confirmed pneumonia

Variables	Hazard Ratio (95% CI)	p value	Adjusted hazard ration (95% CI)	p value
Age above 50 years	1.01 (1.00-1.02)	**0.001****	1.588 (1.131–2.227)	**0.006****
Duration from first symptom to admission	1.22 (1.17–1.26)	**0.001****	2.488 (1.865–3.432)	**0.001****
Creatinine > 1.5 mg/dl	1.47 (1.16–1.83)	**0.002****	0.835 (0.635–1.097)	0.187
D-dimer elevated	1.87 (1.32–2.55)	**0.001****	1.33 (0.857–1.722)	0.071
CRP elevated	1.63 (1.11–2.32)	**0.001****	1.45 (0.951–1.804)	0.152
Lymphocyte count elevated	0.74 (0.59–0.94)	**0.011***	0.531 (0.242–0.511)	**0.001****
Monocyte count decreased	1.01 (1.01–1.01)	**0.001****	1.12 (0.880–1.323)	0.259
Hypertension presence	2.33 (1.89–3.01)	**0.001****	1.801 (0.746–1.606)	0.072
Diabetes presence	1.45 (1.15–1.73)	**0.001****	0.935 (0.655–1.101)	0.134
Bradykinin above 200000 ng/ml	1.001 (1.001–1.001)	**0.001****	2.135 (1.666–2.567)	**0.001****
Galectine − 3 above	1.07 (1.04–1.016)	**0.001****	1.182 (0.991–1.651)	0.244

Discussion

This was prospective cross-sectional study which included 47 adult patients with confirmed SARS-CoV-2 infection and with criteria for hospital treatment who are admitted to tertiary Clinical Center “Bezanijska kosa” during June of 2021. The main inclusion criteria were old above 18 years; confirmed COVID pneumonia; voluntary participation in study. All patients were managed with supportive care and specific pharmacological protocols created by the hospital’s COVID-19 management guidelines committee in accordance with the Ministry of Health Republic of Serbia.

This study was aimed to recognize the major factor risk for severe form of disease and death. Still, there is no exactly information does and how cytokine bradykinin and peptide galectin-3 are involved in the bad prognosis of COVID-19 pneumonia and fatal outcome. These finding could be a promising therapeutical target and independent predictor of patient’s survival.

In the first part of study, we evaluated basic demographic characteristics of patients.

Mean age of survived patients 46.50 years and died patients was 72.53 (Table 1.). Among all groups male gender was predominately distributed and present in high percent.

Regarding the presence of comorbidities in survived and died patients, we observed that the higher percent of patients with hypertension (93.3%), diabetes (20.0%) and obesity (6.67%) was in patients who died in comparison with patients who survived. Other comorbidities, such as coronary disease, cardiomyopathy and chronic kidney disease were more frequently in patients who died, while the chronic obstructive pulmonary disease and asthma were not present neither in groups (Table 1). Previous study recognized established, probable and possible risk factors for severe COVID-19, but still there are some cases with unusually outcomes and prognosis. Very often, possible risk factors could be mixed in the one patient, so the appropriate pharmacological protocol is very hard to find and use. Important, all selected comorbidities from our study is associated with illness in adults of all ages, but the mortality rate and fatal outcome was more frequently in adults above 50 years (30, 31). Definitely, comorbidities do not tell the full story about the risk for severity form and death in COBID-19 confirmed patients.

In the second part, we evaluated the routine and specific laboratory blood test in COVID-19 confirmed patients at admission. Laboratory blood test was significantly altered in survived and died group of patients (Table 2). In died patients we observed that the levels of serum creatinine, uremic acid, direct bilirubin, AST, LDH, CK, hsTnT, chloride ions and C-reactive protein were significantly higher, while the serum concentration of urea was significantly decreased in survived patients in comparison with died patients at admission (Table 2).

Regarding the total blood count and coagulation status, we observed significant differences between survived and died group of patients (Table 3). Statistically significant were higher count of neutrophiles, concentration of D-dimer and activity of fibrinogen in died patients. On the other hand, lower levels of hemoglobin, platelets, lymphocytes, monocytes, activity of Factor IX and Factor XII in died patients (Table 3).

Definitely, it is recognized one algorhytm of laboratory markers that could be a guide for the prognosis and using therapeutical protocols. As a standard of care, baseline blood tests and inflammatory markers are obtained on admission to the hospital. The proper approach for the risk assessment should allow physicians to forecast the patient’s future worsening out of the initial findings on admission. As many times mentioned in the literature, D-dimmer is definitely associated with worse prognosis, as we observed in our study. In that sense, CRP and fibrinogen with altered levels of white blood cells, are the second early diagnostic markers of hyper-coagulopathy and needed treatment with direct factor Xa inhibitors (32–34).

Elevated D-dimer levels suggest extensive thrombin generation and fibrinolysis, and is associated with poor prognosis in COVID-19, which has prompted clinicians to hypothesize that increased D-dimer concentrations are indicative of co-existing venous thromboembolisms that may lead to ventilation-

perfusion mismatch. Also, there are lot of evidence about the high risk for thromboembolism in patients with COVID-19 disease and activating the KKS in plasma which leads to BK overproduction (35).

Also, for each laboratory marker could be very significant to obtain a cut-off values for severity of disease, to reduce the severe or fatal outcome.

Secondary outcomes in survived and died patients were also different (Table 4). Duration of hospital treatment was significantly different, so died patients were longer in hospital treatment in comparison with survived patients with confirmed COVID-19 pneumonia (Table 4). Also, CT score, duration on respirator mechanical ventilation as well as duration in ICU was different. Interestingly, duration from first symptoms to the admission was very similar in survived and died patients (Table 4). As expected, ICU duration was longer in death patients, but interesting is that after the same time prior to admission, survived and died patients had different prognosis. Chaim T et al investigated the hospital length (LOS) among COVID-19 positive patients (36). They concluded that COVID-19 patients LOS vary based on multiple factors, such as older age, comorbidities and disease severity. Definitely, understanding these factors are crucial to improving the prediction accuracy of COVID-19 patient census in hospitals for resource planning and care delivery.

In the final we tried to find responsible molecular mechanism by which COVID-19 pneumonia leads to fatal outcome. We observed definitely elevated oxidative stress in died patients at admission and very high levels of serum bradykinin and galectin-3. In particular, we observed significantly lower levels of nitric oxide and activity of superoxide dismutase in died group of patients, and higher levels of superoxide anion radical and index of lipid peroxidation in died patients in comparison with survived patients (Figs. 1–7) and bradykinin and galectin-3 concentrations were significantly higher in died patients with previously confirmed COVID-19 pneumonia in comparison with survived (Figs. 8 and 9).

Our results are in accordance with the results of previous studies. Garvin et al suggested that bradykinin metabolite, des-Arg9-BK, could contribute to the inflammation, vasodilation, vascular permeability via activation of bradykinin receptors (37). Also, it is known that a possible source of this bradykinin in COVID-19 patients could be bronchiole and alveoli resident mast cells. It is well known that as tissue resident granulocytes, mast cells can synthesize bradykinin via the secretion of heparin, activation of coagulation factor XII, and formation of plasma kallikrein. Therefore, the increase in bradykinin may be due to the increased mast cells density in the lungs of COVID-19 patients (38, 39).

Using correlation analysis we confirmed association between some markers of oxidative stress and cytokine bradykinin and peptide galectine-3 (Table 5). Serum bradykinin was in positive weak correlation with levels of plasma hydrogen peroxide, and in inverse weak correlation with activity of superoxide dismutase. Also, galectin-3 correlate with index of lipid peroxidation in similar manner (Table 5). Interestingly, we have observed critical values of bradykinin in COVID-19 patients, so the levels of serum bradykinin from 200000 to 280000 pg/ml represent a significant borderline between distribution of survived and died patients. These marked values could be a significant diagnostic and prognostic sign

for changing the therapy protocols and preventing fatal outcome (Figs. 10–16). On the other hand, galectin-3 do not have linear dynamic and could not be a sensitive diagnostic or prognostic marker.

Definitely, bradykinin storm was present in died group patients. Unfortunately, the present storm probably induced microvascular permeability, edema and further inflammation and worse prognosis. Our study strongly supports the bradykinin storm hypothesis which underlying in many of COVID-19 fatal outcomes. In that sense, future therapeutical strategies must be focused on reducing a bradykinin serum concentration in COVID-19 patients. Study conducted by Ghahestani et al. suggested that blocking the B2 receptors with icatibant may be good strategy for large BK degradation in COVID-19 patients. As they concluded, this drug also could be able to reduce angioedema and to improve oxygenation in severe forms of disease, and also in controlling the outcomes in patients with COVID-19 pneumonia (40). This phenomenon was described in experimental models also, where are bradykinin and substance P are detected in very high concentrations in animals with stroke and brain injury (41).

Definitely, bradykinin could be a prognostic marker of mortality with linear dynamic and high sensitivity.

Limitation of this study is a number of patients, but we included matched patients with similar clinical features and duration of disease prior to admission. Also, given the different pathophysiology of COVID-19 disease, we plan to include the selected patients with 7 and 28 days follow-up but with mild type of disease, to aprove all these assumptions.

Conclusion

“Bradykinin storm” and oxidative stress in patients with fatal outcome are probably responsible for the overactive inflammatory response in COVID-19 patients and resulting symptoms. Understanding the mechanisms by which the two storms, bradykinin and cytokine, affect the body, separate or together, is crucial to mitigating the pandemic’s effect, saving lives, and producing effective treatments that we presently lack.

Declarations

Funding

None.

Contribution to the Field Statement

In the heart of the pandemic, there was growing concerns about the pathophysiology of COVID positive patients and prognosis. Also, since there are no unique and effective pharmacological protocols for SARS-CoV-2 infected patients, the importance for the knowing the pathophysiological molecular mechanism increasing. The proinflammatory cytokines and peptides such as bradykinin and galectin-3 are dominant in sick patients and may contribute to leaky vasculature and cell necrosis which results in

microvascular endotheliopathy. Also, the course of bradykinin follows free radicals, which are identified in high bioavailability in systemic circulation in patients with COVID-19 pneumonia. The cytokine and bradykinin storm theory could be a possible explanation for the severe forms of COVID-19 disease and symptoms among many organ systems. This study offers a many potential therapeutic targets for prevention multi organ failure in patients with severe forms of COVID-19 viral pneumonia such as inhibitors of bradykinin production.

Conflict of interests

None.

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Tables

Table 1. Demographics characteristics and comorbidity of COVID-19 patients at admission. Statistical significance was confirmed by Mann-Whitney U test or Chi Square Test.

Variables	Survived [n=32]	Died [n=15]	Statistical significance
Age [years], mean [SD]	46.50±13.84	72.53±9.95	**p<0.05***
Gender, n [%] (M/F)	F 31.3% M 68.7%	F 26.7% M 73.3%	**p<0.05***
Hypertension, n [%]	Yes 40.6%	Yes 93.3%	**p<0.05***
Diabetes, n [%]	Yes 15.6%	Yes 20.0 %	**p<0.05***
Morbid Obesity, n [%]	Yes 0%	Yes 6.67%	**p<0.05***
Chronic obstructive pulmonary disease, n [%]	Yes 0%	Yes 0%	p>0.05
Asthma, n [%]	Yes 0%	Yes 0%	p>0.05
Coronary disease, n [%]	Yes 0%	Yes 6.67%	**p<0.05***
Cardiomyopathy, n [%]	Yes 0%	Yes 6.67%	**p<0.05***
Chronic kidney disease, n [%]	Yes 0%	Yes 6.67%	**p<0.05***

Table 2. Laboratory blood test (liver and renal function, inflammatory markers) of COVID-19 patients at admission. Statistical significance was confirmed by Mann-Whitney U test.

Variables	Survived [n=32]	Died [n=15]	Statistical significance
Urea [mmol/l], mean [SD]	46.50±13.84	10.55±4.44	**p<0.05***
Creatinine [mg/dl], mean [SD]	90.08±15.21	124.46±57.22	**p<0.05***
Uremic acid [mg/dl], mean [SD]	271.92±88.17	336.54±158.26	**p<0.05***
Glucose [mmol/l], mean [SD]	7.21±3.01	8.06±4.53	p>0.05
Direct Bilirubin [µmol/l], mean [SD]	2.24±0.68	4.23±2.87	**p<0.05***
Total Bilirubin [µmol/l], mean [SD]	6.47±3.33	8.83±6.16	p>0.05
AST [U/l], mean [SD]	26.67±13.67	53.00±42.68	**p<0.05***
ALT [U/l], mean [SD]	31.50±26.86	31.77±25.01	p>0.05
ALP [U/l], mean [SD]	58.31±13.20	61.38±28.92	p>0.05
LDH [U/l], mean [SD]	351.92±74.89	734.77±278.73	**p<0.05***
CK [U/l], mean [SD]	125.21±97.17	382.15±365.28	**p<0.05***
hsTnT [ng/ml], mean [SD]	6.96±3.52	59.92±12.23	**p<0.05***
gamaGT [U/l], mean [SD]	104.08±62.76	104.92±22.12	p>0.05
K [mmol/l], mean [SD]	4.33±0.40	4.25±0.61	p>0.05
Na [mmol/l], mean [SD]	141.21±1.47	140.23±7.87	p>0.05
Ca [mmol/l], mean [SD]	2.25±0.18	2.08±0.14	p>0.05
P [mmol/l], mean [SD]	1.05±0.21	1.09±0.27	p>0.05
Manganese [mmol/l], mean [SD]	0.84±0.11	0.89±0.18	p>0.05
CL [mmol/l], mean [SD]	2.25±0.18	100.52±6.99	**p<0.05***
Total Proteins [g/l], mean [SD]	67.75±6.43	69.92±6.59	p>0.05
Albumin [g/l], mean [SD]	42.50±4.87	35.54±4.77	p>0.05
CRP [mg/l], mean [SD]	23.58±33.08	167.64±19.93	**p<0.05***

Table 3. Total blood count and Coagulation status of COVID-19 patients at admission. Statistical significance was confirmed by Mann-Whitney U test.

Variables	Survived [n=32]	Died [n=15]	Statistical significance
Leu [x10^9/l], mean [SD]	4.81±1.30	5.52±3.58	p>0.05
Er [x10^{12}/l], mean [SD]	4.67±0.67	3.92±0.77	p>0.05
HGB [g/l], mean [SD]	136.50±14.86	115.31±23.12	**p<0.05***
HCT [l/l], mean [SD]	0.41±0.04	0.38±0.11	p>0.05
MCV [fl], mean [SD]	86.72±3.41	88.75±4.41	p>0.05
TR [x10^9/l], mean [SD]	209.38±87.27	168.54±69.23	**p<0.05***
Neu [x103/µl], mean [SD]	2.85±0.95	4.29±3.63	**p<0.001****
Lym [x103/µl], mean [SD]	1.33±0.56	0.60±0.35	**p<0.001****
Mon [x103/µl], mean [SD]	0.37±0.23	0.14±0.11	**p<0.05***
INR, mean [SD]	0.94±0.06	1.12±0.47	p>0.05
PT, mean [SD]	109.18±8.85	93.58±25.32	p>0.05
aPTT, [s], mean [SD]	26.33±4.18	35.14±14.73	p>0.05
D-dimer [ng/ml], mean [SD]	355.50±372.70	1460.23±1135.17	**p<0.001****
Factor II [ng/ml], mean [SD]	113.52±17.00	93.31±24.44	p>0.05
Factor V [ng/ml], mean [SD]	121.80±21.04	124.06±18.37	p>0.05
Factor VII [mg/ml], mean [SD]	119.88±30.36	96.24±29.31	p>0.05
Factor VIII [ng/ml], mean [SD]	101.31±36.23	113.01±33.86	p>0.05
Factor IX [ng/ml], mean [SD]	120.00±20.19	98.65±33.68	**p<0.05***
Factor X [ng/ml], mean [SD]	111.60±19.11	97.35±33.17	p>0.05
Factor XI [ng/ml], mean [SD]	102.69±31.19	91.57±29.76	p>0.05
Factor XII [ng/ml], mean [SD]	114.20±31.22	77.92±22.66	**p<0.05***
AT III [g/dl], mean [SD]	97.12±12.22	81.02±14.22	p>0.05
Fibrinogen [g/l], mean [SD]	3.95±1.06	5.09±1.43	**p<0.05***

Table 4. Secondary Outcomes of COVID-19 patients. Statistical significance was confirmed by Chi Square Test.

Secondary Outcomes	Survived [n=32]	Died [n=15]	Statistical significance
Duration from first symptoms to admission (days)	7.36±4.15	6.31±3.88	p>0.05
Duration of hospital treatment (days)	7.46±5.53	11.54±4.27	**p<0.05***
CT score	5.10±3.23	20.30±4.14	**p<0.05***
Duration on respirator (days)	0.00	5.00±3.81	**p<0.05***
Duration of mechanical ventilation (days)	0.00	5.00±3.81	**p<0.05***
Duration of N mechanical ventilation (days)	0.00	4.23±2.95	**p<0.05***
Duration in ICU (days)	0.00	8.54±2.99	**p<0.05***

Table 5. Correlation analysis between cytokines and parameters of redox balance in hospitalized patients with confirmed SARS-CoV-2 infection.

Variables		NO^-	H_2O_2	O_2-	TBARS	SOD	CAT	GSH
Bradykinin	Pearson Correlation Coeff.	-0.007	**0.332***	0.259	0.282	**-0.289***	-0.122	0.142
	p value	0.963	**0.023**	0.079	0.055	**0.049**	0.416	0.341
Galectin-3	Pearson Correlation Coeff.	0.075	0.166	0.193	**0.322***	-0.049	-0.224	0.279
	p value	0.623	0.277	0.204	**0.031**	0.749	0.140	0.063

Table 6. COX regression analysis of predictors of factors associated with mortality in patients with COVID-19 confirmed pneumonia

Variables	Hazard Ratio (95% CI)	p value	Adjusted hazard ration (95% CI)	p value
Age above 50 years	1.01 (1.00-1.02)	**0.001****	1.588 (1.131-2.227)	**0.006****
Duration from first symptom to admission	1.22 (1.17-1.26)	**0.001****	2.488 (1.865-3.432)	**0.001****
Creatinine >1.5 mg/dl	1.47 (1.16-1.83)	**0.002****	0.835 (0.635-1.097)	0.187
D-dimer elevated	1.87 (1.32-2.55)	**0.001****	1.33 (0.857-1.722)	0.071
CRP elevated	1.63 (1.11-2.32)	**0.001****	1.45 (0.951-1.804)	0.152
Lymphocyte count elevated	0.74 (0.59-0.94)	**0.011***	0.531 (0.242- 0.511)	**0.001****
Monocyte count decreased	1.01 (1.01-1.01)	**0.001****	1.12 (0.880-1.323)	0.259
Hypertension presence	2.33 (1.89-3.01)	**0.001****	1.801 (0.746-1.606)	0.072
Diabetes presence	1.45 (1.15-1.73)	**0.001****	0.935 (0.655-1.101)	0.134
Bradykinin above 200000 ng/ml	1.001 (1.001-1.001)	**0.001****	2.135 (1.666-2.567)	**0.001****
Galectine -3 above	1.07 (1.04-1.016)	**0.001****	1.182 (0.991-1.651)	0.244

Figures

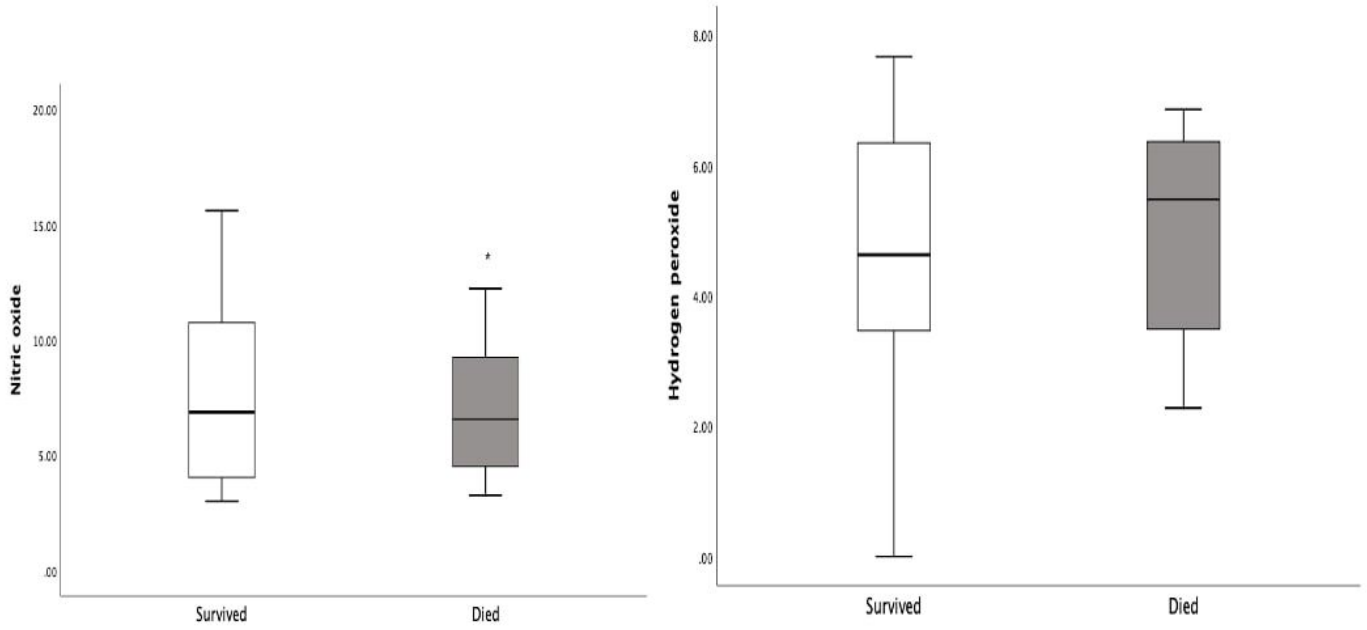

Figure 1

The mean plasma values of nitric oxide (nmol/ml) and hydrogen peroxide (nmol/ml) in survived [n=32] and died patients [n=15] at admission. Statistical significance was confirmed by Mann-Whitney U test.

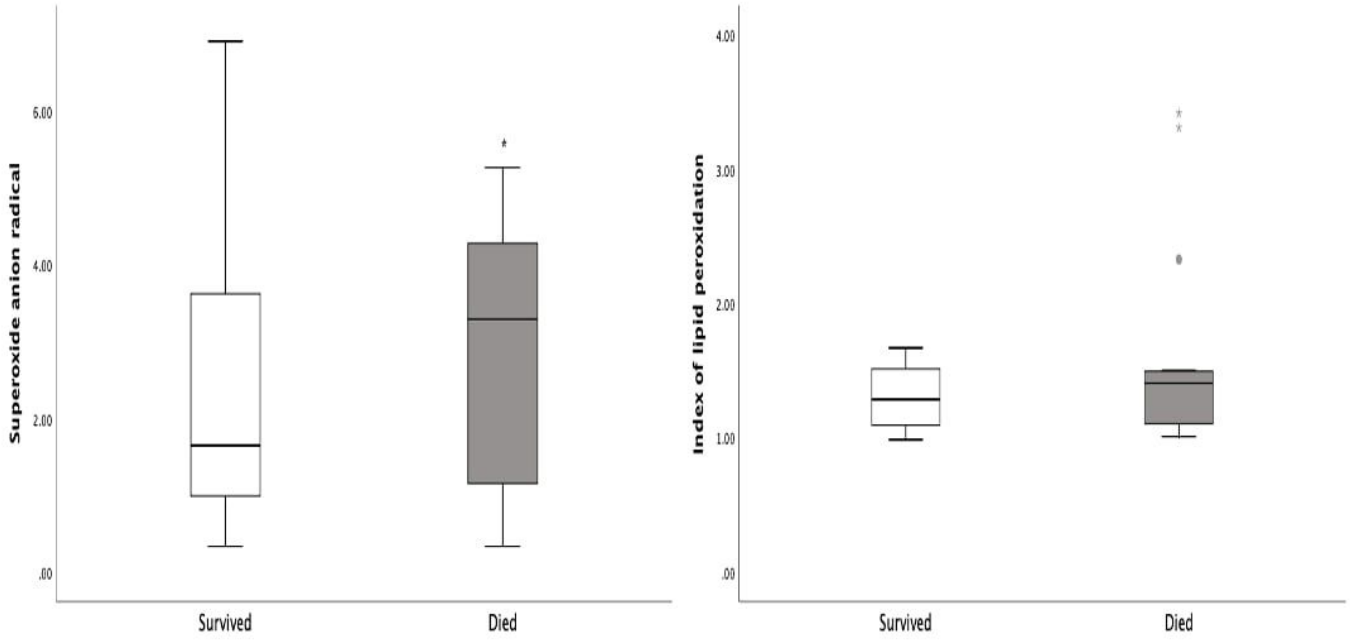

Figure 2

The mean plasma values of superoxide anion radical (nmol/ml) and index of lipid peroxidation measured as TBARS (µmol/ml) in survived [n=32] and died patients [n=15] at admission. Statistical significance was confirmed by Mann-Whitney U test.

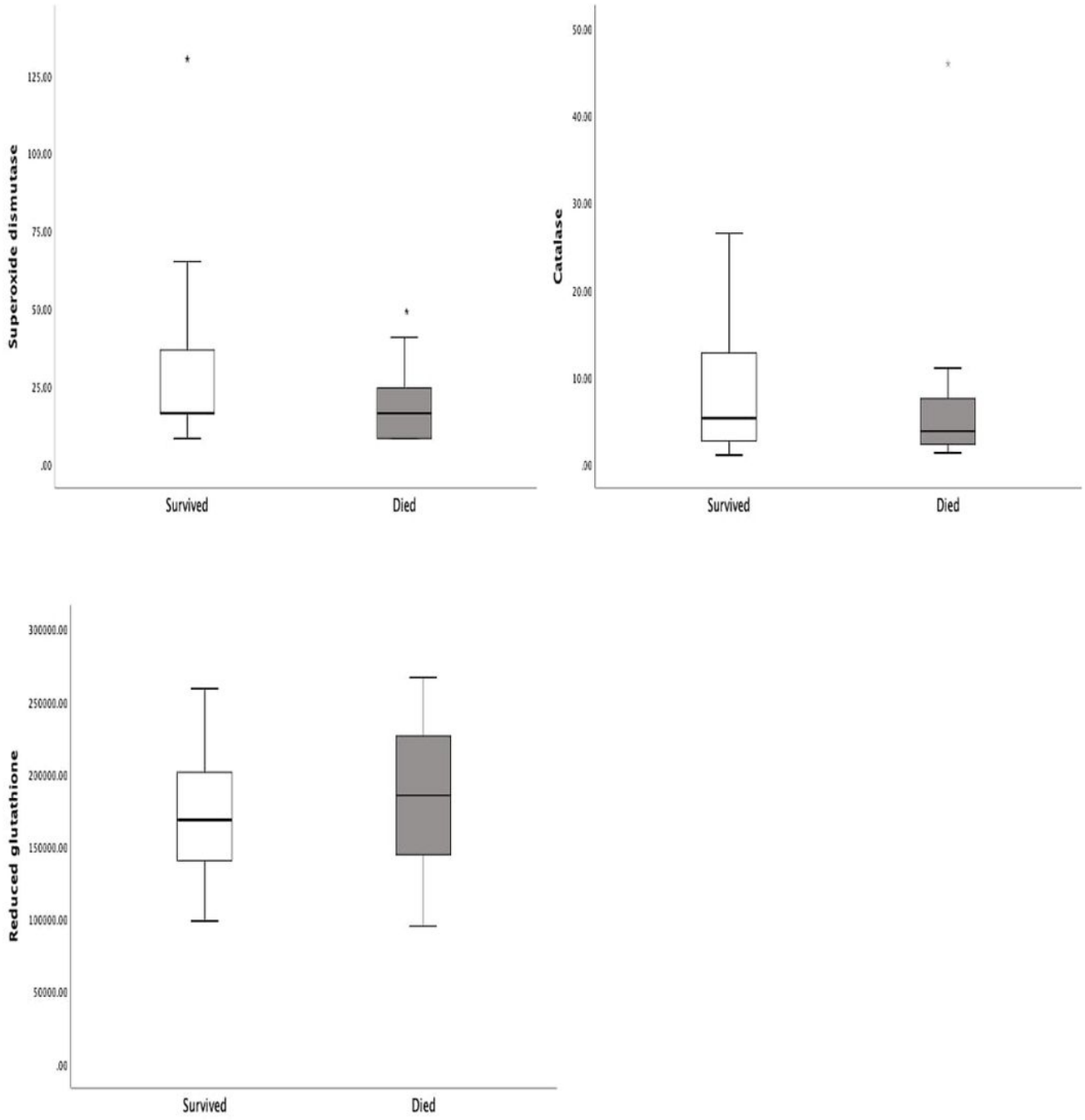

Figure 3

The mean hemolysate activity of superoxide dismutase (U/Hg x 10^9), catalase (U/Hg x 10^9) and reduced glutathione (U/Hg x 10^9) in survived [n=32] and died patients [n=15] at admission. Statistical significance was confirmed by Mann-Whitney U test.

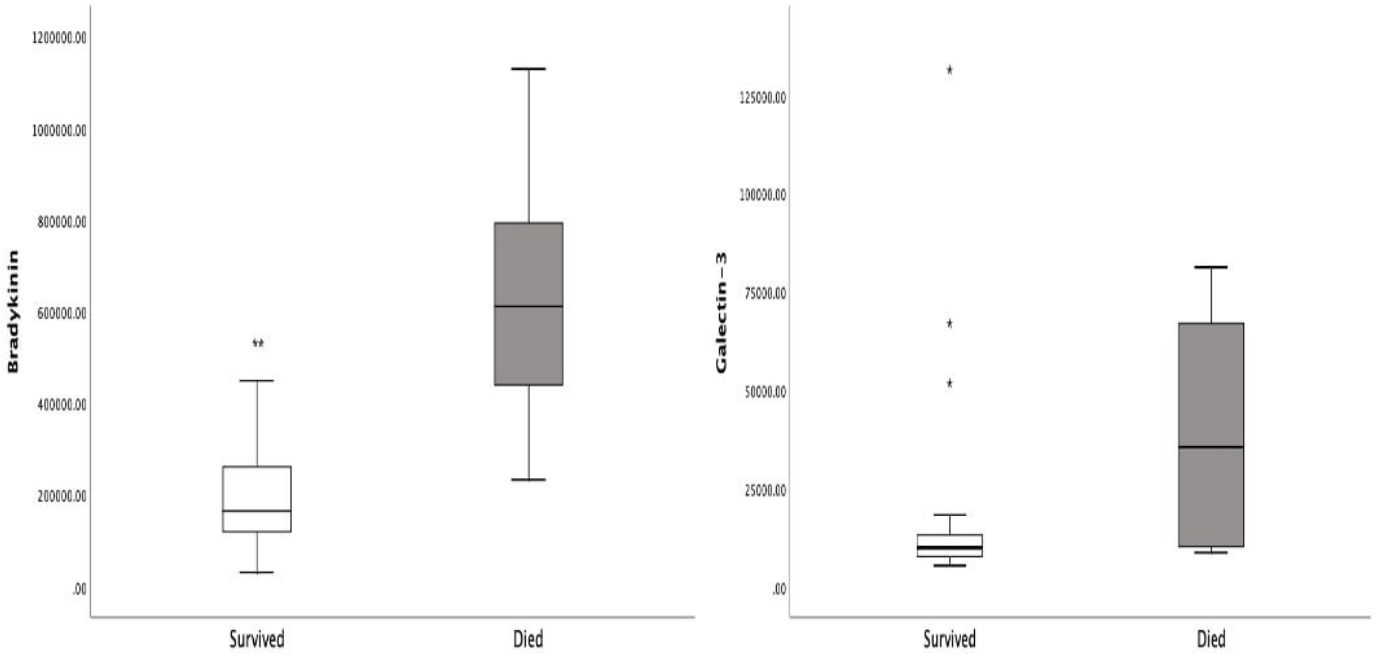

Figure 4

The mean serum concentration of bradykinin (pg/ml) and galectin-3 (ng/ml) in survived [n=32] and died patients [n=15] at admission. Statistical significance was confirmed by Mann-Whitney U test.

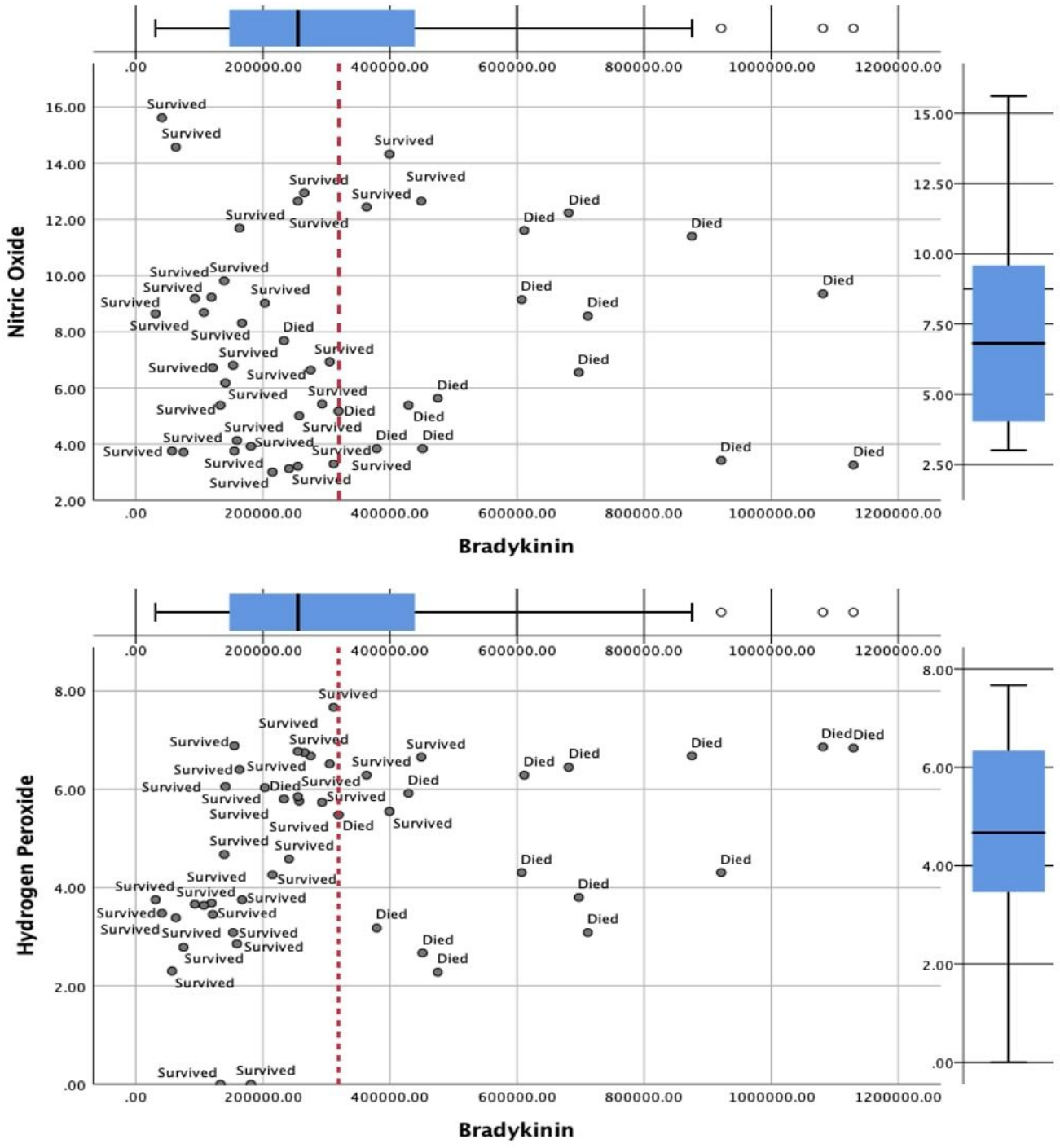

Figure 5

Distribution of survived and died patients in different concentration of nitric oxide/hydrogen peroxide and bradykinin. Red line represents an observed cut-off value of bradykinin [pg/ml] for predicting death in patients with COVID-19 pneumonia

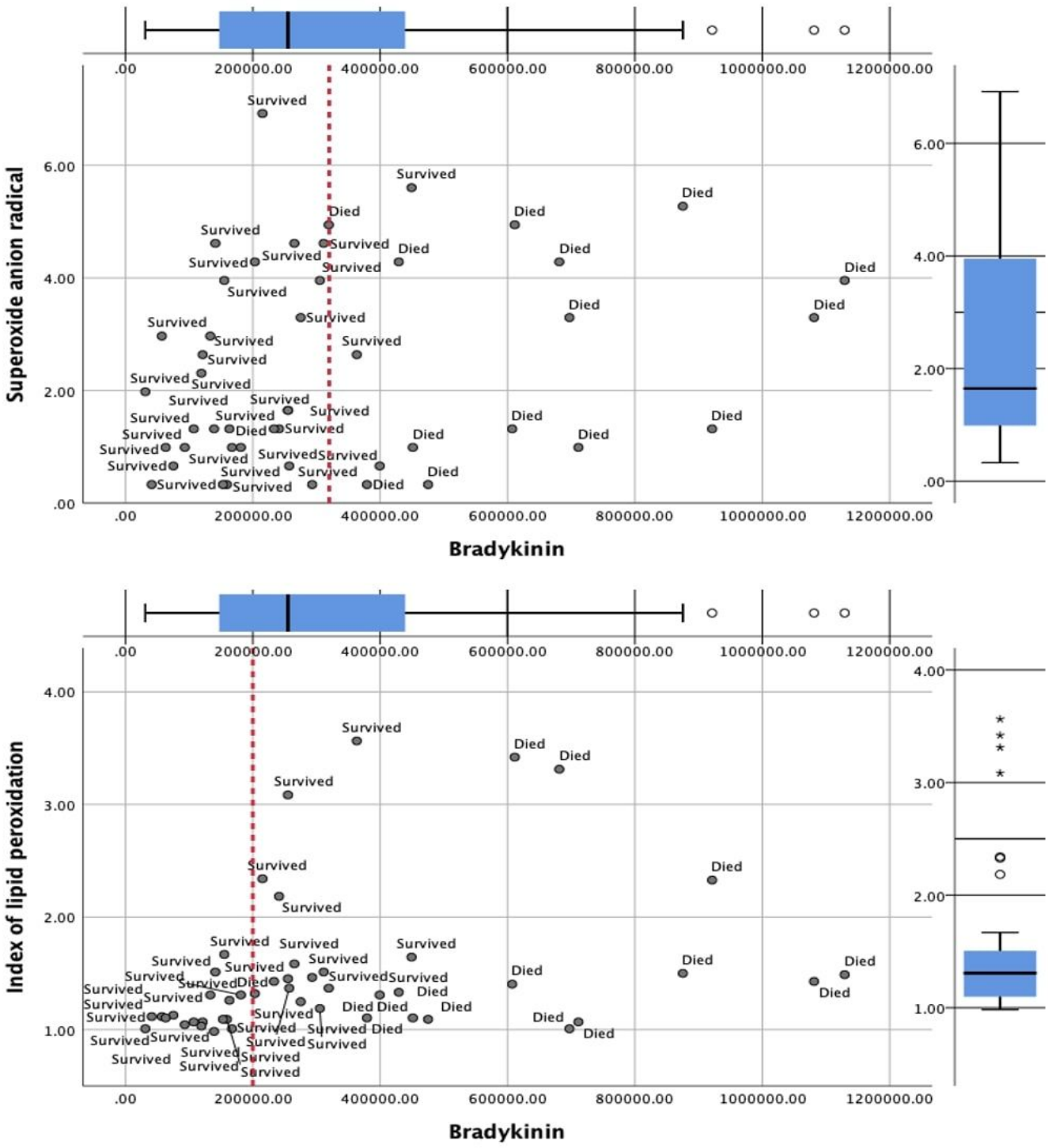

Figure 6

Distribution of survived and died patients in different concentration of superoxide anion radical/ index of lipid peroxidation and bradykinin. Red line represents an observed cut-off value of bradykinin [pg/ml] for predicting death in patients with COVID-19 pneumonia

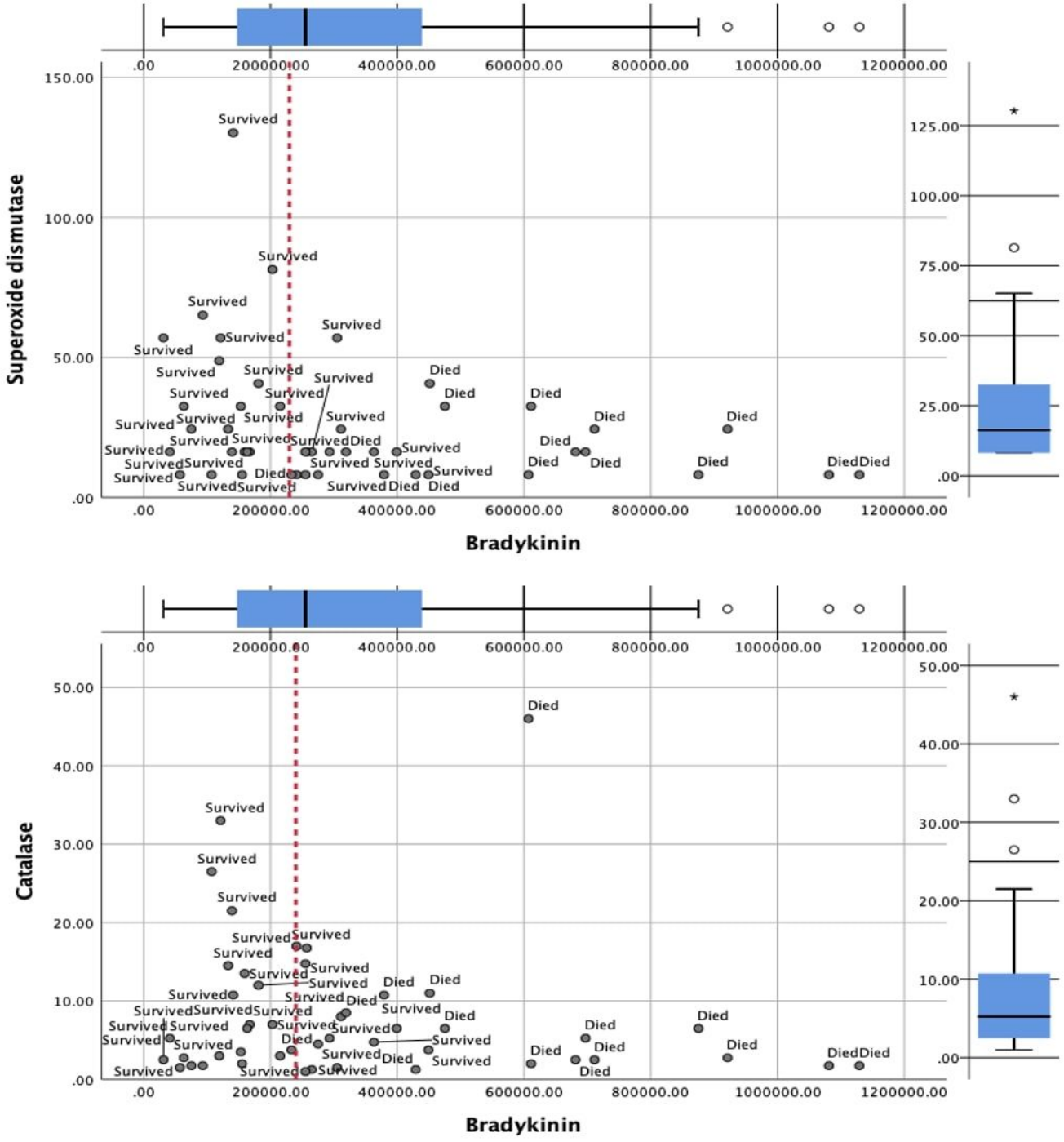

Figure 7

Distribution of survived and died patients in different activity of superoxide dismutase/ catalase and bradykinin [pg/ml]. Red line represents an observed cut-off value of bradykinin for predicting death in patients with COVID-19 pneumonia

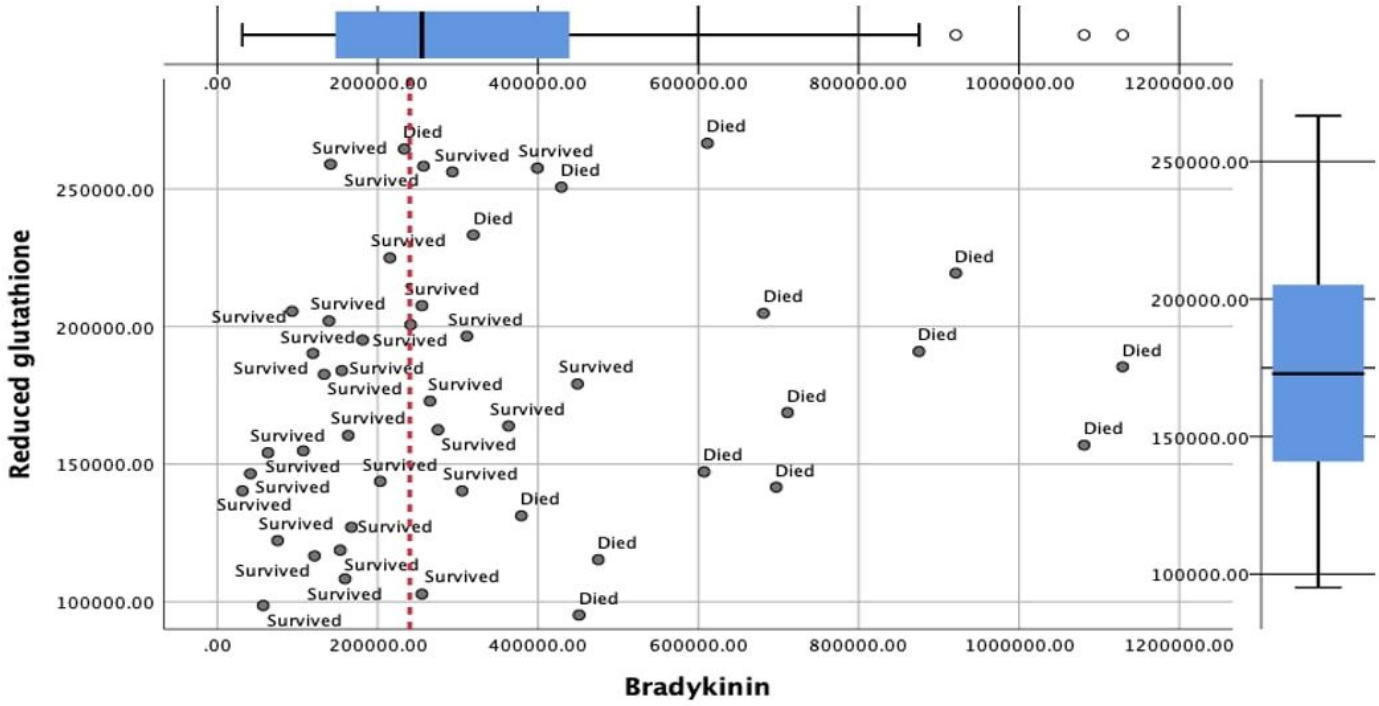

Figure 8

Distribution of survived and died patients in different content of reduced glutathione and bradykinin [pg/ml]. Red line represents an observed cut-off value of bradykinin for predicting death in patients with COVID-19 pneumonia