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Lipid peroxidation as a hallmark of severity in COVID-19 patients

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ABSTRACT

Background: Oxidative stress may be a key player in COVID-19 pathogenesis due to its significant role in response to infections. A defective redox balance has been related to viral pathogenesis developing a massive induction of cell death provoked by oxidative stress. The aim of this study is to perform a complete oxidative stress profile evaluation regarding antioxidant enzymes, total antioxidant capacity and oxidative cell damage in order to characterize its role in diagnosis and severity of this disease.

Methods: Blood samples were obtained from 108 COVID-19 patients and 28 controls and metabolites representative of oxidative stress were assessed. The association between lipid peroxidation and 28-day intubation/death risk was evaluated by multivariable regression analysis. Probability of intubation/death to day-28 was analyzed by using Kaplan-Meier curves and tested with the log-rank test.

Results: Antioxidant enzymes (Superoxide dismutase (SOD) and Catalase) and oxidative cell damage (Carbonyl and Lipid peroxidation (LPO)) levels were significantly higher in COVID-19 patients while total antioxidant capacity (ABTS and FRAP) levels were lower in these patients. The comparison of oxidative stress molecules' levels across COVID-19 severity revealed that only LPO was statistically different between mild and intubated/death COVID-19 patients. COX multivariate regression analysis identified LPO levels over the OOP (LPO>1948.17 μM) as an independent risk factor for 28-day intubation/death in COVID-19 patients [OR: 2.57; 95% CI: 1.10–5.99; $p = 0.029$]. Furthermore, Kaplan-Meier curve analysis revealed that COVID-19 patients showing LPO levels above 1948.17 μM were intubated or died 8.4 days earlier on average (mean survival time 15.4 vs 23.8 days) when assessing 28-day intubation/death risk ($p < 0.001$).

Conclusion: These findings deepen our knowledge of oxidative stress status in SARS-CoV-2 infection, supporting its important role in COVID-19. In fact, higher lipid peroxidation levels are independently associated to a higher risk of intubation or death at 28 days in COVID-19 patients.

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1. Introduction

A new strain of coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was recognized to have emerged in Wuhan, China in December 2019. It is the third coronavirus that causes severe respiratory disease in humans (COVID-19) [1] along with SARS-CoV [2] and Middle East Respiratory Syndrome-coronavirus (MERS-CoV). In most cases causes a mild or no symptomatic respiratory disease but up to 20% of them present serious illness requiring hospitalization [3] with high fever and pneumonia [4], leading to acute respiratory distress syndrome (ARDS) [5]. Severe COVID-19 infection triggers imbalanced and uncontrolled cytokine response, exuberant endothelial inflammatory reactions and vascular thrombosis [6].

Oxidative stress may be a key player in COVID-19 pathogenesis due to its significant role in response to infections [7]. Several studies have reported some viruses' ability to disrupt redox balance of a cell to ensure survival [8]. This defective redox balance has been related to viral pathogenesis developing a massive induction of cell death provoked by oxidative stress [9]. Oxidative stress is a typical phenomenon of infections produced by Respiratory Syncytial Virus (RSV) [10], which induces reactive oxygen species (ROS) production, activating pro-inflammatory cytokines and innate immunity [11]. RSV increases lipid peroxidation and decreases Glutathione (GSH) in human alveolar type II-like epithelial cells and small airway epithelial cells and inhibits Nrf2 pathway activation, decreasing gene expression of protective molecules [12]. Furthermore, an excessive amount of ROS is produced by Influenza infection in several tissues [13] such as endothelium [14] and alveolar epithelium [15]. Influenza virus induces apoptosis and cytotoxicity in alveolar epithelial cells increasing caspase 1 and 3 and IL-8 expression [16]. However, this virus facilitates the nuclear translocation of Nrf2 with subsequent expression of a protective enzyme against oxidative injury in human alveolar epithelial cells [16]. Accordingly, oxidative stress may also profoundly impact COVID-19 pathogenesis, but only few studies have been developed for this purpose [17–19].

In this regard, here we aimed to perform a complete oxidative stress profile evaluation regarding antioxidant enzymes, total antioxidant capacity and oxidative cell damage in plasma samples from a prospective COVID-19 patients' cohort in order to characterize its role in diagnosis and severity of this disease.

2. Materials and methods

2.1. Patient selection

A total of 108 adult patients diagnosed with COVID-19 and admitted at the "Hospital Clínico Universitario de Valladolid" (Valladolid, Spain) were prospectively recruited between 24th of March and 11th of April 2020. Positive result in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection was confirmed in all patients by polymerase chain reaction on nasopharyngeal swabs. Patients with other acute diseases, infections, or chronic terminal illness were excluded. In addition, 28 age- and gender-matched healthy volunteers were also recruited in the same time period. Those 28 control samples were collected during the preanesthetic evaluation for scheduled surgery with a negative PCR result for SARS-CoV-2 infection. The study was approved by the Hospital's Clinical Ethics Committee (CEIm) and the informed consent was obtained from all study participants (cod: PI 20–1717). This study followed the code of ethics of the World Medical Association (Declaration of Helsinki).

2.2. Severity and mortality

Our sample was divided into three groups: i. Controls (n = 28), ii. Mild and moderate COVID-19 patients (n = 76): admitted in ward, iii. Critical (n = 32): mechanical ventilation. Moreover, in terms of mortality there were 20 dead patients (12 of them included in critical group) and 88 alive ones. In order to focus a better approach to disease severity we joined both critical and death COVID-19 patients following another important studies [20]. So that we finally arranged two COVID-19 groups: Intubated or death patients (n = 40) and non-intubated or death (mild) patients (n = 68).

2.3. Biological samples

Plasma samples from each patient were prospectively recruited at 9 a.m. immediately after their first night of hospital admission for preventing circadian variations. Blood was collected in 3.2% sodium citrate tubes and centrifuged at 2000×*g* for 20 min at room temperature. The resulting plasma was aliquoted and directly frozen at −80 °C until used.

2.4. Antioxidant enzymes' levels determination

Superoxide dismutase activity was assessed by using Superoxide Dismutase (SOD) Colorimetric Activity Kit, following the manufacturer's recommendations. In the assay, superoxide (O2-) is provided by xanthine oxidase (XO) catalyzed reaction. O2- reacts with a WST-1 dye to form a colored product. SOD scavenges the O2- thus less O2- is available for the chromogenic reaction. The color intensity at 440 nm is used to determine the SOD activity.

Catalase (CAT) activity was determined by using Catalase (CAT) Activity Assay Kit, following the manufacturer's recommendations. The reaction that CAT decomposes H_2O_2 can be quickly stopped by ammonium molybdate. The residual H_2O_2 reacts with ammonium molybdate to generate a yellowish complex. CAT activity can be calculated by production of the yellowish complex at 405 nm.

2.5. Total antioxidant capacity levels determination

The antioxidant capacity of samples was evaluated by two methods:

FRAP (Ferric Reducing Antioxidant Power): This assay is based on the ability of the sample to allow iron reduction, which is carried out as described by Benzie and Strain. Results will be quantified by absorbance at 595 nm using a standard curve of known Trolox concentrations, following our lab protocol.

ABTS (2,2-azino-bis (3-ethylbenzthioziozline-6-sulfonic acid): This technique is based on the estimation of the antioxidant capacity by performing a colorimetric test using the cationic radical (ABTS). This assay was performed following our lab protocol.

2.6. Oxidative cell damage levels determination

The DNA oxidized guanosine specie, 8-hydroxy-2′-deoxyguanosine (8-OHdG), was quantitatively measured at 450 nm wavelength by using The DetectX® DNA Damage Immunoassay Kit (Arbor Assays, Ann Arbor, MI, USA), following the manufacturer's recommendations.

Lipid peroxidation (LPO) products were analyzed by using the Bioquochem commercial kit ref KB03002 (BQCell™ MTT, Bioquochem, Oviedo, Spain), following the manufacturer's recommendations. Malondialdehyde (MDA) and 4-Hydroxynonenal (HNE) concentrations were measured as an index of lipid peroxidation. Reactions between indoles and aldehydes (MDA and HNE) gives a diindolylalkane (chromophore) whose maximal absorbance is in the 580–620 nm region.

Protein carbonyl was analyzed by using Protein Carbonyl Colorimetric Assay Kit (Tissue and Serum Samples) commercial kit ref E-BC-K117-S (Elabscience Biotechnology Inc, United States), following the manufacturer's recommendations. Protein carbonyl was indirectly calculated by measuring at 370 nm after the precipitate formed between carbonyl group and 2, 4-dinitrophenylhydrazine is dissolved.

2.7. Statistical analysis

Differences between groups were assessed as described in Tamayo-Velasco A et al. [21]. Differences in oxidative stress molecules' levels between groups were assessed using the Mann Whitney *U* test. Those differences across COVID-19 severity were evaluated by the Kruskal–Wallis test, with post hoc tests adjusting for multiple comparisons. The optimal operating point (OOP) of LPO was calculated, being the value for which the point on the curve had the minimum distance to the upper left corner (where sensitivity = 1, and specificity = 1). By Pythagoras' theorem this distance is: Optimal Operating Point (OOP) = $\sqrt{(1-\text{sensitivity})2 + (1-\text{specificity})}$ [22]. COX multivariable logistic regression analysis was employed to evaluate the association between LPO and 28-day intubation/death risk. Variables with a *p*-value <0.1 in the univariate regression analysis were included in the multivariate analysis as adjusting variables. Results derived from the multivariate logistic regression analysis were validated by bootstrapping method using 1000 random samples. We analyzed probability of intubation/death to day-28 in COVID-19 patients based on LPO OOP by using Kaplan-Meier curves and tested with the log-rank test (Mantel-Haenszel). We considered 2-sided *p*-values <0.05 to indicate statistical significance. All data were analyzed using the IBM SPSS 22.0 software (SPSS, Chicago, IL).

2.8. Role of the funding source

None.

3. Results

3.1. Clinical characteristics

A total of 136 patients were registered in the study. Clinical characteristics of patients are shown in Discovery cohort panel of Table 1 in Tamayo-Velasco et al. [21]. In terms of age, there were no differences. Hypertension was the principal comorbidity followed by presence of diabetes, lung disease or coronary disease in both COVID-19 and Non COVID-19 patients. Referred to laboratory assessments, COVID-19 patients had significantly lower lymphocyte count as well as higher C-reactive protein and D-dimer. Both lower platelet and leukocyte count as well as higher neutrophils levels were observed. Non COVID-19 patients associated significant lower in-hospital stay. Finally, 18.5% of 28-day mortality was found in COVID-19 patients while no deaths were recorded across Non COVID-19 patients.

3.2. Oxidative stress levels in COVID-19 disease

The comparison of COVID-19 patients with control group displayed statistically significant differences across all oxidative stress molecules evaluated except for 8-OHdG (Table 1). Antioxidant enzymes (Superoxide dismutase (SOD) and Catalase) and oxidative cell damage (Carbonyl and Lipid peroxidation (LPO)) levels were significantly higher in COVID-19 patients while total antioxidant capacity (ABTS and FRAP) levels were lower in these patients (Table 1 and Fig. 1). The comparison of oxidative stress molecules' levels across COVID-19 severity revealed that only LPO was statistically different between mild and intubated/death COVID-19 patients (Supp File 1 and Fig. 2). A sub-analysis by mortality taking into account only intubated patients did not show statistically significant differences in terms of LPO values ($p = 0.460$).

Table 1
Oxidative stress molecules' levels in COVID-19 and Non-COVID-19 patients. Data are represented as median and interquartile range (IQR).

	Non COVID-19	COVID-19	*p*
SOD (U/mL)	0.15 [0.08]	0.38 [0.42]	< **0.001**
Catalase (U/μL)	0.49 [0.19]	0.67 [0.41]	< **0.001**
ABTS (μM)	2510.47 [437]	2264.99 [525]	< **0.001**
FRAP (μM)	700.67 [251.45]	453.84 [192.30]	< **0.001**
8-OHdG (pg/ml)	7925.78 [4894]	8373.06 [7103]	0.246
Protein Carbonyl (nmol/mg prot)	5.56 [3.68]	10.78 [7.41]	< **0.001**
MDA + HNE (μM)	284.19 [339.84]	2123.62 [2068]	< **0.001**

3.3. Evaluation of 28-day intubation/death risk depending on LPO levels

COX multivariate regression analysis identified LPO levels over the OOP (LPO>1948.17 μM) as an independent risk factor for 28-day intubation/death in COVID-19 patients [OR: 2.57; 95% CI: 1.10–5.99; $p = 0.029$] (Table 2). These results were validated in 1000 samples by Bootstrapping method (Table 3). Kaplan-Meier curve analysis revealed that COVID-19 patients showing LPO levels above 1948.17 μM were intubated or died 8.4 days earlier on average (mean survival time 15.4 vs 23.8 days) when assessing 28-day intubation/death risk ($p < 0.001$) (Fig. 3).

4. Discussion

The findings derived from this study revealed for the very first time that COVID-19 patients showed significantly lower levels of total antioxidant capacity (ABTS and FRAP) and higher levels of antioxidant enzymes (SOD, Catalase) and oxidative cell damage (Carbonyl and Lipid peroxidation (LPO)). Indeed, LPO levels over 1948.17 μM are independently associated with higher 28-day intubation/death risk.

SARS-CoV-2 stimulates reactive oxygen species (ROS) generation [23]. In COVID-19 disease, ACE2 acts as SARS-CoV-2 cellular entry receptor in type II pneumocytes of lung alveoli. ACE2 is responsible for angiotensin II (Ang II) degradation to angiotensin- (1–7) (Ang 1–7) [24]. Ang II produces ROS by stimulating membrane-bound NADPH oxidase [25]. In consequence, Ang II degradation into Ang 1–7 by ACE2 mitigates oxidative stress as it inhibits NADPH oxidase [26]. Indeed, ACE2 bounding to the virus downregulates ACE2, leading to an increased presence of superoxide species and subsequent cell damage, which may include lipid peroxidation, protein carbonylation and DNA oxidation [27], establishing an oxidative stress cycle, and ultimately, increasing the risk of suffering severe COVID-19 illness forms [25]. Our findings suggest a reactive increase of antioxidant enzymes which may be insufficient, leading to a decreased total antioxidant capacity and biomolecules' damage. Those findings differed from previous studies in respiratory viral infections such as RSV [12], hMPV [28] and Rhinovirus [29] which documented a lower expression of antioxidant enzyme levels. However, Kosmider B et al. [16] demonstrated that Influenza virus causes an increase of antioxidant genes' expression, in line with our results.

Lipid peroxidation is a biological free radical chain reaction. The oxidation of unsaturated fatty acids or other lipids results in peroxides of these compounds. Further reactions lead to aldehydes syntheses such as MDA or HNE. Lipid peroxidation affects all cell membranes inducing damage and loss of function [30]. MDA is commonly considered a marker of ferroptosis [31]. Ferroptosis is a form of regulated cell death characterized by iron-dependent lipid peroxidation, which induces cell death [32]. During ferroptosis, an accumulation of polyunsaturated fatty acids (PUFAs) occurs [33]. This implies a lipid peroxidation driven by PUFAs which enhances cell membrane permeability making the cell more sensitive to oxidation [34]. This phenomenon is a critical mechanism in sepsis-induced injuries in mice models. Kang R et al. [35] described that lipid peroxidation in ferroptosis induces pyroptosis, suggesting a link between ferroptosis and other forms of cell death in sepsis. Lipid peroxidation is involved in several disease conditions [36]

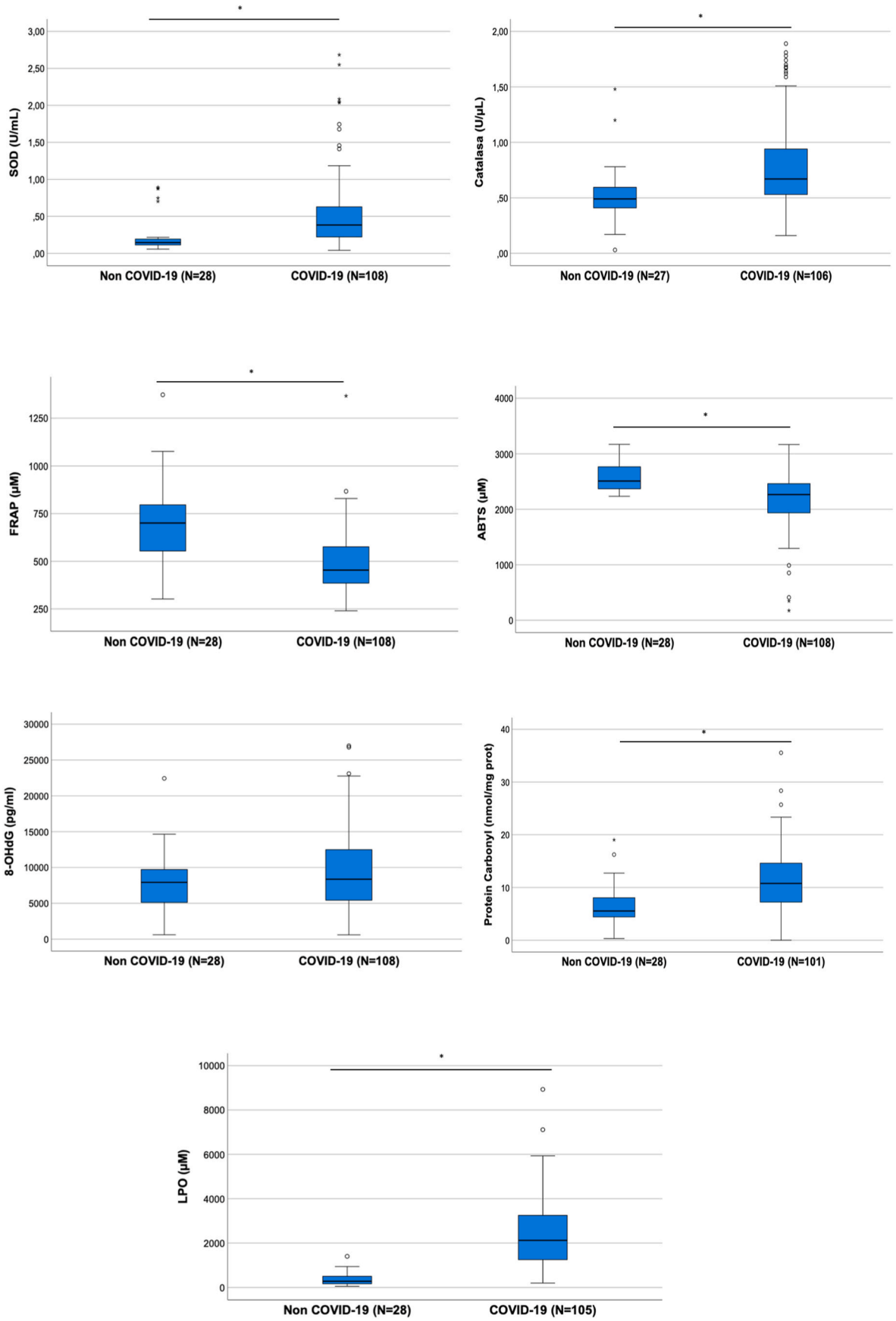

Fig. 1. Box plots showing oxidative stress molecules' levels across groups.

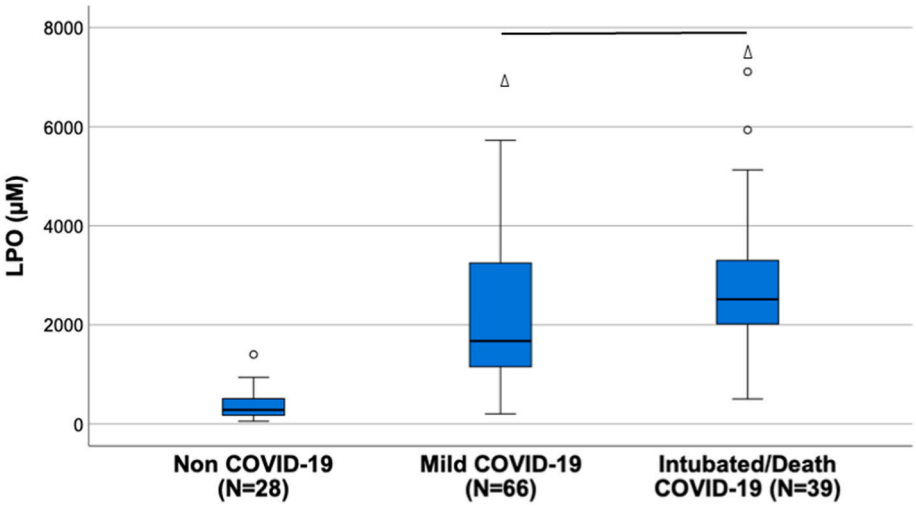

Fig. 2. Lipid peroxidation (LPO) levels across severity. The line represents significant differences between groups. The triangle represents significant differences against the healthy control.

Table 2
Multivariate logistic regression analysis to evaluate the independent association of LPO levels and risk of intubation or death at 28 days.

		OR	95% CI	*p*
Intubated/Death COVID-19 disease	**LPO >1948.17 μM**	**2.57**	**1.10–5.99**	**0.029**
	Obesity	1.19	0.48–2.97	0.702
	Diabetes mellitus	2.81	1.20–6.60	0.017
	Chronic hepatic disease	7.44	1.37–40.23	0.020
	Septic Shock	2.61	1.15–5.92	0.022
	Lymphocytes <875 cells/mm^3	0.22	0.10–0.51	<0.001
	Neutrophils >5480 cells/mm^3	2.30	1.04–5.09	0.041

Table 3
Validation of the multivariate analysis for evaluating the risk of intubation/mortality at 28 days by Bootstrapping method using 1000 random samples.

		B	95% CI	*p*
Intubated/Death COVID-19 disease	**LPO >1948.17 μM**	**0.94**	**0.16–1.96**	**0.015**
	Obesity	0.18	−0.91 – 1.72	0.729
	Diabetes mellitus	1.04	−0.20 – 2.13	0.026
	Chronic hepatic disease	2.01	0.97–3.71	0.005
	Septic Shock	0.96	0.14–2.39	0.046
	Lymphocytes <875 cells/mm^3	−1.50	(-2.71) – (−0.64)	0.002
	Neutrophils >5480 cells/mm^3	0.83	0.13–1.79	0.026

like cardiovascular disease [37] cancer [38], Alzheimer [39] and chronic diseases such as NAFLD [40], Multiple Sclerosis [41], COPD [42] and Diabetes mellitus [43].

In fact, it has a role in both homeostasis and response to stress, such as viral infection [44]. Obesity is one of the most important medical conditions leading to an exponentially increase of SARS-CoV-2 patients' mortality risk [45]. In patients suffering from metabolic disorder and COVID-19, lipid peroxidation produces reactive lipid aldehydes which will affect its prognosis [46]. In this line, our study revealed that lipid peroxidation is related to COVID-19 severity and intubation/death risk. Potje SR et al. [47] in a preliminary study in 20 COVID-19 patients documented the presence of higher levels of lipid peroxidation in COVID-19 patients but they did not find differences across patients' severity, which could be dued to a lower simple size in comparison with our study.

The results derived from this work highlight the importance of oxidative stress mediators in COVID-19, particularly the role of lipid peroxidation in prognosis of these patients. Taking this into account, these observations reinforce the urgent necessity of clinical trials in order to test the security and effectiveness regarding the implementation of antioxidant treatments in COVID-19 [48–50] for improving prognosis in this disease.

Our study has some limitations to be addressed. First, oxidative stress biomarkers were compared only at first hospital admission. Further prospective follow-up studies with serial sampling should validate these results. Second, it was conducted in a single center and should be evaluated in a multicenter fashion design to validate the potential role of lipid peroxidation in predicting intubation/death risk in COVID-19.

5. Conclusions

In summary, our findings deepen our knowledge of oxidative stress status in SARS-CoV-2 infection, supporting its important role in COVID-19. In fact, higher lipid peroxidation levels are independently associated to a greater risk of intubation or death at 28 days in COVID-19 patients. We believe that these findings open a new avenue for designing clinical trials to evaluate the beneficial role of antioxidant treatment in patients suffering from COVID-19.

Author contribution

Conceptualization: M.M-F., R.A., E.T., Á.T.-V.; methodology: M.M-F., R.A., M.H-R, E.G-S, E.T., Á.T.-V.; formal analysis: M.M-F., Á.T.-V.; investigation: M.H-R, E.G-S, P.M – P, H.G-B, L.S-dP, O.G, I.C-F.; resources: H.G.-B., E.T.; data curation: M.H-R, E.G-S, P.M – P, H.G-B, L.S-dP, O.G, I.C-F.; writing: M.M-F., Á.T.-V., E.T., R.A.; original draft preparation: M.M-F., Á.T.-V., E.T., M.H.-R.; writing—review and editing: M.M-F., Á.T.-V., M.H.-R., E.G.-S., R.A.; visualization: M.M-F., Á.T.-V.; supervision: E.T.; project administration: E.T., Á.T.-V.; funding acquisition: E.T., R.A., E.G-S. All authors have read and agreed to the published version of the manuscript.

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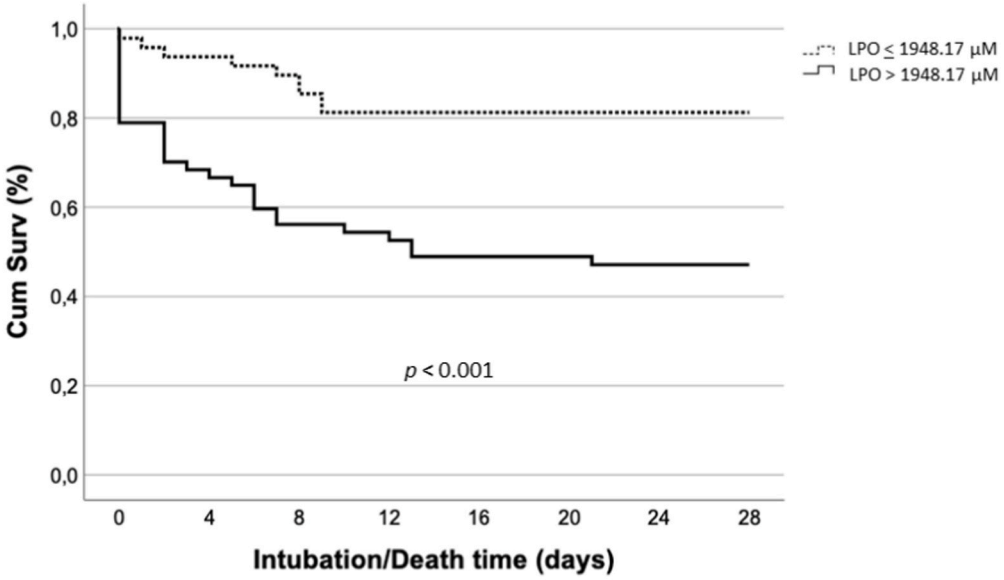

	Number at risk							
LPO ≤1948.17 µM	47	45	41	39	39	39	39	39
LPO >1948.17 µM	45	38	32	29	27	26	26	26
	0	4	8	12	16	20	24	28

Fig. 3. Kaplan-Meier curve analysis showing LPO association with 28-day intubation/death risk.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102181.

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