Intestinal Barrier Biomarker ZO1 and Endotoxin Are Increased in Blood of Patients With COVID-19-associated Pneumonia

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Abstract. *Background/Aim: The present study was undertaken to investigate (i) whether hospitalized patients with COVID-19 pneumonia present intestinal barrier dysfunction with consequent translocation of endotoxin into the systemic circulation and (ii) whether intestinal barrier biomarkers have any prognostic role in terms of progression to severe respiratory failure. Patients and Methods: In this prospective study, 22 patients with COVID-19-associated pneumonia and 19 patients with non-COVID-19-related community-acquired pneumonia (CAP group) were studied while 12 healthy persons comprised the control group. Blood samples were collected on admission and analysed for serum levels of endotoxin and zonula occludens-1 (ZO1). Clinical courses regarding progression to severe respiratory failure (SRF) requiring mechanical ventilation were recorded. Results: Patients with COVID-19-associated pneumonia and patients with CAP presented significantly higher serum endotoxin and ZO1 concentrations on admission as compared to healthy controls. There was no difference in endotoxin levels between patients with COVID-19-related pneumonia and patients with CAP. In patients with COVID-19-related pneumonia, serum endotoxin concentrations were positively correlated with C-reactive protein and ferritin values. There were no significant differences in serum endotoxin and ZO1 concentrations between patients with severe and not severe COVID-19-related pneumonia, nor between patients who developed SRF and those who did not Conclusion: Patients with COVID-19-related pneumonia present intestinal barrier dysfunction leading to systemic endotoxemia. Admission values of endotoxin and ZO1 do not have any prognostic role for progression to SRF.*

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Although the vast majority of patients infected with SARS-CoV-2 will suffer from a mild respiratory illness, some patients develop severe complications, such as acute respiratory distress syndrome, renal and hepatic dysfunction, coagulopathy with thromboses and acute cardiac injury, increasing the risk of mortality (1). In these severely ill patients with multiple organ dysfunction syndrome, usually no infectious bacterial focus is identified and blood cultures are negative but the clinical picture is compatible with sepsis, supporting the concept of viral sepsis (2).

The intestine plays a central role in the pathophysiological sequence of events that lead from sepsis to multiple organ dysfunction, being characterized as the 'motor' of sepsis (3). Enterocytes in the small intestine and colon express the angiotensin-converting enzyme 2 receptor and can therefore be infected by the SARS-CoV-2 virus (4). A multicenter study with 204 patients with COVID-19 from Hubei, China, showed that half of the patients experienced gastrointestinal symptoms and one-third had diarrhoea. In addition, SARS-CoV-2 RNA is commonly detected in faeces from patients with COVID-19, with a 3-fold increased risk of detection in those with diarrhoea (5). Previous studies have provided evidence for intestinal microbiota alterations, while endotoxemia and circulating bacteriome have been detected in critically ill patients with COVID-19 (6, 7). Bacterial and endotoxin translocation might activate the release of cytokines and other proinflammatory mediators, producing structural and functional deleterious effects on remote

Table I. *Baseline characteristics in patients with non-COVID-19-related community-acquired pneumonia (CAP) and COVID-19-related pneumonia and progression to severe respiratory failure (SRF).*

Characteristic		CAP (n=19)	COVID-19-related pneumonia (n=22)	*p*-Value
Gender, n	Male/female	9/10	11/11	0.86
Age, years	Median (IQR)	60 (55-66)	64 (55-69)	0.28
CRP, mg/dl	Median (IQR)	9 (3-15)	5 (2-13)	0.18
D-Dimers, μg/ml	Median (IQR)	0.9 (0.4-2.9)	1.7 (0.8-3)	0.17
Ferritin, ng/dl	Median (IQR)	345 (195-928)	328 (181-1574)	0.77
Fibrinogen, mg/dl	Mean±SD	561±200	619±108	0.20
Severe pneumonia, n	Yes	8/19	9/22	0.93
Positive blood culture, n	Yes	0/19	0/22	-
Progression to SRF, n	Yes	4/19	7/22	0.43

CRP: C-Reactive protein; SD: standard deviation.

organs, thus promoting the development of viral sepsis and multiple organ dysfunction syndrome.

The present study was undertaken to investigate (i) whether hospitalized patients with COVID-19-related pneumonia present intestinal epithelial barrier dysfunction with consequent translocation of endotoxin into the systemic circulation and (ii) whether intestinal barrier biomarkers play any prognostic role in terms of progression to severe respiratory failure (SRF).

Patients and Methods

Patients. We prospectively studied 22 consecutive patients hospitalized at the University Hospital of Patras from March 3 to May 3, 2020, diagnosed with COVID-19-related pneumonia according to established criteria, requiring confirmation of SARS-CoV-2 infection by positive real-time reverse transcription-polymerase chain reaction assay of a nasopharyngeal swab sample (8). The exclusion criteria were as follows: Malignancy, rheumatic diseases, renal diseases, gastrointestinal diseases (*e.g.* celiac disease, inflammatory bowel disease, gastrointestinal bleeding or intestinal surgery in the previous 4 weeks), infections during the previous 4 weeks, alcohol abuse during the previous 4 weeks, elevated serum or urine amylase and treatment during the previous month with medications that might act on tight junction (TJ) regulation such as antibiotics, corticosteroids, nonsteroidal anti-inflammatory drugs and antioxidants (vitamins C and E, allopurinol, *N*-acetyl-cysteine) (9). Nineteen age- and sex-matched patients with non-COVID-19 (negative molecular testing of respiratory secretions for SARS-CoV-2) community-acquired pneumonia (CAP) of the same severity and 12 healthy persons were also included for comparisons. CAP was diagnosed for patients who were not hospitalized for the previous 90 days and presented with all the following: (a) At least two out of four clinical signs compatible with a lower respiratory tract infection *i.e.,* dyspnoea, purulent expectoration, cough and auscultatory rales; and (b) new infiltrate on chest X-ray. Blood was sampled for analyses of investigated parameters within 24 h of admission. Classification of pneumonia severity was based on the American Thoracic Society guidelines (10). Patients with COVID-19-related pneumonia were treated according to the National Institutes of Health and the National (Greek) Public Health Organization treatment guidelines (8). They were followed-up for 14 days for development of severe

respiratory failure (SRF) (defined as PO_2/FiO_2 <200) requiring invasive or non-invasive mechanical ventilation. Patients with SRF underwent computed tomographic pulmonary angiography for exclusion of thromboembolic disease. Patients were enrolled in the context of an infectious diseases and sepsis protocol approved by the Regional Research Ethics Committee (9632/17-05-2016). Our study was carried out in accordance with the ethical guidelines of the 2003 Declaration of Helsinki.

Serum endotoxin and zonula occludens-1 (ZO1) measurements. The concentrations of endotoxin and ZO1 in the serum of patients with COVID-19-related pneumonia or CAP and healthy controls were measured by enzyme-linked immunosorbent assay using commercially available kits Endotoxin (cat#abx051541; Abbexa Ltd, Cambridge Science Park, Cambridge, UK) (range=0.015-2 EU/ml, sensitivity<0.005), ZO1 (TJP1; cat#EH15434; Finetest, Wuhan Fine BiotechCo, Wuhan, PR China) (range=0.156-10 ng/ml, sensitivity=0.094 ng/ml) as per the manufacturer's instructions.

Statistical analyses. Data were analysed using the SPSS statistical package for Windows (version 25.0; IBM, Armonk, NY, USA) and GraphPad Prism (version 9.1.0, GraphPad Software Inc., San Diego, CA, USA). Normality of data was tested using the Shapiro-Wilk test. All parameters exhibited a non-normal distribution except fibrinogen. Comparisons were performed using the nonparametric analysis of variance (Kruskal-Wallis test) followed by a *post-hoc* Mann-Whitney *U*-test (non-normally distributed data) or with one-way analysis of variance followed by *post-hoc* Student's *t*-test (normally distributed data). Subanalyses of patients with COVID-19-related pneumonia (severe/non-severe, progressors to SRF or not) were performed with the Mann-Whitney *U*-test. Results are expressed as the median (interquartile range) for non-normally distributed data or the mean±standard deviation for normally distributed data. The chi-squared test, with Yates' correction if required, was used to compare the proportional data. Correlations were estimated by a nonparametric Spearman correlation test. All tests were two-tailed and a *p*-value of less than 0.05 was considered significant.

Results

Patient characteristics. The baseline characteristics of the patients with COVID-19-related pneumonia and those with
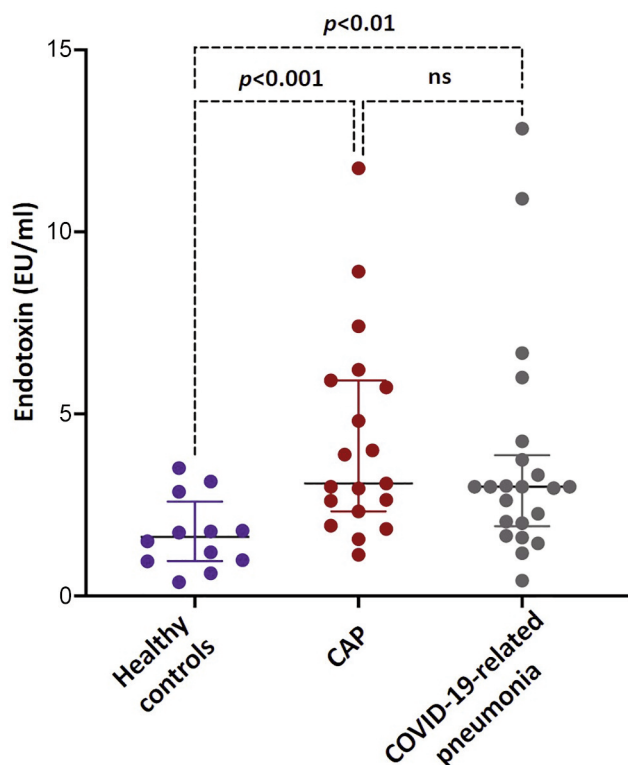
Figure 1. *Serum endotoxin levels in healthy controls, patients with non-COVID-19-related community-acquired pneumonia (CAP) and those with COVID-19-related pneumonia. Lines and bars represent median and interquartile range, respectively ns: Non-significant.*

CAP, including age, sex, CRP, ferritin, D-dimers, fibrinogen, as well as progression to SRF are shown in Table I. There were no statistically significant differences in age, gender, CRP, D-dimers, fibrinogen and ferritin levels between patients who presented CAP and those with COVID-19-related pneumonia. All blood cultures that were taken on admission were negative. Progression to SRF was observed in 7/22 patients with COVID-19-related pneumonia and in 4/19 with CAP ($p>0.05$).

Endotoxin. Patients with COVID-19-related pneumonia and those with CAP presented significantly higher serum endotoxin concentrations as compared to healthy controls ($p<0.01$ and $p<0.001$, respectively) (Figure 1). There was no statistically significant difference in endotoxin levels between patients with COVID-19-related pneumonia and those with CAP. In addition, there were no statistically significant differences in serum endotoxin concentrations between patients with severe and those with not severe COVID-19-related pneumonia (Figure 2A upper panel), nor between patients who progressed to SRF and those who did not (Figure 2A lower panel).

ZO1. Patients with COVID-19-related pneumonia and patients with non-COVID-19 CAP presented significantly higher levels of serum ZO1 compared to healthy controls ($p<0.001$, respectively) (Figure 3). There was no statistically significant difference in ZO1 levels between patients with COVID-19-related pneumonia and those with CAP. There were no statistically significant differences in the levels of ZO1 between patients with severe and those with not severe COVID-19-related pneumonia (Figure 2B upper panel) nor between patients who progressed to SRF and those who did not (Figure 2B lower panel).

Correlations. In patients with COVID-19-related pneumonia, serum endotoxin concentrations were positively correlated with CRP (r=0.52, $p<0.05$) and ferritin values (r=0.54, $p<0.05$) (Figure 4).

Discussion

In this COVID-19-related pneumonia cohort with 22 patients, we have shown that there was significant systemic release of endotoxin, comparable to that in non-COVID-related CAP. Previous studies have shown that CAP induces significant endotoxemia, which is of greater magnitude than that observed in other infections such as urinary, intra-abdominal and ventilator-associated pneumonia (11). Considering that the bacterial aetiology of CAP implicates mainly Gram-positive pathogens, while endotoxin is a compound of the cell wall of Gram-negative bacteria, it is reasonable to assume that blood endotoxin is translocated from the gut. In the present study, the fact that all patients with COVID-19-related pneumonia had negative blood cultures simultaneously with endotoxemia detection also points towards the gut origin of systemic endotoxemia.

Previous clinical studies have provided evidence for systemic endotoxemia in patients with severe COVID-19-related pneumonia admitted to intensive care units (7). In the present study, we found no difference in systemic endotoxin levels between patients with severe and non-severe COVID-19-related pneumonia, which indicates that gut-barrier integrity is affected early in the disease course. Endotoxin levels on admission were correlated with the inflammatory and prognostic markers CRP and ferritin. However, endotoxin levels on admission were not found to have any prognostic role, according to the presented results, since there was no difference between patients who progressed or not to SRF. A potential explanation of this finding might be that progression to SRF, which is usually associated with the development of an hyperinflammatory state, is not only dependent on the magnitude of inflammatory stimuli (*e.g.* endotoxin) but principally on the host inflammatory response (12). It has been shown that there is a complex interaction between the S protein of SARS-CoV-2 and endotoxin, which can lead to activation
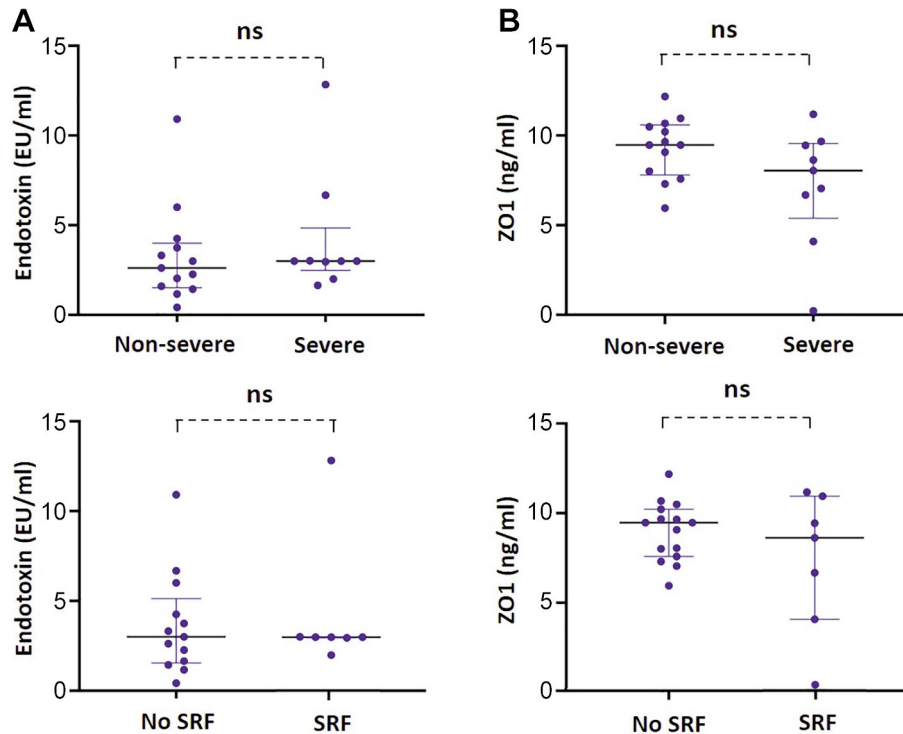
Figure 2. *Comparison of serum levels of endotoxin (A) and ZO1 (B) between patients with non-severe and severe COVID-19-related pneumonia on admission (upper panels) and between patients with and without progression to severe respiratory failure (SRF) requiring mechanical ventilation (lower panels). Lines and bars represent median and interquartile range, respectively. ns: Non-significant.*

of pro-inflammatory nuclear factor kappa B in monocytes, thus promoting the subsequent cytokine response (13).

The mechanism of intestinal barrier dysfunction in COVID-19-related pneumonia leading to systemic translocation of endotoxin might be multifactorial involving biological (gut microbiota), mechanical (intestinal epithelial cells and TJs) and immunological barriers (14). It has been demonstrated that patients with COVID-19-related gut-barrier dysfunction present significant alterations of their gut microbiota (6). To the best of our knowledge, this is the first study showing that the integrity of the intestinal paracellular barrier in patients with COVID-19-related pneumonia is impaired, as evidenced by their significantly higher serum ZO1 levels compared to healthy controls. The gut microbiota and the intestinal epithelium interact in a continuous so-called cross-talk because gut bacteria synthesize short-chain fatty acids such as butyrate, propionate and acetate, which are rich sources of energy for intestinal epithelial cells, while production of pathogen-associated molecular patterns contribute to gut immunomodulation, which interacts with both the innate and adaptive immune systems of the gut (15). Therefore, alterations of the gut microbiota in COVID-19 might represent a contributing factor implicated in impaired enterocyte TJ integrity, either through reduced energy supply to enterocytes or through the injurious effects of locally and systemically
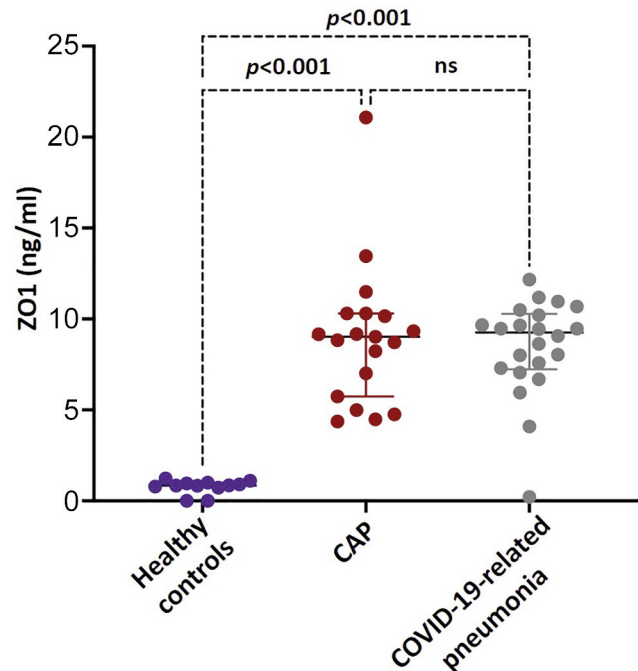

Figure 3. *Serum zonula occludens-1 (ZO-1) levels in healthy controls, patients with non-COVID-19-related community-acquired pneumonia (CAP) and those with COVID-19-related pneumonia. Lines and bars represent median and interquartile range, respectively. ns: Non-significant.*
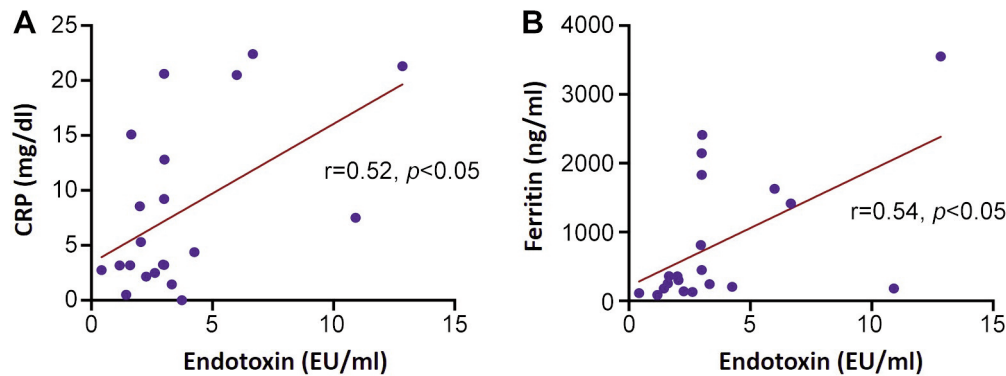
Figure 4. *Correlations of serum levels of endotoxin with C-reactive protein (CRP) (A) and ferritin (B) in patients with COVID-19-related pneumonia.*

produced proinflammatory cytokines (16). ZO1 is a 210-225 kDa phosphoprotein that interacts with the TJ proteins occludin, claudins and junctional adhesion molecule, as well as ZO2, ZO3, afadin-6, cingulin and the actin cytoskeleton (16). Therefore, it plays a key role in bringing several components responsible for the paracellular barrier together and in connecting TJ proteins to the cytoskeleton (17). ZO1 was shown to be increased in serum in several gastrointestinal and non-gastrointestinal diseases associated with increased intestinal paracellular permeability due to TJ dysfunction, such as in coeliac disease, inflammatory bowel disease, obesity, diabetes type 1, rheumatoid arthritis and sepsis (18-21). According to our findings, serum ZO1 cannot be used to distinguish patients with COVID-19-related pneumonia according to their disease severity, nor does it appear to have a prognostic role for progression to SRF. Dysfunction of the paracellular function of the gut is an important determinant of gut-barrier failure and endotoxin translocation, but several other co-factors come into play when considering the potential development of the hyperinflammatory response termed a 'cytokine storm', which is associated with adverse outcomes (3, 22). In a recent small clinical study, we showed that increased circulating pro-coagulant phospholipids in patients with COVID-19-related pneumonia had a prognostic role for progression of patients to SRF (23).

Some limitations of the current study should be acknowledged. Firstly, a limited number of patients from one center were studied, which is a drawback especially when investigating the potential prognostic role of intestinal barrier biomarkers. Secondly, investigated biomarkers of gut-barrier function were evaluated only at one time point (on admission); therefore, this study does not provide information on their longitudinal dynamics, which have important pathophysiological value, especially for patients who consequently developed SRF. Thirdly, we did not directly assess the potential derangement of intestinal integrity by obtaining appropriate intestinal biopsy specimens with upper gastrointestinal endoscopy but instead chose to evaluate a non-invasive biomarker of barrier integrity.

In conclusion, the present study provides evidence that patients with COVID-19-related pneumonia present intestinal barrier dysfunction leading to systemic endotoxemia. Evaluated biomarkers of gut-barrier dysfunction and bacterial translocation on admission appear to have no prognostic role for progression to SRF. However, the role of gut-barrier dysfunction and bacterial translocation should not be neglected as an important component of the pathophysiology in such patients. The results of this pilot study should be further examined in larger prospective studies, potentially also examining the value of therapeutic measures aiming at preservation of gut-barrier integrity.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

SFA designed the study; DP, TC, CC and IO collected the samples; MM supervised clinical handling of patients; A-LdL performed the sample analyses; AM supervised laboratory analyses; DA and SM performed the statistical analysis and prepared the output of results; SFA wrote the article; and CC, MM and AM critically revised the article. All Authors have accepted responsibility for the entire content of this article and approved its submission.

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