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# ORIGINAL RESEARCH—BASIC

## Intestinal Alkaline Phosphatase Activity and Efficiency Are Altered in Severe COVID-19 Patients

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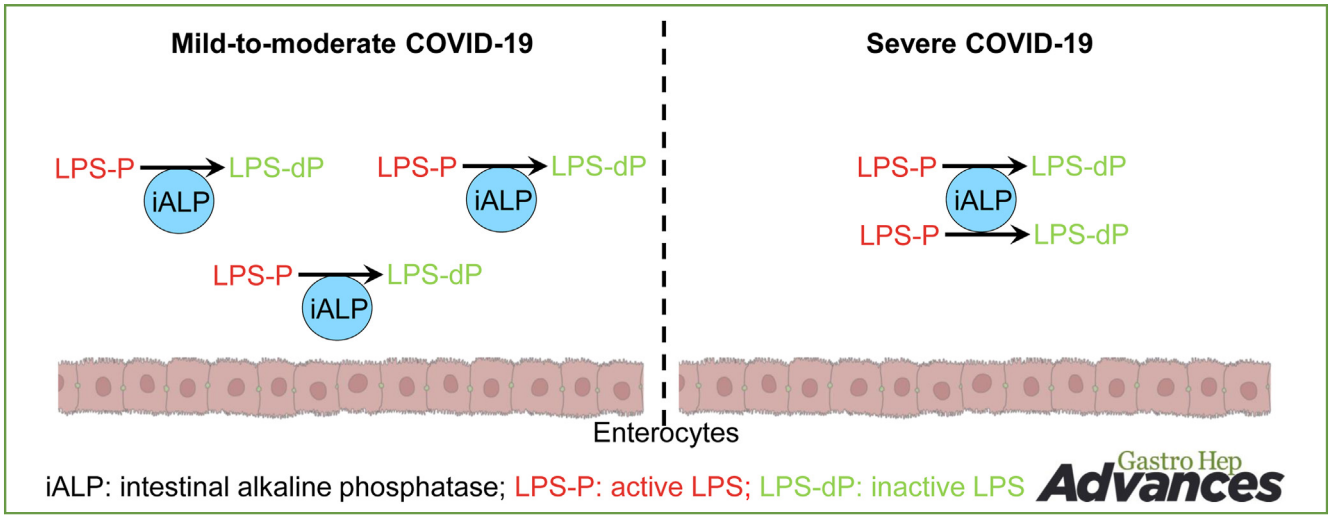


**BACKGROUND AND AIMS:** Although gut inflammation and dysbiosis have been implicated in the pathophysiology of severe cases of coronavirus disease 2019 (COVID-19), the role of intestinal anti-inflammatory enzymes, such as alkaline phosphatase, is still underexplored. Therefore, the aim of this study was to compare intestinal alkaline phosphatase (iALP) activity and its proinflammatory substrate - bacterial lipopolysaccharide (LPS) - concentration between mild-to-moderate and severe COVID-19 patients. **METHODS:** Stool samples collected from 53 mild-to-moderate and 57 severe adult COVID-19 patients, previously enrolled in a national multicentre cross-sectional study (NCT04355741), were analysed for iALP activity and LPS concentration. **RESULTS:** iALP activity decreased by 40% in severe compared to mild-to-moderate COVID-19 patients (median [interquartile range] of 120.6 [25.2–593.1] nmol pNP/min/g of protein vs 202.8 [102.1–676.1] nmol pNP/min/g of protein; $P = .04$) after adjustment for clinical and gut microbiota parameters. Regarding fecal LPS, its concentration was found to be decreased in severe patients (mean ± standard error of mean of 18,118 ± 1225 EU/g of feces vs 22,508 ± 1203 EU/g of feces; $P = .01$), although this parameter did not correlate with plasma levels of C-reactive protein ($P = .08$), a sensitive biomarker of systemic inflammation. In contrast, fecal ALP activity / LPS concentration ratio, an indicator of iALP efficiency, was found to be increased in severe compared to mild-to-moderate COVID-19 patients ($P = .04$). **CONCLUSION:** Changes in iALP kinetic parameters found in severe COVID-19 patients may represent a potential mechanism to counterbalance alterations in gut homeostasis (*eg* inflammation and dysbiosis) associated with COVID-19 severity.

*Keywords:* Alkaline Phosphatase; Coronavirus Disease 2019; Gut; Inflammation

## Introduction

Consistent evidence implicates the involvement of the gastrointestinal tract in the pathophysiology of coronavirus disease 2019 (COVID-19).[1,2] Gastrointestinal symptoms, most commonly diarrhea, can affect up to 50%

***Abbreviations used in this paper:*** **COVID-19, coronavirus disease 2019; EU, endotoxin units; iALP, intestinal alkaline phosphatase; LPS, lipopolysaccharide; pNP, *p*-nitrophenol; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.**

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of COVID-19 patients,[3] and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been demonstrated to infect and replicate in human enterocytes.[4] Moreover, several studies have reported that COVID-19 patients, particularly the most severe cases, have an altered gut microbiota composition, characterized by: a) a reduction in bacterial diversity; b) depletion of beneficial butyrate-producing bacteria abundance belonging to *Lacnhospiraceae* and *Ruminococaceae* families and to *Roseburia, Faecalibacterium, Eubacterium,* and *Bifidobacterium* genera; and c) an enrichment in opportunistic pathogens abundance including *Actinomyces, Ralstonia*, *Clostridium hathewayi, Bacteroides nordii, Candida,* and *Aspergillus*.[5–8] These microbiota alterations seem to persist even 6 months after COVID-19 recovery.[8] Furthermore, a pronounced gut inflammatory response has also been detected in COVID-19 patients, as evidenced by elevated concentrations of calprotectin[3] and decreased concentrations of the anti-inflammatory short-chain fatty acid butyrate[9] found in fecal samples of these patients.

Intestinal alkaline phosphatase (iALP) is an ectoenzyme mainly expressed in the brush border membrane of enterocytes.[10] This enzyme can be largely released into the intestinal lumen in a functionally active form,[11] where it can dephosphorylate pathogen associated molecular patterns, in particular bacterial lipopolysaccharides (LPSs), thereby preventing and reducing intestinal inflammation and promoting epithelial integrity.[10,12] Butyrate, whose intestinal levels are mainly derived from commensal bacteria fermentation of dietary fiber,[13] is a well-known activator of iALP explaining, at least in part, its recognized anti-inflammatory properties at intestinal level.[14,15] Considering the anti-inflammatory role of iALP and that severe COVID-19 patients show marked gut dysbiosis and inflammation, it was hypothesized that iALP activity is decreased in these patients. Therefore, the aim of this study was to compare iALP activity and its proinflammatory substrate, LPS, concentration among mild-to-moderate and severe COVID-19 patients previously enrolled in a national multicentre cross-sectional study (NCT04355741).[7] Data obtained in this work will contribute to unveil the kinetic changes that occur in intestinal anti-inflammatory enzymes according to COVID-19 severity.

# Methods

## Subject recruitment and stool samples collection

Subject recruitment and stool samples collection protocols have already been published.[7] In brief, 115 COVID-19 patients were recruited from 6 geographically different Portuguese centres during the first wave of the pandemic in Portugal (from April 21$^{st}$ to July 1$^{st}$ 2020). Inclusion criteria included: age $\geq$18 years and a positive reverse transcriptase-polymerase chain reaction test for SARS-CoV-2 in a nasopharyngeal swab. Immediately after recruitment, participating centres collected patients' clinical and demographic data, and categorised them according to COVID-19 severity as mild, moderate, or severe (using the World Health Organisation Clinical Progression Scale).[16] Additionally, a single stool sample per patient was collected using an appropriate collection kit (EasySampler, ALPCO, Salem, NH) containing ribonucleic acid (RNA) later (Sigma-Aldrich, St. Louis, MO), and 5% Triton X-100 (Merck, Darmstadt, Germany) to inactivate SARS-CoV-2. Samples were stored at −80 °C until processing. SARS-CoV-2 RNA detection and the relative abundance of Gram-negative bacteria genera in feces was performed according to the methodology described by Moreira-Rosario et al.[7]

The ethic committee from Faculdade de Ciências Médicas, NOVA Medical School, Universidade Nova de Lisboa, as well the ethic committees and institutional review boards from participating centers approved the study. Written and informed consent was obtained from all patients or their proxies prior to inclusion in the study. The study was registered at ClinicalTrials.gov with the accession number NCT04355741.

## Fecal ALP activity

Thirty-five mg of each stool sample were homogenized in 1750 $\mu$L extraction buffer (1 mM $MgCl_2$ and 10 mM Tris-HCl, pH 8.0). After centrifugation (100,00× g for 20 minutes at 4 °C), supernatants were collected, mixed thoroughly with dilution buffer (1 mM $MgCl_2$ and 200 mM Tris-base, pH 10.4) at a 1:1 ratio, and used to determine ALP activity and total proteins concentration. Regarding total ALP activity, 990 $\mu$L of diluted supernatants where incubated with 10 $\mu$L p-nitrophenylphosphate (5 mM) for 5 minutes at 37 °C. After that, 5 mL of ice cold NaOH (0.02 M) was added, and samples were kept on ice for 10 minutes. After 15 minutes at room temperature, the *p*-nitrophenol (pNP) generated from the enzymatic hydrolysis of p-nitrophenylphosphate was quantified spectrophotometrically at 410 nm. To specifically determine the activity of iALP isoenzyme, diluted supernatants were preincubated for 20 minutes with 30 mM L-phenylalanine, a specific inhibitor of iALP,[17] before incubation with p-nitrophenylphosphate. Total and L-phenylalanine-inhibitable ALP activity were quantified in duplicate, and iALP activity was estimated by the difference between these 2 components. Proteins concentration of diluted supernatants was determined using the EZQ protein quantitation kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. ALP activity was expressed in nmol pNP/min/g of protein.

## Fecal LPS quantification

Quantification of LPS was performed using the Chromo-LAL reagent kit according to manufacturer's instructions (Associates of Cape Cod, Inc., Falmouth, MA). This kit quantifies essentially, although not exclusively, LPS in the phosphorylated form.[18,19] Briefly, 100 mg of each stool sample was homogenized in 1 mL of sterile saline solution (NaCl 0.9 %). After that, homogenates were centrifuged (100,00× g for 10 min at 4 °C), and supernatants were collected. This step was performed twice. Supernatants were subsequently filtered using 0.22 $\mu$m sterile syringe filters and incubated with Chromo-LAL (at a 1:1 ratio) at 37 °C. Absorbance was measured at 405 nm every 10 s and LPS concentration was expressed in endotoxin units (EU) per g of feces.

## Statistics

Statistical analysis was performed using GraphPad Prism version 9.4.0 (GraphPad Software, San Diego, CA). Data are

**Table 1.** Clinical and demographic characteristics of COVID-19 patients

| Characteristics | Total $n = 115$ | Mild-to-moderate (score 1–5)[a] $n = 56$ | Severe (score 6–9)[a] $n = 59$ | *P* Value |
|---|---|---|---|---|
| Age, y, median [IQR] | 68 [52–76] | 70 [51–76] | 66 [53–76] | .909[b] |
| Male sex, n (%) | 73 (63.5) | 30 (53.4) | 43 (72.9) | .032[c] |
| BMI, kg/m$^2$, n (%) | | | | |
| ≤ 24.9 | 36 (34.3) | 15 (32.6) | 21 (35.6) | .749[c] |
| ≥ 25.0 | 69 (65.7) | 31 (67.4) | 38 (64.4) | |
| Pneumonia SARS-CoV-2, n (%) | 84 (83.2) | 26 (61.9) | 58 (98.3) | <.0001[c] |
| C-reactive protein, mg/L, median [IQR] | 40.5 [16.5–123.4] | 29.4 [10.0–70.2] | 50.0 [20.4–148.5] | .037[b] |
| Mortality, n (%) | 20 (19.4) | 7 (14.0) | 13 (24.5) | .262[c] |
| Comorbidities, n (%) | | | | |
| Overweight or obesity | 69 (65.7) | 31 (67.4) | 38 (64.4) | .749[c] |
| Hypertension | 67 (62.0) | 31 (63.3) | 36 (61.0) | .811[c] |
| Diabetes | 45 (42.1) | 16 (33.3) | 29 (49.2) | .099[c] |
| Chronic respiratory disease | 21 (19.6) | 7(14.6) | 14 (23.7) | .236[c] |
| Pharmacological therapy, n (%) | | | | |
| Non-antibiotic therapy prior to COVID-19 diagnosis (last 6 mo) | 86 (86.9) | 37 (92.5) | 49 (84.5) | .429[c] |
| Proton pump inhibitors during the course of COVID-19 | 74 (69.8) | 32 (68.1) | 42 (71.2) | .730[c] |
| Laxatives during the course of COVID-19 | 4 (5.1) | 2 (5.0) | 2 (5.2) | .958[c] |
| Antibiotic therapy prior to COVID-19 diagnosis (last 6 mo) | 42 (38.9) | 22 (44.9) | 20 (33.9) | .243[c] |
| Antibiotic therapy during the course of COVID-19 | 92 (85.2) | 39 (79.6) | 53 (89.8) | .136[c] |

IQR, interquartile range.
[a]Patients were classified according to the WHO, Clinical Progression Scale, which provides a measure of disease severity ranging from 0 (not infected with SARS-CoV-2) to 10 (dead).
[b]Mann–Whitney test.
[c]Chi-square test.

presented as numbers and percentages for categorical variables, as mean ± standard error of the mean for normally distributed continuous variables and as median (interquartile range) for nonnormally distributed continuous variables. The D'Agostino-Pearson omnibus test was used to assess normality. Chi-square test was used to compare categorical variables between mild-to-moderate and severe COVID-19 patients; and unpaired Student's *t* and Mann–Whitney tests were used to compare normally and non-normally distributed continuous variables, respectively, between mild-to-moderate and severe patients. Multivariate regression analysis was performed to identify independent clinical predictors of fecal ALP activity in COVID-19 patients. Correlation analyses between fecal LPS concentrations or fecal ALP activity / LPS concentration ratios and clinical or demographic parameters were performed using Pearson or Spearman correlation coefficients for normally or nonnormally distributed continuous variables, respectively, and point-biserial correlation coefficients for binary categorical variables. Differences were considered to be statistically significant when $P < .05$.

## Results

### Clinical and demographic characteristics of COVID-19 patients

Clinical and demographic characteristics of COVID-19 patients are summarized in Table 1. Briefly, 115 adults, of which 64% were men, with a median age of 68 years were included in this study. Overweight or obesity (66%), hypertension (62%), and diabetes (42%) were the most common comorbidities among patients. Regarding pharmacological therapy, 39% of patients reported antibiotics use during the 6 months prior to COVID-19 diagnosis and 85% reported it during the course of COVID-19. Moreover, 87% of patients reported nonantibiotic chronic therapy prior to COVID-19 diagnosis. According to the World Health Organisation guidelines for COVID-19 severity,[16] 56 patients were classified as mild-to-moderate and 59 as severe. Clinical and demographic characteristics of both groups were similar, except for the following 3 parameters that were significantly higher in severe patients: percentage of male gender (73% vs 53%; $P = .03$), prevalence of SARS-CoV-2 pneumonia (98% vs 62%; $P < .0001$), and median plasma concentrations of C-reactive protein (50 mg/L vs 29 mg/L; $P = .04$).

### Fecal ALP activity decreases in severe COVID-19 patients

From the initial 115 COVID-19 patients, a sufficient amount of stools from 110 patients (53 mild-to-moderate and 57 severe) was obtained to assess total ALP activity. As shown in Figure 1A, total ALP activity decreased by 40% in severe compared to mild-to-moderate patients (median [interquartile range] of 120.6 [25.2–593.1] nmol pNP/min/g

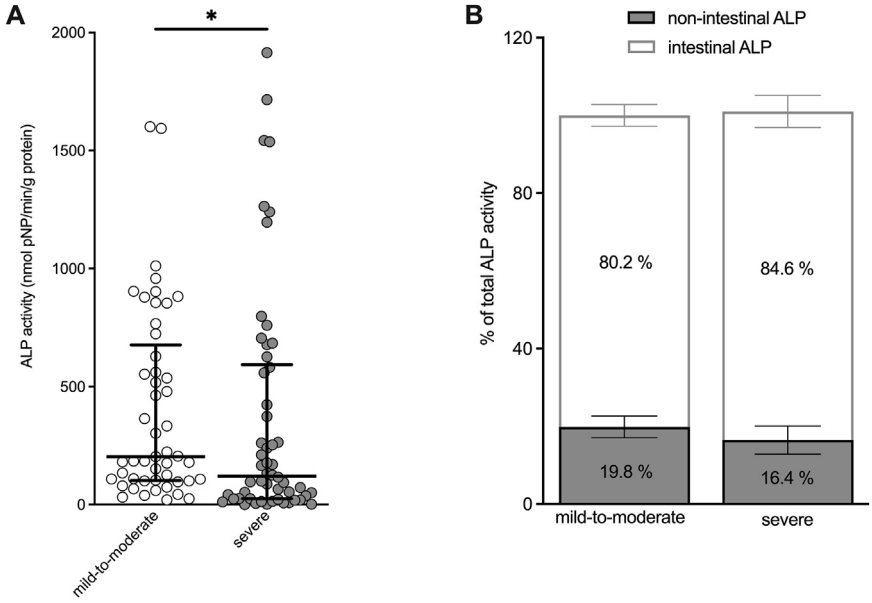


**Figure 1.** iALP activity decreases in severe COVID-19 patients. (A) Total ALP activity in stools of mild-to-moderate (n = 53) and severe (n = 57) COVID-19 patients. Data are presented as median with interquartile range. (B) Effect of L-Phe 30 mM on ALP activity in stools of mild-to-moderate (n = 6) and severe (n = 6) COVID-19 patients. Data are presented as mean ± SEM. *$P < .05$ (unpaired Mann–Whitney test). ALP, alkaline phosphatase; pNP, *p*-nitrophenol; SEM, standard error of the mean.

of protein vs 202.8 [102.1–676.1] nmol pNP/min/g of protein; $P = .04$). Using a multivariate regression model, this decrease was found to be independent of clinical and microbiota parameters previously shown to be significantly associated with a higher severity of COVID-19 in this study population.[7] Specifically, after adjustment for male gender ($P = .0002$), antibiotics use 6 months prior to COVID-19 diagnosis ($P < .0001$), microbiota diversity (expressed by the Shannon diversity Index) ($P = .0004$) and *Roseburia* abundance ($P = .046$), fecal ALP activity was predicted to decrease 122 nmol pNP/min/g protein in severe compared to mild-to-moderate COVID-19 patients (Table 2).

## Fecal ALP activity in COVID-19 patients is due to iALP

To identify the main isoenzyme of ALP present in stools of COVID-19 patients, ALP activity was assessed in the presence of L-phenylalanine, a specific iALP inhibitor,[17] in a subset of randomly selected severe ($n = 6$) and mild-to-moderate ($n = 6$) patients, due to the limited amount of stools available for most patients. In both groups, ALP activity was equally and largely (by more than 80%) inhibited by L-phenylalanine ($P = .42$), suggesting that, independently from severity, the large majority of fecal ALP measured in COVID-19 patients corresponds to iALP activity (Figure 1B).

## Fecal LPS concentration decreases in severe COVID-19 patients

Since one of the main functions of iALP is to inactivate bacterial LPS through dephosphorylation of its lipid A moiety,[11] phosphorylated LPS concentration was quantified in stool samples of COVID-19 patients. Fecal phosphorylated LPS concentration was observed to be 20% lower in severe compared to mild-to-moderate patients (mean ± standard error of mean of 18,118 ± 1225 EU/g of feces vs 22,508 ± 1203 EU/g of feces; $P = .01$) (Figure 2A).

Besides iALP function, the concentration of fecal phosphorylated LPS depends on the abundance of gram-negative bacteria,[20] since LPS is a central component of their outer membranes.[21] Taking this into consideration, the relative abundance of gram-negative bacteria, at genus level, was quantified in stool samples of COVID-19 patients. As shown in Figure 2B, the fecal abundance of gram-negative bacteria was similar in both mild-to-moderate and severe COVID-19 patients ($P = .01$), suggesting that a change in iALP kinetics

**Table 2.** Multivariate Regression Analysis Predicting an Association Between Fecal ALP Activity and COVID-19 Severity After Adjustments for Clinical and Microbiota Parameters

| Predictor variables | Estimate | 95% CI | *P* value | Goodness of fit |
|---|---|---|---|---|
| Intercept | 57.46 | −28.81 to 143.7 | .1892 | $R^2 = 0.32$ (weighted) |
| Severity | −122.0 | −184.6 to −59.47 | .0002 | |
| Male sex | 286.0 | 141.7 to 430.2 | .0002 | |
| Antibiotic therapy (last 6 mo) | −209.5 | −299.6 to −119.3 | <.0001 | |
| Shannon's index | 156.8 | 71.95 to 241.6 | .0004 | |
| *Roseburia* r.a. | 3173 | 53.35 to 6292 | .0463 | |

CI, confidence interval; r.a., relative abundance.

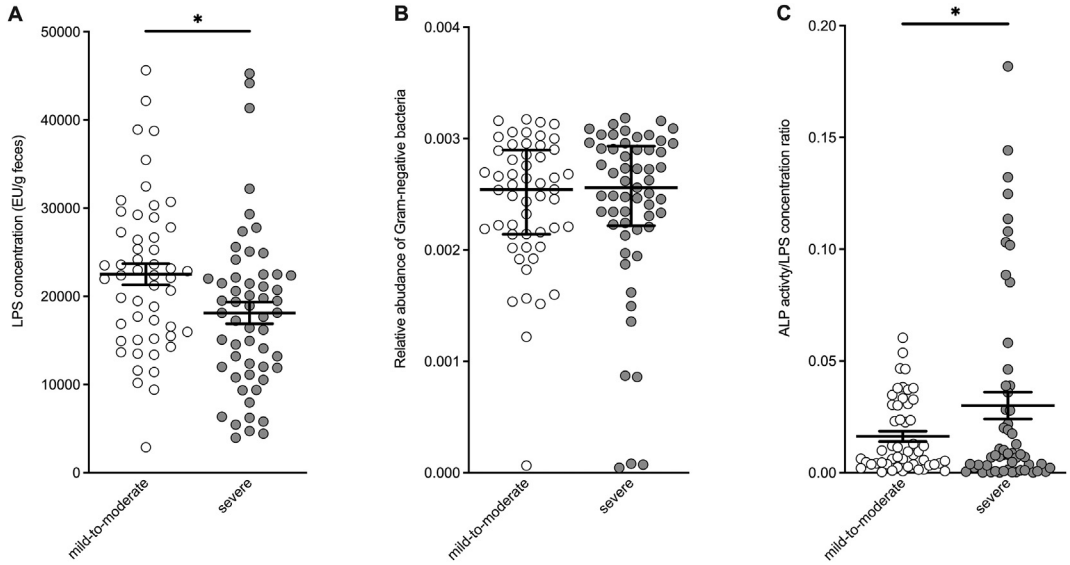


**Figure 2.** iALP efficiency increases in severe COVID-19 patients. (A) Phosphorylated LPS concentration in stools of mild-to-moderate (n = 53) and severe (n = 57) COVID-19 patients. Data are presented as mean ± SEM. *$P < .05$ (unpaired Student's *t* test). (B) Relative abundance of gram-negative bacteria, at genus level, in stools of mild-to-moderate (n = 53) and severe (n = 57) COVID-19 patients. Data are presented as median with interquartile range. *$P < .05$ (unpaired Mann–Whitney test). (C) Total ALP activity / LPS concentration ratio in stools of mild-to-moderate (n = 53) and severe (n = 57) COVID-19 patients. Data are presented as mean ± SEM. *$P < .05$ (unpaired Student's *t* test). ALP, alkaline phosphatase; EU, endotoxin units; LPS, lipopolysaccharide.

is most probably the main determinant of the reduction of phosphorylated LPS concentration observed in severe patients.

### *Fecal ALP efficiency increases in severe COVID-19 patients*

The fecal ALP activity / phosphorylated LPS concentration ratio, as a proxy indicator of iALP relative efficiency, was calculated for each COVID-19 patient. This ratio was found to be significantly higher in severe compared to mild-to-moderate COVID-19 patients ($P = .04$), suggesting the presence of a more efficient iALP in the former group of patients (Figure 2C). Even though fecal SARS-CoV-2 RNA was detected in 40% of COVID-19 patients,[7] iALP relative efficiency did not correlate with the presence of SARS-CoV-2 in stools of either severe ($r = 0.015$, $P = .92$) or mild-to-moderate ($r = -0.088$, $P = .52$) COVID-19 patients.

## Discussion

A reduction in iALP activity or expression has been associated with intestinal inflammation,[12,22] dysbiosis (lower abundance of beneficial commensal bacteria[23,24] and higher abundance of opportunistic pathogens[24,25]) and, consequently, disruption of epithelial integrity.[12] Similar intestinal alterations have also been described in severe COVID-19 patients (see Introduction), raising the hypothesis that iALP activity would be decreased in this group of patients. Taking this into account, this study aimed to compare fecal ALP activity and its proinflammatory substrate - LPS - concentration between mild-to-moderate and severe COVID-19 patients, previously enrolled in a national multicentre cross-sectional study.[7] Confirming the hypothesis raised, results from this study show that fecal ALP activity decreased by 40% in severe compared to mild-to-moderate patients, after adjustments for clinical (male sex and antibiotics use) and microbiota (bacterial diversity and commensal bacteria abundance) parameters. In agreement with previous studies conducted in non-COVID-19 patients,[10,26] the intestinal isoenzyme accounts for the large majority (around 80%) of the total ALP activity detected in stools of both severe and mild-to-moderate COVID-19 patients. However, the presence of a residual activity (less than 20%) of, most probably, ALP of bacterial origin[27,28] cannot be excluded.

To the best of our knowledge, this is the first study to assess iALP activity in COVID-19 patients. Previous studies[29–31] only assessed the activity of serum ALP, which reflects mainly the activity of the tissue-nonspecific isoenzyme of hepatic origin, whose presence was reported to be absent in human stools.[10,26] Serum ALP activity was found to be unaltered[29,31] or increased[30] in severe compared to mild-to-moderate COVID-19 patients. This discrepancy might be explained by the different types of liver injury induced by SARS-CoV-2, being some types more prone to cause an elevation of serum ALP activity than others. On the other hand, the decrease in iALP activity observed in severe COVID-19 patients in the present study, might be explained by a decrease in the intestinal concentration of butyrate (a potent inducer of ALP activity[15]), since a lower abundance of butyrate-producing *Lacnhospiraceae* and *Roseburia* has been previously observed in stools of this group of patients

compared to mild-to-moderate ones.[7] Furthermore, Zhang et al.[9] recently demonstrated that fecal butyrate concentration and the microbial pathways involved in their synthesis, were found to be depleted in severe COVID-19 patients.

One of the most important roles of iALP is to detoxify bacterial LPS through dephosphorylation of its lipid A constituent,[11,12] thereby inhibiting its ability to activate toll-like receptors 4 and stimulate proinflammatory cytokines secretion.[32] In this study, it was observed, for the first time, that phosphorylated LPS concentration decreased by 20% in stools of severe compared to mild-to-moderate COVID-19 patients. Other studies that assessed total LPS, demonstrated that its concentration was increased in the plasma of severe COVID-19 patients.[33,34] Altogether, these data suggest that a higher severity of COVID-19 seems to be associated with an increased translocation of LPS from the intestinal lumen to the blood circulation,[33,35] most probably due to a disruption of the intestinal barrier integrity.[5,33] However, in this study, fecal concentrations of LPS did not correlate with plasma levels of C-reactive protein, a sensitive biomarker of systemic inflammation,[36] neither in severe ($r = -0.033$; $P = .81$) nor in mild-to-moderate ($r = -0.138$; $P = .42$) COVID-19 patients, suggesting that the decrease in intestinal LPS concentration, and its eventual translocation into the circulation, is unlikely to be the main determinant of systemic inflammation in COVID-19 patients. Corroborating this, Effenberger et al.[3] showed that fecal concentrations of calprotectin, a marker of intestinal inflammation, did not correlate with circulating levels of C-reactive protein in patients with different degrees of COVID-19 severity.

Even though both intestinal ALP activity and LPS concentration were lower in severe COVID-19 patients, this group seems to have a more catalytically efficient iALP, as indicated by their higher fecal ALP activity / phosphorylated LPS concentration ratio in comparison to mild-to-moderate patients. This may eventually reflect a mechanism, independent of Gram-negative bacteria abundance, to counterbalance the reduced iALP activity present in severe COVID-19 patients with a dysbiotic gut microbiota, and probably explains why intestinal LPS concentrations were not associated with circulating levels of C-reactive protein. Moreover, although iALP efficiency was not associated with the presence of SARS-CoV-2 in stools, this result must be interpreted with caution, since only a single stool sample per patient was collected. Therefore, it cannot be ruled out that patients that tested negative for SARS-CoV-2 in stools could become positive throughout the course of COVID-19.

Despite the overall consistency of results obtained, this study has some limitations. First, iALP activity and fecal LPS concentration were not determined before SARS-CoV-2 infection. Secondly, due to the limited volume of blood collected after patients' hospital admission, nonroutine biochemical analyses, such as serum LPS and LPS-binding protein concentrations, could not be performed. And thirdly, iALP activity is influenced by age, sex, body mass index, antibiotics use, and diet.[12] Although both severe and mild-to-moderate COVID-19 patients were well matched for these parameters, and iALP activity data remained unaltered after adjusting for them, patients' dietary records were not collected. It is, however, unlikely that diet would have a significant impact on the results of this study, since meals served at all participating centres were standardized and aligned with hospital dietary recommendations for the Portuguese healthcare system.[37]

## Conclusion

This work supports the view that changes in kinetic parameters of intestinal anti-inflammatory enzymes, such as ALP, may occur in response to alterations in gut homeostasis, *eg* "inflammatory dysbiosis," associated with COVID-19 severity.

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**Ethical Statement:**
The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

**Data Transparency Statement:**
Deidentified individual participant data supporting the findings of this study are indefinitely available within the article or from the corresponding author upon reasonable request.

**Reporting Guidelines:**
Helsinki Declaration.