The relationship between gut and nasopharyngeal microbiome composition can predict the severity of COVID-19.

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ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) is a respiratory illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that displays great variability in clinical phenotype. Many factors have been described to be correlated with its severity but no specific determinants of infection outcome have been identified yet, maybe due the complex pathogenic mechanisms. The microbiota could play a key role in the infection and in the progression and outcome of the disease. Hence, SARS-CoV-2 infection has been associated with nasopharyngeal and gut dysbiosis and higher abundance of opportunistic pathogens. **Methods:** To identify new prognostic markers for the disease, a multicenter prospective observational cohort study was carried out in COVID-19 patients that were divided in three cohorts according to their symptomatology: mild (n=24), moderate (n=51) and severe/critical (n=31). Faecal and nasopharyngeal samples were taken and the microbiota was analysed. **Results:** Microbiota composition could be associated with the severity of the symptoms and the linear discriminant analysis identified the genera *Mycoplasma* and *Prevotella* as severity biomarkers in nasopharyngeal samples, and *Allistipes*, *Enterococcus* and *Escherichia* in faecal samples. Moreover, *M. salivarium* was defined as a unique microorganism in COVID-19 patients' nasopharyngeal microbiota while *P. bivia* and *P. timonensis* were defined in faecal microbiota. A connection between faecal and nasopharyngeal microbiota in COVID-19 patients was also identified as a strong positive correlation between *P. timonensis* (faeces) towards *P. dentalis* and *M. salivarium* (nasopharyngeal) was found in critically ill patients. **Conclusions:** This ratio could be used as a novel prognostic biomarker for severe COVID-19 patients.

Keywords: COVID-19; Gut microbiota; Nasopharyngeal microbiota; SARS-CoV-2; Severity.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a respiratory illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The data reported in November 2023 revealed that almost 700 million people have been infected with the virus [1]. Even though the majority of COVID-19 cases are mild, disease has been also shown to cause long-term effects on human health. Therefore, a remarkable feature of SARS-CoV-2 infection is the great variability in clinical phenotype among infected people. Many factors can correlate with COVID-19 disease severity, including age, gender, body mass index, previous comorbidities, immune responses, and genetics [4-6], but, unfortunately, the determinants of infection outcome and the pathogenic mechanisms are not completely understood yet [3].

SARS-CoV-2 primarily infects the respiratory tract by binding to angiotensin-converting enzyme 2 (ACE2) receptor [7], and a growing body of evidence suggests that it can also infect other organs since viral particles and nucleic acids have been found in various biological samples, like sputum, bronchoalveolar lavage fluid, faeces, blood, and urine [8-10]. Thus, ACE2 has been detected by single-cell RNA sequencing in various organs and tissues, like the gastrointestinal tract, where they are highly expressed [11], suggesting a substantial involvement of the gastrointestinal tract in the pathogenesis of the disease, including the ability of SARS-CoV-2 to infect and replicate in intestinal enterocytes [12], increased expression of the viral entry receptor (ACE2 receptor) and several membrane-bound serine proteases (such as transmembrane protease serine 2 (TMPRSS2) and TMPRSS4) in intestinal epithelial cells [13].

Moreover, SARS-CoV-2 infection has been extensively reported to induce dysbiosis the in the respiratory tract and the colon [17-20], characterized by increased presence of opportunistic pathogens, including *Staphylococcus*, *Corynebacterium* and *Acinetobacter* bacteria [14, 15], which can raise the risk of secondary infections, morbidity and mortality [16]. Thus, it is evident that there is a relevant connection between the microbiome from the respiratory and gastrointestinal tracts and the development and progression of this disease, and also the recovery processes [14, 20]. However, there is limited understanding of its precise association with the establishment of different

104 symptomatic profiles in this condition, and to date, few studies have focused on the
105 relationships between the severity of COVID-19 and the microbiome composition of the
106 nasopharyngeal and intestinal tracts contemplated simultaneously.
107 Considering that the emergence of mutations and variants has caused several additional
108 waves of infection and threatens to compromise the efficacy of existing vaccines and
109 anti-viral drugs [2], new therapeutic approaches and prognostic tools are necessary.
110 Therefore, the characterization of the nasopharyngeal and intestinal microbiome will
111 allow identifying predictive biomarkers for the diagnosis and prognosis of the disease,
112 as well as possible therapeutic targets in the management of SARS-CoV-2.
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115 **MATERIALS AND METHODS**

116 **Ethics approval.**

117 The study was conducted in accordance with the declaration of Helsinki and the
118 protocol approved by the Clinical Research Ethics Committee of Granada (CEIC) (ID
119 of the approval omicovid-19 1133-N-20). All patients provided written informed
120 consent before being included in the study. The samples were managed by the
121 ibs.GRANADA Biobank following the protocols approved by the Andalusian
122 Biomedical Research Ethics Coordinating Committee.

123 **Subject recruitment and sample collection**
124 A multicentre prospective observational cohort study was carried out between
125 September 2020 and July 2021. Patients with SARS-CoV-2 infection were recruited
126 from the University Hospital San Cecilio, the University Hospital Virgen de las Nieves,
127 and the Primary Care centres, Salvador Caballero and Las Gabias in Granada (Spain).
128 These patients were laboratory-confirmed SARS-CoV-2 positive by quantitative reverse
129 transcription polymerase chain reaction (RT-qPCR) performed on nasopharyngeal
130 swabs collected by healthcare practitioners. Patients were classified in three groups
131 based on severity profile following the described guidelines [21] mild cohort (n=24),
132 subjects with moderate symptomatology (n=51) and severe/critically ill patients (n=31).
133 Mild illness included individuals who have any of the various signs and symptoms of
134 COVID-19 (e.g., fever, cough, sore throat, malaise, headache, muscle pain, nausea,

vomiting, diarrhoea, loss of taste and smell) but do not have shortness of breath, dyspnoea, or abnormal chest imaging. Moderate cases were those showing fever and respiratory symptoms with radiological findings of pneumonia. Severe group was composed of patients with any of the following criteria: respiratory distress (≥30 breaths/min), oxygen saturation ≤93% at rest, arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) ≤300 mmHg, respiratory failure and requiring mechanical ventilation, shock, and with other organ failures that required intensive care. Healthcare staff collected Nasopharyngeal swabs and stools samples from patients while asymptomatic patients provided stools self-sampled at home. Stools and nasopharyngeal swabs were collected in collection tubes containing preservative media (OMNIgene®•GUT, DNAGENOTEK®, Ottawa, Ontario, Canada) and stored at −80°C until processing.

Microbial DNA extraction, library preparation and next generation sequencing

For all faecal and nasopharyngeal samples, DNA was isolated according to the protocol reported by *Rodríguez-Nogales et al.* [22] and using Qiagen Allprep PowerFecal DNA kit (Qiagen, Hilden, Germany). DNA was quantified using Qubit dsDNA HS assay kit (Yeason Biotechnology, Shanghai, China) and total DNA was amplified by targeting variable regions V4-V5 of the bacterial 16 S rRNA gene. Quality control of amplified products was achieved by running a high-throughput Invitrogen 96-well-E-gel (Thermo Fisher Scientific, Waltham, MA, USA). PCR products from the same samples were pooled in one plate and normalised with the high-throughput Invitrogen SequalPrep 96-well Plate kit. Then, the samples were pooled into a library prior to sequencing. Lastly, Next-Generation Sequencing (NGS) techniques were performed using an Illumina MiSeq machine.

Bioinformatic tools and statistical analysis

Bioinformatic analysis of demultiplexed raw data from nasopharyngeal and stool microbiota samples was performed with QIIME2 software (open access, Northern Arizona University, Flagstaff, AZ, USA). Trimming and filtering taking into account their quality scores before specific taxa identification achieved quality control of the samples. DADA2 software was employed to carry out denoising steps and to obtain

amplicon sequence variants (ASVs). SILVA reference database was used for taxonomic assignment [23]. The remaining analyses were performed R software [24].

For numerical clinical variables analysis, data was displayed as mean ± SD when it followed a normal distribution and median and interquartile range were represented for non-normal distributions. Categorical variables were set out as percentages. In these cases, statistical differences were calculated by ANOVA and Kruskal Wallis test for numerical variables and Fisher's exact test for categorical.

Alpha and beta diversity and relative abundance were appraised with the Phyloseq package. Normality and homogeneity of variance were examined by the Nortest and LeveneTest packages, respectively. When these assumptions were reached, an ANOVA test was carried out. Otherwise, the Kruskal Wallis test was employed.

Beta diversity differences were analysed with a Permutational Multivariate Analysis of Variance (PERMANOVA) included in the Vegan package. Euler and microbial packages were utilised for constructing Venn diagrams and to perform linear discriminant analysis (LDA) effect size (LEfSe) with an LDA score of 3. The Corrplot package was applied for correlation analysis using the Spearman's correlation coefficient.

RESULTS

Study patients characteristics

A total of 106 patients (52 women and 54 men) who had laboratory confirmation of SARS-CoV-2 infection were included in the present study. The patients had a median age of 54 years (range, 40 to 68). Based on the clinical spectrum criteria reported in the COVID-19 treatment guidelines, patients were categorised into 3 cohorts: mild symptomatology (24 patients), moderate illness and hospitalised in Respiratory Unit (51 patients) and severe symptomatology and admitted in the intensive care units (ICU) (31 patients) (**Table 1**). As expected, the age of the patients significantly increased with the severity of the symptoms, and therefore, the patients included in the severe symptoms group were significantly older than those with mild or moderate symptoms **(Table 1**). Patient inclusion was carried out evenly in terms of gender; nevertheless, a gender-related impact on the clinical course of these patients can be observed since the group of patients with severe symptoms was predominantly composed of males when compared

with the mild illness group **(Table 1)**. Correspondingly, the clinical course of patients classified according to severity was different. Most mild patients showed symptoms of a mild respiratory infection, but a third of them also displayed dyspnoea and low oxygen saturation, and a quarter reported the existence of gastrointestinal complaints (like stomach ache, digestive discomfort or diarrhoea); and a low percentage of patients (4%) reported high respiratory and heart rates. Moderate and severe patients showed higher frequencies of the evaluated symptoms: dyspnoea, low oxygen saturation and increased respiratory or heart rates ($p < 0.05$). However, no significant differences were observed in the percentage of the gastrointestinal complaints among the three groups of patients (**Table 1**). When the different comorbidities were considered, only those patients with severe symptoms showed a higher percentage of cardiomyopathy compared to those from mild or moderate symptomatology ($p < 0.05$). Additionally, no significant differences were found in the prevalence of the other pathologies among groups. Regarding the counts of lymphocytes and neutrophils did not show meaningful differences between the three groups of patients. However, the plasmatic determinations of platelets, D-dimer, ferritin and C reactive protein correlated with the severity of the symptoms, being the severe group significantly different ($p<0.05$) (**Table 1**).

	Mild (n=24)	Moderate (n=51)	Severe (n=31)
Clinical variables			
Age (Years)	43 ± 12 [a]	54 ± 14 [b]	62 ± 11 [c]
Gender (Male)	33% [a]	47% [b]	71% [c]
Symptoms			
Dyspnoea (Yes)	33% [a]	75% [b]	84% [b]
Gastrointestinal alteration (Yes)	26%	33%	33%
Respiratory rate (≥ 20 bpm)	4% [a]	22% [a]	63% [b]
SpO_2 (Low)	33% [a]	43% [a]	71% [b]
Heart rate (≥ 100 bpm)	4% [a]	27% [b]	55% [c]
Comorbidities			
Obesity (Yes)	26%	27%	32%
Diabetes (Yes)	20%	18%	26%
Asthma (Yes)	3%	6%	7%
Cardiomyopathy (Yes)	5% [a]	6% [a]	42% [b]
Plasma determinations			
Lymphocytes (10^3/µL)	1.1 ± 0.6	1.4 ± 0.6	1.2 ± 2.7
Neutrophils (10^3/µL)	6 [5.5;6.6]	6.4 [4.2;8.6]	7.9 [5.4;10.9]
Platelets (10^3/µL)	329.6 ± 8.5 [a]	257.4 ± 115 [b]	276.4 ± 93 [b]
D dimer (mg/L)	0.39 [0.2;0.8] [a]	0.6 [0.3;1] [a]	1.6 [0.9;4.3] [b]
Ferritin (ng/L)	157 [126;179] [a]	487 [274;1027] [b]	829 [488;1376] [c]
C reactive protein (mg/L)	3.4 [2.6;4] [a]	18.2 [7.8;41.9] [b]	162 [65;210] [c]

Table 1. Clinical data description of enrolled patients. Normal distributions are represented as mean ± SD while non normal distributions are represented by median and interquartile range. Categorical variables are represented with percentage. Groups with different letters statistically differ ($p < 0.05$).

Bacterial composition differs between sample type and severity index in SARS-CoV-2 infected patients

Nasopharyngeal swabs and faeces were obtained from all the patients included in the study in the first seven days after symptom onset, and used for characterization of the microbiota composition. Microbiome diversity showed alterations that could be associated with the disease severity (**Figure 1**). Specifically, the α-diversity in the nasopharyngeal microbiota was reduced in the moderate and severe groups, in comparison with the mild group although it was only significant in patients with moderate symptoms (**Figure 1A**). Conversely, when α-diversity was examined in stool samples, no significant modifications were observed between groups (**Figure 1B**). On the other hand, ß-diversity analysis revealed statistical differences between groups for both samples, nasopharyngeal swabs and stools ($p < 0.001$) (**Figure 1C** and **1D**). Nasopharyngeal microbial populations could be grouped based on the severity of the symptoms and appear like three distinct and separate clusters corresponding to the patients with mild, moderate and severe symptoms (**Figure 1C**). Remarkably, faecal microbial communities of patients with severe symptoms differed significantly from those of mild and moderate ill patients using the unweighted Bray-Curtis metric, which compares samples based on bacterial presence-absence information (**Figure 1D**).

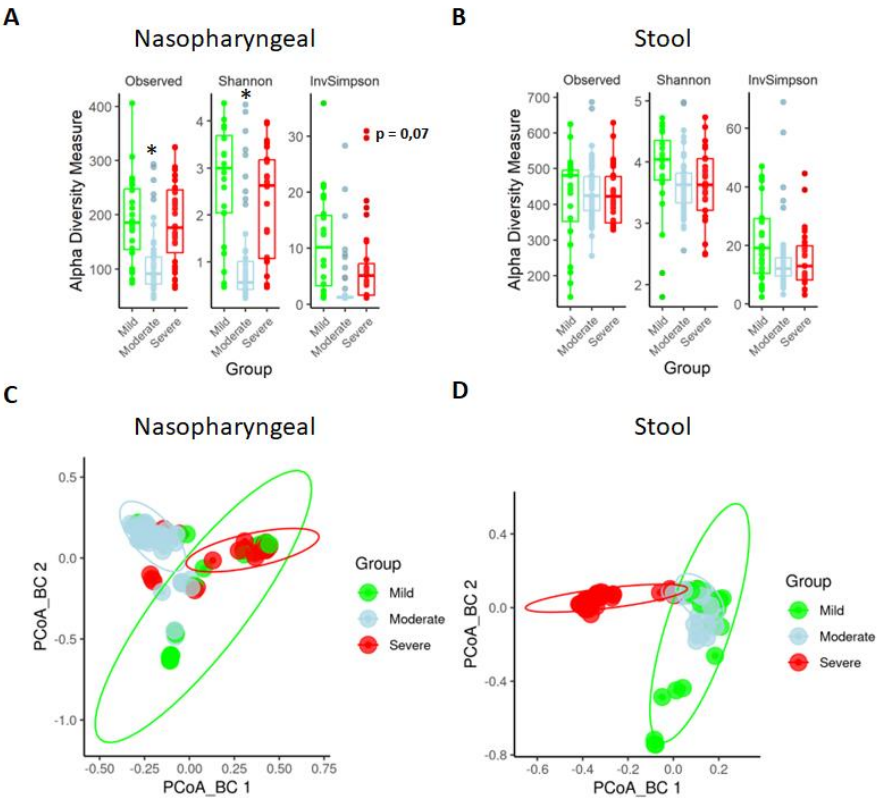

Figure 1. Nasopharyngeal and gut microbiota composition is modified depending on the severity of COVID-19 symptoms. (A) Alpha diversity analysis of nasopharyngeal swab samples microbiota. **(B)** Alpha diversity analysis of stool samples microbiota. **(C)** PCoA for Bray-Curtis index of nasopharyngeal swab microbiota. **(D)** PCoA for Bray-Curtis index of stool samples microbiota. Values are represented as mean ± SD. Significant differences are represented as * = $p < 0.05$.

Similarly, the characterization of microbiota composition revealed heterogeneity in the microbiota profile associated with severity and disease progression in these patients (**Figure Sx**). At phylum level, in nasopharyngeal microbiota, the abundance of *Bacillota* was increased while the abundance of *Bacteroidota* and *Actinobacteroidota* was reduced in patients with severe symptomatology (**Figure 2A**). Conversely, the three groups presented a more homogeneous distribution of faecal microbiota than the nasopharyngeal one, being the most abundant phyla *Bacillota* and *Bacteroidota* (**Figure 2B**). Only the patients that had a worse prognosis showed a decrease of abundance in *Bacteroidota* (**Figure 2B**).

At genus level, symptom severity was associated with a higher number of detected genera (**Figure 2C-D**). Of note, the nasopharyngeal microbiome composition revealed significant differences between groups in genus abundance. The mild group presented significantly higher abundance of *Alistipes*, *Muribaculaceae* and *Lachnospiraceae* ($p < 0.001$), the moderate group showed a significant increase in *Alcaligenes* and *Pseudorobacter* ($p < 0.001$) while the severe group had significantly higher relative

abundance of *Acinetobacter*, *Actinomyces*, *Anaerococcus*, *Atopobium*, *Campylobacter*, *Dolosigranulum*, *Enterobacter*, *Enterococcus*, *Finegoldia*, *Fusobacterium*, *Gemella*, *Haemophilus*, *Lawsonella*, *Leptotrichia*, *Megasphaera*, *Neisseria*, *Serratia*, *Rotia* and *Veillonella* ($p < 0.001$) (**Figure 2C**). However, a reduction in the number of detected genera was observed in stool samples as symptom severity increased (**Figure 2D**). Concretely, mild patients showed more presence of *Barnesiella*, *Muribaculaceae* and different members of the *Clostridia* class (*Clostridia*, *Coprococcus*, *Dorea*, *Lachnospiraceae*, *Roseburia* and *Ruminococcus*) ($p < 0.001$). Although moderate ill patients presented different genera, only *Streptococcus* was significantly increased in this group ($p < 0.001$). Remarkably, *Anaerococcus*, *Dialister*, *Lachnocostridium* or *Peptoniphilus* were more abundant in patients with severe symptoms ($p < 0.001$) (**Figure 2D**).

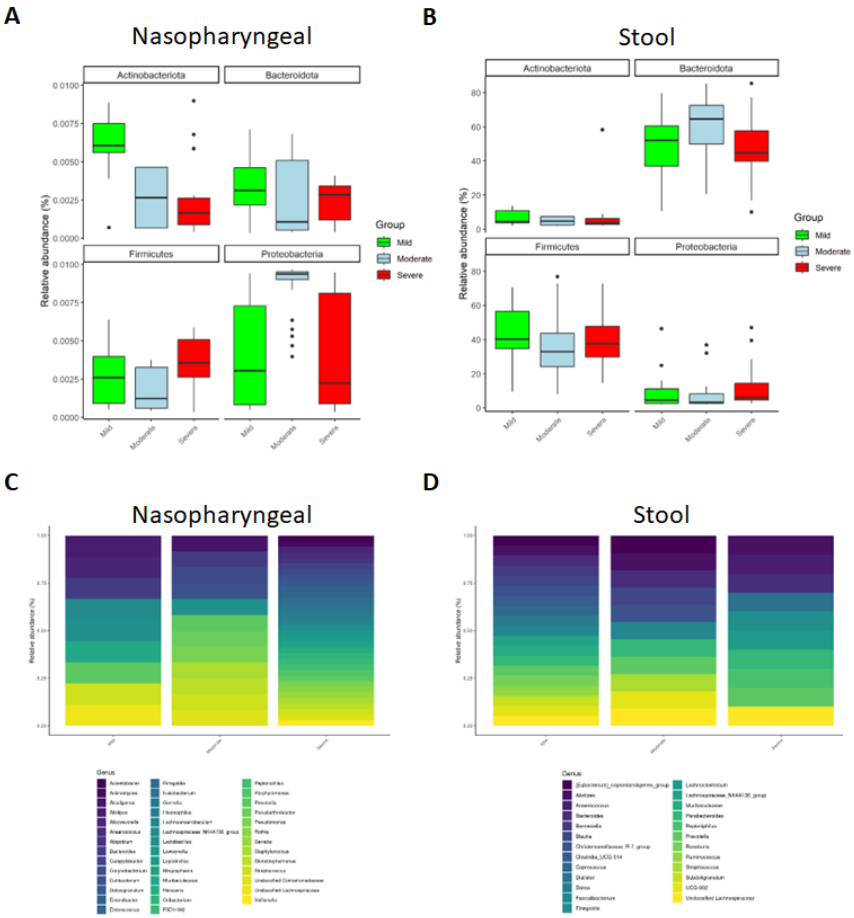

Figure 2. Microbiota composition of nasopharyngeal and stool samples at phylum level is slightly modified by COVID-19 symptoms severity. In contrast, at genus level, severity increases the total amount of detected bacteria in nasopharyngeal swabs while in stool samples it is reduced. (A) Representation of the most abundant phyla in nasopharyngeal swab samples. **(B)** Representation of the most abundant phyla in stool samples. **(C)** Taxa identification of the most abundant genera in nasopharyngeal swab samples. **(D)** Taxa identification of the most abundant genera in stool samples.

Differences in bacteria abundance could be used as biomarkers to predict disease severity and outcome in SARS-CoV-2 infection

We investigated if some specific taxa could contribute to the severity of the symptoms. ASVs were evaluated to determine core taxa along with the specific bacteria of each group of patients and samples (**Figure 3A,B**). In nasopharyngeal swabs, Venn diagram analysis revealed that the three groups of study shared 51 core taxa. 60 specific bacteria were identified in patients with mild symptoms while 32 were seen in patients with moderate symptoms and 8 in patients with severe symptoms. In stool, 159 core taxa were shared by the three groups of patients, being 27 specific for mild patients symptoms, 33 for moderate patients and 27 for severe patients (For more details see **Table S1**).

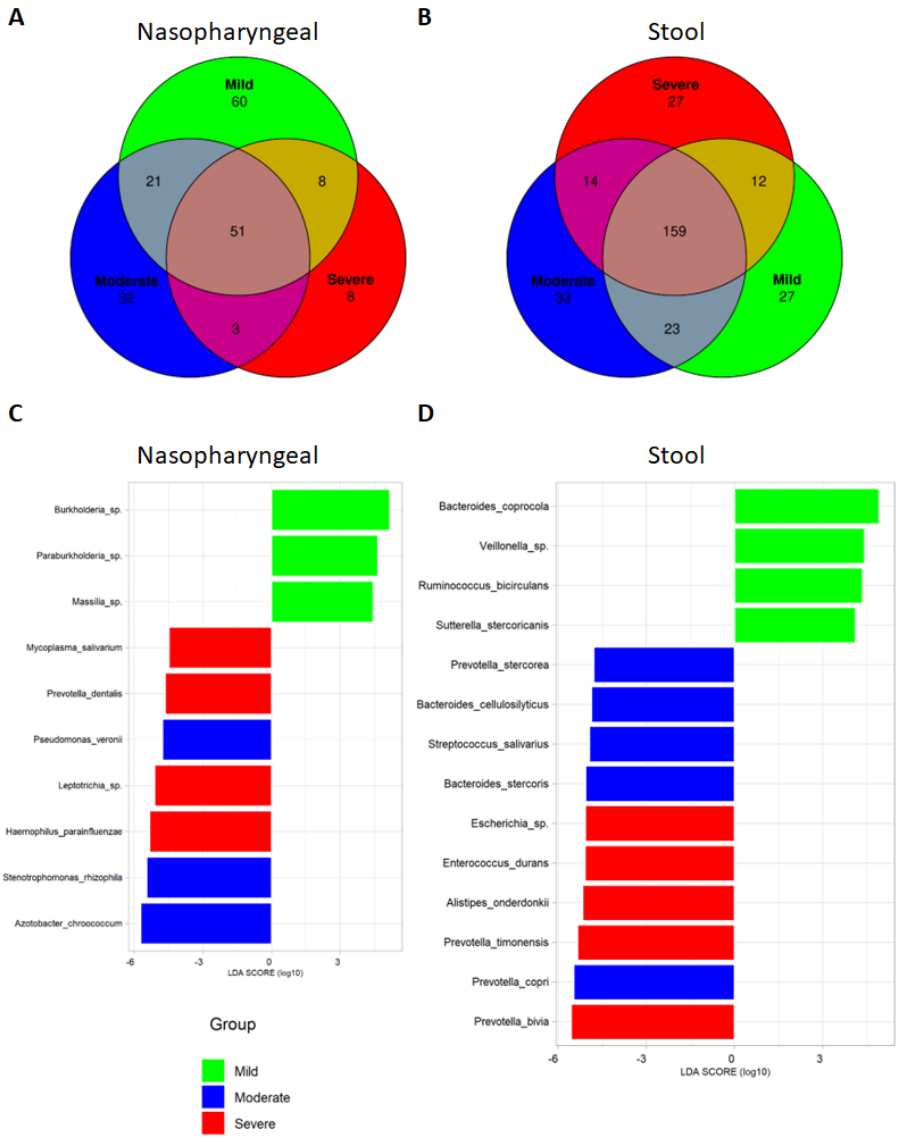

Figure 3. Differential analysis expression of microbiota composition from nasopharyngeal and stool samples revealed the presence of specific bacteria related to COVID-19 severity index. (A) Venn diagram showing ASVs distribution in nasopharyngeal swab samples. **(B)** Venn diagram showing ASVs distribution in stool samples. **(C)** LEfSe plot of taxonomic biomarkers present in nasopharyngeal swab samples (p value = 0.01 and LDA value = 4). **(D)** LEfSe plot of taxonomic biomarkers present in stool samples (p value = 0.01 and LDA value =4).

Besides, the linear discriminant analysis (LEfSe) was performed to identify differential microorganisms for each group of patients (**Figure 3C,D**). In nasopharyngeal samples, *Burkholderia sp.*, *Paraburkholderia sp.* and *Massilia sp.* were identified in mild patients; *Pseudomonas veronii*, *Stenotrophomonas rhizophila* and *Azotobacter chroococcum* in moderate patients; and *Mycoplasma salivarium*, *Prevotella dentalis*, *Leptotrichia* and *Haemophilus parainfluenzae* in severe patients. In stool samples, *Bacteroides coprocola*, *Veillonella sp.*, *Ruminococcus bicirculans* and *Sutterella stercoricanis* were identified as predictors of mild condition; *Prevotella stercorea*, *Bacteroides cellulosilyficus*, *Streptococcus salivarus*, *Bacteroides stercoris* and

Prevotella copri as predictors of moderate symptoms; and *Escherichia, Enterococcus durans, Alistipes onderdonkii, Prevotella timonensis* and *Prevotella bivia* as markers of severe condition.

To further assess the role of these potential biomarkers in the prediction of COVID-19 severity, a correlation analysis was performed (**Figure 4**). In summary, biomarkers for mild symptomatology (*B. coprocola*, *R. bicirculans*, *S. stercoricanis* and *Veillonella sp.*) presented a negative correlation profile with the different clinical features or biochemical parameters evaluated. In contrast, biomarkers associated with severe symptoms in nasopharyngeal swabs (*M. salivarium* and *Leptotrichia*) showed a positive correlation with D dimer and cardiomyopathy, respectively. In addition, the other two biomarkers linked to the highest severity (*H. parainfluenzae* and *P. dentalis*) also showed a tendency related to CRP, D dimer and cardiomyopathy (**Figure 4A**). Interestingly, similar results were found in stool samples. Severe biomarkers revealed a positive correlation towards D dimer and CRP levels, especially *P. bivia* and *P. timonensis*. These two bacteria also presented a positive association with ferritin levels, age and respiratory rate, and a negative correlation with lymphocyte count (**Figure 4B**).

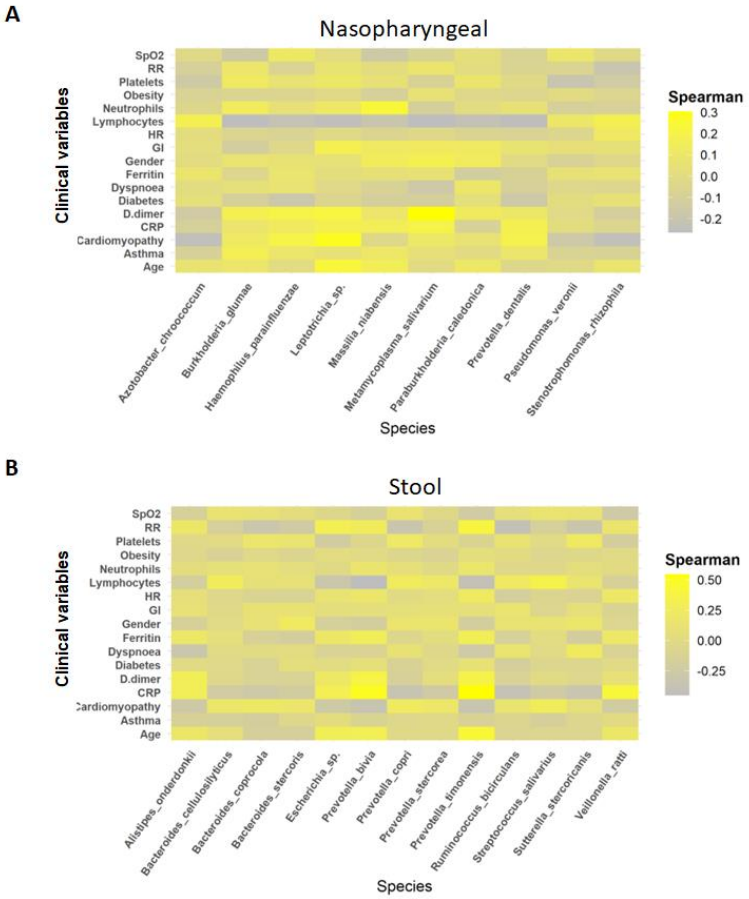

Figure 4. Whereas mild biomarkers showed negative correlations towards clinical variables, severe biomarkers presented positive correlations. (A) Correlation plot of nasopharyngeal swab biomarkers and clinical variables. **(B)** Correlation plot of stool samples biomarkers and clinical variables. RR: respiratory rate; HR: heart rate; GI: gastrointestinal alterations.

Identification of a novel microbiome-based COVID-19 prognosis approach.

Considering the LEfSe results, we propose a new approach to predict disease severity in patients suffering SARS-CoV-2 infection based on establishing a pattern of nasopharyngeal-gut microbiota. The Spearman's correlation analysis revealed no important associations between nasopharyngeal and faecal microbiota in mild and moderate groups (**Figure 5A,B**). However, in patients with severe symptoms the Spearman's rho coefficient showed a significant positive correlation between *P. timonensis* towards *P. dentalis* and *M. salivarium* (**Figure 5C**). Consequently, the ratio between the abundance of these bacteria could serve as reliable predictors of severity of COVID-19. The results revealed a significant increase in the ratios *P. timonensis* / *M. salivarium* and *P. timonensis*/*P. dentalis* in patients with severe symptoms compared to those with mild or moderate symptoms **(Figure 5D,E**).

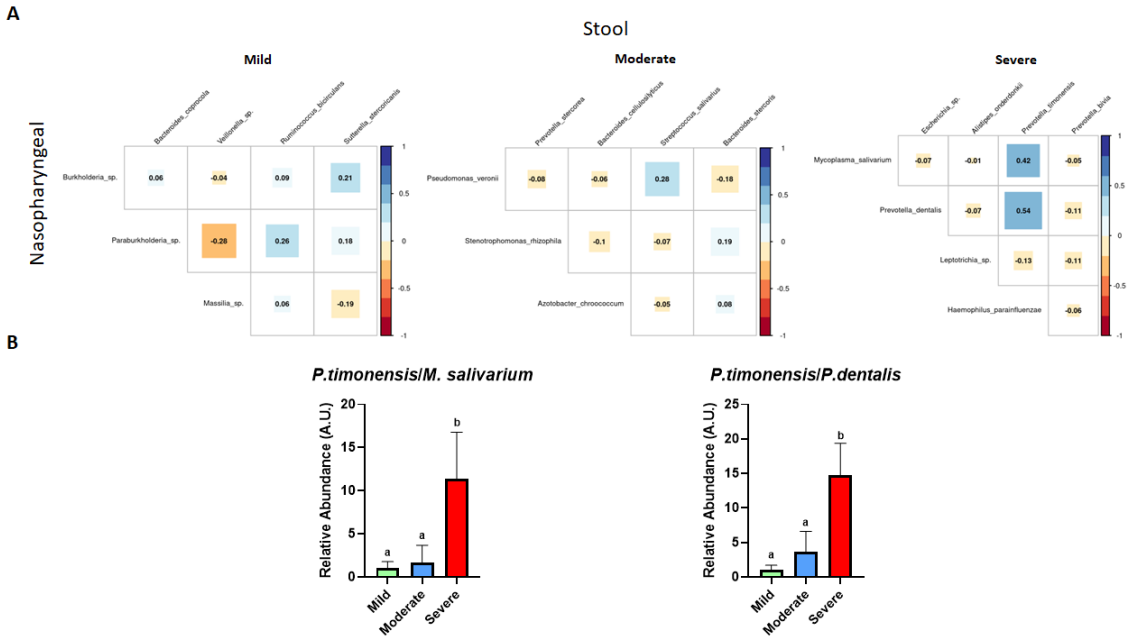

Figure 5. The existence of a relationship between the abundance of nasopharyngeal severe biomarkers and stool severe biomarkers allow the employment of an abundance ratio between them as a new tool for predicting COVID-19 severity. (A) Correlation plot among biomarkers found in nasopharyngeal swab and stool samples in each condition (mild, moderate and severe from left to right) (**B**) Ratio of the abundance between *P. timonensis* (stool) and *M. salivarium* and *P.dentalis* (nasopharyngeal swab) biomarkers. Groups with different letters statistically differ ($p < 0.05$).

DISCUSSION

Recent findings have evidenced the prominent role of the microbiome in viral infections, and it can either promote or supress viral them [25, 26]. In fact, different studies have explored the interplay between the host microbiota and SARS-CoV-2 infection [27, 28]. However, few studies have investigated the nasopharyngeal-faecal axis as a potential biomarker of severity in patients infected with SARS-CoV-2. Hence, the present study provides several nasopharyngeal and gut microbiota-based biomarkers that could help to predict COVID-19 severity.

According to the National Institutes of Health (NIH), COVID-19 severity is classified depending on the associated symptoms [21] that include age, gender, D-dimer levels, dyspnoea and higher SpO_2 score, which are predictors of worse disease progression [29]. The current study confirmed that older age as well as a higher percentage of dyspnoea; increased heart and respiratory rates together with lower oxygen saturation were associated with severe symptoms. In fact, ageing is related to immune response decline as well as higher incidence of systemic, chronic and low-grade inflammation called inflammaging. Gender is also considered a risk factor, as a recent meta-analysis has shown that men tended to have higher risk of developing severe symptoms, being hospitalised, admitted to the intensive care units and die [34] for more severe disease. The results in the present study support these previous studies as it is found that men and women are disproportionately affected since males suffered from more severe disease than females, including higher ICU admission rates, dyspnoea, increased heart rate. Sex disparities in symptoms severity has been attributed to higher rates of hazardous behaviours and existence of comorbidities, such as cardiomyopathy, in males than in females. In fact, the incidence of cardiovascular complications in COVID-19 pathology appears to be associated with sex and gender differences, thus contributing to the greater severity and poorer outcomes of the SARS-CoV2-mediated disease in male patients compared to women [35]. This relationship has also been demonstrated in this study since a higher rate of cardiovascular condition is evidenced in male patients as the severity of symptoms increases. In this context, there is few and controversial sex-stratified data investigating the role of cardiovascular complications in the prognosis and outcome of COVID-19 disease in men and women. However, it has been reported

that women exhibit higher expression and activation of angiotensin type 2 (AT2) receptors, which have been associated with a more robust anti-inflammatory immune response against SARS-CoV-2 infection and are also involved in the control of blood pressure and renal function, thereby providing protection for cardiovascular complications in female patients [36]. Remarkably, the extent of cardiac cell mortality is more noticeable in males than in females under various conditions [35], and this could be linked to an augmented protection against cardiovascular complications in female patients. Additionally, women produce high levels of regenerative white blood cells and epoxy-eicosatrienoic acids, which display antihypertensive and anti-inflammatory properties on blood vessels [37]. Consequently, this leads to restricted cardiac remodelling and a more effective restoration of functionality [38]**.** Regarding some biochemical parameters typically described as biomarkers for COVID-19 severity (D-dimer, ferritin and CRP) [39, 40], the findings obtained in this study found that those groups of patients with moderate or severe symptoms showed significantly increased levels of these plasma parameters, which are consistent with previous reports [41-43].

The association of then microbiota composition with these clinical variables has been widely studied [15] and it is well described that the microbiota can modulate host immunity and physiological functions [47], Consequently, the microbiota could be key in the clinical phenotype of these patients although the specific contribution of the microbiota to the progression of the infection and a poor prognosis is not yet fully understood. This study addresses for the first time the implication of nasopharyngeal and faecal microbiota in the prognosis of COVID-19. Firstly, when the alpha and beta diversity were evaluated, the results revealed only substantial changes in richness and Shannon diversity index in nasopharyngeal microbiome associated with severe symptoms. In this sense, controversial results have been previously reported, and although most of the studies have proposed that SARS-CoV-2 infection is associated with lower microbial diversity in nasopharyngeal samples [14, 48, 49], others did not find differences in alpha diversity composition among groups with different symptomatology [50, 51].

Furthermore, in terms of beta diversity, previous studies have reported modifications in the microbiome composition from the respiratory or gastrointestinal tract in COVID-19 patients when compared to healthy subjects [19, 52]. In the present study, in both

nasopharyngeal swabs and stools, every group of patients presented distinctly differentiated clusters. As previously reported, COVID-19 disease severity is more dependent on the presence or absence of certain bacteria rather than alterations in bacterial diversity and richness [14, 53]. Supporting this, characterization of bacterial microbiota composition at phyla and genera levels for nasopharyngeal and stool samples indicated a more evident association between changes on them and the severity of the disease. Specifically, in nasopharyngeal swabs, the presence of *Bacteroidota* and *Actinobacteriota* has been previously linked to a better prognosis of SARS-CoV-2 infection, since these bacteria have been proposed to exert beneficial effects by preventing respiratory diseases, including COVID-19 [54-58]. Moreover, in nasopharyngeal samples, the abundance of *Bacillota* and *Pesudomonadota* was increased in patients with severe symptomatology, thus supporting previous studies in which higher counts of *Bacillota* (*Staphylococcus* sp. and *Streptococcus* sp.) and *Pesudomonadota* (*Pseudomonas* sp.) were associated with moderate and severe symptoms of COVID-19 [59].

The evaluation of genera abundance composition showed that *Alistipes* and *Muribaculaceae* were highly abundant in mild patients. While these bacteria have been well characterised in gut microbiota, little information regarding their presence in nasopharyngeal microbiota has been provided up to date. Different experimental studies in mice have suggested their role in viral infections. Thus, *Muribaculaceae* was found in the lung microbiota in SARS-CoV-2 infected mice that were treated with a selective inhibitor of the main protease (M^{pro}) [60]. In the case of *Alistipes*, in a study conducted in children infected with respiratory syncytial virus (RVS), these bacteria were more abundant in the nasopharyngeal microbiota of non RVS-infected subjects [61]. Overall, these genera could be associated with a protective role against viral infection, and their higher presence in nasopharyngeal samples from mild COVID-19 patients could prevent the progression to severe disease.

Interestingly, mild patients have also shown a higher content of *Lactobacillus*, similarly to that reported previously in asymptomatic COVID-19 patients [62]. Of note, it is well described that the microorganisms forming the protective microbiota are fundamentally represented by *Lactobacillus* species. Correspondingly, the use of *Lactobacillus* strains as probiotics for preventing viral infections has been previously explored [63], and hence, its administration in SARS-CoV-2 infection could be considered to avoid complications. The increased presence of other genera, such as *Corynebacterium*,

Acinetobacter, *Staphylococcus* and *Veillonella*, positively correlated with the severity of SARS-CoV-2 infection. This correlation is supported by previous studies in which these genera were associated with both disease severity and systemic inflammation [14, 64]. In addition, higher abundance of *Enterococcus* was observed in severe patients, thus confirming other studies in critically ill patients [65].

In contrast to the findings in the nasopharyngeal microbiota, the analysis at phylum level in stool samples did not reveal notable modifications among patient groups. However, in mild ill patients, different genera from *Clostridia* class (*Clostridia*, *Coprococcus*, *Dorea*, *Lachnospiraceae*, *Roseburia* and *Ruminococcus*), *Barnesiella* and *Muribaculaceae* were identified as highly abundant. While the class *Clostridia* was associated with a reduced production of proinflammatory cytokines in COVID-19 patients and in those who recovered from the infection [66, 67], *Barnesiella* prevents colonisation by antibiotic-resistant bacteria such as *Enterococcus*, which is involved in bloodstream infection in critically ill COVID-19 patients [68, 69]. Furthermore, studies performed in mice have shown that *Muribaculaceae* abundance is reduced in mice coinfected with different respiratory viruses, suggesting that it may play a protective role under viral infection [70]. Therefore, it seems that under SARS-CoV-2 infection, the reduction of *Barnesiella, Clostridia* and *Muribaculaceae* members were associated with more severe symptoms. Nonetheless, the detected genera for the severe illness group, *Lachnocostridum*, *Anaerococcus* and *Peptoniphilus*, have been recognised as opportunistic pathogens and could contribute to a poor prognosis through inducing gut inflammation [71].

Regarding these differences, both nasopharyngeal and gut microbiota composition could be used to identify specific bacteria to predict COVID-19 severity. In the present study, unique ASVs for each condition were identified. For nasopharyngeal samples, species belonging to the genus *Lactobacillus* (*L. fermentum* or *L. reuteri*) or *Prevotella* (*P. pallens, P. ori* and *P. shahii*) have been identified. The role of *Prevotella sp.* in COVID-19 infection has not been clearly elucidated. Published microbiome analysis have revealed that its abundance was higher in mild patients [72], although others have suggested that it could be a biomarker of critical phenotype in COVID-19 patients [73, 74]. In spite of the controversial results, the results obtained in this study would confirm the potential use of this species as a biomarker for mild symptomatology. Interestingly, *Anaerococcus prevotii* was one of the exclusive species found in stools in mild patients.

This species has been linked to lower inflammation in COVID-19 patients [75]. Conversely, *Coprobacillus cateniformis* was solely found in severe patients, which could be involved in the development of a worse condition in these patients through ACE2 upregulation [18].

Even though these bacteria are unique for each group, LEfSe was performed to obtain specific biomarkers [76]. For mild patients, *Burkholderia* and *Paraburkholderia* were identified in nasopharyngeal swabs and *B. coprocola* and *R. bicirculans* in stool samples. Although the information regarding the first two species in humans is limited, a few studies have reported their presence in the commensal human microbiota [77, 78]. Contrarily, *B. coprocola* and *R. bicirculans* have been found in both healthy and COVID-19 patients [79] although the abundance of *R. bicirculans* was reduced in infected subjects [80].

In patients with moderate symptoms, *P. veronii* was detected in nasopharyngeal samples whereas *P. stercorea*, *B. cellulosilyficus*, *B. stercoris* and *P. copri* were identified in stool samples. In general, these findings agree with previous studies in COVID-19 patients [81]. Thus, *Xu et al.* found that infected patients showed higher abundance of *B. cellulosilyficus* [82], whereas *B. stercoris* and *P. copri* were associated with ACE2 upregulation and increased proinflammatory cytokine production, respectively, in COVID-19 patients [79, 83].

In critically ill patients, the biomarkers found for nasopharyngeal microbiota were *M. salivarium*, *P. dentalis*, *Leptotrichia* and *H. parainfluenzae.* In stool samples, *Escherichia sp.*, *E. durans*, *P. timonensis* and *P. bivia* were the species recognised as biomarkers. In general, all of them have been observed in the microbiota of SARS-CoV-2 infected patients. Moreover, both *P. bivia* and *P. timonensis* have been defined as unique microorganisms in COVID-19 patients' microbiota [79, 84], whilst *M. salivarium*, *H. parainfluenzae* and *E. durans* were related to a higher abundance and poor outcome in these patients [85-87]. Of note, the use of these bacteria as biomarkers of severity in SARS-CoV-2 infection is further supported by the fact that these species exhibited positive correlations with various clinical variables. Specifically, *M. salivarium*, *H. parainfluenzae*, *P. dentalis*, *P. bivia* and *P. timonensis* showed positive correlation with ferritin, CRP and D-dimer levels, as well as cardiomyopathy and respiratory rates. Several studies have revealed both the relationship between CRP levels, gut microbiota and COVID-19 severity [88], as well as the positive correlation of specific bacteria with D-dimer, CRP and the levels of pro-inflammatory mediators in

plasma [89]. When considering *A. onderdonkii*, it has been reported that this bacteria do not aggravate the symptomatology of the COVID-19 patients due to its anti-inflammatory properties [18]; however, there are conflicting evidences regarding its pathogenicity that indicate that *A. onderdonkii* may have protective effects against some diseases, including liver fibrosis, colitis and cardiovascular disease, as well as in cancer immunotherapy, while it may be *involved* in colorectal cancer development and affective disorders like depression [90]. Moreover, *Alistipes* is a relatively recent subdivision genus of the *Bacteriodota*, which is commonly associated with chronic intestinal inflammation [90]. Therefore, taking into account that *Zuo T et al.* employed a different methodology to analyse microbiota composition from stool samples [18], *A. onderdonkii* could be considered as a biomarker of severe condition in SARS-CoV-2 infected subjects.

Finally, the implication of a connection between faecal and nasopharyngeal microbiota in COVID-19 patients has been previously proposed [91]. In the present study, and to maximise the potential use of these biomarkers, the relationship of specific bacteria from nasopharyngeal and stool samples was analysed. Concretely, a strong positive correlation between *P. timonensis* (stool) towards *P. dentalis* and *M. salivarium* (nasopharyngeal) was found in severe condition. Accordingly, the ratio of the abundance of these species was also significantly increased within the highest severity of this condition. As a result, the ratio proposed in this study could be used as a novel predictor to identify critically ill COVID-19 patients as the ratio *Bacillota* and *Bacteroidetes* has been used as a marker of dysbiosis [92]. In this case, this ratio *P. timonensis*/*P. dentalis* and *M. Salivarium* could be a prognostic tool for severe SARS-CoV-2, and an increase in it could be associated with a higher risk to develop a severe condition.

CONCLUSION

This inter-individual variability between the COVID-19 patients could contribute to the different symptomatology observed. This study has identified a correlation between changes in the nasopharyngeal and stool microbiota with COVID-19 severity. A novel biomarker linked to severity of COVID-19 infection has been described based on changes in the abundance of bacterial species in nasopharyngeal and faecal samples. This knowledge can support the design of novel therapeutic strategies to mitigate adverse outcomes. Further investigations are imperative to explore how the association

between nasopharyngeal and faecal microbiota can be modulated to uncover its role in enhancing immune health, preventing or treating SARS-CoV-2 infections, and fostering immunity.

AUTHOR CONTRIBUTIONS

Benita Martín-Castaño, Margarita Martínez-Zaldívar, Emilio Mota, Fernando Cobo, Concepcion Morales-García, Marta Alvarez-Estevez, Federico García, Silvia Merlos, Paula García-Flores, Manuel Colmenero-Ruiz, José Hernández-Quero, María Nuñez were involved in the sample collection. Patricia Diez-Echave, Jorge García-García, Alba Rodríguez-Nogales, Maria Elena Rodríguez-Cabezas, Laura Hidalgo-García, Antonio Jesús Ruiz-Malagon, José Alberto Molina-Tijeras, María Jesús Rodríguez-Sojo and Anaïs Redruello were involved in the processing of simples and obtaining results. Rocio Morón and Emilio Fernández-Varón had access to the data and were involved in the conception and data analysis and interpretation. Alba Rodríguez-Nogales, Javier Martin, Maria Elena Rodriguez-Cabezas, Benita Martín-Castaño, Rocio Morón, Jorge García-García, Ángel Carazo and Julio Gálvez had access to the data and were involved in the conception and design of the work, data analysis and interpretation, critical revision of the article and final approval before submission. All authors reviewed the final manuscript and agreed to be account able for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

All authors declare no interest.

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DATA AVAILABILITY STATEMENT

Participant data cannot be made publicly available due to the sensitive nature of the personal health data and privacy and confidentiality reasons. However, under certain

conditions, these data could be accessible for statistical and scientific research. For further information, please contact the corresponding authors.

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