

# Original Research

## MicroRNA 205-5p and COVID-19 adverse outcomes: Potential molecular biomarker and regulator of the immune response

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### Impact statement

Even after the advent of vaccination, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) still circulates and causes death worldwide. Epigenetic mechanisms, such as gene expression regulation by microRNAs, may be at the basis of the individual immunological responses associated with coronavirus disease 2019 (COVID-19), and also related to the increased risk of poor outcomes in some people. In this way, the identification of new serum prognostic biomarkers at the time of the hospitalization is a feasible approach to improving patient care, and also filling gaps in the knowledge of the molecular pathways of the disease that are still not completely understood.

### Abstract

Coronavirus disease 2019 (COVID-19) is an acute respiratory infection caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The uncontrolled systemic inflammatory response, resulting from the release of large amounts of pro-inflammatory cytokines, is the main mechanism behind severe acute respiratory syndrome and multiple organ failure, the two main causes of death in COVID-19. Epigenetic mechanisms, such as gene expression regulation by microRNAs (miRs), may be at the basis of the immunological changes associated with COVID-19. Therefore, the main objective of the study was to evaluate whether the expression of miRNAs upon hospital admission could predict the risk of fatal COVID-19. To evaluate the level of circulating miRNAs, we used serum samples of COVID-19 patients collected upon hospital admission. Screening of differentially expressed miRNAs in fatal COVID-19 was performed by miRNA-Seq and the validation of miRNAs by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The Mann-Whitney test and receiver operating characteristic (ROC) curve were used to validate the miRNAs, whose potential signaling pathways and biological processes were identified through an *in silico* approach. A cohort of 100 COVID-19

patients was included in this study. By comparing the circulating levels of miRs between survivors and patients who died due to complications of the infection, we found that the expression of *miR-205-5p* was increased in those who died during hospitalization, and the expression of both *miR-205-5p* (area under the curve [AUC]=0.62, 95% confidence interval [CI]=0.5–0.7,  $P=0.03$ ) and *miR-206* (AUC=0.62, 95% CI=0.5–0.7,  $P=0.03$ ) was increased in those who later evolved to severe forms of the disease (AUC=0.70, 95% CI=0.6–0.8,  $P=0.002$ ).“*In silico*” analysis revealed that *miR-205-5p* has the potential to enhance the activation of NLRP3 inflammasome and to inhibit vascular endothelial growth factor (VEGF) pathways. Impaired innate immune response against SARS-CoV-2 may be explained by epigenetic mechanisms, which could form early biomarkers of adverse outcomes.

**Keywords:** COVID-19, SARS-CoV-2, microRNAs

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### Introduction

Since 2019, the novel coronavirus disease 2019 (COVID-19) has spread worldwide leading to global health instability

and millions of deaths.<sup>1,2</sup> Even after the advent of vaccines, new COVID-19 fatal cases are still being reported.<sup>3</sup> Clinical manifestations of COVID-19 range from mild flu-like symptoms to multiple organ failure and death, and the prediction

of fatal cases remains challenging.<sup>4,5</sup> These differences in the individual responses to the disease have been attributed to genetic and epigenetic variations.<sup>3,6</sup>

MiRs consist of small, non-coding, single-stranded RNA molecules that play an important role in the post-transcriptional regulation of gene expression mainly by direct binding to messenger RNAs (mRNAs).<sup>7,8</sup> Furthermore, miRs are actively secreted into the bloodstream, participating in intercellular communication between several cells and tissues and possibly orchestrating pathological processes by mechanisms of endocrine-genetic signaling.<sup>9</sup>

In the last decade, miRNAs have been associated with several diseases, including infections such as HIV<sup>10</sup> and hepatitis C.<sup>11</sup> However, despite miRs have been pointed as epigenetic modulators of immune response against COVID-19<sup>12,13</sup> and other diseases caused by coronaviruses,<sup>14</sup> the association between circulating miRs and the risk of death in COVID-19 remains to be explored. Therefore, the aim of this study was to evaluate whether the expression of specific miRs upon hospital admission could predict the risk of fatal COVID-19.

## Materials and methods

### Selection and clinical features of COVID-19 patients

This study cohort comprised adult patients with confirmed diagnosis of COVID-19, admitted to the UNICAMP Clinical Hospital, one of the largest public hospitals in South America, due to COVID-19 manifestations. Patients were enrolled from January to March 2021, during the second wave of the COVID-19 in Brazil. Patients of both genders aged between 18 and 98 years were eligible to the study. The diagnosis of COVID-19 was confirmed by real-time polymerase chain reaction (RT-PCR). Patients with two negative severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RT-PCR results and no radiological findings were considered COVID-19 negative and were excluded from the study. We also excluded patients with cancer; organ transplantation; systemic chronic infections; pregnant women; neurodegenerative, autoimmune, chronic kidney, or hepatic diseases; or patients within less than 24 h of hospitalization.

COVID-19 patients' serum samples were obtained from the clinical laboratory of UNICAMP Clinical Hospital, to where the patients' blood samples were sent for routine analysis. Only the blood samples collected on the first day of hospitalization were considered for this study.

Patients were followed up from the day of admission until the day of discharge or death. All demographic and clinical data were daily retrieved from electronic medical records during the hospitalization period. Two outcomes were recorded: critical COVID-19 and death during hospitalization.

### Isolation and identification of miRs differentially expressed in fatal COVID-19 by next-generation sequencing

First, we extracted total RNA from the serum samples using the miRNeasy Serum/Plasma Kit (Cat. No. / ID: 217184; Qiagen), and by next-generation sequencing (NGS) approach,

we screened dysregulated miRNAs in fatal COVID-19 compared to survivors. MiRs Library kit (Cat. No. / ID: 331502; Qiagen) was used to obtain complementary DNA (cDNA) libraries, which were sequenced in a MiSeq sequencer using the Reagent Kit v3, 150-cycle (MS-102-3001; Illumina). A total of 2,000,000–5,000,000 reads per sample were obtained. All procedures were performed according to the manufacturers' recommendations and the library quality control accessed by concentration (Qubit Fluorometric Quantitation; Thermo Fisher Scientific) and size (2100 Bioanalyzer Instrument; Agilent).

### NGS differential expression data analysis between fatal COVID-19 and survivors

Data generated by NGS were aligned against the reference genome and normalized using the DESeq2 method in GeneGlobe Data Analysis Center (Qiagen) software. Next, we performed differential expression analyses between the two groups (fatal COVID-19 and survivors) according to the unique molecular index (UMI) count in the samples. We considered differentially expressed miRs with fold change  $\pm 2$  and a value of  $P < 0.01$ . In addition, we also performed the differential expression analyses as previously described<sup>14</sup> using R script analysis (R CoreTeam, 2017) and we considered adjusted  $P$  value (adj.  $P$ )  $< 0.05$  to detect differentially expressed miRs between the two groups. Finally, we considered miRs differentially expressed between fatal COVID-19 and survivors, only those miRs that showed differential expression in both methods.

### In silico prediction of miR targets and biological function validation

Differentially expressed miR predicted target genes were accessed using Ingenuity Pathway Analysis (IPA<sup>®</sup>, Qiagen bioinformatics) and miRWalk 3.0 software. Gene set enrichment analysis was submitted to DAVID annotation (<https://david.ncifcrf.gov/>) tool, and enriched pathways were considered when the  $P$  value was  $< 0.05$ .

### Validation of differentially expressed miRs in an expanded cohort by quantitative polymerase chain reaction

RNA extracted from COVID-19 patients' serum samples (miRNeasy Serum/Plasma Kit, Cat. No. / ID: 217184; Qiagen) was reversely transcribed into cDNA using miRCURY LNA RT kit (Cat. No. / ID: 339340; Qiagen). Quantitative polymerase chain reaction (qPCR) expression was quantified using the ABI 7500 Sequence Detection System (Applied Biosystems) after SYBR green master mix addition (miRCURY LNA SYBR Green PCR Kit, Cat. No. / ID: 339306; Qiagen). We used the Pfaffl method<sup>15</sup> to calculate the relative miRNA expression, using endogenous *hsa-miR-16-5p* (GeneGlobe ID – YP00205702) as a reference gene and exogenous *cel-miR-39* (GeneGlobe ID – YP00203952) as an interplate calibrator. All reactions were performed in duplicates and primers were validated by the supplier in wet lab with estimated PCR efficiency of 2 (miRCURY LNA miRNA PCR Assay, Cat. No. / ID: 339346, *hsa-miR-133a-3p*

– GeneGlobe ID – YP00204788, hsa-miR-206 – GeneGlobe ID – YP00206073, hsa-miR-205-5p – GeneGlobe ID – YP00204487, hsa-miR-22-3p – GeneGlobe ID – YP00204606, hsa-miR-4516 – GeneGlobe ID – YP02112882, hsa-miR-184 – GeneGlobe ID – YP00204601; Qiagen). Negative controls were included on each run and produced no amplification.

### Statistical analysis

We performed descriptive analyses to describe patients' baseline characteristics and clinical outcomes. Categorical variables were expressed in absolute number and percentage. Numerical variables with normal distribution were expressed as mean and standard deviation, and numerical variables with non-normal distribution were expressed as median and interquartile range (IQR). MiR expression levels were compared between severe and non-severe COVID-19, and fatal COVID-19 and survivors using the Mann–Whitney test. Their discriminatory capacity to distinguish between the two groups was accessed by receiver operating characteristic (ROC) analyses. GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for graph plotting, and SPSS version 25.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

**Table 1.** Demographic and clinical data of COVID-19 patients selected for the screening cohort.

Clinical and demographic features	Survivors (n = 10)	Fatal COVID-19 (n = 7)
Age in years, median (IQR)	59 (50.2–66.2)	55 (45.0–65.0)
Male, n (%)	6 (60.0)	5 (71.4)
Previous diseases, n (%)		
Hypertension	6 (60.0)	3 (42.9)
Diabetes	2 (20.0)	2 (28.6)
Obesity	3 (30.0)	3 (42.9)

IQR: interquartile range; n: absolute number; COVID-19: coronavirus disease 2019.

## Results

### Screening cohort

The screening cohort for the analysis of the circulating differentially expressed miRs was composed of 10 survivors and 7 fatal COVID-19 patients. In both groups, most of the patients were middle-aged men (survivors' median age = 59 years, fatal COVID-19 median age = 55 years) with history of a previous disease, such as hypertension, diabetes, or obesity. Clinical and demographic features of the screening cohort were described in Table 1.

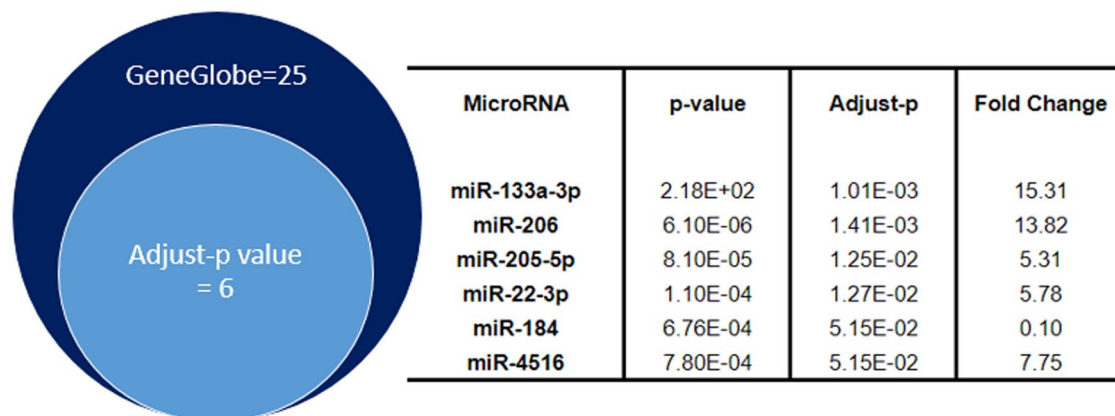
### miR differential expression in the screening cohort patients.

By an NGS analysis approach, we found a total of 25 miRs differentially expressed between survivors and fatal COVID-19 patients when considering  $P < 0.01$  in GeneGlobe analysis (data not showed). Among them, only miR-133a-3p, miR-206, miR-205-5p, miR-22-3p, miR-184, and miR-4516 remained dysregulated when the adj.  $P$  of the sample UMI count (adj.  $P < 0.05$ ) was considered. These results are illustrated in Figure 1, in which the top-six miRNAs differentially expressed in fatal COVID-19 were ranked according to the adj.  $P$ .

### Patient inclusion and exclusion criteria

A total of 100 hospitalized patients due to COVID-19-related respiratory symptoms composed our validation cohort. The inclusion and exclusion criteria of the patients are detailed in Figure 2.

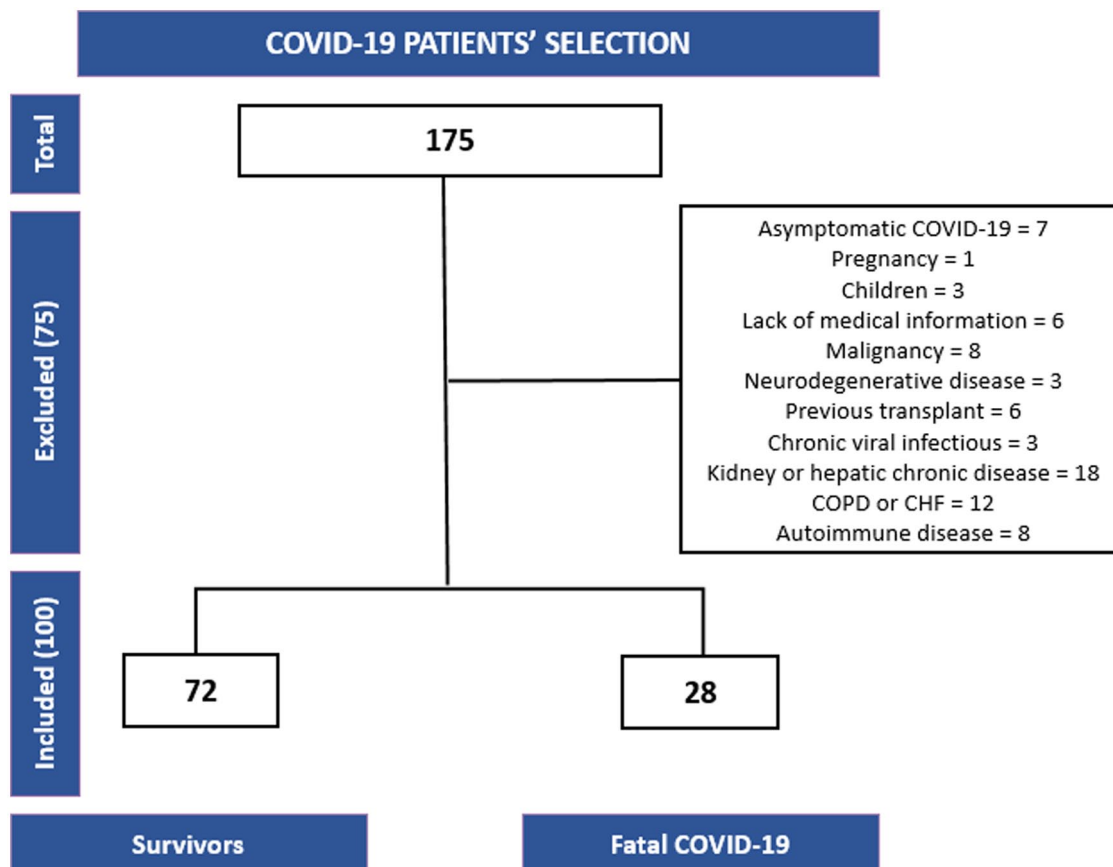
**Patient clinical and demographic features.** The validation cohort was composed of 100 hospitalized patients due to COVID-19. Among those, 28 patients died due to COVID-19 complications in the hospital, 27 were critical COVID-19 survivors, and 45 patients had non-critical COVID-19. Patient demographic and clinical features were described according to their outcome (non-critical patients, critical



**Figure 1.** miRNAs differentially expressed in survivors and fatal COVID-19 detected by NGS in COVID-19 patients.

miRNAs: microRNAs; COVID-19: coronavirus disease 2019; NGS: next-generation sequencing.

Using GeneGlobe analysis, a total of 25 dysregulated miRNAs were detected in fatal ( $P < 0.01$ ) COVID-19 patients compared to the survivors. This result was graphically represented by the dark-blue circle on the left side. Six of these 25 miRNAs, miR-133a-3p, miR-206, has-miR-205-5p, miR-22-3p, miR-184, and miR4516 (pale-blue circle on the left side) remained differentially expressed between the two groups (adj.  $P < 0.05$ ) when we performed the adjustment of the  $P$  value of the expression of miRNAs in R script analyses. Statistical characterization of these six miRNAs is described on the right side of this figure.



**Figure 2.** Flowchart of COVID-19 patient selection, inclusion, and reasons for exclusion. COPD: chronic obstructive pulmonary disease; CHF: chronic heart failure; COVID-19: coronavirus disease 2019.

**Table 2.** Demographic and clinical data of the entire COVID-19 cohort.

Clinical and demographic features	Non-critical survivors (n=45)	Critical survivors (n=27)	Fatal COVID-19 (n=28)
Age in years, median (IQR)	57.0 (42.0–66.0)	54.0 (44.0–63.0)	61.5 (50.2–71.3)
Male, n (%)	21 (46.6)	16 (59.2)	18 (64.3)
Previous diseases, n (%)			
Hypertension	16 (35.5)	10 (37.0)	18 (64.3)
Diabetes	13 (28.8)	7 (29.9)	13 (46.4)
Obesity	10 (22.2)	10 (37.3)	12 (42.8)
Cardiovascular diseases <sup>a</sup>	4 (8.8)	3 (11.1)	7 (25.0)

IQR: interquartile range; n: absolute number; COVID-19: coronavirus disease 2019.  
<sup>a</sup>Cardiovascular diseases=previous heart attack, arrhythmia, and heart failure.

COVID-19 survivors, or fatal COVID-19) in Table 2. Non-survivors were slightly older than critical COVID-19 survivors and non-critical COVID-19 (median age: 61.5 years versus 57.0 and 54.0 years, respectively). Also, individuals with hypertension (64.3%), diabetes (46.4%), obesity (24.8%), and cardiovascular diseases (25.0%) were more prevalent among the non-survivors group, but this difference was not observed between critical COVID-19 survivors and non-critical patients.

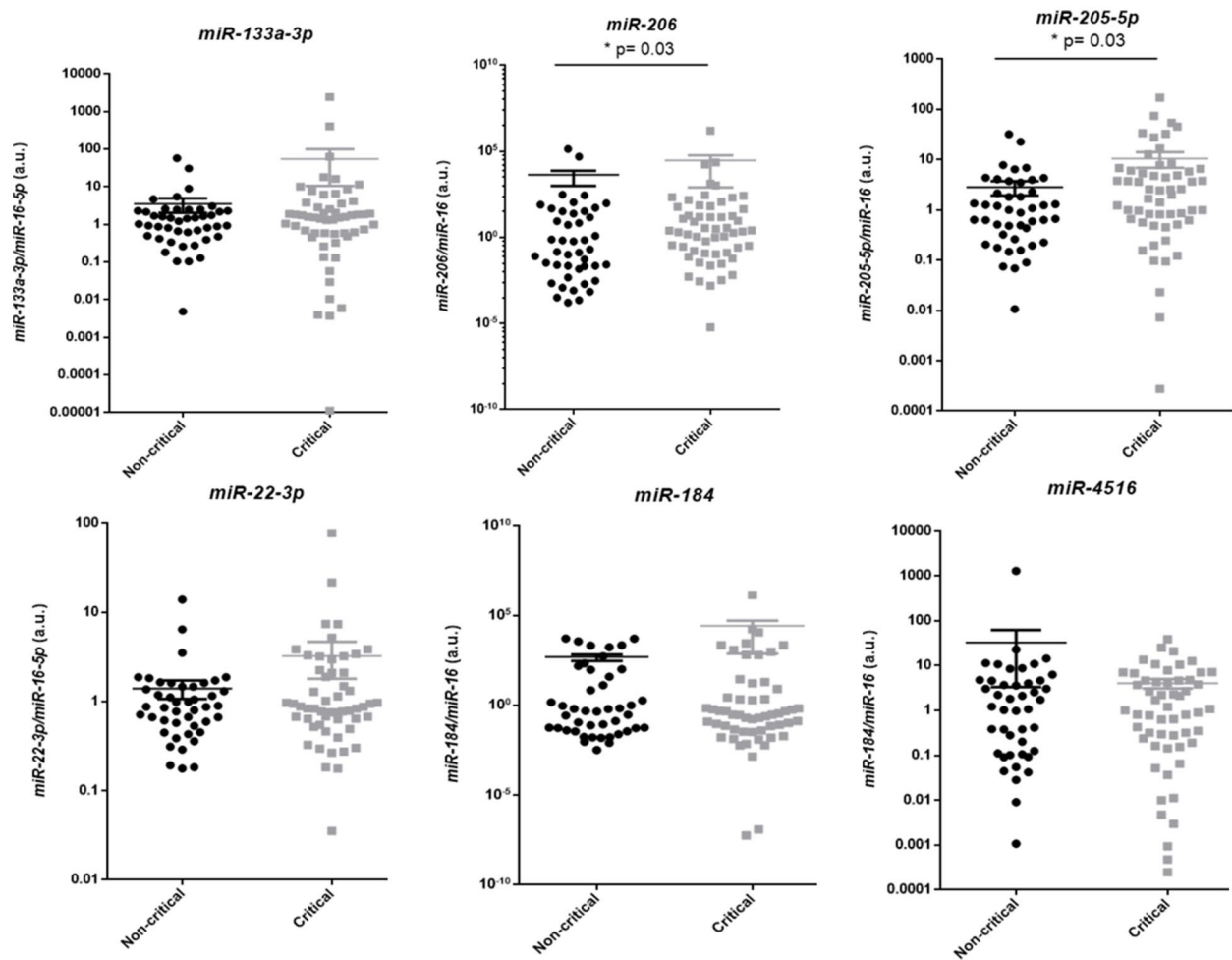
### Association between differentially expressed miRs and critical COVID-19

Serum levels of the top-six miRs identified by NGS analysis were validated in the validation cohort of COVID-19 patients

by qPCR. To evaluate if the top-six miRs were related to an increased risk of the development of critical COVID-19 forms, we first compared the miR expression levels in serum of patients who needed intensive care and/or died at any time of the hospitalization period (critical survivors and fatal COVID-19,  $n=55$ ) with the serum levels of patients who were treated on the ward and survived (non-critical survivors,  $n=45$ ).

As a result, we found that both *miR-205-5p* ( $P=0.03$ ) and *miR-206* ( $P=0.03$ ) were upregulated at the beginning of the hospitalization in patients who presented critical COVID-19 compared to non-critical ones (Figure 3).

ROC curve analysis (Figure 4) showed that *miR-205-5p* was capable of discriminating patients at higher risk to develop a critical COVID-19, from non-critical disease (area under the



**Figure 3.** miR validation in the expanded cohort of COVID-19 patients, comparing critical versus non-critical patients.

miR: microRNA; COVID-19: coronavirus disease 2019; IQR: interquartile range.

MiR-133a-3p (non-critical:  $n=45$ , median  $[M]=1.1$ , IQR<sup>95</sup>=0.5–2.3; critical COVID-19:  $n=55$ ,  $M=0.5$ , IQR=1.5–3.5;  $P=0.5$ ). MiR-206 (non-critical:  $n=45$ ,  $M=0.2$ , IQR=0.01–30.8; critical COVID-19:  $n=55$ ,  $M=2.5$ , IQR=0.1–59.8; \*  $P=0.03$ ). MiR-205-5p (non-critical:  $n=45$ ,  $M=0.9$ , IQR=0.3–3.2; critical COVID-19:  $n=55$ ,  $M=2.4$ , IQR=0.6–6.1; \*  $P=0.03$ ). MiR-22-3p (non-critical:  $n=45$ ,  $M=0.9$ , IQR=0.5–1.4; critical COVID-19:  $n=55$ ,  $M=0.8$ , IQR=0.6–2.1;  $P=0.57$ ). MiR-184 (non-critical:  $n=45$ ,  $M=0.4$ , IQR=0.04–83.2; critical COVID-19:  $n=55$ ,  $M=0.3$ , IQR=0.03–8.2;  $P=0.69$ ). MiR-4516 (non-critical:  $n=45$ ,  $M=1.5$ , IQR=0.1–4.6; critical COVID-19:  $n=55$ ,  $M=1.0$ , IQR=0.1–4.6;  $P=0.83$ ).

curve [AUC]=0.62, 95% confidence interval [CI]=0.5–0.7,  $P=0.03$ ) with 73.3% of specificity and 50.0% of sensitivity. Moreover, patients with *miR-205-5p* serum expression levels superior to 2.37 (cutoff value = 2.37) were 3 times more likely to evolve to critical COVID-19 (odds ratio [OR]=2.75; 95% CI=1.18–6.43) than patients with lower *miR-205-5p* levels. Also, *miR-206* presented a significant capacity to discriminate patients at higher risk to develop a critical form of the disease (AUC=0.62, 95% CI=0.5–0.7,  $P=0.03$ ) with 44.4% specificity and 79.6% sensitivity. In addition, patients who showed *miR-206* serum expression levels superior than 0.10 (cutoff value=0.10) were 3 times more likely to evolve to COVID-19 critical forms (OR=3.12; 95% CI=1.3–10.6) as compared to patients with lower levels of *miR-206*.

### Association between differentially expressed miRs and fatal COVID-19

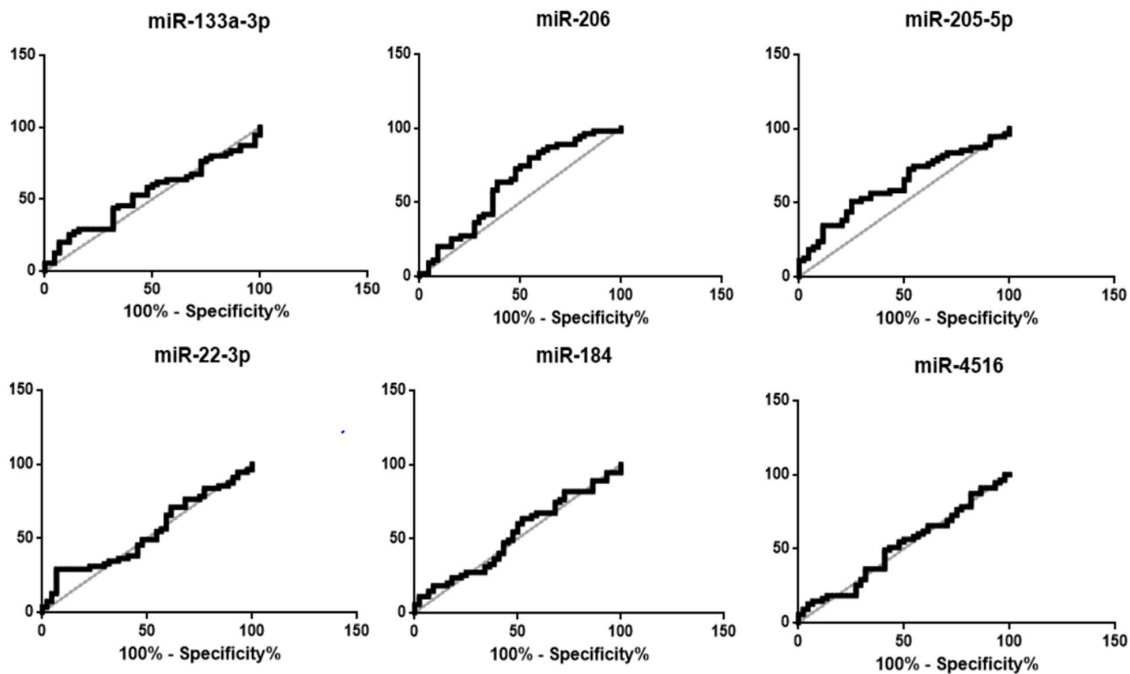
From the six dysregulated miRs detected in the screening cohort, only *miR-205-5p* remained differentially expressed in fatal COVID-19 compared to the survivors (non-critical and critical) when the expand cohort was tested. The expression

of *miR-205-5p* was higher in fatal COVID-19 ( $n=28$ ,  $M=3.2$ , IQR=0.8–14.6) compared to survivors ( $n=72$ ,  $M=1.0$ , IQR=0.4–3.7,  $P=0.03$ ), while the relative expression of *miR-133a-3p*, *miR-206*, *miR-22-3p*, *miR-184*, and *miR-4516* was similar between groups (Figure 5).

When death is considered as the outcome, ROC curve analysis showed that *miR-205-5p* levels upon hospital admission were capable of discriminating patients who died due to COVID-19 complications from survivors (AUC=0.70, IQR=0.6–0.8,  $P=0.002$ ), with 84.5% specificity and 42.9% sensitivity (Figure 6). Patients with *miR-205-5p* serum expression levels superior to 4.6 (cutoff value=4.4) had around 4 times increased risk to die due to COVID-19 complications as compared to those with lower *miR-205-5p* levels (OR=4.09, 95% CI=1.52–10.97).

### Association between demographic and clinical characteristics of COVID-19 patients and the relative expression of miR-205-5p and miR-206

To investigate whether circulating *miR-205-5p* and *miR-206* were independently associated with an increased risk



**Figure 4.** ROC curve analysis of miR relative expressions in the expanded cohort of COVID-19 patients, considering critical COVID-19 as outcome.

ROC: receiver operating characteristic; COVID-19: coronavirus disease 2019; AUC: area under the curve; CI: confidence interval.

MiR-133a-3p (AUC=0.53, 95% CI=0.4–0.6,  $P=0.54$ ); MiR-206 (AUC=0.62, 95% CI=0.5–0.7,  $P=0.03$ ); MiR-205-5p (AUC=0.62, 95% CI=0.5–0.7,  $P=0.03$ ); MiR-22-3p (AUC=0.52, 95% CI=0.4–0.6,  $P=0.57$ ); MiR-184 (AUC=0.52, 95% CI=0.4–0.6,  $P=0.6$ ); MiR-4516 (AUC=0.51, 95% CI=0.4–0.6,  $P=0.83$ ).

of critical COVID-19 or death, we analyzed the correlation between patient age, sex, previous diseases, and *miR-205-5p* or *miR-206* expression levels. No significant correlation was found between age and levels of *miR-205-5p* (correlation coefficient [ $R$ ]=0.1,  $P=0.3$ ) or *miR-206* ( $R=0.02$ ,  $P=0.8$ ). Also, *miR-205-5p* levels were similar between male ( $n=57$ ,  $M=0.9$ , IQR=0.3–4.2) and female patients ( $n=43$ ,  $M=1.4$ , IQR=0.6–5.0;  $P=0.23$ ), and between patients without comorbidities ( $n=30$ ,  $M=1.3$ , IQR=0.5–3.6) and those with previous diseases ( $n=70$ ,  $M=1.1$ , IQR: 0.4–5.5;  $P=0.64$ ). Similar results were found for *miR-206* (male:  $n=57$ ,  $M=1.5$ , IQR=0.02–25.6; female:  $n=43$ ,  $M=0.7$ , IQR=0.05–52.3;  $P=0.68$  / no previous comorbidities:  $n=30$ ,  $M=5.3$ , IQR=0.1–67.1; previous comorbidities:  $n=70$ ,  $M=0.9$ , IQR: 0.02–21.2;  $P=0.39$ ), which may indicate that basal conditions did not affect these miR serum expression levels.

**In silico prediction of *miR-205-5p* target genes.** As *miR-205-5p* expression levels were associated with critical and fatal COVID-19, an *in silico* analysis was performed to investigate possible biological pathways related to investigate the possible biological role of this miRNA on patients' aggressive outcomes. We found that *miR-205-5p* may regulate over 400 different genes, most of them involved in molecular pathways of inflammation, immunological response, viral diseases, cellular metabolism, and cardiovascular risk factors. The results of gene enrichment analysis are shown in Figure 7.

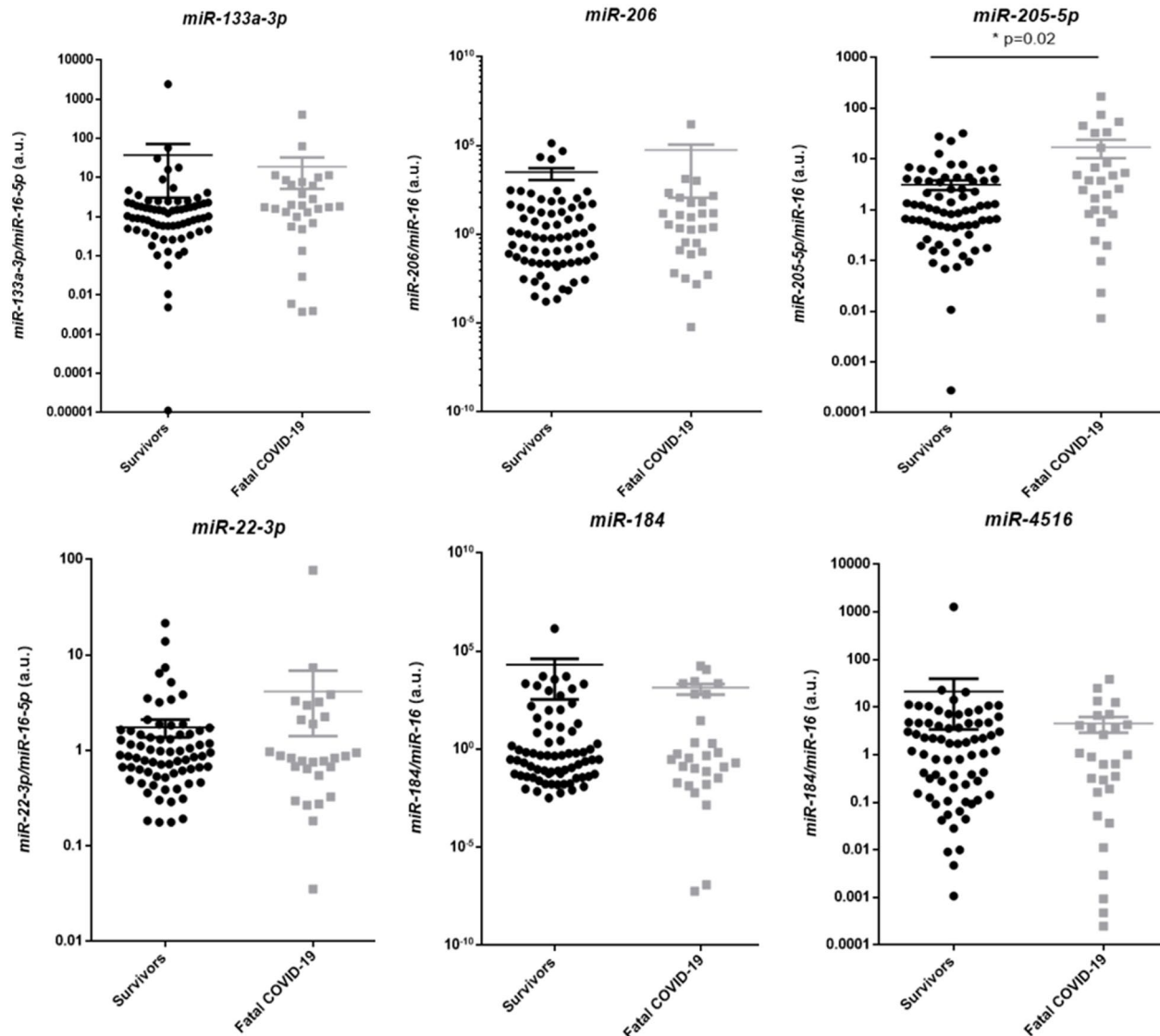
## Discussion

In this study, we evaluated the association between serum levels of miRNAs and COVID-19 severity in hospitalized patients. Compared to non-critical survivors, patients

who needed intensive support to recover from COVID-19-related complications presented higher expression of circulating *miR-205-5p* and *miR-206* upon hospital admission. The increased expression of *miR-205-5p* was also linked to a high mortality risk. Moreover, patients' baseline characteristics apparently did not affect the expression of these miRNAs, which may indicate that circulating *miR-205-5p* and *miR-206* expression levels could be a potential independent predictor of COVID-19 outcome in the beginning of the hospitalization period.

In the last 10 years, levels of miRNAs in peripheral blood samples have been pointed as regulators of gene expression and intercellular messengers in pathological states. In previous studies, *miR-205-5p* was described as a regulator of the angiogenic process in non-small cell and squamous lung cancer progression, and also as an influencer of the inflammatory responses in airway diseases.<sup>16–19</sup>

A decade ago, Suojalehto *et al.*<sup>17</sup> demonstrated that patients with symptomatic allergic rhinitis and asthma presented higher expression of *miR-205-5p* in nasal mucosa compared to non-symptomatic patients. These high levels of *miR-205-5p* were significantly correlated with an increased release of the cytokines interleukin (IL)-4, IL-5, and IL-13 by the activation of T-helper 2 (Th2) immune response, which may indicate that *miR-205-5p* participates in the establishment of tissue inflammation and clinical deterioration. In line with these findings, a recent study showed that *miR-205-5p* knockdown provides symptomatic relief in a mice model of allergic rhinitis and also minimizes the production of pro-inflammatory cytokines in nasal mucosa. This decreased inflammatory response allied with the induced lower expression of *miR-205-5p* was attributed to the regulation of the B-cell lymphoma 6 (BCL6) by *miR-205-5p* activity.<sup>20</sup>



**Figure 5.** miR validation in the expanded cohort of COVID-19 patients, comparing survivors with fatal COVID-19 patients.

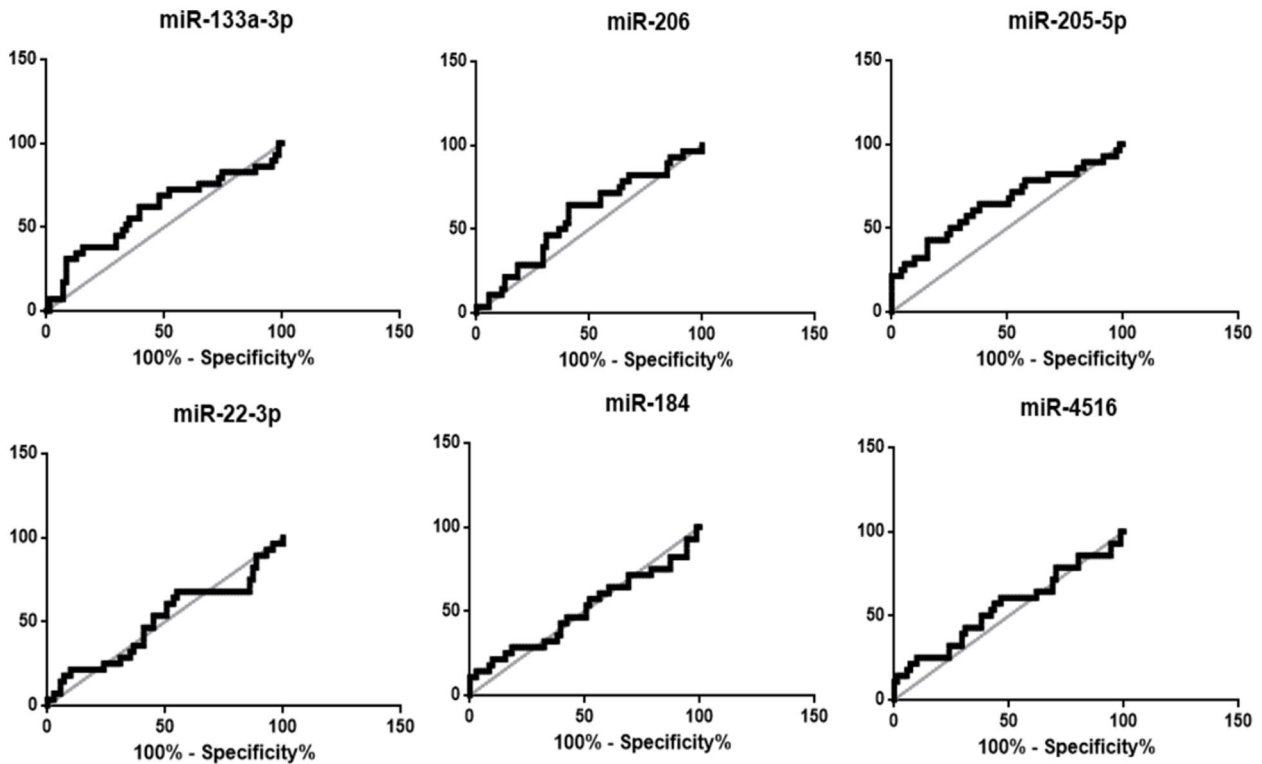
miR: microRNA; COVID-19: coronavirus disease 2019; IQR: interquartile range; n: absolute number; M: median.

MiR-133a-3p (survivors:  $n=72$ ,  $M=1.05$ ,  $IQR=0.5-2.3$ ; fatal COVID-19:  $n=28$ ,  $M=1.8$ ,  $IQR=0.6-7.2$ ;  $P=0.1$ ). MiR-206 (survivors:  $n=72$ ,  $M=0.7$ ,  $IQR=0.02-32.9$ ; fatal COVID-19:  $n=28$ ,  $M=3.1$ ,  $IQR=0.1-64.0$ ;  $P=0.24$ ). MiR-205-5p (survivors:  $n=72$ ,  $M=1.0$ ,  $IQR=0.4-3.7$ ; fatal COVID-19:  $n=28$ ,  $M=3.2$ ,  $IQR=0.8-14.6$ ;  $*P=0.02$ ). MiR-22-3p (survivors:  $n=72$ ,  $M=0.9$ ,  $IQR=0.6-1.5$ ; fatal COVID-19:  $n=28$ ,  $M=0.8$ ,  $IQR=0.6-2.2$ ;  $P=0.96$ ). MiR-184 (survivors:  $n=72$ ,  $M=0.30$ ,  $IQR=0.04-13.4$ ; fatal COVID-19:  $n=28$ ,  $M=0.33$ ,  $IQR=0.02-484.1$ ,  $P=0.94$ ). MiR-4516 (survivors:  $n=72$ ,  $M=1.7$ ,  $IQR=0.2-4.6$ ; fatal COVID-19:  $n=28$ ,  $M=0.7$ ,  $IQR=0.1-4.3$ ;  $P=0.43$ ).

BCL6 is a transcriptional regulator that acts mainly targeting the promoter regions of genes related to the immunological responses in T cells, B cells, macrophages, mast cells, and airway epithelial cells.<sup>20,21</sup> As a consequence of BCL6 regulation, a suppressed Th2 response with less production of IL-4, IL-5, and IL-13 cytokines and IgE is observed, as well as a decrease in the amount of macrophages producing IL-1 $\beta$  and IL-18.<sup>20</sup> This negative regulation of the inflammation responses by BCL6 occurs mainly due to the inhibition of the inflammasome linked to the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3).<sup>20</sup> In fact, the authors also observed that together with symptomatic relief and less expression of immunological markers, miR-205-5p knockdown results in higher BCL6 protein and mRNA levels while decreasing NLRP3 levels.<sup>20</sup> NLRP3 inflammasome is a complex of proteins that, once activated, induces the apoptosis-associated

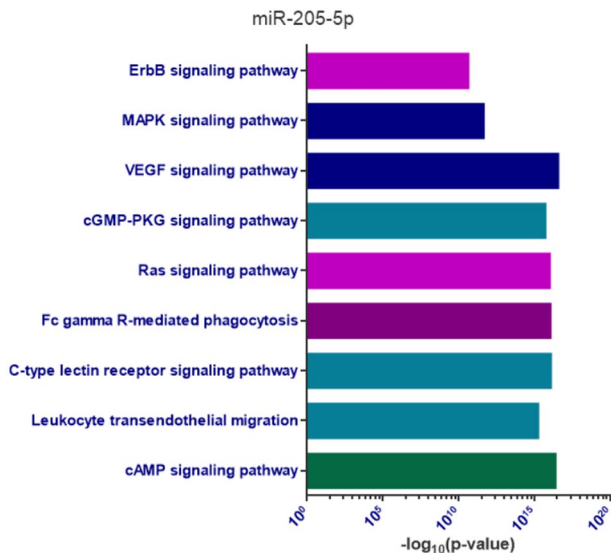
speck-like protein containing a CARD (ASC), an inflammasome adapter presented mainly in T cells and macrophage nucleus. As a consequence of ASC induction, the caspase-1 cleavage may occur, enhancing IL-1 $\beta$  and IL-18 maturation, which supports the Th2 immunological response.<sup>22</sup> Also, this inflammatory effect related to the NLRP3 activation was already described in bronchus and epithelial mucous cells of asthmatic patients as an important factor related to lung inflammation and damage.<sup>23</sup>

In the context of COVID-19, significant evidence supports the NLRP3 inflammasome activation as an essential step in developing tissue inflammatory response to SARS-CoV-2 infection in the lungs.<sup>24-27</sup> The main hypothesis is that the N viral protein can directly bind to the NLRP3 complex, starting the release of IL-1 $\beta$  that culminates in the cytokine storm. In fact, both levels of NLRP3 proteins and IL-1 $\beta$  were found significantly increased in patients who presented SARS or



**Figure 6.** ROC curve analysis of miRNA relative expressions in the expanded cohort of COVID-19 patients, considering fatal COVID-19 as outcome. ROC: receiver operating characteristic; COVID-19: coronavirus disease 2019; AUC: area under the curve; CI: confidence interval.

MiR-133a-3p (AUC=0.61, 95% CI=0.5–0.7,  $P=0.08$ ); MiR-206 (AUC=0.57, 95% CI=0.4–0.7,  $P=0.25$ ); MiR-205-5p (AUC=0.70, 95% CI=0.6–0.8,  $P=0.002^{**}$ ); MiR-22-3p (AUC=0.52, 95% CI=0.4–0.6,  $P=0.75$ ); MiR-184 (AUC=0.54, 95% CI=0.4–0.7,  $P=0.5$ ); MiR-4516 (AUC=0.53, 95% CI=0.4–0.6,  $P=0.63$ ).



**Figure 7.** Gene enrichment analysis of miR-205-5p predict targets. In this figure, biological pathways enriched ( $P < 0.05$ ) by the predicted targets of miR-205-5p are represented, emphasizing processes related to inflammation, immunological response, other viral diseases, cardiovascular risk factors, and cellular metabolism.

multiple organ failure due to the infection.<sup>25</sup> Mice models of COVID-19-related NLRP3 activation showed that this process could be crucial during inflammation development and closely related to pulmonary injury, hypercoagulability state, and apoptotic signaling of the infected cells.<sup>24–26</sup> The same events were also observed in other coronaviruses.<sup>28</sup>

Other studies have shown that genetic variants of the NLRP3 inflammasome can influence the individual response to SARS-CoV-2 infection, which emphasizes that the activation of NLRP3 plays a key role during the inflammatory response.<sup>29,30</sup> Yet, this is the first time that the highly expressed circulating miRNA was proposed as a positive regulator of NLRP3 during COVID-19 due to the inhibition of the BCL6 repressor, and as a biomarker of COVID-19 severity.

In addition to the pro-inflammatory effect of *miR-205-5p* by increasing NLRP3 expression, this *miR* has been previously implicated in antiangiogenic effects in diabetes-induced limb ulcer.<sup>30</sup> High levels of circulating *miR-205-5p* have been found to decrease protein levels of vascular endothelial growth factor A (VEGFA) through direct binding on its 3'UTR mRNA in “in vitro” and “in vivo” models.<sup>31</sup> VEGFA is one of the most important members of the VEGF family,<sup>32</sup> and its angiogenic activity is strongly induced by hypoxia.<sup>33</sup>

However, in the context of critical COVID-19, the antiangiogenic role of *miR-205-5p* through the downregulation of VEGFA seems to be controversial. Despite being a key factor for tissue repair during inflammatory disease, the angiogenic process is considered an inducer of pulmonary endothelial damage in severe COVID-19.<sup>34</sup> Previous studies have demonstrated that patients who had severe lung injuries and organ failure presented high levels of VEGF family members in tissues (mainly VEGFA) and inflammatory cytokines. This induction of VEGFA tissue expression was attributed mainly to the cytokine storm process and to the COVID-19-related hypoxia state, which can induce a

pro-angiogenic response. However, this angiogenic stimulus appears to be responsible for an increase of endothelial lung cell permeability that further impairs endothelium function and triggers tissue factor (TF) release, which leads to the establishment of a pro-thrombotic environment,<sup>35–38</sup> once this interaction, in theory, could minimize the angiogenesis process and avoid organ damage. Thus, considering that our results showed that *miR-205-5p* is present in the serum of patients who presented non-fatal and fatal COVID-19, but significantly upregulated in the last cohort, we hypothesize that *miR-205-5p* could be involved in the COVID-19 pathophysiology both as an inducer of inflammation and as a cell regulator released in response to VEGFA over-expression. However, we cannot exclude the possibility that the increase in *miR-205-5p* expression may be a consequence of an increase in VEGFA levels.

In addition, we found that *miR-206* was upregulated in serum samples of patients who needed intensive care to recover, but not in fatal COVID-19 or non-critical patients. *miR-206* is considered a “miomiR” due to its enrichment in myocardial and vascular tissue. Also, several studies in cancer have demonstrated that *miR-206* acts as an important antiangiogenic factor due to the downregulation of the VEGFA-linked axis.<sup>39–42</sup> Although the antiangiogenic role of *miR-206* in the COVID-19 context was not yet explored, we hypothesize that the increase in serum levels of this miR in patients who present critical, but non-fatal, forms of COVID-19 occurred as a compensatory mechanism, to control the exacerbated angiogenesis process provoked by the hyper-inflammatory state caused by SARS-CoV-2 infection. Yet, further investigation is needed to clarify this point.

Our study has some limitations that should be highlighted. Although our results showed an association between high levels of circulating *miR-205-5p* and *miR-206* upon hospital admission and increased risk of adverse outcomes, the potential of these miRs as predictive clinical biomarkers has to be confirmed in other cohorts. Besides, the post-transcriptional regulation of molecular pathways involved in COVID-19 pathophysiology by *miR-205-5p* activity remains to be proved by functional assays approaches, and once all our data were related to circulating miRNAs, the expression of *miR-205-5p* at tissue levels during the disease remains unknown. Finally, all demographic and clinical data reported in this article were obtained from medical records.

In conclusion, our study demonstrated that circulating *miR-205-5p* and *miR-206* expression levels are capable of discriminating patients at higher risk to develop critical or fatal COVID-19 upon hospital admission. These results also indicate that epigenetic mechanisms may regulate the immunological response in COVID-19.

#### AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; CdOV conducted the experiments.

#### DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

#### ETHICAL APPROVAL

This study was approved by the Local Research Ethics Committee (CAAE 30648520.6.0000.5404).

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