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Evaluation of MicroRNA Expression Pattern (miR-28, miR-181a, miR-34a, and miR-31) in Patients with COVID-19 Admitted to ICU and Diabetic COVID-19 Patients

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Keywords
SARS-CoV-2 · COVID-19 · microRNA · Diabetes · Biomarker

Abstract

Introduction: MicroRNAs, or miRNAs, with regulatory performance in inflammatory responses and infection are the prevalent manifestations of severe coronavirus disease (COVID-19). This study aimed to evaluate whether PBMC miRNAs are diagnostic biomarkers to screen the ICU COVID-19 and diabetic COVID-19 subjects. ***Methods:*** Candidate miRNAs were selected through previous studies, and then the PBMC levels of selected miRNAs (miR-28, miR-31, miR-34a, and miR-181a) were measured via quantitative reverse transcription PCR. The diagnostic value of miRNAs was determined by the receiver operating characteristic (ROC) curve. The bioinformatics analysis was utilized to predict the DEM genes and relevant bio-functions. ***Results:*** The COVID-19 patients admitted to ICU had significantly greater levels of selected miRNAs compared to non-hospitalized COVID-19 and healthy people. Besides, the mean miR-28 and miR-34a expression levels in the diabetic COVID-19 group were significantly upregulated when compared with the non-diabetic COVID-19 group. ROC analyses demonstrated the role of miR-28, miR-34a, and miR-181a as new biomarkers to discriminate the non-hospitalized COVID-19 group from the COVID-19 patients admitted to ICU samples, and also miR-34a can probably act as a useful biomarker for screening

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diabetic COVID-19 patients. Using bioinformatics analyses, we found the performance of target transcripts in many bioprocesses and diverse metabolic routes such as the regulation of multiple inflammatory parameters. ***Discussion:*** The difference in miRNA expression patterns between the studied groups suggested that miR-28, miR-34a, and miR-181a could be helpful as potent biomarkers for diagnosing and controlling COVID-19.

Introduction

Coronavirus 2019, known as COVID-19, as a novel beta-coronavirus is the infectious agent of a highly contagious and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. COVID-19 became a serious outbreak with a mortality rate of about 2% and spread worldwide [2]. COVID-19 disease presents with numerous complications, ranging from the common cold symptoms [3]. Most patients with COVID-19 have asymptomatic, mild-moderate signs and approximately 15% of affected people experience severe symptoms and need hospitalization [4]. In 5% of cases, the status of patients becomes critical so that they may need ICU admission [5]. The mortality rate of ICU patients is 25–50% [6]. Aging, viral strains, comorbidities such as obesity, diabetes, and host genetics, and the type of immune response all have prominent activities in the disease severity and death rate [7]. Reportedly, diabetic COVID-19 patients are at greater risk of ICU admission, with a greater risk of death [8]. In the current situation, a diagnosis of COVID-19 progression in patients predominantly relies on clinical manifestation, and so finding the identification of noninvasive biomarkers for the early prediction of critical condition deterioration is a challenging task.

Earlier transcriptomic investigations on COVID-19 focused on protein-coding transcripts with the aid of profiling the mRNA expression to determine the functional roles and patterns of resultant proteins [9]. It has been found that ˂3% of human genome is responsible for coding proteins [10]; however, most transcriptomic investigations on COVID-19 analyzed protein-coding transcripts with the aid of profiling the mRNA expression to determine the functional roles and patterns of resultant proteins [9]. Non-coding regions are often so-called "junk DNA" among which microRNAs are a cluster of highly conserved non-coding RNAs (18–25 nucleotides long) that can regulate physiological processes via targeting their target mRNAs expression at post-transcriptional levels [11, 12]. Circulating miRNAs can serve as diagnostic and prognostic biomarkers of cancer and other diseases such as viral respiratory infections and inflammation-related diseases [13–15]. Furthermore, miRNAs can facilitate favorable conditions for viral replication by regulation of host gene expression [16], and they may regulate pathogenesis of viruses by targeting the coding regions of the viral genome [17]. Besides, viral proteins can control host immune response through miRNA expression dysregulation [18]. Hence, the potential of manipulating miRNA expression as a therapeutic strategy in viral diseases has largely been explored.

Recently, the interaction of cellular miRNAs with the SARS-CoV-2 attachment to the host target cell receptor was reported and suggested their targeted regulation may have substantial therapeutic impact in COVID-19. Besides, both the host-encoded and virus-encoded miRNAs have been proposed most recently as potent biomarkers for COVID-19 [19]. Furthermore, the patients exhibited immune system dysregulation in severe infection of SARS-CoV-2 [20, 21], which is characterized by hyperinflammation. Evidence has shown that miRNAs, in addition to influencing cellular mechanisms such as differentiation, apoptosis, also regulated and modulated the immune responses against infectious agents [22]. Several human miRNAs, including miR-34a/b, were predicted to target SARS-CoV-2 genes based on data from bioinformatics analyses [23]. It has also been observed that the expression level of miR-34a-5p was upregulated during the early stage of COVID-19 and was significantly associated with inflammatory factors. Also, it has been suggested that miR-34a may play a crucial role in regulating endothelial dysfunction and the inflammatory response in COVID-19 patients with thrombosis and significant lung injury [24, 25]. Analysis of interaction between host cell miRNAs with ACE2 and TMPRSS2, SARS-CoV-2 cell entry depends on these proteins, demonstrated that miR-181a and miR-28 could regulate SARS-CoV-2 entry via targeting ACE2 and TMPRSS2, respectively [26, 27]. So, these cellular miRNAs probably can be promising targets for COVID-19 therapy. Some miRNA expression levels (e.g., miR-28, miR-181a, miR-34a, and miR-31) potentially appear to change during infections and inflammations, which is a decrease or increase in expression [28]. Under this premise, the present work compared the expression pattern of selected cellular miRNAs (miR-28, miR-181a, miR-34a, and miR-31) which are directly or indirectly involved in inflammatory pathway and/or interact with SARS-CoV-2 receptor [27, 29], between patients with

COVID-19 admitted to ICU with non-hospitalized patients and also between diabetic COVID-19 group with non-diabetic COVID-19 patients.

Methods and Study Population

Patients Selection

From January 2022 to April 2022, one hundred individuals with SARS-CoV-2 referred to Hazrat Rasoul Hospital of Tehran affiliated to Iran University of Medical Sciences were enrolled in this cross-sectional research. Fifty of these people were treated on an outpatient basis and did not have any particular problems (group 2), and another fifty were admitted to ICU ward of the hospital due to specific clinical manifestations (group 3). The peripheral blood samples were taken from these participants and also fifty healthy controls (group 1). Also, COVID-19 subjects were divided into two groups: diabetic (n = 30) and non-diabetic (n = 70), and then the expression pattern of selected miRNAs was compared between diabetic and non-diabetic COVID-19 groups. The Ethics Committee of Iran University of Medical Sciences reviewed and approved all aspects of this research (IR.IUMS.REC.1400.1022).

All clinical and laboratory information of individuals with verified COVID-19 using RT-PCR was extracted from electronic medical records. This information includes physical signs, demographic data, laboratory results, underlying comorbidities, medical history, and admission to the ICU. None of the studied patients and healthy individuals who entered the study as controls had co-infection with *Mycobacterium tuberculosis*, human cytomegalovirus, human immunodeficiency virus, hepatitis B virus, and hepatitis C virus.

Separation of Peripheral Blood Mononuclear Cells and Extraction of Total RNA

Five milliliters of blood was taken from each participant and transferred into a sterile vacutainer tube containing ethylenediaminetetraacetic acid. The separation of peripheral blood mononuclear cells (PBMCs) was based on a standard guideline of Ficoll-Hypaque (Lymphoprep, Oslo, Norway) gradient centrifugation technique on the basis of the manufacturer's procedure. The PBMC specimens were washed more than twice using phosphate-buffered saline (pH = 7.2–7.4), followed by re-suspension in RNA maintenance solution (200 μL, RNALater: Ambion Inc., Austin, TX, USA) and freezing at −80°C. The total RNA of PBMC samples was isolated via the miRNeasy Mini Kit (reference 217,004, Qiagen, CA), according to the manufacturer's procedure, and afterward, the integrity and purity of the extracted RNA was examined by the NanoDrop device (Thermo Fisher Scientific, Wilmington, MA, USA).

MicroRNA Expression Analysis

To determine miR-28, miR-31, miR-34a, and miR-181a expression, synthesis of complementary DNA was generated on 5 μg of the total RNA via miScript® II RT Kit (Qiagen, Germany) on the basis of the manufacturer's protocol [15]. The present research investigated the expression pattern of four selected miRNAs in PBMC samples of SARS-CoV-2-infected patients and healthy controls.

The real-time PCR was done via miScript SYBR Green PCR Kit (Qiagen, Valencia, CA; #218073), on the basis of the manufacturer's guidelines. The thermal profile of this assay was performed for 3 min at 95°C, and then 40 cycles for 15 s at 95°C, for 20 s at 60°C, and for 25 s at 72°C employing the Rotor-Gene® Q RT-PCR (Qiagen, Hilden, Germany). It is noteworthy that melting curve analysis was performed from 55 to 99°C. The levels of miR-28, miR-31, miR-34a, and miR-181a expression were normalized to Snord47 RNA (reference RNA) and Livak [30] method was used for calculation of the fold change. All the reactions in this survey were done in triplicate.

Target Gene Predictions for miR-28, miR-181a, miR-34a, and miR-31

The prediction of target mRNAs of selected miRNAs was done according to web-based prediction tools including miRWalk (http://mirwalk.umm.uni-heidelberg.de/), miRDB (www.mirdb.org/), TargetScan (http://www.targetscan.org), and DIANA-microTCDS (https://dianalab.ece.uth.gr/html/dianauniverse/index.php?r=microT_CDS). Experimentally confirmed miRNA targets are uploaded on these databases, presenting the updated targets. Predicted target genes for each of the miRNAs expressed differently were combined from four various sites. By deleting duplicates, the Venn plot (https://bioinfogp.cnb.csic.es/tools/venny/) was utilized for the analysis of overlap genes.

Protein-Protein Interactions Network Formation

Online database of STRING (http://string-db.org, version 11.5) was utilized to analyze the PPI network of overlapping target genes. The threshold value was considered to be the interaction score of ≥0.4. Each node is representative of one gene, and the width of the edge between the nodes indicates their interaction and the degree indicates the count of edges.

Pathway Enrichment Analysis

The prominent bio-functions of these miRNAs were determined by exploring the Molecular Signatures Database (MSigDB) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Hallmark pathways via the Enrichr online analysis tool. The significance level of differences in the enrichment was considered to be $p < 0.05$.

Statistical Analysis

At least three independent measurements of miRNAs were conducted. GraphPad Prism version 6.0 and SPSS version 16.0 software were performed to analyze all data. Following the preprocessing of qPCR data, the differences in miRNA expression level were analyzed by Mann-Whitney U and Kruskal-Wallis tests among patients with COVID-19 admitted to ICU, non-hospitalized patients, and healthy control group and among the diabetic COVID-19 group, non-diabetic COVID-19 patients' group, and healthy control group. To assess the diagnostic and prognostic accuracy of miR-28, miR-31, miR-34a, and miR-181a, the receiver operating characteristic (ROC) curve analysis was conducted and the AUC was calculated. Also, Spearman's correlation coefficients were computed to analyze the association between miRNA expression levels and the laboratory data of the studied participants. Benjamini and Hochberg procedure was applied to control the false discovery rate, followed by the calculation of adjusted p values.

Table 1. Demographic parameters of research units

Parameters	Male	Female	Total	*p* value
Healthy controls				
n, (%)	27 (54.0)	23 (46.0)	50 (100.0)	–
Age	37.4±13.8 (23–68)	33.6±9.7 (24–60)	35.6±12.1 (23–68)	0.391 Mann-Whitney U test
Non-hospitalized patients with COVID-19				
n, (%)	26 (52.0)	24 (48.0)	50 (100.0)	–
Age	38.1±8.4 (28–53)	33.0±10.8 (22–63)	35.6±9.9 (22–63)	0.006[a] Mann-Whitney U test
Patients with COVID-19 admitted to ICU				
n, (%)	34 (68.0)	16 (32.0)	50 (100.0)	–
Age	59.0±11.9 (18–72)	72.1±10.4 (56–92)	63.1±13.0 (18–92)	<0.001[a] Mann-Whitney U Test

[a]Statistically significant.

Results

Characteristics of Research Units

One hundred consecutive COVID-19 research units were admitted to Hazrat Rasoul hospital in Tehran, Iran (related to IUMS), and 50 healthy people (group 1) were included in the current cross-sectional research. Fifty COVID-19 research units were treated on an outpatient basis and did not have any particular problems (group 2), and another 50 were admitted to ICU ward of the hospital due to specific clinical manifestations (group 3).

The mean age was calculated to be 35.6 ± 9.9 (ranging between 22 and 63 years) for patients in group 2, 63.1 ± 13.0 (ranging between 18 and 92 years) for the group 3 patients, and 35.6 ± 12.1 (ranging between 23 and 68 years) for healthy people (Table 1). In the groups 2, 3 of patients, and healthy controls, 26 (52%), 34 (68%), and 27 (54%) were males, sequentially (Table 1). Table 2 compares the laboratory data of group 1 and group 2 patients (Mann-Whitney U test). The serum levels of CRP (p value <0.001), ALP (p value <0.001), CPK (p value <0.001), LDH (p value <0.001), ALT (p value <0.001), AST (p value <0.001), FBS (p value <0.001), PTT (p value = 0.004), INR (p value = 0.001), K (p value = 0.004), and Cr (p value <0.001) in the patients with COVID-19 admitted to ICU or group 3 were significantly higher than that in non-hospitalized patients with COVID-19 or group 2. Furthermore, the levels of Hct (p value = 0.012), platelet (p value = 0.001), Na (p value <0.001), Ca (p value <0.001), and Ph (p value = 0.02) were significantly lower in group 2 compared with group 2. In addition, the comparison of clinical characteristics between group 2 and group 3 patients was performed by Fisher's exact test. The most common complaints in both groups 2 and 3 were fever (76% and 80%, respectively), skeletal pain (76% and 80%, respectively), and headache (76% and 80%, respectively). The occurrence of chest pain, weakness, and sputum cough were more frequent in group 3 patients than in group 2 patients, while gastrointestinal symptoms, runny nose, and nose cape were more often in group 2 patients (Table 3). The laboratory data and clinical characteristics of the studied subjects and also healthy controls are listed in Tables 2 and 3.

Expression Pattern of Selected miRNAs Is Significantly Different between Healthy and COVID-19 Subjects

PBMC samples were collected before starting any therapy against COVID-19 from 50 non-hospitalized COVID-19 patients, 50 patients with COVID-19 admitted to ICU, and 50 healthy individuals. The levels of selected miRNAs expression were determined. Based on the results, the non-hospitalized COVID-19 patients had significantly lower levels of PBMC miR-34a and miR-181a when compared with healthy subjects ($p = 0.002$ and 0.0002, sequentially), but no significant difference was found in miR-28 and miR-31 expression level between them ($p > 0.99$, and 0.17, sequentially). Also, the mean expression levels of miR-28, miR-34a, and miR-181a in PBMC were significantly elevated in the COVID-19 patients in ICU group compared to control group ($p < 0.0001$ for all). Besides, the mean fold changes of miR-28, -31, -34a, and miR-181a were elevated significantly in the COVID-19 patients in ICU group when compared with non-hospitalized COVID-19 group (6.65 ± 3.57 vs. 0.18 ± 2.06, $p < 0.0001$; −0.07 ± 2.84 vs. −1.22 ± 3.1, $p = 0.004$; 1.48 ± 1.31 vs. −1.4 ± 2.01, $p < 0.0001$; and 3.5 ± 3.7 vs. −2.2 ± 2.1, $p < 0.0001$; sequentially). The miRNA expression profile in the control group in the COVID-19 patients in ICU group and non-hospitalized COVID-19 group is shown in Figure 1a–d.

According to correlation analysis, miR-28 and miR-181a had a positive correlation with CRP (Rs = 0.8, $p < 0.0001$, Rs = 0.74, $p < 0.0001$, sequentially), ALT (Rs = 0.58,

Table 2. Comparison of laboratory data between group 2 and group 3 patients

Parameters	Group 2	Group 3	*p* value
WBC	7.8±1.5 (4.1–10.3)	9.6±6.9 (2.6–32.4)	0.725
RBC	4.4±0.5 (3.4–5.4)	4.3±1.0 (2.4–7.0)	0.604
Hb	13.8±1.2 (11.6–15.4)	12.9±3.0 (6.9–20.2)	0.085
Hct	42.1±4.0 (34–49)	38.7±7.8 (23–59)	0.012[a]
Platelet	241±106 (105–437)	178±124 (11–485)	0.001[a]
INR	1.1±0.1 (0.8–1.3)	1.4±1.0 (1.0–5.3)	0.001[a]
PTT	30.4±4.0 (25–39)	38.7±19.4 (25–103)	0.004[a]
FBS	84.2±10.5 (69–110)	202.6±131.9 (77–512)	<0.001[a]
Urea	21.2±5.7 (14–35)	25.3±11.5 (12–58)	0.324
Cr	1.1±0.4 (0.5–2.4)	1.6±1.0 (0.8–4.8)	<0.001[a]
AST	17.5±9.1 (9–33)	69.0±61.4 (10–377)	<0.001[a]
ALT	19.5±9.8 (10–39)	60.6±39.0 (11–205)	<0.001[a]
LDH	235.7±92 (109–439)	593±281 (189–1243)	<0.001[a]
CPK	60.7±34.2 (23–143)	336±743 (26–3200)	<0.001[a]
ALP	91.2±38.0 (41–140)	325±241 (96–1037)	<0.001[a]
Na	140±2.9 (136–148)	136±6.2 (124–147)	<0.001[a]
K	4.0±0.5 (3.4–5.3)	4.3±0.6 (3.5–5.7)	0.004
Ca	9.9±0.7 (8.9–11.2)	8.9±1.3 (2.7–10.8)	<0.001[a]
Ph	3.9±0.5 (2.8–4.9)	3.4±0.9 (1.6–4.3)	0.026[a]
CRP	8.4±4.1 (2–19)	36.2±14.0 (19–53)	<0.001[a]
Vitamin D	21.5±10.5 (9–44)	22.6±13.2 (4.0–45)	0.777

Group 2, non-hospitalized patients with COVID-19; group 3, patients with COVID-19 admitted to ICU. WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; PT/INR Test, prothrombin time and international normalized ratio; PTT, partial thromboplastin time; FBS, fast blood sugar; Cr, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CPK, creatine phosphokinase; ALP, alkaline phosphatase; Na, sodium; K, potassium; Ca, calcium; Ph, phosphorus; CRP, C-reactive protein. [a]Statistically significant.

Table 3. Comparison of the clinical characteristic between group 2 and group 3

Parameters	Group 2, *n* (%)	Group 3, *n* (%)	*p* value
Fever	38 (76.0)	40 (80.0)	0.810
Weakness	7 (14.0)	18 (36.0)	0.039[a]
Confusion	12 (24.0)	10 (20.0)	0.810
Headache	38 (76.0)	35 (70.0)	0.653
Chills	29 (58.0)	20 (40.0)	0.109
Skeletal pain	38 (76.0)	38 (76.0)	1.000
Dry cough	28 (56.0)	29 (58.0)	1.000
Sputum cough	6 (12.0)	16 (32.0)	0.028[a]
Chest pain	13 (26.0)	27 (54.0)	0.008[a]
Shortness of breath	13 (26.0)	20 (40.0)	0.202
Runny nose	25 (50.0)	10 (40.0)	0.003[a]
Cape of nose	26 (52.0)	14 (28.0)	0.024[a]
Deceased smell	7 (14.0)	5 (10.0)	0.760
Deceased taste	7 (14.0)	6 (12.0)	1.000
Gastrointestinal symptom	31 (62.0)	20 (40.0)	0.045[a]
Bleeding stomach	3 (6.0)	7 (14.0)	0.318

Group 2, non-hospitalized patients with COVID-19; Group 3, patients with COVID-19 admitted to ICU. [a]Statistically significant.

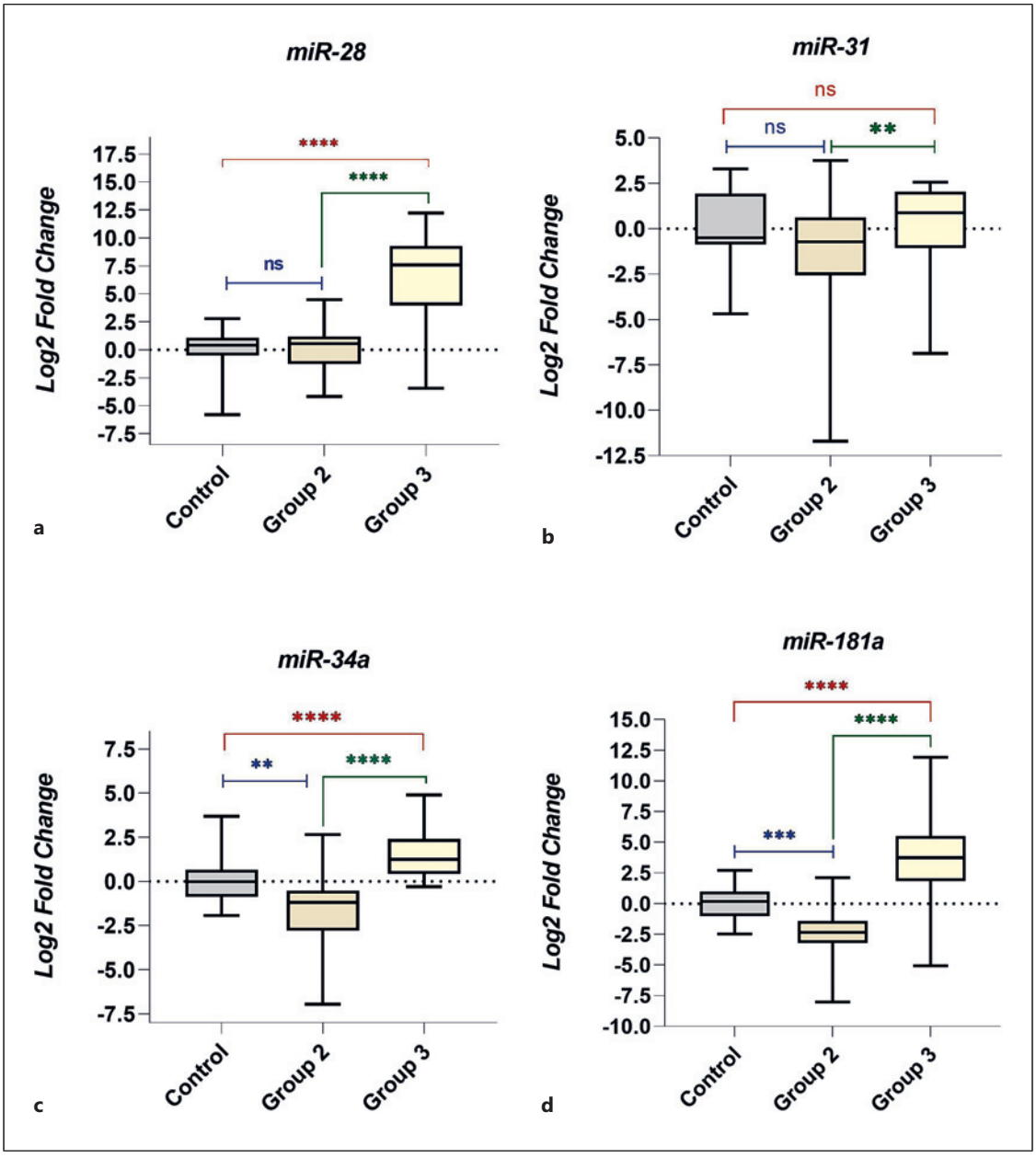

Fig. 1. Comparison of miR-28, miR-31, miR-34a, and miR-181a expression levels between COVID-19 patients admitted to ICU (CP-ICU as group 3), non-hospitalized COVID-19 patients (NHCP as group 2), and healthy subjects (group 1).

$p = 0.0009$, Rs = 0.41, $p = 0.018$, sequentially), and AST (Rs = 0.51, $p = 0.001$, Rs = 0.4, $p = 0.024$, sequentially) (Table 4). Overall, miR-28 has a positive correlation with AST, ALT, and LDH and a negative correlation with Ca and pH and hence may serve as a potential biomarker. Also, there is a good correlation between miR-181a and CRP, indicating that this miRNA maybe can act as a prognostic factor in the inflammatory response to severe COVID-19. However, since there was no significant positive or negative correlation between miR-31 and laboratory findings, it seems to have no prognostic value. Also, ROC curve analysis showed that miR-28 (AUC: 0.92, $p < 0.0001$), miR-181a (AUC: 0.89, $p < 0.0001$), and miR-34a (AUC: 0.89, $p < 0.0001$) can serve as useful markers for discriminating non-hospitalized COVID-19 group from the COVID-19 patients admitted to the ICU (Fig. 2a–c).

Table 4. Values of the spearman's rank correlation coefficients between the miRNA expression level (miR-28, miR-31, miR-34a, and miR-181a) with the laboratory data

	miR-28 Rs (*p* value)	miR-31 Rs (*p* value)	miR-34a Rs (*p* value)	miR-181a Rs (*p* value)
WBC	0.18 (0.06)	0.16 (0.59)	0.18 (0.57)	0.2 (0.055)
RBC	−0.04 (0.08)	0.1 (0.1)	−0.03 (0.6)	−0.02 (0.7)
Hb	−0.18 (0.06)	0.08 (0.3)	−0.09 (0.3)	−0.1 (0.1)
Hct	−0.2 (0.055)	0.04 (0.5)	−0.13 (0.08)	−0.16 (0.59)
Platelet	−0.21 (0.053)	−0.12 (0.1)	−0.16 (0.59)	−0.21 (0.053)
INR	0.22 (0.052)	0.06 (0.4)	0.04 (0.5)	0.13 (0.08)
PTT	0.33 (0.033)	0.07 (0.3)	0.16 (0.59)	0.2 (0.05)
FBS	0.59 (<0.0001)	−0.1 (0.2)	0.5 (0.006)	0.21 (0.053)
Urea	0.31 (0.041)	−0.01 (0.8)	0.16 (0.059)	0.3 (0.047)
Cr	0.43 (0.015)	−0.11 (0.2)	0.15 (0.06)	0.34 (0.036)
AST	0.51 (0.001)	0.15 (0.06)	0.29 (0.04)	0.04 (0.5)
ALT	0.58 (0.0009)	0.14 (0.07)	0.31 (0.04)	0.01 (0.8)
LDH	0.586 (0.0001)	0.02 (0.7)	0.47 (0.002)	0.17 (0.058)
CPK	0.23 (0.051)	0.14 (0.07)	0.12 (0.1)	0.18 (0.057)
ALP	0.58 (0.0009)	0.1 (0.1)	0.32 (0.036)	0.49 (0.007)
Na	−0.37 (0.02)	−0.017 (0.8)	−0.1 (0.1)	−0.19 (0.056)
K	0.35 (0.03)	0.06 (0.4)	0.36 (0.029)	0.15 (0.06)
Ca	−0.4 (0.019)	−0.03 (0.6)	−0.22 (0.52)	−0.35 (0.032)
Ph	−0.37 (0.02)	0.15 (0.59)	−0.12 (0.1)	−0.35 (0.032)
CRP	0.8 (<0.0001)	0.035 (0.6)	0.31 (0.041)	0.74 (<0.0001)
Vitamin D	−0.05 (0.09)	0.04 (0.8)	0.05 (0.5)	0.0108 (0.8)

A p value >0.05 is not statistically significant.

Expression Pattern of miR-28, -34a, and -181a Is Significantly Different between Diabetic and Non-Diabetic Patients with COVID-19

When the selected miRNAs were compared for expression pattern among the groups with respect to internal control, the results demonstrated a significant difference in the miR-28, miR-34a, and miR-181a expression levels between diabetic COVID-19 and non-diabetic COVID-19 groups ($p < 0.05$). Kruskal-Wallis test results revealed significantly higher mean fold change of miR-28, miR-34a, and miR-181a in diabetic COVID-19 group than in non-diabetic COVID-19 group (5.1 ± 3.7 vs. 2.6 ± 4.4, $p = 0.005$; 1.09 ± 1.2 vs. −0.41 ± 2.3, $p = 0.0007$; and 2.11 ± 3.32 vs. 0.24 ± 4.5, $p = 0.004$; sequentially). However, no significant difference was seen in the miR-31 expression level between these groups ($p = 0.21$). The fold changes of selected miRNAs in the control, diabetic COVID-19, and non-diabetic COVID-19 samples are shown in Figure 3a–d.

According to correlation analysis (Table 4), a moderately statistically significant correlation was found between miR-28 and miR-34a with FBS (Rs = 59, $p < 0.0001$ and Rs = 5, $p = 0.006$, sequentially). Besides, a significant correlation was positively there between miR-28, miR-34a, and miR-181a with LDH (Rs = 0.586, $p = 0.0001$; Rs = 0.47, $p = 0.002$; and Rs = 0.43, $p = 0.015$, sequentially). Therefore, miR-28 and miR-34a may serve as potential biomarkers in diabetic COVID-19 patients.

To investigate the potential of the PBMC level of miR-28, miR-31, miR-34a, and miR-181a as biomarkers of diabetic COVID-19 patients, we carried out an ROC analysis of data from the qPCR results. The analysis of ROC curve displayed that the PBMC miR-34a level probably could serve as a potential biomarker for screening diabetic and non-diabetic COVID-19 patients with the AUC value of 0.7 ($p = 0.0009$). Besides, the levels of miR-28 (AUC: 0.89, $p < 0.0001$), miR-34a (AUC: 0.69, $p = 0.002$), and miR-181 (AUC: 0.76, $p = 0.0001$) in PBMC seem useful in distinguishing diabetic COVID-19 cases from healthy control subjects (Fig. 4a–c).

Predicted Target Genes and Visualization of Genes Network

The target genes of miR-28, miR-31, miR-34a, and miR-181a were obtained from four online databases. After duplicates were removed by overlapping analysis, we found 18, 98, 125, and 16 potential target genes for miR-28, miR-31, miR-34a, and miR-181a, sequentially, and the result was indicated by the Venn plot (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000529985).

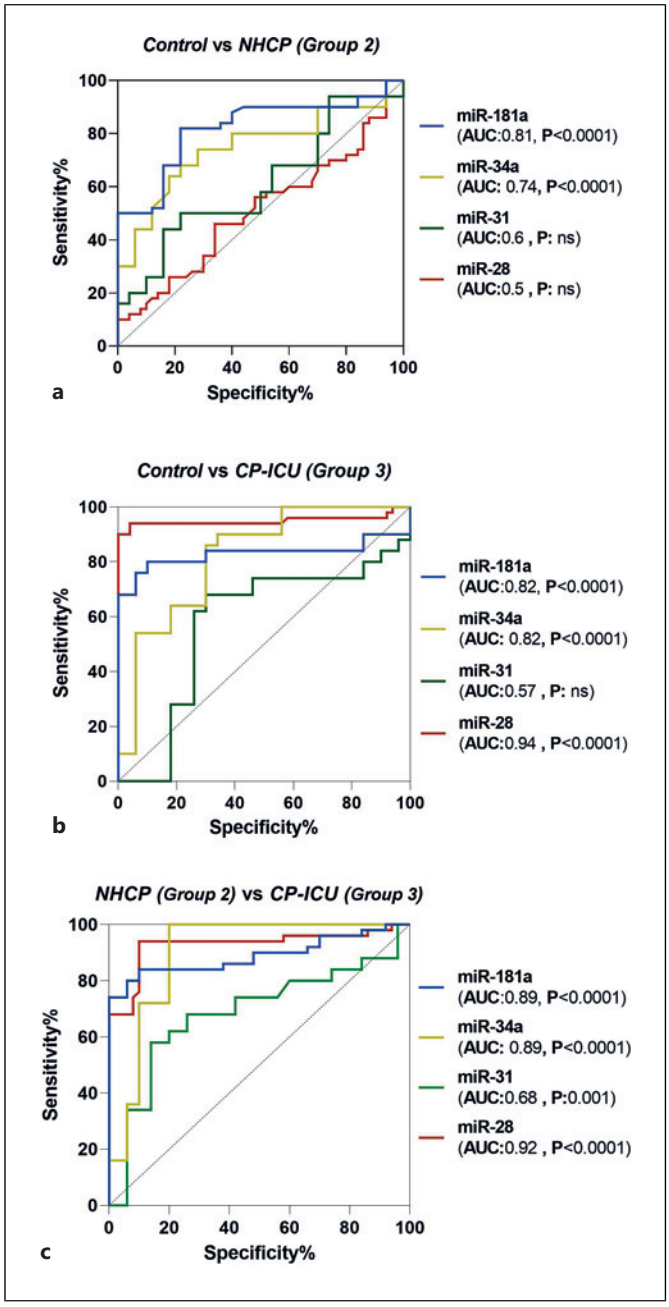

Fig. 2. ROC curve analysis using the PBMC levels of miR-28, miR-31, miR-34a, and miR-181a for distinguishing COVID-19 patients admitted to ICU (CP-ICU), non-hospitalized COVID-19 patients (NHCP), and healthy individuals.

To analyze the interaction between the identified common targets of miRNAs, PPI network was constructed using STRING. This pathway consists of 255 nodes and 254 edges with a confidence score of ≥0.4. Network nodes are the representatives of genes and edges displaying protein-protein relations (online suppl. Fig. 2).

To identify pathway enrichment, we uploaded 255 target genes to Enrichr database to find out MSigDB Hallmark and KEGG pathways. The top 10 enriched KEGG pathways of selected target were shown in online supplementary Figure 3, which includedi pathogenic *Escherichia coli* infection, platelet activation, circadian rhythm, longevity-regulating pathway, long-term potentiation, C-type lectin receptor signaling pathway, adipocytokine signaling pathway, amphetamine addiction, oxytocin signaling pathway, and Cushing syndrome ($p < 0.05$). Based on the MSigDB Hallmark pathway enrichment analysis, the targeted genes primarily were enriched in inflammatory response, TGF-beta signaling, hypoxia, PI3K/AKT/mTOR signaling, heme metabolism, and mitotic spindle ($p < 0.05$, online suppl. Fig. 3). Overall, similar to the previous experimental study, these results displayed that target genes of selected miRNAs are probably involved in inflammation process.

Discussion

The study findings proposed both virus-encoded and host-encoded miRNAs as potent biomarkers for COVID-19 in the recent years [19]. Besides, the patients exhibited immune system dysregulation in severe infection of SARS-CoV-2 [20, 21], which is characterized by hyper-inflammation. Under this premise, four selected miRNAs which are directly or indirectly involved in inflammatory pathway (online suppl. Fig. 3) and/or interact with SARS-CoV-2 receptor were investigated in this study. In addition, the diagnostic potential of these miRNAs as biomarkers was examined.

Severe infections of SARS-CoV-2 need ICU admission where the rate of death is variable between 25 and 50% [31, 32]. In COVID-19 patients admitted to the ICU, troponin, D-dimer, and CRP levels, as important prognostic factors, are associated with higher hospital mortality and a higher incidence of venous thromboembolism (VTE) [33]. In addition, in the last years, circulating miRNAs have been suggested as new potential biomarkers of infectious diseases and as useful biomarkers for clinical use which may improve diagnostic accuracy of clinical biomarkers [14, 15, 19]. For example, Jiang et al. [34] suggested that co-determination of circulating miRNA-320a/b and D-dimer can enhance the diagnostic power of deep venous thrombosis [34]. On another side, the miRNA differential expression has a link with a vascular pathology of COVID-19 with the classification of

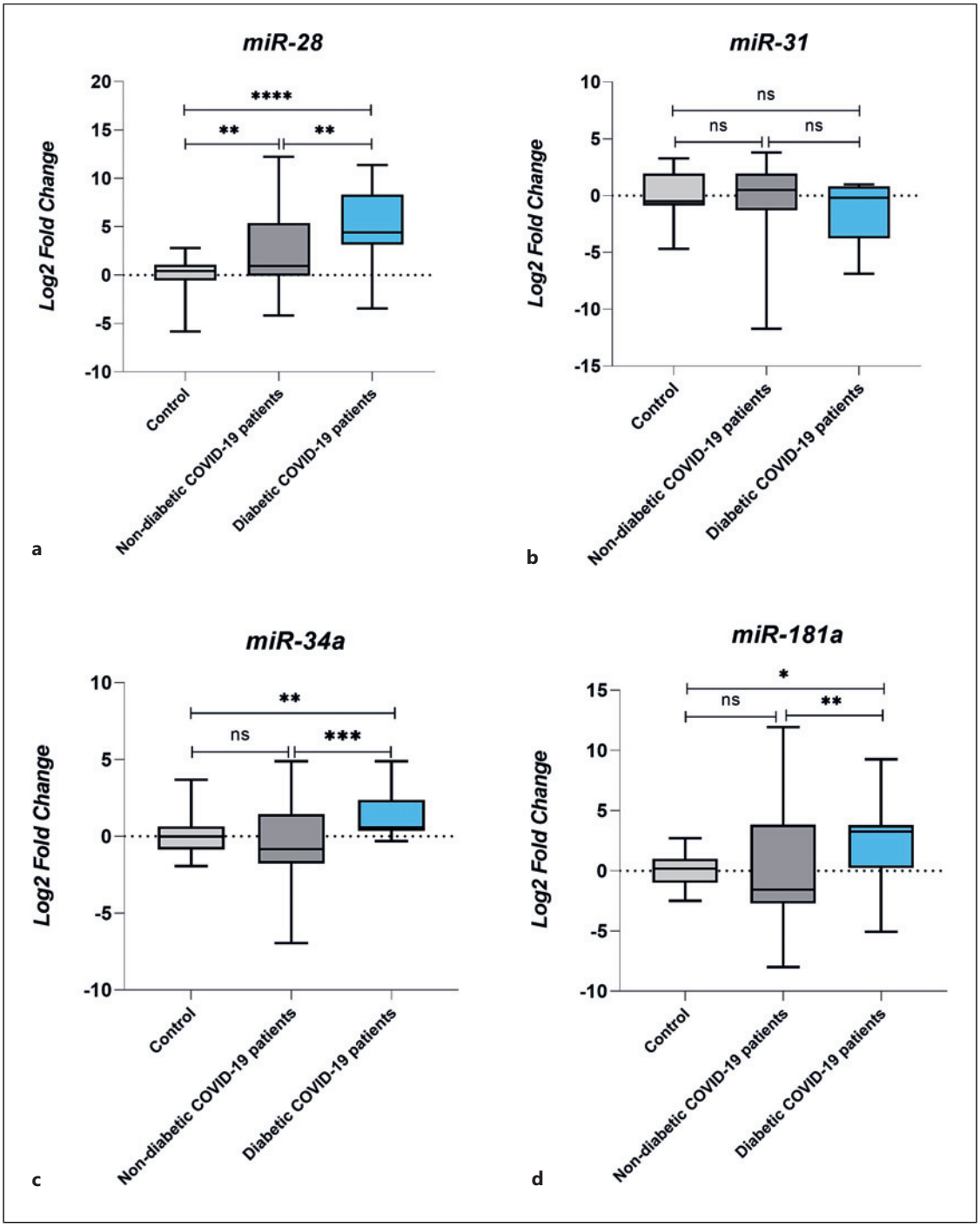

Fig. 3. Comparison of the expression levels of miR-28, miR-31, miR-34a, and miR-181a between diabetic COVID-19 patients, non-diabetic COVID-19 patients, and healthy individuals.

COVID-19 patients on the basis of D-dimer levels [35]. Recently, Gambardella et al. [36] observed that in the circulating exosomes of COVID-19 patients with high D-dimer level, a significant downregulation was observed in miR-103a, miR-145, and miR-885 and a significant upregulation in miR-424. Also, it has been shown that the expression of miR-28-3p [37] and miR-34a [38] was upregulated significantly in the plasma samples of

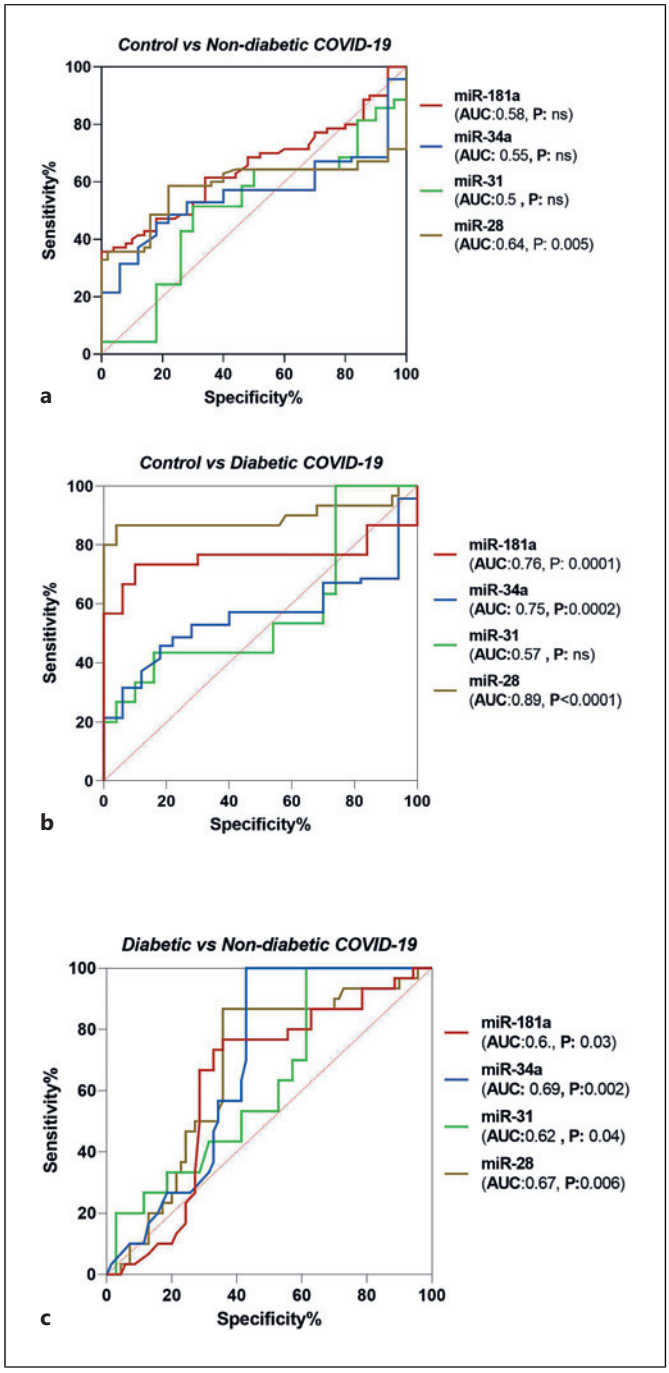

Fig. 4. ROC curve analysis using the PBMC levels of miR-28, miR-31, miR-34a, and miR-181a for distinguishing diabetic COVID-19 patients, non-diabetic COVID-19 patients, and healthy individuals.

non-COVID-19 VTE subjects and non-COVID-19 pulmonary embolism, sequentially. Furthermore, miR-34a [39] and miR-181a [40] have a key performance in the progression of thrombin in VTE through targeting F8 [41] and factor XI [42], sequentially. So, the regulation of such miRNAs is important in clot-associated disorders. In the present study, it was observed which the mean expression levels of miR-28, miR-31, miR-34a, and miR-181a in the ICU COVID-19 patients with greater D-dimer levels were significantly higher than the non-hospitalized COVID-19 patients with normal D-dimer levels (Fig. 1). Based on the ROC analysis, miR-28 (AUC: 0.92, $p < 0.0001$), miR-34a (AUC: 0.9, $p < 0.0001$), and miR-181a (AUC: 0.91, $p < 0.0001$) may be potent biomarkers for discriminating the non-hospitalized COVID-19 patients from ICU COVID-19 subjects (Fig. 2). Therefore, there is a need for further research to clarify the functional implications of miRNAs and the potential use of these miRNAs as biomarker in VTE associated with COVID-19 disease.

The risk of critical and severe COVID-19 was higher in those with comorbidities like diabetes mellitus (commonly referred to as diabetes) [43]. Infections, especially those of the skin, genitourinary tract, and respiratory system, are known to be more common in patients with diabetes [44]. Diabetes creates a hyperglycemic environment that promotes immunological dysfunction in some ways, which can result in the downregulation of interleukin production after the infection and increase the virulence of certain pathogens [44, 45]. Patients with diabetes not only have a greater risk of infection but also a higher incidence of infection-related hospitalizations and infection-related death. Diabetes mellitus was one of the most prevalent comorbidities reported in clinical data of COVID-19 patients [44]. It was first assumed that diabetic patients are at risk of progression to severe COVID-19; however, most of this research has been conducted with patients who had been hospitalized, or even patients admitted to ICU, indicating a more advanced stage of COVID-19 [44, 45]. Besides, a meta-analysis study showed that diabetes may not raise the incidence of SARS-CoV-2 infection but may worsen the outcome of diseases [46]. The relation between diabetes and increased mortality from both acute and chronic diseases, including viral infections, is supported by these results [47].

D-dimer levels were higher in those with COVID-19 and hyperglycemia [48–50]. Moreover, decreased hyperglycemia has been reported to be associated with decreased D-dimer [49]. Thus, hyperglycemia may contribute to the production of thrombosis, which is mostly seen in COVID-19 [51, 52]. Similarly, we also found significantly higher mean level of D-dimer in the diabetic COVID-19 group when compared with the non-diabetic COVID-19 group (1667.9 ± 1864 μg/L vs.

1001.4 ± 972.1 μg/L, sequentially; $p = 0.021$). However, underlying mechanisms are still not elucidated, and further in-depth studies are warranted.

In addition, patients having diabetes display poor prognosis with SARS-CoV-2 infection because of oscillation in blood glucose and complications of metabolic processes [53]. Since miRNAs can potentially contribute to the pathogenesis of diabetes and have unique expression patterns in diabetes subjects, thus will be candidates as potent biomarkers for the prognosis and diagnosis of diabetic COVID-19 patients [54, 55]. Recently, a gene-miRNA interaction network was used to clarify molecular pathway of SARS-CoV-2 infection and its genetic relationship with diabetes via bioinformatics with the aid of transcriptomic data of pancreatic islet cells, lung epithelium cells, and PBMCs [56]. Then, 11 miRNAs related to 19 differentially expressed genes (such as miR-34a-5p) were detected during this work; the significance of such miRNAs can be attributed to the sharing of pathogenic consequences between diabetes and COVID-19 [56]. Also, Zhao et al. [57] reported that miR-34a-5p elevates high glucose-induced apoptosis in cardiomyocytes through a decrease in anti-apoptotic BCL2 protein. Hence, the miR-34a-5p upregulation is higher in the diabetic status to elevate the apoptosis [58]. We observed in our study significantly higher miR-34a expression level in diabetic COVID-19 group than in the non-diabetic COVID-19 and control groups ($p = 0.0005$ and 0.007, sequentially). On the basis of ROC analysis, this miRNA probably can be a new potent biomarker to distinguish diabetic COVID-19 patients from non-diabetic COVID-19 subjects (AC: 0.7, $p = 0.0009$).

Disintegrin and metalloproteases (ADAMs) play a role in ectodomain shedding of enzymes like membrane-bound ACE2. The ACE2 can be broken down by ADAM17 to liberate the ACE2 ectodomain into circulation [59, 60]. Elevated ectodomain ACE2 may impair renin-angiotensin system imbalance (ACE/ACE2) in those with certain underlying conditions like diabetes [60]. ACE2 downregulation occurs via viral transcription and endocytosis plus SARS-CoV [61, 62]. Decreased expression of ADAM17 suppresses ACE2 shedding, but ADAM17 complementary DNA introduction can restore SARS-S-mediated ACE2 shedding [63]. High shed ACE2 levels decrease SARS-CoV infection [59, 64]. According to Xu and Li [27], the miR-28-3p possibly has a regulatory performance in ADAM17-mediated ACE2 ectodomain shedding in SARS-Cov-2 infection. Their result clarified an inhibitory role for miR-28-3p toward ADAM17-mediated CE2 ectodomain shedding in 293 T cells exposed to SARS-CoV-2 S-protein, highlighting potent therapeutic function of miR-28-3p mimic to prevent and treat patients with SARS-CoV-2 infection [27]. Besides, investigators have reported both increased and decreased miR-28-3p expression in diabetic patients [65]; however, the mechanism of action of miR-28-3p has been yet unclear in the onset of diabetes. According to the result of our study, the miR-28 expression level was significantly greater in the diabetic COVID-19 patients and the COVID-19 patients admitted to the ICU. As a result, miR-28 is implicated in the COVID-19 progression by reducing ACE2 shedding. Therefore, further research should be conducted to clarify the functional implications of such miRNAs between severe and diabetic COVID-19.

Lung epithelial cells highly express ACE2; thus, miRNAs (e.g., miR-181a) that are implicated in the regulation of this receptor expression in the respiratory system could be robust agents to manage the SARS-CoV-2 infection in lung tissue [66]. The miR-181a as a tumor suppressor miRNA can be seen in non-small cell lung cancer by declining IL-17 [67]. The miR-181a declines the ACE2 expression indirectly by targeting renin-angiotensin system [68]. The ACE2-mediated alterations are important in cardiomyocytes of diabetics during SARS-CoV-2 infection, so targeting miRNAs capable of regulating ACE2 can be helpful in COVID-19 patients [69]. The miR-181a has been shown to target the mRNA of ACE2 [70]. Accordingly, this miRNA overexpression can be a therapeutic candidate for COVID-19 [71]. It has been reported that miR-181a-5p expression was significantly lower in diabetics than in those with normal glucose tolerance [72, 73]. Also, miR-181a-5p impedes the TNFα-mediated insulin resistance in adipocytes by modulating the expression of S6K and PTEN [73]. However, we observed lower mean miR-181a expression level in the diabetic COVID-19 group (fold change: −0.15) when compared with the non-diabetic COVID-19 patients (fold change: 1.57), but it is not statistically significant ($p = 0.075$). Thus, further investigation is suggested to clarify the interaction of SARS-CoV-2 with miR-181a in diabetic COVID-19 patients.

Conclusion

This was the first work reporting differentially expressed miRNA profiles (miR-28, miR-31, miR-34a, and miR-181a) between the diabetic COVID-19 group with non-diabetic COVID-19 patients and also between patients with COVID-19 admitted to ICU with non-hospitalized patients. Overall, our findings revealed that the selected miRNA expression levels were

significantly higher in the PBMC samples of patients with COVID-19 admitted to ICU when compared with non-hospitalized COVID-19. Also, the mean expression levels of miR-28 and miR-34a in the diabetic COVID-19 group were significantly upregulated when compared with the non-diabetic COVID-19 group. Using bioinformatics analyses, we found the performance of target transcripts in many bioprocesses and diverse metabolic routes such as regulation of multiple inflammatory parameters (online suppl. Fig. 3). ROC curve analysis in studied groups showed that miR-28, miR-34a, and miR-181a can serve as useful biomarkers for discriminating non-hospitalized COVID-19 group from the ICU COVID-19 patients, and miR-34a can also be a new biomarker to screen diabetic COVID-19 patients from non-diabetic COVID-19. The study limitations were small sample size and no other respiratory conditions as controls, so there is a need for further research with larger sample size to explore the diagnostic power of such miRNAs. In the current study, mean ages of the patients admitted to ICU and non-hospitalized patients were 63.1 ± 13.0 and 35.6 ± 9.9, respectively. This may affect our findings. Therefore, we suggest that the interpretation of our findings should be considered with caution.

Statement of Ethics

The research was directed ethically according to the World Medical Association Declaration of Helsinki. The study subjects have given their written informed consent, and this study protocol was approved by the Medical Ethics Committee of Iran University of Medical sciences, approval number [IR.IUMS.REC.1400.1022].

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

AliReza Khatami, Javid Sadri Nahand, Mohammad Karimzadeh, and Sara Chavoshpour carried out the experiment and analyzed the data. Seyed Jalal Kiani, Tahereh Donyavi, and Khadijeh Khanaliha co-wrote the manuscript. Hamed Mirzaei, Mohammad Taghizadieh, and Saeed Kalantari drafted and designed the experiment and supervised the research. Farah Bokharaei-Salim supervised the research.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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