The Role of miRNAs in Patients with COVID-19 in Babylon Province

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Abstract

This study explores the role of microRNAs (miRNAs) in patients with COVID-19 in Babylon Province, focusing on their potential as biomarkers for disease progression and clinical outcomes. miRNAs are small non-coding RNAs that regulate gene expression and have been implicated in various biological processes, including immune response and inflammation. We conducted a cross-sectional analysis of COVID-19 patients to assess the expression levels of specific miRNAs and their association with disease severity, inflammation markers, and patient demographics. Our findings reveal distinct miRNA profiles that correlate with adverse outcomes in COVID-19, suggesting their potential utility in predicting disease severity and guiding therapeutic strategies. This work highlights the need for further investigation into the functional roles of miRNAs in COVID-19 pathophysiology and their possible application as biomarkers for monitoring disease progression and treatment response.

The study found a significant increase in miR-423 and miR-195 levels in COVID-19 patients compared to the control group. The miR-23a level was notably reduced in COVID-19 patients compared to the control group.

Keywords: miRNAs, COVID-19, biomarkers, Babylon Province, disease progression, inflammation, gene expression,

Introduction:

Coronaviruses are enveloped viruses possess a positive-sense single-stranded RNA genome ranging from 26 to 32 kb in length. Coronaviruses are classified under the subfamily Orthocoronavirinae within the family Coronaviridae. Coronavirus members within the subfamily are categorized into four genera, namely Alphacoronavirus, Betacoronavirus,

Gammacoronavirus, and Deltacoronavirus, based on differences in genome sequence and serological reactions. a positive-sense single-stranded RNA genome 26–32 kb in length1. Coronaviruses belong to the Coronaviridae subfamily Orthocoronavirinae. According to variations in the genome sequence and serological reactions, coronavirus members in the subfamily are classified into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus (Weiss and Navas, 2005).

Coronaviruses exhibit high genetic recombination and mutation rates, leading to their ecological diversity (Cui *et al.*, 2019). These viruses exhibit host versatility, infecting a diverse range of hosts including avian and cetacean species. They are associated with mild respiratory symptoms akin to those of the common cold (Fung and Liu, 2019). SARS-CoV, MERS-CoV, and SARS-CoV-2 can cause severe respiratory diseases in humans, which may lead to high mortality rates (de Wit *et al.*, 2016; Mas-Ubillus *et al.*, 2022).

miRNAs, which are 18-25 nucleotides in length, are noncoding RNAs that can degrade or inhibit the translation of targeted mRNAs. MiRNAs function as cellular observers (Fani *et al.*, 2021). The precise mechanistic functions of cellular miRNAs in viral infections remain incompletely comprehended. Cellular miRNA is generated during the initial phase of viral infections as a result of the antiviral reaction (Fani *et al.*, 2018). miRNAs can inhibit viral translation by attachment to the 3′-UTR of the viral genome or targeting SARS-CoV-2 receptors, structural or nonstructural proteins. This process does not impact human gene expression (Fani *et al*., 2021). Host miRNA expression affects viral pathogenesis by interfering with T cells and immune responses to viral infections. Several miRNAs are associated with elevated levels of plasma cytokine storms, including TNF-α, IL-1β, IL-6, miR-146a, miR-146b, and IL-8, in patients with acute respiratory distress syndrome and COPD. Reducing miRNA expression may improve COVID-19 severity by downregulating pro-inflammatory cytokines and increasing apoptosis protein expression. The miRNAs' expression may provide valuable diagnostic information for SARS-CoV-2 infection (Guterres *et al*., 2020). Therefore, comprehending the function of cellular miRNAs in COVID-19 is essential for the development of potential therapeutics (Fani *et al*., 2021).

The Aim of This Study

This study aims to examine the potential association between COVID-19 and miRNAs in Iraqi patients.

The purpose of this study was to evaluate the expression profile of specific miRNAs, namely miR-423-5p, miR-23a-3p, and miR-195-5p. The miRNA expression levels are detected via high-throughput sequencing.

Materials:

miRNA Gene Expression Primers

In this study, qPCR primers for miRNAs (miR-423-5p, miR-23a-3p, and miR-195-5p) were designed using the Sanger Center miRNA database Registry to select miRNA sequences and the miRNA Primer Design Tool (http://www.srnaprimerdb.com). The qPCR housekeeping

gene (GAPDH) was designed for this study using the NCBI-Database and Primer3 plus design online. The primers were provided by Macrogen Company in Korea, as shown in the table below.

Table (1): The qPCR Primers for miRNA.

Primer	Sequence (5'-3')		Genbank Sequence Code
miR-423-5p qPCR primer	F	AACAAGTGAGGGGCAGAGAG	MIMAT0004748
	R	GTCGTATCCAGTGCAGGGT	
RT primer (specific) miR-423-5p	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACAAAGTC		
miR-23a-3p qPCR primer	F	AACACGCATCACATTGCCAG	MIMAT0000078
	R	GTCGTATCCAGTGCAGGGT	
RT primer (specific) miR-23a-3p	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACGGAAAT		
miR-195-5p qPCR primer	F	AACCGGTAGCAGCACAGAA	MIMAT0000461
	R	GTCGTATCCAGTGCAGGGT	
RT primer (specific) miR-195-5p	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACGCCAAT		
GAPDH qPCR primer	F	TCTGACTTCAACAGCGACAC	NM_001256799.3
	R	TGACAAAGTGGTCGTTGAGG	

Methods

Subjects of The Study

Blood samples were collected from a total of 100 patients diagnosed by RT-PCR, who were either attended (mild cases) or admitted (severe cases) to Al-Imam Al-Sadiq Hospital and Merjan Medical City in Babylon province, Republic of Iraq between February and March 2022. The study collected patient samples and divided them into two groups based on the severity of infection. One group consisted of 50 patients with mild symptoms, while the other group consisted of 50 patients with severe symptoms. Fifty healthy individuals were selected as control samples. The samples were stored at a temperature of -20 ℃.

Anti-coagulant tubes (EDTA tube) were used to collect whole blood samples, which were then stored at -20 ℃ for molecular analysis. A volume of 200 µl of RNAlater was combined with 200 µl of anticoagulated blood obtained from an EDTA tube. The resulting mixture was placed in an Eppendorf tube for the purpose of preserving and stabilizing RNA samples.

Study Design

This is a case-control study design.

Inclusion Criteria

People with diabetes and hypertension of any age and sex were included.

Exclusion Criteria

This study excluded Individuals who were afflicted with autoimmune disorders.

Ethical Approval

The present study's samples will be procured subsequent to obtaining verbal ethical clearance from the Ethics Committees of Babylon health office unit in Babylon province. Furthermore, it is imperative that the present investigation receives approval from the ethical research committee at the College of Medicine, Babylon University, as well as the Babylon Health Directorate. Prior to their participation in the study, all patients and healthy control subjects were provided with counseling and verbally consented to their inclusion in the research. The present investigation is being conducted with the authorization of Babylon University, specifically the College of Medicine, and the General Health Directorate of Babylon Province.

Blood Samples Collection

For the purposes of molecular analysis, whole blood samples were drawn into an EDTA tube and frozen at -20 degrees Celsius. To preserve and transport RNA samples, 200 µl of RNAlater was mixed with 200 µl of anticoagulated blood from an EDTA tube into an Eppendorf tube.

Stem Loop RT-qPCR

The study used the stem loop RT-qPCR method to quantify the expression of miR-423-5p, miR-23a-3p, and miR-195-5p in blood samples from COVID-19 patients and healthy controls. The normalization of the data was performed using the GAPDH housekeeping gene. The Real-Time PCR technique was utilized for this purpose, following the protocol described by Ryan *et al.* (2021).

Total RNA Extraction

The extraction of total RNA from blood samples was carried out utilizing the AccuZolTM reagent kit, following the manufacturer's instructions.

Estimation Total RNA Yield and Quality

The concentration and estimated purity of the extracted total RNA was measured using a Nanodrop spectrophotometer (Thermo scientific. USA), which reads the absorbance at (260 / 280 nm).

DNase I Treatment

The total RNA that was extracted undergo treatment with DNase I enzyme in order to eliminate any residual genomic DNA. This was achieved by utilizing a DNase I enzyme kit and following the protocol outlined in the instructions provided by Promega company, USA.

cDNA Synthesis

cDNA Synthesis for miRNA

DNase-I treated RNA samples were utilized in the miRNA cDNA synthesis step , which was carried out with the use of an M-MLV Reverse Transcriptase kit and carried out in accordance with the instructions provided by the manufacturer.

cDNA Synthesis for GAPDH Gene

DNase-I treated RNA samples were also used cDNA synthesis step for GAPDH gene by using M-MLV Reverse Transcriptase kit and done according to company instruction.

qPCR Master Mix Preparation

miRNA qPCR Master Mix Preparation

The mRNA qPCR master mix was prepared utilizing the GoTaq® qPCR master mix kit, which relies on the detection of gene amplification through SYBER Green dye in a Real-Time PCR system.

miRNA qPCR Thermocycler Conditions

Subsequently, the qPCR plate was loaded and subjected to the thermocycler protocol outlined in the table below:

Table (2): Thermocycler conditions in cDNA synthesis for GAPDH Gene.

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	10min	1
Denaturation	95 °C	20 sec	45
Annealing/ Extension Detection (Scan)	60 °C	30 sec	

GAPDH qPCR Master Mix Preparation

The GAPDH qPCR master mix was prepared utilizing the GoTaq® qPCR master mix kit, which relies on the detection of gene amplification through SYBR Green dye in a Real-Time PCR system.

GAPDH qPCR Thermocycler Conditions

Subsequently, the qPCR plate was loaded and subjected to the thermocycler protocol outlined in the following table:

Table (3): Standard protocol in cDNA synthesis for GAPDH Gene.

qPCR Step	Temperature	Time	Repeat Cycle
Initial Denaturation	95 °C	10min	1
Denaturation	95 °C	20 sec	45
Annealing / Extention Detection (Scan)	58 °C	30 sec	

Data Analysis of qPCR

The gene expression levels (fold change) of the target and housekeeping genes obtained from q RT-PCR data were analyzed using the ΔCT Method with a reference gene, as described by Livak and Schmittgen in 2001. The equation is as follows:

Gene expression ratio (reference/target) = $2^{CT(reference) - CT(target)}$

Results and Discussion

Comparison of micro-RNA expression level between patients and control groups

Comparison of micro-RNA expression level between patients and control groups is shown in tabl3 3.4. The level of miR-423 was significantly higher in patients with COVID-19 in comparison with control group, 1.81 (6.12) versus 0.45 (1.89), respectively ($p < 0.001$), as shown in figure 3.3. In addition, the level of miR-195 was significantly higher in patients with COVID-19 in comparison with control group, 10.74 (17.68) versus 6.95 (10.02), respectively ($p < 0.001$), as shown in figure 3.4. However, the level of miR-23a was significantly lower in patients with COVID-19 in comparison with control group, 8.33 (14.71) versus 19.13 (23.69), respectively ($p < 0.001$), as shown in figure 3.5.

Table (4): Comparison of micro-RNA expression level between patients and control groups

Characteristic	Patients group *n* = 100	Control group *n* = 50	*p*	Interpretation
miR-423				
Median (IQR)	1.81 (6.12)	0.45 (1.89)	<0.001 M	Significant
Range	0.02 -28.32	0.03 -13.73		
miR-195				
Median (IQR)	10.74 (17.68)	6.95 (10.02)	0.005 M	Significant
Range	1.18 -177	1.05 -29.86		
miR-23a				
Median (IQR)	8.33 (14.71)	19.13 (23.69)	<0.001 M	Significant
Range	0.39 -142.67	3.48 -90.56		

n: number of cases; **IQR**: inter-quartile range; **M:** Mann Whitney U test.

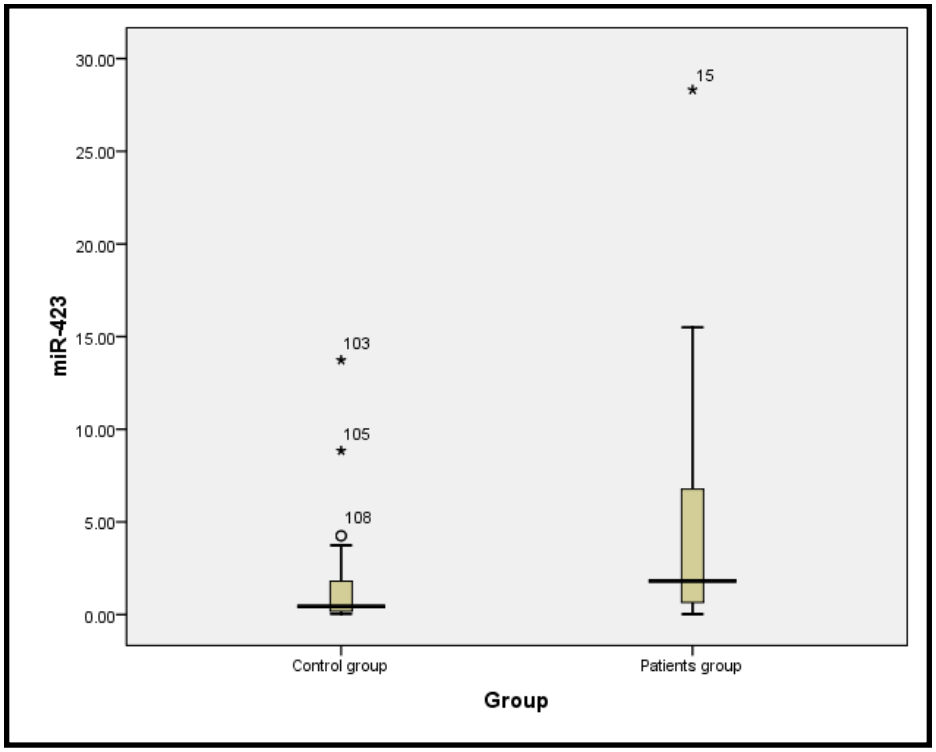

Figure (1): Box plot showing comparison of miR-423 expression between patients' group and control group.

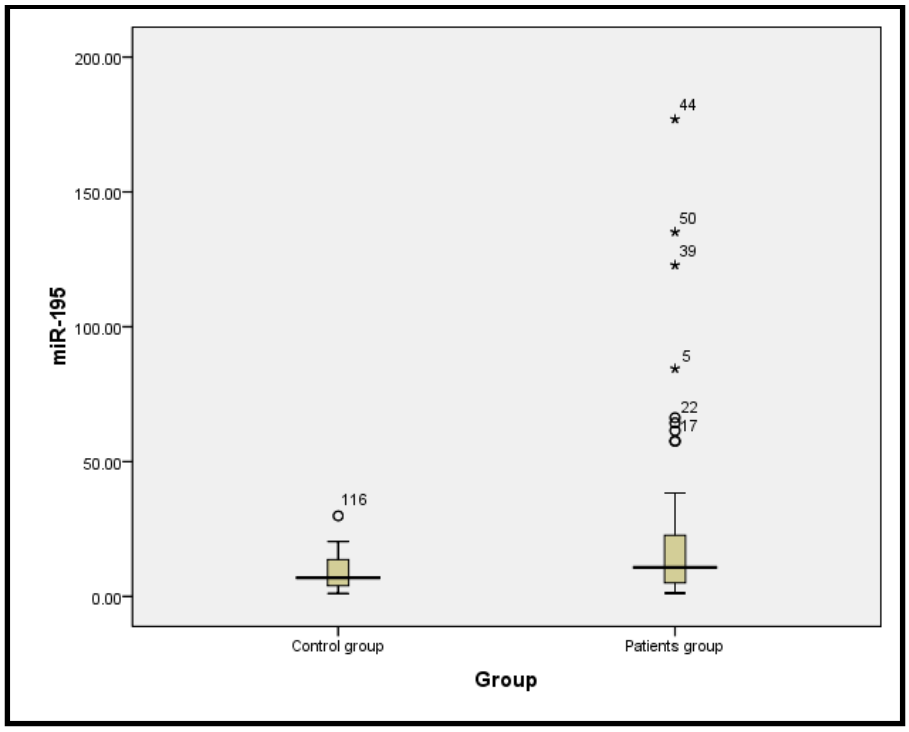

Figure (2): Box plot showing comparison of miR-195 expression between patients' group and control group.

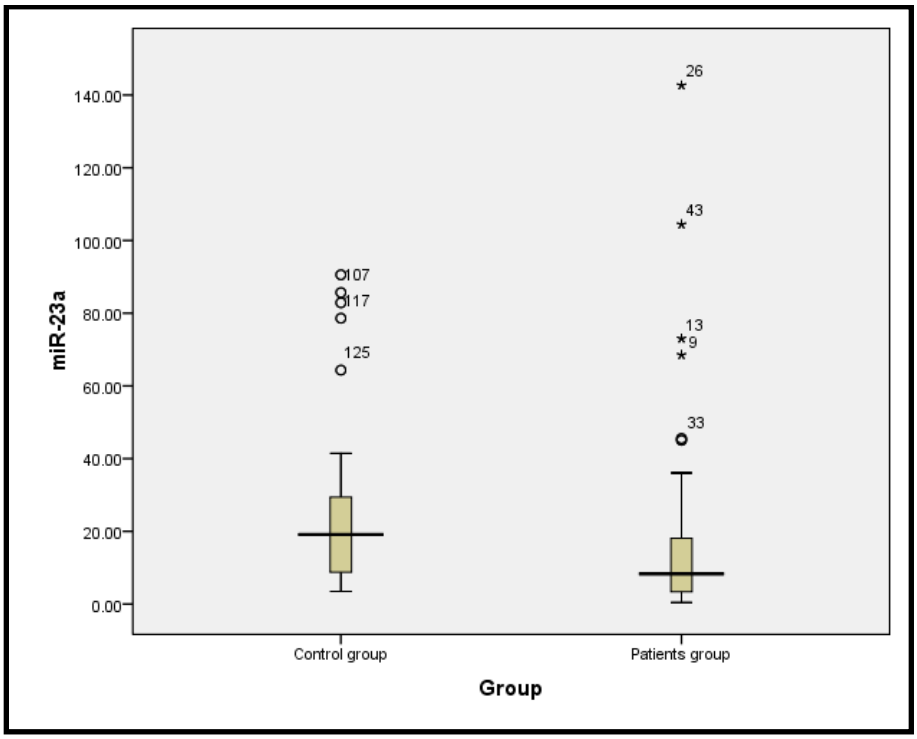

Figure (3): Box plot showing comparison of miR-23a expression between patients' group and control group.

Understanding the host response to SARS-CoV-2 infection can help with patient care and understanding viral pathogenesis. But it's still unclear how the SARS-CoV-2 infection affects the host-encoded microRNA (miRNA) response (Farr *et al*., 2021). In the current study, the levels of miR-423 and miR-195 were significantly higher in patients with COVID-19 in comparison with control group; however, the level of miR-23a was significantly lower in patients with COVID-19 in comparison with control group. According to the study of (Farr *et al*., 2021), the levels of 55 miRs were studied in 10 patients with COVID-19 and compared to 10 age and gender matched control group; they observed that miR-423 and miR-195 were significantly higher and that miR-23a was significantly lower in patients in comparison with control, thus we agree with their results.

It should be emphasized that significant changes in the levels of individual miRs is not specific because it has been reported that increased expression of circulating miR-423-5p is observed during heart failure (Tijsen *et al*., 2010) and pulmonary tuberculosis (Tu *et al*., 2019). Increases in the amount of miR-195-5p in the blood have been linked to osteosarcoma, autism, and gestational diabetes mellitus (Mundalil *et al*., 2014 ; Lian *et al*., 2015 ; Wang *et al*., 2020). A four-miRNA signature that may accurately detect HIV-1 infection includes miR-195-5p, which is interestingly found to have enhanced plasma expression during HIV-1 infection (Biswas *et al*., 2019).

However, in our study, the measurement of the levels of three miRs simultaneously may provide strong diagnostic evidence to the state of SARS-Cov-2 infection and become an auxiliary diagnostic tool with relatively high accuracy rate in patients with clinically suspected manifestations.

Viral RNA is the target of the current COVID-19 molecular assays for detection. Unfortunately, a somewhat high viral load for SARS-CoV-2 is necessary for even the most sophisticated contemporary molecular diagnostic methods (such as PCR or LAMP amplifying viral RNA) to reliably detect infection (Kucirka *et al*., 2020). Since the viral load is still low during the early presymptomatic phase of the disease (incubation period), their sensitivity is weak. It is challenging to diagnose infections in many cases that are pre-symptomatic and in some cases that are asymptomatic because the overall sensitivity of current PCR testing has been reported to be as low as 30-70% (Ai *et al*., 2020; Kanne *et al*., 2020).

The significance of miRNAs in COVID-19 pathogenesis is little understood, despite the fact that host responses to infection are known to be crucial in the diverse outcomes of SARS-CoV-2 infection. Therefore, future histopathological studies in conjunction with serum evaluation of miRs levels may help revealing such pathogenic role.

References:

Ai T, Yang Z, Hou H, Zhan C, Chen C, Lv W, et al. Correlation of Chest CT and RT-PCR Testing in Coronavirus Disease 2019 (COVID-19) in China: A Report of 1014 Cases. Radiology. 2020:200642.

Biswas S, Haleyurgirisetty M, Lee S, Hewlett I, Devadas K. Development and validation of plasma miRNA biomarker signature panel for the detection of early HIV-1 infection. EBioMedicine. 2019;43:307–16.

Cascella M, Rajnik M, Aleem A, *et al.* Features, Evaluation, and Treatment of Coronavirus (COVID-19) [Updated 2023 Jan 9]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan.

Cui, J., Li, F. & Shi, Z.-L. Origin and evolution of pathogenic coronaviruses. Nat. Rev. Microbiol. 17, 181–192 (2019).

de Wit, E., van Doremalen, N., Falzarano, D. & Munster, V. J. SARS and MERS: recent insights into emerging coronaviruses. Nat. Rev. Microbiol. 14, 523–534 (2016).

Fani M, Zandi M, Rezayi M, Khodadad N, Langari H, Amiri I. The role of microRNAs in the viral infections. Curr. Pharm. Des. 24(39), 4659–4667 (2018).

Fani, M., Zandi, M., Ebrahimi, S., Soltani, S., & Abbasi, S. (2021). The role of miRNAs in COVID-19 disease. Future Virology.

Farr, R. J., Rootes, C. L., Rowntree, L. C., Nguyen, T. H. O., Hensen, L., Kedzierski, L., Cheng, A. C., Kedzierska, K., Au, G. G., Marsh, G. A., Vasan, S. S., Foo, C. H., Cowled, C., & Stewart, C. R. (2021). Altered microRNA expression in COVID-19 patients enables identification of SARS-CoV-2 infection. PLoS pathogens, 17(7), e1009759.

Fung, T. S. & Liu, D. X. Human coronavirus: host-pathogen interaction. Annu. Rev. Microbiol. 73, 529–557 (2019).

Guterres A, De Azeredo Lima CH, Miranda RL, Gadelha MR. What is the potential function of microRNAs as biomarkers and therapeutic targets in COVID-19? Infection, Genetics and Evolution 85, 104417 (2020).

Kanne JP, Little BP, Chung JH, Elicker BM, Ketai LH. Essentials for Radiologists on COVID-19: An Update-Radiology Scientific Expert Panel. Radiology. 2020:200527.

Kucirka LM, Lauer SA, Laeyendecker O, Boon D, Lessler J. Variation in False-Negative Rate of Reverse Transcriptase Polymerase Chain Reaction-Based SARS-CoV-2 Tests by Time Since Exposure. Ann Intern Med. 2020;173(4):262–7.

Lian F, Cui Y, Zhou C, Gao K, Wu L. Identification of a plasma four-microRNA panel as potential noninvasive biomarker for osteosarcoma. PLoS One. 2015;10(3):e0121499.

Mas-Ubillus, G., Ortiz, P. J., Huaringa-Marcelo, J., Sarzo-Miranda, P., Muñoz-Aguirre, P., Diaz-Ramos, A., Arribasplata-Purizaca, K., Mendoza, D., Rojas-Poma, J., Marcelo-Ruiz, C., Ayala-Diaz, P., Hidalgo-Arroyo, E., & Tupia-Cespedes, L. (2022). High mortality among hospitalized adult patients with COVID-19 pneumonia in Peru: A single centre retrospective cohort study. PloS one, 17(3).

Mundalil Vasu M, Anitha A, Thanseem I, Suzuki K, Yamada K, Takahashi T, et al. Serum microRNA profiles in children with autism. Mol Autism. 2014;5:40.

Tijsen AJ, Creemers EE, Moerland PD, de Windt LJ, van der Wal AC, Kok WE, et al. MiR423-5p as a circulating biomarker for heart failure. Circ Res. 2010;106(6):1035–9.

Tu H, Yang S, Jiang T, Wei L, Shi L, Liu C, et al. Elevated pulmonary tuberculosis biomarker miR-423-5p plays critical role in the occurrence of active TB by inhibiting autophagosome-lysosome fusion. Emerg Microbes Infect. 2019;8(1):448–60.

Wang J, Pan Y, Dai F, Wang F, Qiu H, Huang X. Serum miR-195-5p is upregulated in gestational diabetes mellitus. J Clin Lab Anal. 2020;34(8):e23325.

Weiss, S. R. & Navas-Martin, S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiol. Mol. Biol. Rev. 69, 635–664 (2005).