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Correlation between MicroRNA-155 Expression and Some Inflammatory Biomarkers of Covid-19 Infection

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Abstract: Coronavirus disease is still an interesting topic of study, although the influence of the pandemic has been limited. MicroRNA-155, or miR-155, is a small RNA molecule that plays an important role during inflammation in many diseases at epigenetic levels. So, we aim, in this study, to investigate the expression of microRNA-155 in coronavirus disease 2019 patients and the healthy control groups and determine the relationship of miR-155 with some disease biomarkers (CRP, D-dimer, and ferritin levels). Two hundred thirty five (235) samples were selected from 120 patients with covid-19 and 115 apparently healthy individuals of different sexes and ages. All the patients are severe cases of covid-19. MicroRNA-155 expression was performed using the real-time PCR technique (RT-PCR). CRP was determined by immunofluorescence assay, D-dimer was calculated using enzyme-linked fluorescent assay, and ferritin levels were estimated depending on the immunoturbidimetric assay. After statistical analysis was done, results showed a highly significant overexpression of miR-155 in patients. It is about twofold and half higher in patients than in healthy control individuals. Also, C-reactive protein, D-dimer, and ferritin show elevated scores in covid-19 patients. All the parameters studied in this research could be considered biomarkers for the critical cases of the disease. miR-155 can be invested in as a promising therapeutic agent because this inflammatory microRNA is correlated with the severity of the disease. Further investigations about the exact role of microRNA-155 during covid-19 should be done.

Key words: Covid-19, CRP, D-dimer, Ferrtin, miR-155.

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Introduction

Coronavirus disease, first reported in 2019, considered a worldwide health emergency by the World Health Organization (WHO) (1). Worldwide, there are millions of cases and thousands of deaths. However, the signs and symptoms of the disease are mild and may be asymptomatic. Some cases of covid-19 cause severe pneumonia and may develop into pulmonary distress and even death (2). The novel type of coronavirus is called SARS-CoV-2 which cause the disease (Covid-19). The virus was recognized in China for the first time (3). In Iraq, the pandemic went through four main waves of SARS-CoV-2 infections. The third wave was the largest daily infection report in a period between June 2021 and January 2022, while the most deadly wave was the first wave between June 2020 and December 2020, concentrated in Babylon and

Thiqar provinces. Vaccination played a role in changing the waves statistics (4, 33). The critical-severe patients of covid-19 usually suffer from hyperinflammation and cytokine storms (5). The characteristics of cytokine storms and uncontrolled inflammation are the increased levels of C-reactive protein (CRP), D-dimer, and hyperferritinemia of the serum (6). In Iraq, during the pandemic of SARS-CoV-2, many studies confirmed that CRP, D-dimer, and ferritin levels were raised in the case of critical coronavirus disease 2019 symptoms (7). So, these biomarkers are important in diagnosing the severity of the disease and predicting possible further complications that may occur during the infection.

MicroRNA molecules are small sized RNA molecule with an average of 22 bases. MicroRNAs have the ability to degrade messenger RNA (mRNA) and suppress the translation of proteins. They are complementarity in sequence with their targeted mRNA and induce gene expression in certain circumstances (8). MicroRNAs (miRNAs) are involved in biological processes and considered as potential biomarkers for many diseases (9,30). MiRNAs have been involved in some pulmonary diseases and have also contributed to the development of the lung and homeostasis (10). Many researches revealed the role of microRNAs during viral infection and demonstrated the different expressions of microRNAs according to viral infection (11). One of the microRNAs that are known to have pivotal effect in the regulation of immunity and inflammation in the lung and many other organs is microRNA-155 (miR-155) (12). In viral infections, microRNA-155 is considered a multi-role miRNA that strictly regulates all the types of immune responses (innate, humoral, and cellular)(13).

Therefore, the goal of this study is to evaluate the expression of microRNA-155 in positive SARS-Cov-2 infected persons and negative control healthy individuals to search for the impact of this molecule in the pathogenesis and severity of coronavirus disease-2019 and to determine its validity as a novel biomarker for the examination of coronavirus disease-2019 and the critical cases of infection. Also, to investigate whether there is a relationship between the MicroRNA-155 expression and the inflammatory biomarkers CRP, D-dimer, and ferritin levels in the plasma.

Materials and methods

Patients and sampling

The present study was performed during the period between October 2022 and June 2023. It includes two types of samples (blood and sputum). They are collected from Ibn Al-Khatteeb Hospital in Baghdad, Iraq. The Ethical Committee at the Department of Biology in the University of Baghdad and the Iraqi Ministry of Health accepted the study proposal and protocol (reference: CSEC/0922/0083). A total of 235 samples were obtained from 120 patients with coronavirus disease (covid-19) and 115 apparently healthy

individuals of different sexes and ages. All the patients are hospitalized cases of covid-19. From the confirmed positively infected persons, 5 ml of blood was drawn and placed in a gel tube for serum collection. Then, serum was yield by centrifugation (3000 rpm for 10 minutes). The supernatants were collected carefully and frozen at -20°C. Nasopharyngeal and nasal swabs were also collected by swabbing the area of the pharynx from patients and controls. Swabs were repeated and agitated to confirm the presence of epithelial cells in the sample. Then, centrifuge at 5000 g/min for 5 min was applied. The supernatant was omitted. For MicroRNA-155 expression determination, swabs were placed in Trizol reagent to protect the RNA in the sample.

Extraction and quantitation of total RNA

After sample collection, ReliaPrep, Viral Nucleic Acid Extraction Kit (Promega) was used to extract and purify RNA from sputum. An aliquot of sample 300 µl was added to 600 µl of TRIZOL reagent in a 1.5 ml micro-centrifuge tube, and incubated (10 minutes, 25˚C) for complete dissociation of the nucleoprotein complex. Proteinase K Solution (20 µl) and Cell Lysis Buffer (200 µl) were added, respectively, to each 1.5 ml tube. Samples were incubated at 56°C for 10 minutes using a heat block device. Then, 250µl of 100% isopropanol was added and placed in a vortex (10 seconds). Contents of the tube were placed in ReliaPrep Binding Column and centrifuge applied for 1 minute at maximum speed (1200 rpm). Column Wash Solution (500µl) and centrifuge (3 minutes, 1200 rpm) were applied. The remains are discarded. The last step was repeated two times for three washes of achievement. The elution step requires adding 60 µl of nuclease-free water to the column, followed by centrifugation for 1 minute (1200 rpm). The silica tube (ReliaPrepTM Binding Column) was discarded, and the elutes were saved in 1.5-ml tubes for processing. After the extraction of RNA from the samples, the concentration of RNA obtained is calculated using a quantum fluorometer device. A volume of 1–20µl of an unknown sample was added to 200µl of QuantiFluor RNA Dye working solution in 0.5 ml PCR tubes. Then, the tube was placed in a vortex and protected from light. The fluorescence of the unknown sample was measured using the quantum fluorometer. The number displayed represents the concentration of the original sample.

Primer design

In the present study, the microRNA molecule (mir-155) was targeted by itself but not the gene that expresses it. The sequence of mir-155 was obtained and targeted using three types of primers. All the primers for Micorna-155 were delivered from Macrogen, Korea. They include: forward primer (complementary to the cDNA sequence of mirr-155), oligo-DT primer (adaptor primer), and universal primer (reverse).

The sequences of the primers in Table (1).

Table (1): Showing the sequences of Mir-155 primers.

Primer	Sequence
Stemloop primer (adaptor)	3′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACAACCCC -5′
Forward primer	3′- CGCGCGTTAATGCTAATC-5′
Universal reverse primer	5′-CCA GTG CAG GGT CCG AGG TA-3′

Conversion of RNA to cDNA

Luna-Script RT Super-Mix Kit (Biolabs, UK) was used for conversion of extracted RNA to cDNA. Lyophilized primers were liquefied by 200 µl of free nuclease water in order to prepare the stock solution. The working solution was prepared at a ratio of 1:10 of stock solution to free nuclease water. The reaction mixture was placed on ice. After dispensing aliquots of this mixture into the microtubes, the RNA sample was affixed. Reaction mixture was incubated at 25 °C for 2 minutes (primary annealing), 55 °C for 15 minutes (reverse transcriptase, cDNA synthesis), followed by 90 °C for 1 minute (heat inactivation) for one cycle only.

Performing RT-PCR

Sacycler, Saccac, Italy: Quantitative RT-PCR (qPCR) technology was carried out using the BioLabs, England-based LunaScript RT Master Mix Kit (5X). The RT-PCR mixture included 0.5 µl of each forward primer and reverse primer and 10 µl of the master mix. After adding 5 µl of cDNA from each sample, primers and reverse primers, 4 µl of nuclease-free water was also added to bring the volume up to 20 µl. The endogenous control was the U6 gene. A qPCR was run for one minute at 95°C to activate the polymerase. This was followed by 45 cycles of denaturing the double-stranded cDNA for 15 seconds at 95°C and annealing for 20 seconds at 60°C with channel scanning. Based on the separation properties of double-stranded cDNA during cycles with increasing denaturing TM, melting curve analysis was carried out. The Ct value of miRNA-155 was standardized to the U6 reference gene, and the expression of miRNA-155 was assessed by the relative quantitative method using the comparative Ct formula and folding $=2^{-\Delta\Delta CT}$ analysis (38).

C- reactive protein (CRP) level determination

C-reactive protein (CRP) level is determined by using the hs-CRP+CRP Fast Test Kit, which is dependent on the immunofluorescence assay. This test can be done with the aid of the Getein 1100 immunofluorescence quantitative analyzer. It is used for the estimation of CRP levels in serum, plasma and whole blood. For plasma samples, EDTA can be used as anticoagulants. Samples may be stored for up to 7 days at 2~8°C or at -20°C for 6 months before testing. Before testing, frozen samples left to reach 25˚C and homogenized. At room temperature, 10 µL of sample was transferred into the tube of sample diluent, mixed gently. Then, 100 µL of sample mixture was transferred into the suitable port of the sample on the test card. This card was inserted into the Getein1100 device. The reaction time duration is three minutes. After reaction time finished, results was obtained on the screen of the device (37).

D-Dimer level determination

Using the ELFA method (Enzyme Linked Fluorescent Assay), VIDAS® D-Dimer Exclusion IITM (DEX2) is an automated quantitative test designed to be used on the VIDAS family of instruments for the immunoenzymatic detection of fibrin degradation products (FbDP) in human plasma (sodium citrate). The assay's basic idea combines final fluorescence detection (ELFA) with a two-step enzyme immunoassay sandwich technique. The pipetting tool and solid phase are combined in the Solid Phase Receptacle (SPR), which has an anti-FbDP monoclonal antibody adsorbed on its surface. The sealed single-use reagent strips include pre-dispensed, ready-to-use test reagents. For every sample, control, or calibrator that needed to be examined, one DEX2 strip and one DEX2SPR (Solid Phase Receptacle) were utilized. DEX2SPR and the DEX2 strip were positioned on the VIDAS preparation/loading tray. To increase reliability, 200 µL of each calibrator, control, and sample were vortexed to separate the pellet from the plasma. The strips were placed on the instrument in the proper locations. The apparatus initiated the test automatically, following the operator's instructions. Within 20 minutes, the assay results are available (34, 35)

Ferrtin level determination

UsingRoche/Hitachi Cobas C 3 11 equipment, Tina- quant ferritin Gen. 4 kit is used to quantitatively determine the amount of ferritin in human serum. The assay's basic idea is based on a particle- enhanced immunoturbidimetric assay,in which latex particles coated with anti-ferritin antibodies agglutinate with human ferritin. Turbidimetric analysis is used to determine the precipitate at 570/800 nm (36).

Statistical work

The statistical analysis was carried out using Microsoft Office Excel (2010) for data visualization and the SPSS Statistical software (Version 26; IBM, SPSS) for data collection. The receiver operating characteristic (ROC) curve was retained. Comparing regularly distributed data, shown as mean±SD: quantitative variables between the groups under study, was done using independent student samples (t-test). When comparing qualitative characteristics between groups under study, the Pearson chi-square test ($\chi 2$) is utilized. Utilizing the Pearson correlation (r) test, one may ascertain the associations among variables. A P-value of $P<0.05$ and $P<0.01$ was considered the statistical significance level.

Results and discussion

Sex and age groups distributions

The individuals included in this research were selected randomly. The number of male patients was 132 (56.4%) and the number of female patients was 102 (43.6%). The control group was 52.2% males and 47.8% females. The results show non-significant differences between male and female patients suffering from severe covid-19 (Table 2) and (Figure 1). Also, the ages of the studied individuals were divided into three groups (group1: from 20 to 40 years, group 2: from 41to 60 years, and group 3: older than 60 years). Statistical results also show non-significant differences between the age groups of the studied population (Table 3 and Figure 2).

Table (2): Distribution of Sex between patients and control groups.

Gender		Studied groups		P - value
		Controls	Patients	
Male	N	47	132	P = 0.497 Non significant (P>0.05)
	%	52.2%	56.4%	
Female	N	43	102	
	%	47.8%	43.6%	
Total	N	90	234	
	%	100%	100%	

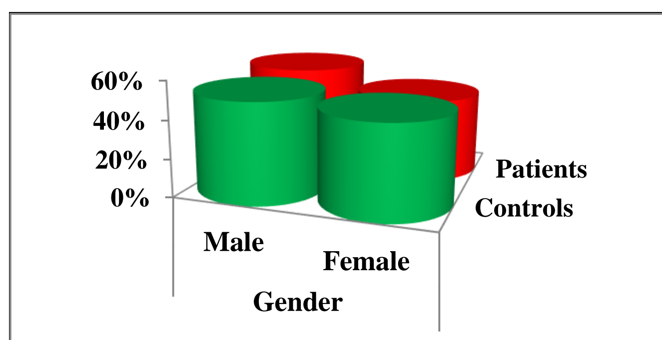

Figure (1): Distributions of Sex groups.

Table (3): Distribution of Age groups between patients and control.

Age groups / Year		Studied groups		P - value
		Controls	Patients	
20 - 40	N	56	124	P = 0.256 Non significant (P>0.05)
	%	62.2%	53%	
41 - 60	N	32	99	
	%	35.6%	42.3%	
> 60	N	2	11	
	%	2.2%	4.7%	
Total	N	90	234	
	%	100%	100%	

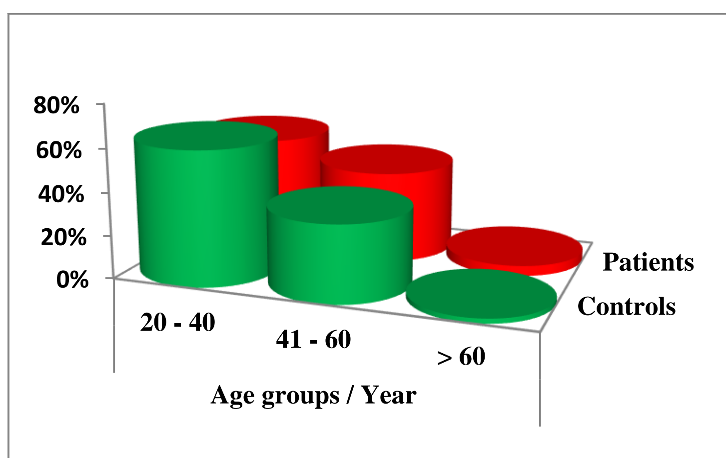

Figure (2): Distributions of the three age groups between patients and control.

MicroRNA-155 expression

A real-time PCR procedure was applied to nasopharyngeal samples from patients and the control group to evaluate the expression of Microrna-155 in both groups. *The U6* gene was used in the procedure for standardization of the *Ct* value (endogenous control). MiR-155 expression was calculated by relative quantitative method. The comparative *Ct* formula and folding =2^-ΔΔCT analysis was used. The results of RT-PCR revealed that MicroRNA-155 expression in critical covid-19 patients was elevated. Apparently healthy control individuals show lower results of expression. The mean fold number of MicroRNA-155 expression in covid-19 patients was 4.167970, while in control, it was only 1.631186. Differences between the expression of MiR-155 in patients and in healthy control individuals was highly significant (P = 0.00, P<0.01), (Table 4 and Figure 3).

Table (4): Distribution of microRNA-155 expression between patients and control.

Tested groups		No.	Mean	Std. Deviation	Std. Error	P - value
Mir-155	**Controls**	115	31.991	1.6709	.1558	P = 0.006 Highly sign. (P<0.01)
	Patients	120	30.692	4.7642	.4349	
	Total	235				
U6	**Controls**	115	17.043	1.2575	.1173	P = 0.00 Highly sign. (P<0.01)
	Patients	120	18.496	3.3351	.3045	
	Total	235				
ΔCT	**Controls**	115	14.948	1.4253	.1329	P = 0.00 Highly sign. (P<0.01)
	Patients	120	13.884	1.6117	.1471	
	Total	235				
ΔΔCT	**Controls**	115	-.052	1.4253	.1329	P = 0.261 Non-sign. (P>0.05)
	Patients	120	-.483	3.8381	.3504	
	Total	235				
Fold	**Controls**	115	1.631186	1.6556037	.1543858	P = 0.00 Highly sign. (P<0.01)
	Patients	120	4.167970	5.7691001	.5266444	
	Total	235				

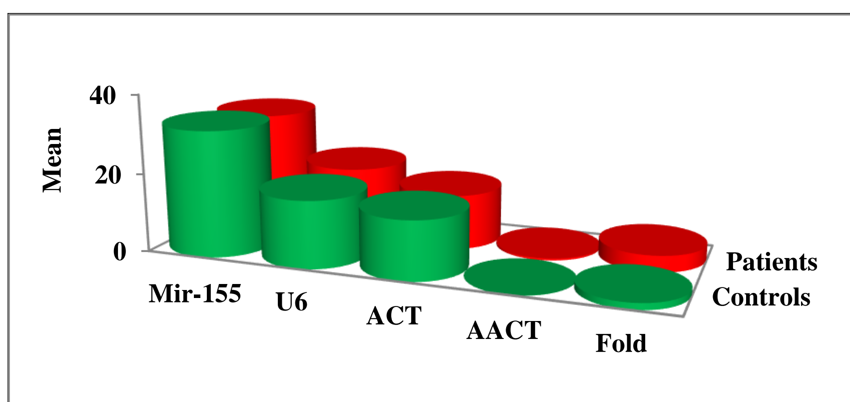

Figure (3): Distrbution of microRNA-155 expression between patients and control.

The biomarkers (CRP, D-dimer and ferritin)

CRP, D-dimer, and ferritin levels were determined in current research. The number of individuals tested for each biomarker (CRP and D-dimer) was 234 covid-19 patients and 90 apparently healthy control

individuals, while ferritin levels were tested for 90 confirmed SARS-COV-2 patients and 90 healthy controls. Highly significant differences between patients and healthy group was reported (P<0.01) for the three tested parameters (Table 5 and Figure 4).

Table (5): Results of some biomarkers in Covid-19 patients andl healthy groups.

Studied groups		N	Mean	Std. Deviation	Std. Error	P - value
Ferritin (ng/ml)	**Controls**	90	108.3778	66.70002	7.03080	P = 0.00 Highly significant (P<0.01)
	Patients	90	342.6463	286.64397	30.21493	
	Total	180				
CRP (ng/ml)	**Controls**	90	5.0837	7.22865	0.76197	P = 0.00 Highly significant (P<0.01)
	Patients	234	41.6138	33.55380	2.19348	
	Total	324				
D- dimer (ng /ml)	**Controls**	90	210.6875	146.71278	15.46489	P = 0.00 Highly significant (P<0.01)
	Patients	234	1131.0598	2318.95040	151.59453	
	Total	324				

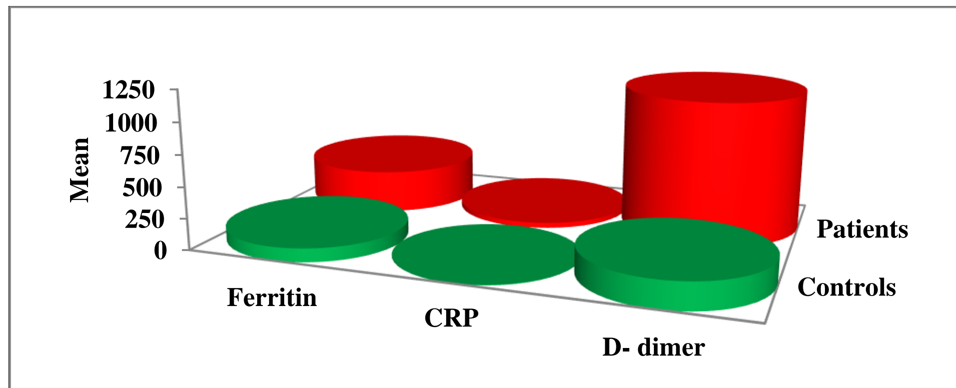

Figure (4): Results of C-reactive protein, D-dimer and ferrtin levels tests in Covid-19 patients and control healthy groups.

Correlation study

Significant negative correlation was reported between CRP results tested in serum and microRNA-155 expression present in nasopharyngeal swabs. All the other correlations between the studied biomarkers are non-significant (Table 6).

Table (6): Correlation between microRNA-155 and different biomarkers (CRP, D-dimer, and ferritin) levels.

Pearson Correlation		Age / Year	Mir-155 Expression
CRP (ng/ml)	**r**	**-.041**	**-0.265**
	P - value	**.533**	**0.005**
	significance	**NS**	**S**
D- dimer (ng/ml)	**r**	**-.007**	**0.032**
	P - value	**.910**	**0.761**
	significance	**NS**	**NS**
Ferritin (ng/ml)	**r**	**.004**	**-0.141**
	P - value	**.971**	**0.185**
	significance	**NS**	**NS**

During the pandemic, many researchers discussed the association among the infection of SARS-CoV-2 and the sex and age of the individuals (14). The severity of the disease and its correlations with these parameters were quite interesting in the first weeks and months of the pandemic. The present study dealt with the sex and age of our studied groups and found that there are no valuable differences between male and female suffering from severe covid-19. The ages of the studied groups were divided into three groups, ranging from 20 to above 60 years old. Also, the results show non-significant differences between the studied groups of severe or critical cases of covid-19. Our results were compatible with another study performed in Iraq. It discussed SARS-Cov-2 variants according to age and sex of the studied groups and showed non-significant differences (32).

MicroRNAs, short non-coding RNAs, are overexpressed during different health conditions. The exact role of microRNAs in different diseases is still challenging. According to various articles, microRNA-155 incorporated in the regulation of the immune response against viral infections (15). Current investigations revealed that microRNA-155 (mirr-155) expressed in covid-19 more than in healthy control individuals. This result is compatible with many other recent research studies. In December 2023, researchers from Saudi Arabia published an article confirming that miR-155 is distinguishable for covid-19 and could be used as a biomarker because of the high accuracy (specificity and sensitivity) of the test(16). Another study proposed that miR-155 affects Th17/Treg in the infected patients of covid-19 and suggested that microRNA-155 is a valuable prognostic marker in covid-19 (17). Also, miR-155 could be used as a biomarker for the pathogenesis and severity of the disease (18). Many approaches were taken to find a suitable therapy for the disease. Bautista-Becerril *et al.* (19) suggested that microRNA-155, among other microRNAs, is considered a possible therapeutic target (19). So, the higher expression of microRNA-155 in severe covid-19 cases could be used as a biomarker for the disease and a promising therapeutic agent for critical cases to reduce mortality ratio.

Some vital markers increased during the SARS-CoV-2 infection. C-reactive protein, D-dimer, and ferritin in plasma/serum are most common laboratory markers (20). CRP is considered an inflammatory biomarker and an indicator of tissue damage that is elevated during the early period of disease (21,). During the viral infection, ferritin, a biomarker of stored iron, increased in serum (22). Patients with hard pulmonary distress had elevated ferritin more than patients with mild respiratory distress (23). D-dimer is an indicator of disease severity (24). The present study significantly reported that C-reactive protein, D-dimer, and ferritin score higher levels than healthy control individuals.A study in China dealing with 113 patients, during the pandemic, observed that D-dimer was massively higher in deceased individuals than in recovered individuals(25). Elevated C-reactive protein (CRP) was also reported during the SARS-Cov-2 infection by Tanu S. (2020) (26). Also, increased ferritin levels reported as a risk factor for patients of covid-19 (27). Iraqi researchers of covid-19 indicated that CRP was not increased in children

but only increased in adults with critical cases. They show, in their study, an association between coronavirus disease and CRP and ferritin levels (28).So, many researchers agreed that the levels of these lab. parameters were elevated in severe cases of covid-19.

The present results show a significant negative correlation between CRP levels in serum and mirR-155 expression. Also, the present study revealed non-significant correlations between microRNA-155, D-dimer, and ferritin levels. The same result was reported by Kolarz B. *et al.* in 2020. They determined CRP levels in the serum of rheumatoid arthritis patients and confirmed the negative correlation. The same study reported that there is no valuable relationship between D-dimer or ferritin and microRNA-155 (29). However, another study showed a highly positive correlation between plasma microRNA-155, CRP, and D-dimer and a significant correlation with ferritin levels (18). These correlations are conflicting and suggest that the exact role that microRNA-155 plays in pathogenesis and immunity during covid-19 is still unclear and requires further investigation.

Conclusion

The biomarkers (CRP, D-dimer, and ferritin) increased during critical coronavirus disease 2019 and can be considered a biomarker of the severity of the disease. MicroRNA-155 (miR-115) was significantly overexpressed in severe COVID-19 cases. It was about twofold and a half higher than in normal, healthy individuals. The exact role of microRNA-155 in pathogenesis, inflammation and immunity is still challenging, but it could be used as a biomarker for the severity of coronavirus disease 2019 and a promising therapeutic agent.

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