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ORIGINAL ARTICLE

# miRNA-223-3p, miRNA- 2909 and Cytokines Expression in COVID-19 Patients Treated with Ivermectin

**Noha A. Rezk[1*], Ashraf Elsayed Sileem[2], Doaa Mostafa Gad[2], Amr Osama Khalil[3]**
[1]Medical Biochemistry Department, Faculty of Medicine, Zagazig University, Egypt
[2]Chest Department, Faculty of Medicine, Zagazig University, Egypt
[3]Radiology Department, Faculty of Medicine, Zagazig University, Egypt

***Corresponding author:**
**Noha Rezk**
Medical Biochemistry Department, Faculty of Medicine, Zagazig University
Email: nonnarezk@yahoo.com

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## ABSTRACT

**Background:** The role of Ivermectin in improving the outcome of coronavirus disease of 2019 (COVID-19) symptoms was reported in several studies, while its effect on the pro-inflammatory cytokines triggering the cytokine storm is still not investigated. This study aimed to investigate the role of Ivermectin on the proinflammatory cytokines in Covid-19 patients and correlated the results with the expression of miR-2909, miR-223-3p.

**Methods:** Three hundred and twenty hospitalized patients with confirmed moderate-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection were selected. The patients were divided into 2 groups: Group I treated with the Egyptian protocol of COVID-19 including (Ivermectin plus hydroxychloroquine). Group II was treated with the Egyptian protocol, including hydroxychloroquine and no Ivermectin. IL-6, IL-1b, procalcitonin, and gene expression of miR-2909, miR-223-3p, and Toll-like receptor 4 were done by real-time Polymerase Chain Reaction (PCR).

**Results:** Patients treated with COVID-19 protocol including Ivermectin showed a significant decrease of cytokines levels (IL-6, IL-1, and procalcitonin), when compared with the other group, the cytokines levels improvement were positively correlated with miR-2029 expression and negatively correlated with the expression of miR-223-3p. Moderate ill COVID-19 patients treated with Ivermectin showed a significant decline in mortality rate and duration of hospital stay.

**Conclusions:** Ivermectin is an effective drug in improving the outcome of SARS-CoV-2 patients with a significant decrease in mortality rate through decreasing cytokines expression via controlling miR-2029, miR-233-3p expressions.

**Keywords:** SARS-CoV-2; Ivermectin; miR-223-3p; miR-2029; Covid-19; Cytokines

## INTRODUCTION

On the 11th of March of the year 2020, the World Health Organization (WHO) declared coronavirus disease of 2019 (Covid-19) a pandemic announcing the beginning of a worldwide health emergency, the first to be seen in contemporary history. By the end of May 2021, the total cases were around 175 million and 3.79 million deaths. In Egypt, the number of announced cases reached 272 thousand with 15,547 deaths [1]. Since the emerging of the pandemic there has been momentous progress in comprehending the pathogenesis of the virus and how it works, however, its rapid spread and the fatality rate are still not sufficiently contained with the emerging of new variants worldwide.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, the cause of COVID-19, is described to have 2 stages, firstly the entrance of the virus in the tissues and cells which causes the main symptoms and complications [2,3], secondly the "cytokine storm" which is an inflammatory stage instigated by stimulation of STAT-3, IL-6, IL-8, G-CSF, NF-kB, and others pro-inflammatory genes. The attachment of angiotensin-converting enzyme-related carboxypeptidase-2 (ACE2) receptor to the SARS-CoV-2 spike protein is the first signal in the inflammatory stage leading to the activation of the ACE/Ang II/AT1R axis followed by stimulation of IL-6/STATs axis causing NF-κB hyper-activation [4,5]. This

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hyper-activation is the trigger of the cytokine storm which causes acute respiratory distress syndrome (ARDS) by affecting the lung tissues in severe cases, and it is positively correlated with the severity of the disease [6].

Several studies detected the increase of several inflammatory cytokines and chemokines in coronavirus infection, mainly IL6, IL1β, and IL8, and their increase was correlated with the severity of the infections [7,8]. Tackling these inflammatory cytokines is the basis of many clinical trials to abort the cytokine storm. Toll-like receptor (TLR) 4 identifies then connects to lipopolysaccharide (LPS) to start signaling pathways by MyD88-dependent and/or MyD88-independent cascades, leading to the stimulation of NF-κB [9].

The immune and inflammatory promoting genes are expressed with the help of the inducible transcription factor NF-κB, TLR 4 mediated signaling uses NF-κB activation to stimulate IL-6 and TNF-α expression [10]. It was reported that TLR4 can activate the cytokine's production, therefore its inhibition by neutralizing antibodies may cause a decrease in the production of the inflammatory factors inside the cell [9]. Several studies explored the relationship between the inflammatory signaling pathways and the complication of the COVID-19 leading to the notion to investigate the role of MicroRNAs (miRNAs) in the pathogenesis of COVID-19[7,8,9,10].

miRNA is a small non-coding RNA formed of about 19 to 25 nucleotides that functions as gene expression regulators via controlling mRNAs, causing either repression of mRNA translation or post-translation modifications [11]. miRNAs and RNAs complex regulate several biological processes including differentiation, apoptosis, and cell cycle [12]. miRNAs regulate TLR mediated signaling by many mechanisms like attacking the TLR signaling components; or by triggering RNA-sensing TLRs. miR-223 is one of the main miRNAs involved in regulating inflammatory and immune reactions inside the cell by directly targeting TLR mediated signaling and regulating IL-6 pathways [13]. While miR-2909 can be induced by the activation of NF-κB in peripheral blood mononuclear cells [14]. Inflammatory cytokines as TNF-α and IL6 production can be affected by Krueppel-like factor (KLF) 4, which is targeted by miR-2909[15].

The invention of novel medicines might not be the ideal logic with the increasing number of infections and deaths per minute therefore re-purposing of drugs is the more rational method for quick results. An anti-parasitic FDA-approved drug named Ivermectin used to treat Lymphatic Filariasis [16] is one of these repurposing drugs. The antiviral effect of Ivermectin was studied in some in vitro studies [17]. It was reported to control RNA virus infection as West Nile and influenza virus by inhibiting the viral proteins and the host nuclear import [18], moreover It was found to have a role in modulation of the inflammatory process and the levels of Transforming Growth Factor-Beta 1 (TGFβ) and Vascular Endothelial Growth Factor (VEGF) [19]. In recent studies, it was proposed to be effective against SARS-CoV-2 infection by both in vitro [20] and in vivo [21] trials, on the other hand, another trial reported that it didn't significantly affect the course of early COVID-19 [22]. The mechanism of action of ivermectin in decreasing the complication of COVID-19 and improving its outcome is still not investigated. We aimed to investigate the role of ivermectin in Covid-19 infection, its effect on the inflammatory cytokines and if its action is related to two of the most recent miRNAs implicated in the development of inflammatory reactions miR-223-3P, and miR-2909.

## METHODS

**Subjects:**

Three hundred and twenty hospitalized patients with confirmed moderate SARS-CoV-2 infection were selected. COVID-19 infection diagnosis was confirmed with at least one positive real-time PCR result from the nasopharyngeal/oropharyngeal swab.

According to the New Coronavirus Pneumonia Prevention and Control Program (6th edition),

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COVID cases were divided into four categories: mild, moderate, severe, critical pneumonia [23]. The patients were divided into 2 groups:

**Group I**: 160 patients with moderate COVID-19 infection treated with management protocol as issued by the Egyptian protocol of COVID-19 November 2020 for moderately ill patients including Ivermectin 6 mg (36 mg on day 0 -3-6) [24], plus hydroxychloroquine (400 mg / 12 hours for one day followed by 200 mg every 12 hours for 9 days) [25].

**Group II:** 160 patients with moderate COVID-19 infection treated with management protocol as issued by the Egyptian protocol of COVID-19 May 2020 for moderate ill patients including hydroxychloroquine (400 mg / 12 hours for one day followed by 200 mg every 12 hours for 9 days) [26].

Patients younger than 18 years, as well as pregnant and lactated females, were excluded from the study. Moderate cases were selected according to the following criteria: fever, respiratory tract symptoms, GIT symptoms, and pneumonia manifestations seen in chest imaging.

The two groups were followed up daily clinically and by laboratory investigations for 2 weeks and by radiological imaging after 2 weeks. They were followed up according to the time of SARS-CoV-2 swab negative result, disease progression to severe cases (pneumonia to severe respiratory distress syndrome), duration of hospital stay, clinical, radiological improvement, and mortality rate.

After hospital release, the patients were followed up for another 2 weeks by regular phone calls daily until they returned to their usual state of health or were readmitted to the hospital.

Criteria of severe COVID-19 cases: Respiratory rate over 30/min, blood oxygen saturation < 93%, required an FIO2 of 50% or more, lung infiltrates over 50% of the lung or rapid progression within 1- 2 days, patients need high flow oxygen either noninvasive or invasive mechanical ventilation.

Two consecutive nasopharyngeal/ oropharyngeal swabs with negative PCR results for SARS-CoV-2 taken 2 days apart, or clinical and laboratory test improvement, were considered as an endpoint.

Written informed consent was obtained from all participants, the study was approved by the research ethical committee of Faculty of Medicine, Zagazig University. The study was done according to The Code of Ethics of the world Medical Association (Declaration of Helsinki) for studies involving humans.

**Methods:**

**Biochemical measurement**

Ten ml of peripheral blood was obtained from all the patients on admission and after one week of treatment. 4 mL blood was obtained on EDTA-containing tubes, and 6 mL remained for 10 minutes to clot, and after that was centrifuged. The serum samples were isolated and stored at - 20◦C till the assay. Complete Blood Count (CBC) was done by hematology analyzer (mindray, BC 20s), C reactive protein (CRP) (Atlas Biotech), Ferritin (ELISA, Pishtaz diagnostic kit), D dimer (Unicell- quantitative rapid test), Lactate dehydrogenase (LDH) (Spectrum, REF 283003), interleukin-6 (IL-6), pro-calcitonin (PCT) and IL-1b were estimated by enzyme-linked immunosorbent assay ELIZA (R&D) was done on admission and repeated after one week of treatment.

**TLR-4 quantitative real-time polymerase chain reaction**

Total RNA was extracted from white blood cells using [GenElute TM Mammalian Total RNA Miniprep Kit; Sigma–Aldrich, USA]. Isolated RNA was reverse transcribed using an RT–PCR kit [TOYOBO, Osaka, Japan] according to the manufacturer's instructions. To assess mRNA expression of LTR4, qRT–PCR was done using SYBR green on a [StratageneTMMx3005P] qPCR instrument [Agilent Technologies]. Primers used forward 5'- TGGACCTGCGATTTAATCCC 3'- and reverse 5' GTCTGGATTTCAGAGCAGGA 3' for TLR4, F 5'- TGCTGTCTCTGAGTTTGATGTATCT-3' and R5'- TCTCTGCTCCCCACCTCTATAG -3' for β2m. Reactions were done as follows: 95° C for

30 sec, 40 cycles of 95° C for 5 sec, and 60° C for 34 sec. Relative expression of genes was calculated according to the ΔCt method [27].

**miRNA extraction assay**

Isolation of total RNA was done according to the manufacturer's instructions of the miRNeasy extraction kit (Qiagen, Valencia, CA) by using 250 µl of QIAzol lysis reagent for 5 min at room temperature. Then, 200 µL of chloroform was added and vortexed for 15 sec, incubating the samples for 3 min at room temperature, then centrifuged at 14,000 x g at 4◦C for 15 min. 600 µL were pipetted in miRNeasy Mini spin column and centrifuged at 8000 x g for 60 sec followed by addition of buffer RWT then buffer RPE before centrifugation at 8000 x g for 2 min. 20µL of eluted miRNA was reverse transcribed by incubation for 30 minutes at 25 °C, 30 minutes at 42 °C, 5 minutes at 85 °C, and afterward kept up at 4◦C using the miRNeasy Reverse Transcription Kit (Qiagen, Valencia, CA) as indicated by the manufacturer's instructions [28].

**Real Time-PCR quantification of miR-2909, miR-223-3p**

Quantitative real-time polymerase chain reaction test using SYBR Green PCR kit (Qiagen, Hilden, Germany) on (StratageneTMMx3005P) qPCR instrument (Agilent Technologies) was performed. The sequence of the primers used were h-miR-223-3p forward 5'-ACACTCCAGCTGGGTGTCAGTTTGTCAAAT-3', reverse 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGGGGTAT-3' and h-miR-2909 F 5'ACACTCCAGCTGGGGTTAGGGCCAACATC-3', R 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCAAGAGA-3'. The reactions were done as follow: 50˚C for 2 min, 95˚C for 10 min; and 40 cycles of 95˚C for 30 s, and 60˚C for 30 s. The relative expression of miR-223-3P, miR-2909, was calculated in relation to the level of internal control U6. The data were analyzed using the ΔCt method [27].

**Statistical analysis:**

Data were analyzed with SPSS version 15.0 (statistical package for the Social Science, Chicago, IL). Differences in the frequencies in the studied groups were analyzed using the chi-square (X2) test. Levels of serum miRNA were expressed as mean ± SD. The significant difference between the groups was determined using Student's t-test. Pearson correlation analysis was employed to assess the correlation between different parameters in the studied subjects. A difference was significant at the P-value <0.05. P-value was adjusted after Bonferroni correction.

## RESULTS

**Demographic data of the patients and control groups**

The studied groups did not differ as regards age, sex, smoking, comorbidities, symptoms, and biochemical parameters at the time of admission ($p > 0.05$) (Table 1).

**Serum levels of different parameters in the studied groups one week after treatment.**

We detected that IL-6, Il-1b, and procalcitonin were significantly increased in group II (hydroxychloroquine only) compared to group I (Ivermectin plus hydroxychloroquine) ($p = 0.001, 0.11, 0.008$ respectively). Also, Toll-like receptor expression and miR-2029 expression were significantly increased in group II compared to group I ($p= 0.01. 0.03$), while, miR-223-3p gene expression was significantly decreased in group II compared to group I ($p = 0.023$) (Table 2).

**Correlation between different parameters in the studied groups.**

We observed that miR-223-3p expression level was inversely correlated with IL-6, IL-1b, procalcitonin and TLR-4 in the studied groups ($r2 = 0. 91, 0.82, 0.79, 0.93$), while, miR-2029 expression level was positively correlated with IL-6, IL-1b, procalcitonin and TLR-4 in the studied groups ($r2 = -0.83, -0.77, - 0.89, - 0.94$) (Table 3).

**Prognosis of the disease in the studied groups**

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We observed a statistically significant improvement associated with a significant rapid SARS-CoV-2 RT-PCR conversion to a negative result in group I compared to group II after 2 weeks of treatment (p= 0.013, 0.01 respectively). After 4 weeks of observation, we detected a significant decrease in the number of subjects who were readmitted to the hospital in group I compared to group II (p= 0.041) (Table 4).

**Table 1:** Clinical characteristics of the studied groups

| | **Group I: COVID-19 cases treated with hydroxychloroquine and Ivermectin (n=160)** | **Group II: COVID-19 cases treated with hydroxychloroquine (n=160)** | ***P*** |
|---|---|---|---|
| **Age** | 50.9± 14.7 | 53.1± 12.6 | 0.15 |
| **Sex** | | | |
| Female n (%) | 83 (51.8) | 80 (50) | 0.13 |
| Male n (%) | 77 (48.2) | 80 (50) | |
| **Smoker n (%)** | 43 (26.9) | 45 (28.1) | 0.23 |
| **Comorbidities** | | | |
| Diabetes n (%) | 34/113 (30.3) | 35/132 (26.5) | 0.89 |
| Renal n (%) | 11/113 (10.2) | 17/132 (12.8) | 0.82 |
| Cardiac n (%) | 10/113 (8.1) | 14 /132 (10.6) | 0.78 |
| Obesity n (%) | 14/113 (12.3) | 15 /132 (11.4) | 0.91 |
| Hypertension n (%) | 12/113 (10.8) | 11/132 (8.4) | 0.85 |
| Cancer n (%) | 9/113 (7.9) | 8 /132 (6.1) | 0.9 |
| Thyroid n (%) | 1/113 (0.9) | 3 /132 (2.3) | 0.6 |
| Neurologic n (%) | 1/113 (0.9) | 4 /132 (3.0) | 0.57 |
| Pulmonary n (%) | 21/113 (18.6) | 25/132 (18.9) | 0.96 |
| **Presenting symptoms** | | | |
| Fever n (%) | 130 (81.2) | 121 (75.6) | 0.1 |
| Cough n (%) | 96 (60) | 101 (63.1) | 0.3 |
| Fever plus cough n (%) | 69 (43.1) | 74 (46.3) | 0.34 |
| Dyspnea n (%) | 34 (21.3) | 30 (18.7) | 0.26 |
| Headache n (%) | 58 (36.3) | 62 (38.8) | 0.4 |
| Loss of appetite n (%) | 51 (31.9) | 46 (28.8) | 0.39 |
| Nausea n (%) | 21 (13.1) | 17 (10.6) | 0.25 |
| Diarrhea n (%) | 18 (11.3) | 25 (15.6) | 0.2 |
| Abdominal pain n (%) | 9 ( 5.6) | 12 (7.5) | 0.21 |
| Chest pain n (%) | 16 (10) | 13 (8.1) | 0.22 |
| Anosmia n (%) | 4 (2.5) | 3 (1.9) | 0.13 |
| **Biochemical profile** | | | |
| CRP (mg/L) | 49.4 ± 14.3 | 51.6 ± 17.3 | 0.86 |
| Ferritin (ng/ml) | 171±18.5 | 169.4±13.6 | 0.76 |
| D dimer (µg/ml) | 5.0±2.1 | 4.4±1.8 | 0.80 |
| Lactate dehydrogenase (U/L) | 304 ±105.8 | 297 ±101.8 | 0. 6 |
| Lymphocyte count / $mm^3$ | 1.05 ±0.56 | 1.18 ±0.61 | 0.73 |
| Total leucocytic count/$mm^3$ | 4.79 ± 2.36 | 5.09 ± 2.27 | 0.71 |
| IL-6 (pg/ml) | 72.5±20.2 | 76.3±18.2 | 0.56 |

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| | Group I: COVID-19 cases treated with hydroxychloroquine and Ivermectin (n=160) | Group II: COVID-19 cases treated with hydroxychloroquine (n=160) | *P* |
|---|---|---|---|
| Il-1b (pg/ml) | 45.7± 8.2 | 40.5± 7.8 | 0.43 |
| Procalcitonin (ng/ml) | 0.64±0.14 | 0.75±0.15 | 0.61 |

**Table 2:** Serum levels of different parameters in the studied groups one week after treatment

| | Group I :COVID-19 cases treated with Ivermectin and hydroxychloroquine (N=160) | Group II :COVID-19 cases treated with hydroxychloroquine (N=160) | *p* |
|---|---|---|---|
| IL-6 (pg/ml) | 34.6±9.8 | 53.5±14.2 | 0.001* |
| Il-1b (pg/ml) | 15.6±4.3 | 25.7± 4.2 | 0.011* |
| Procalcitonin (ng/ml) | 0.16±0.03 | 0.44±0.11 | 0.008* |
| TLR4 expression | 0.89± 0.11 | 1.40±0.20 | 0.01* |
| miR-223-3p expression | 0.74±0.10 | 0.52±0.03 | 0.03* |
| miR-2909 expression | 0.93 ± 0.09 | 1.35 ±0.16 | 0.026* |
| CRP (mg/L) | 16.8 ± 2.1 | 30.3 ± 3.6 | <0.001* |
| Ferritin (ng/ml) | 98.8 ± 21.4 | 101.5±15.3 | 0.5 |
| D dimer (µg/ml) | 0.7 ± 0.16 | 1.4 ± 0.21 | <0.001* |
| Lactate dehydrogenase (U/L) | 176 ±45.8 | 185 ±51.8 | 0.61 |
| Lymphocyte count / $mm^3$ | 1.48 ±0.51 | 1.15 ±0.46 | 0.01* |
| Total leucocytic count/$mm^3$ | 6.07 ± 2.17 | 4.29 ± 2.36 | 0.01* |

*****A difference was significant at the P-value <0.05. P-value was adjusted after Bonferroni correction.

**Table 3:** Correlation between plasma miR-233-3p, miR-2029 expression and different parameters in the studied subjects

| | miR-223-3p expression | | miR-2029 expression | |
|---|---|---|---|---|
| | $r^2$ | p | $r^2$ | p |
| IL-6 (pg/ml) | -0.83 | <0.001 | 0.91 | <0.001* |
| Il-1b (pg/ml) | -0.77 | <0.01 | 0.82 | <0.001* |

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| | | | | |
|---|---|---|---|---|
| Pro-calcitonin (ng/ml) | -0.89 | <0.001 | 0.79 | <0.01* |
| TLR4 expression | -0.94 | <0.001 | 0.93 | <0.001* |

**Table 4:** Prognostic markers of Covid-19 outcome in the studied groups after 2 weeks

| | Group I: COVID-19 cases treated with Ivermectin and hydroxychloroquine (N=160) | Group II: COVID-19 cases not treated with hydroxychloroquine (N=158**) | *p* |
|---|---|---|---|
| **Clinical outcomes** | | | 0. 013* |
| **Improved n (%)** | 152 (95) | 142 (90.6) | |
| **Progressed n (%)** | 8 (5) | 16 (9.4) | |
| **RT-PCR conversion (days)** | 6.4 ± 1.2 | 8.8 ± 3.5 | 0.01* |

*A difference was significant at the P-value <0.05. P-value was adjusted after Bonferroni correction.
** Number of cases decreased due to the death of 2 patients

**Table 5:** Prognostic markers of Covid-19 outcome in the studied groups after 4 weeks

| | Group I: COVID-19 cases treated with Ivermectin and hydroxychloroquine. (N=145**) | Group II: COVID-19 cases treated with hydroxychloroquine (N=138**) | *p* |
|---|---|---|---|
| **Clinical outcomes** | | | 0. 041* |
| **Return to usual health n (%)** | 131 (90.4) | 118 (85.5) | |
| **Readmitted to hospital n (%)** | 14 (9.6) | 20 (14.5) | |

*****A difference was significant at the P-value <0.05. P-value was adjusted after Bonferroni correction.
**We could not reach all the 160 individuals in each group, so the number written is the number of cases we successfully reconnected with**.**

## DISCUSSION

The role of Ivermectin in improving the outcome of COVID-19 symptoms was studied in several studies, some of which stated its efficiency in improving the outcome of Coronavirus infection [20,21,29], while others question its value as an effective treatment of COVID-19 cases [22]. Ivermectin has drawn global awareness for its role in the management of COVID-19; it was listed in the management protocol for COVID-19 patients in many countries including Peru, Bolivia, Paraguay, Colombia [30], and Egypt [25]. After incorporating Ivermectin in the Egyptian protocols for the management of COVID-19 [25], this study tried to assess its impact on the cytokine storm, which is connected to many symptoms and complications of Coronavirus infection, and as microRNAs are documented to be implicated in the expression of chemokine, cytokines, and growth factors [31], It investigated the expression of two of the most important miRNAs implicated in the TLR4/TLR2/NF-κB/STAT3 signaling pathway [32], which is one pathway correlated with the cytokine storm and correlated their levels with

the main inflammatory cytokines to deduce the mechanism of Ivermectin actions.

There was a statistically significant improvement associated with a significant decline in mortality rate and duration of hospital stay in groups I (Ivermectin plus hydroxychloroquine) compared to group II (hydroxychloroquine only), also, there was significant rapid SARS-CoV-2 RT-PCR conversion to a negative result in group I compared to group II, these results confirmed what other researchers concluded in their previous trials including, Chowdhury et al. [33], Rajter et al. [34], Behera et al. [35] and our colleague Waheed Shouman [36] who reported that ivermectin has a highly significant role in the protection against SARS-CoV-2 infection and that there was no mortality or serious adverse events because of ivermectin in the intervention group. However, a study by López-Medina et al. [22] didn't find any significant clinical benefit of ivermectin on COVID-19 cases.

An in-vitro study by Clay et al. [20] reported the reduction of SARS-CoV-2 viral RNA by approximately 5000-fold when 5-μM ivermectin was added to the infected cells, however, another study stated that the in-vitro used concentrations are hard to be reached in human plasma and lungs [37] and it may be not safe [38]. Meanwhile, Rajter et al. [34] confirmed that a dose of 200 μg/kg was effective in improving the outcome of severe coronavirus infection.

Our study found that IL-6, IL-1b, pro-calcitonin, and Toll-like receptor expression were significantly increased in group II compared to group I proving our hypothesis that Ivermectin has a role in tackling the cytokine storm by interfering with the proinflammatory cytokine these results are in congruence with the findings of Yan et al. [39] who reported that ivermectin is an effectual suppressor of inflammation and can be used in the treatment of allergic diseases by decreasing the recruitment of immune cells and diminishing the cytokines production, Zhang et al. [40] proved that ivermectin can inhibit lipopolysaccharide (LPS)-induced inflammation by blocking NF-kB pathway and decrease the production of tumor necrosis factor-alpha (TNF-alpha), IL-6 and interleukin-1ss (IL-1ss) and suppress translocation of NF-kB. Sohn et al. [41] detected a link joining TLR4 signaling and inflammation in COVID-19 and leading to the development of new therapeutic methods pursuing TLR4-mediated inflammation.

miR-2029 expression was detected to be significantly higher in group II compared to group I, while, miR-223-3p gene expression was significantly decreased in group II compared to group I, meanwhile miR-223-3p expression level was inversely correlated with IL-6, IL-1b, procalcitonin in the studied groups, while, miR-202 expression level was positively correlated with IL-6, IL-1b, procalcitonin in the studied groups, As far as we know this is the first study investigating miR-2029 and miR-223-3p expression and correlating their levels with Ivermectin use and COVID-19 infection , Wu et al. [32] detected the relation between Lipopolysaccharide (LPS), miR-223-3P, miR-2909 and pro-inflammatory cytokines via TLR 4/TLR2/ NF-κB/ STAT 3 signaling pathway where LPS decrease the expression of both miRNAs while IL-6 stimulation reduce miR-223-3P expression leading to the activation of STAT3 which directly targeted TLR4 increasing the pro-inflammatory cytokine production, while miR-2909, NF-κB dependent, pursued Krueppel-like factor 4 regulating the production of pro-inflammatory cytokines. miR-223 has been reported to controls STAT3 expression and IL-6 expression [42], IL-6 signaling regulation occurs by making STAT3 increase TLR dependent inflammatory reactions [43]. It was reported that miR-223 restrain IL-6 and TNF-α expression in macrophages infected with Helicobacter pylori [44]. TLR4/NF-κB pathway can be negatively regulated by miR-223 which can up-regulate PI3K/AKT signaling pathway leading to TLR4/MAPK/NF-κB pathway block [45]. miR-2909 was proved to regulate genes implicated in inflammation and immunity, and that it is induced by the activation of NF-κB [14],

increasing its expression led to increased IL-6 and TNF-α production by targeting KLF4 [15].

**Conclusions**

In this study, we showed that Ivermectin is an effective drug in improving the outcome of SARS-CoV-2 patients with a significant decrease in mortality rate through decreasing cytokines expression via controlling miR-2029, miR-233-3p expressions.

**Competing interests**: No conflict of interest.

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**Ethical approval (including reference number):** Zagazig University ethical committee and Faculty of Medicine International Review Board (IRB) approved this study (ZU IRP #7890/26-8-2020)

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