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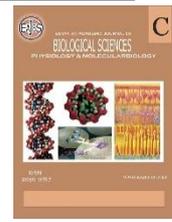
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## Could Targeting miRNA-142-5p Be a Therapeutic Strategy Against COVID-19 Through Activation of The Anti-Inflammatory Nrf2/Keap1 Pathway?

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### ABSTRACT

**Background:** COVID-19 is a highly pathogenic and transmittable viral infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2). COVID-19 infection has a wide range of severity ranging from an asymptomatic form to a severe form with acute respiratory distress which may lead to death. Nuclear factor erythroid 2-related factor 2 (Nrf2) is the main transcription factor that controls the antioxidant defense system which plays an important role in minimizing the symptoms induced by the cytokine storm in COVID-19. It has been found that miRNAs are involved in the regulation of different viral infections, however, the association between miRNAs and COVID-19 remains to be investigated. **Aim:** we aimed to investigate the role of miR-142-5p in modulating the inflammatory response in COVID-19 patients through the regulation of Nrf2/Keap1 pathway. **Methods:** The study was performed on 50 subjects that were divided into 2 groups: 15 healthy control subjects and 35 COVID-19 patients that were subdivided into 20 patients from the ward and 15 from intensive care unit (ICU). Peripheral blood was drawn from all subjects for estimation of gene expression of miRNA-142-5p, ACE2, NF-k $\beta$ , Nrf2, Keap-1, and HO-1 by QRT-PCR. **Results:** The COVID-19 patients showed a highly significant increase in miRNA-142-5p, Keap1, ACE2 and NF-k $\beta$  gene expression levels and a highly significant decreased expression levels of Nrf2 and HO-1 as compared to the control group. A highly significant negative correlation was detected between the miRNA-142-5p and Nrf2 expression levels in COVID-19 patients. **Conclusion:** Such data suggest that miRNA-142-5p could affect the severity of cytokine storm through its effect on Nrf2/Keap1 axis. Targeting miRNA-142-5p could be a potential therapeutic approach against COVID-19.

### INTRODUCTION

Acute respiratory distress syndrome (ARDS), due to SARS-CoV-2 infection, occurs as a result of dysregulation of the host response, followed by alveolar cells damaging and fibrosis of the lung (Cuadrado *et al.*, 2020). The renin-angiotensin system (RAS) and angiotensin-converting enzyme 2 (ACE2) have been found to play a critical role in ARDS caused by SARS infection as it has been confirmed that ACE2 is the cell receptor for SARS-CoV-2 (Ni *et al.*, 2020, Li *et al.*, 2003).

Simply, the binding domain of the receptor of the spike glycoprotein binds to the tip of ACE2 subdomain I (Wrapp *et al.*, 2020, Song *et al.*, 2018, Li *et al.*, 2005). Fusion of the membrane of the virus and the host cell is enhanced after binding, and subsequently, viral RNA is released into the cytoplasm, causing infection.

Cytokine storm, an exacerbation release of pro-inflammatory cytokines, represents the COVID-19 most aggressive presentation (Singh *et al.*, 2021). It was found that inflammation is increased by oxidative stress (Kudo *et al.*, 2012, Wu *et al.*, 2021), and the body's antioxidant defense mechanism aids in opposing the symptoms caused by the cytokine storm in COVID-19. So, in such conditions, it is preferable to boost the release of antioxidants (Singh *et al.*, 2021).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a main regulatory transcription factor that exerts an important role in regulating the activity of the antioxidant system with anti-inflammatory activity. At normal conditions, Nrf2 is kept in the cytosol in an inactive form through its association with its inhibitor protein Keap1 (Kelch-like ECH-associated protein 1), which targets Nrf2 for degradation in the proteasome (Hayes and Dinkova-Kostova, 2014). On the other hand, in response to oxidative stress, inactivation of Keap1 occurs, leading to translocation of Nrf2 into the nucleus where it binds to the antioxidant response element (ARE) causing antioxidant genes upregulation. Generally, the genes which are controlled by Nrf2 protect against cell death induced by stress, and thus it has been suggested that Nrf2 is the main regulator of infection-induced tissue damage (Soares and Ribeiro, 2015). Nrf2 is considered an important inflammatory response regulator (Thimmulappa *et al.*, 2006, Thimmulappa *et al.*, 2006), where it functions as a repressor of transcription of the

inflammatory genes in murine macrophages, mostly interleukin (IL-) 1 $\beta$  (Kobayashi *et al.*, 2016).

Novel reports demonstrated that SARS-CoV-2 inhibits Nrf2, confirming that the virus causes deprivation of the host cells of an important cytoprotective pathway, so it is important to know the underlying mechanism and when and how this occurs during the viral infection process. (Olagnier *et al.*, 2020). Lately, it has been confirmed that microRNAs (miRNAs), the short non-coding and highly conserved RNAs with about 18-25 nucleotides length, are implicated in the indirect or direct epigenetic Nrf2 signaling regulation (Padmavathi and Ramkumar, 2021). The severity and the therapeutic outcomes of various immune-related diseases have been linked to miRNAs dysregulation (Mingqiang *et al.*, 2019). Strikingly, novel studies have demonstrated that miRNAs are implicated in viral infection regulation and host defense (Li *et al.*, 2020, Bandopadhyay and Bharadwaj, 2020).

The link between miRNAs and COVID-19 remains to be investigated. The differential expression of miRNAs detected in COVID-19 patients may play a role in the regulation of the viral replication and immune responses during viral infection. A novel study elucidated that there is upregulation of a total of 35 miRNAs and downregulation of 38 miRNAs in the peripheral blood of COVID-19 patients. The miRNA-142-5p was one of the top genes found to be upregulated (Li *et al.*, 2020). Interestingly, Nrf2 was identified as miR-142-5p target gene, with a negative correlation detected between the expression of miR-142-5p and Nrf2 and its signaling pathway in non-alcoholic fatty liver disease (Teimouri *et al.*, 2020). Accordingly, in the present study, we aimed to investigate the role of miR-142-5p in modulating the inflammatory response in COVID-19

patients through the regulation of Nrf2/Keap1 pathway.

**SUBJECTS AND METHODS**

This study was carried out in compliance with the Declaration of Helsinki, and approved by Cairo University's local ethical committee. Written informed consent from all the participants was obtained.

A total of 35 COVID-19 patients (21 Males and 14 females; with ages ranging from 22 to 85 years), and 15 healthy individuals were included in this study. All patients were enrolled from the Internal medicine hospital, Faculty of Medicine, Cairo University during the period between January 2021 to September 2021. The COVID-19 patients were diagnosed using RT-qPCR Detection Kit (Taq Path™ COVID-19 CE-IVD RT-PCR; Thermo Fisher) and underwent a comprehensive history taking, in addition to, thorough clinical examination and routine laboratory investigations. The patients were classified according to the severity of the disease into 15 critically ill, intensive care unit (ICU) patients and 20 moderately ill patients on general medical wards.

Peripheral blood (10 mL) was drawn into EDTA anticoagulated tubes (BD Vacutainer) from COVID-19 patients at the time of diagnosis and from healthy

volunteers and kept at 4 °C until further processing (within two hours of collection). Plasma samples were subjected to a two-step centrifugation protocol (2500 ×g and 16,000 ×g; 10–10 min, 4 °C) to obtain plasma. After separation, the cell-free plasma samples were homogenized, aliquoted, and stored at –80 °C until further analysis.

**Estimation of Gene Expression of miRNA-142-5P, ACE2, NF-kβ, Nrf2, Keap-1, and HO-1 by QRT-PCR:**

Total RNA extraction was performed from plasma samples using GeneJET RNA Purification Kit (Thermo Fisher Scientific, Inc.) following the instruction. Checking of RNA quality was done using NanoDrop® 1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). Then, synthesis of complementary DNA (cDNA) was done using the High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific) according to the instructions. Subsequently, real time-qPCR was performed for amplification of the genes of interest [*miRNA-142-5P*, *ACE2*, *nuclear factor-kappa beta (NF-kβ)*, *Nrf2*, *Keap-1*, and *heme oxygenase-1 (HO-1)*], using SYBR Premix Ex Taq™ II (Perfect Real Time, TaKaRa, Japan) using the following primers shown in Table (1).

**Table 1:** Primer sequence of the studied genes:

Parameter	Primer sequence	Gene bank accession number
<b>miRNA-142-5P</b>	F: 5'-CCGGTCA TAAAGTAGAAAGC- 3' R: 5'-GTGCAGGGTCCGAGGT- 3'	<a href="#">MIMAT0000433</a>
<b>ACE2</b>	F: 5' -TCCATTGGTCTTCTGTCACCCG- 3' R: 5' -AGACCATCCACCTCCACTTCTC- 3'	<a href="#">NM_021804.2</a>
<b>NF-kβ</b>	F: 5' -GCAGCACTACTTCTTGACCACC- 3' R: 5' -TCTGCTCCTGAGCATTGACGTC- 3'	<a href="#">NM_001165412.1</a>
<b>Nrf2</b>	F: 5' -GAACTTGATGCCGTTTCAGCC- 3' R: 5' -GTCTCCACAAGGAAAGTGAATC- 3'	<a href="#">XM_011419592.1</a>
<b>Keap-1</b>	F: 5' –GAGTCCAAGAAGTGCTCTAAG- 3' R: 5' -GTCAGGATCATAGCACTCAA- 3'	<a href="#">XM_011433564.1</a>
<b>HO-1</b>	F: 5' –AGGCTGAGAATGCCGAGTTC- 3' R: 5' –TGTGGTACAAGGACGCCATC- 3'	<a href="#">NM_001004027.1</a>

PCR reaction conditions were as follows: 95°C for 5 min, then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The  $2^{-\Delta\Delta Ct}$  method was applied to quantitatively analyze the results with normalization to U6 snRNA for miRNA-142-5p and GAPDH for the rest of the genes as internal controls.

#### Statistical Analysis:

Descriptive statistics were held and numerical variables were presented as median + Interquartile range (IQR) in the condition when data were non-normally distributed or as mean  $\pm$  standard deviation (SD) in the case of normally distributed data. Shapiro-Wilk test was used for testing the normality of the numerical variables. A comparison was done

between the two independent groups utilizing the Wilcoxon rank-sum test (Mann-Whitney) or the independent samples t-test as appropriate. Comparison of categorical variables was done using the Chi-square test. P values  $<0.05$  were considered significant. The analysis was performed using STATA 15.1 software (Chan, 2003).

## RESULTS

### Comparisons of the Demographic Features in All Studied Groups:

As shown in Table (2), regarding age and sex, there is no statistically significant difference between the studied groups ( $p$  value  $\geq 0.05$ ), indicating that the studied groups are properly matched.

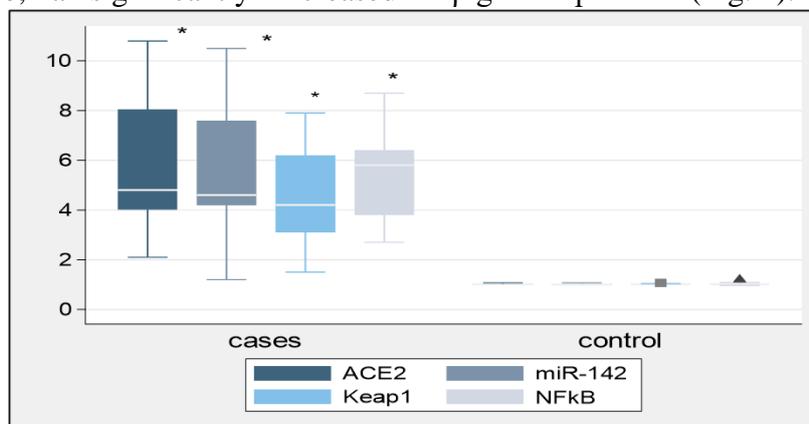
**Table 2:** Demographic data among the studied groups.

	Control (n=15)	COVID-19 patients		P-value
		Ward patients (n=20)	ICU patients (n=15)	
Age (years)	48.27 $\pm$ 17.51	54.9 $\pm$ 13.98	61.67 $\pm$ 14.95	0.05
Sex:				
Male	8 (53.33%)	11 (55%)	10 (66.67%)	0.7
Female	7 (46.67%)	9 (45%)	5 (33.33%)	

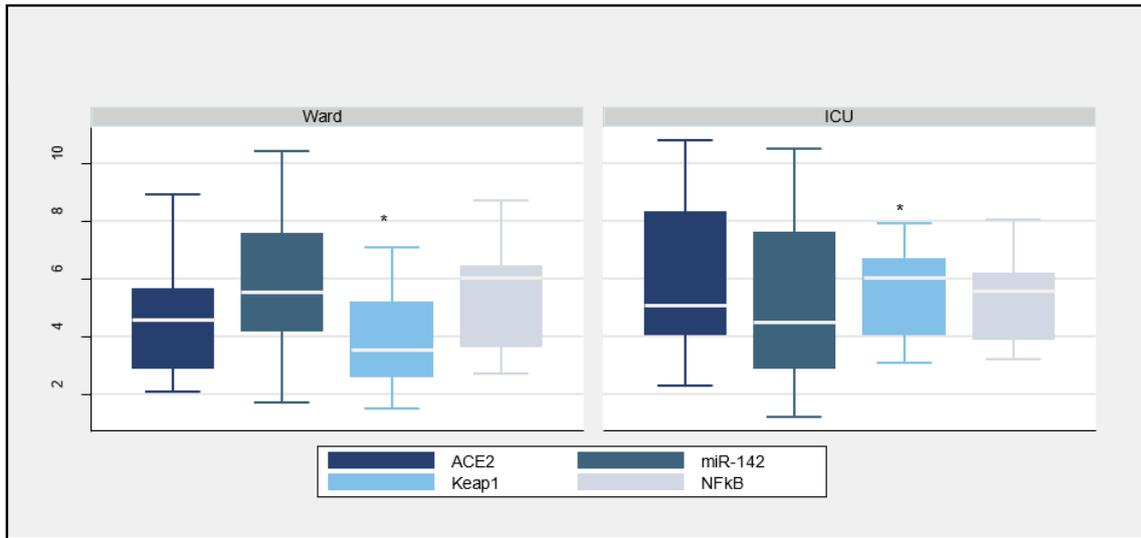
Data are expressed as mean  $\pm$  SD. P-value  $< 0.05$  is considered statistically significant.

As we show in Figure (1), there is a highly significant increase in miRNA-142-5p, Keap1, ACE2 and NF- $\kappa$ B gene expression levels in COVID-19 patients as compared to the control subjects ( $p < 0.001$ ). Also, a significantly increased

expression of Keap1 gene ( $p = 0.01$ ) is seen in the ICU patients compared to the ward patients, but no significant difference is seen between the two patient subgroups regarding miRNA-142-5p, ACE2, and NF- $\kappa$ B gene expression (Fig. 2).



**Fig. 1:** Median (IQR) of the fold change of ACE2, miRNA-142-5p, Keap1 and NF- $\kappa$ B gene expression among the studied groups. \* Denotes a highly significant difference versus the control group ( $p$  value  $< 0.001$ ).

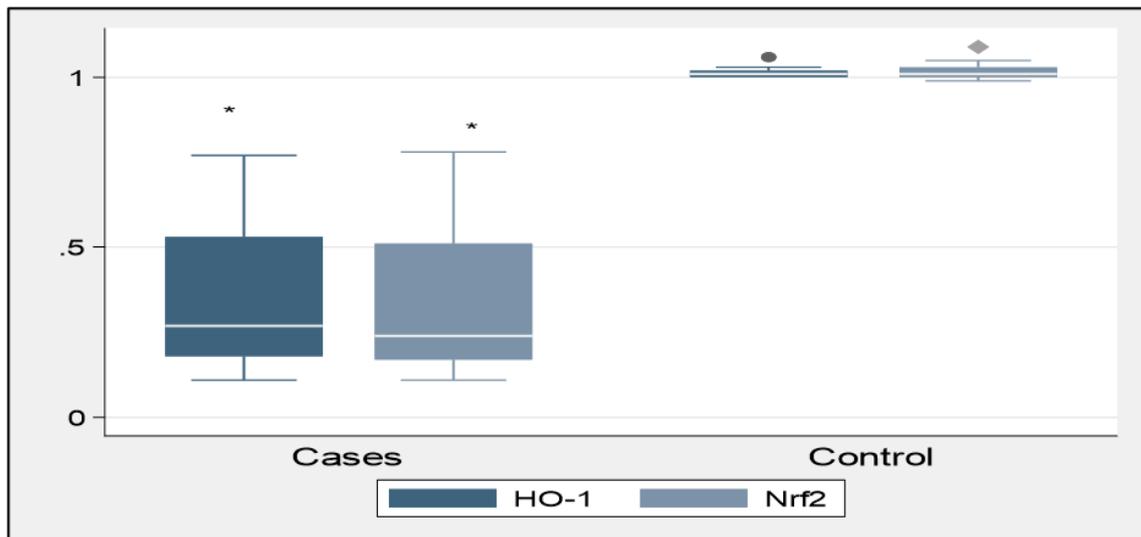


**Fig. 2:** Comparison of the median (IQR) of the fold change of ACE2, miRNA-142-5p, Keap1 and NF-kβ gene expression between the ward and ICU patients. \* Denotes significant difference versus the ward group (p value < 0.05).

**Effect of up-regulated miRNA-142-5p on Nrf2/ HO-1 pathway:**

There is a highly significant decrease in the gene expression levels of Nrf2 and HO-1 in COVID-19 patients as

compared to the control subjects (p < 0.001), with no significant difference between the ward and ICU residents (Fig. 3).

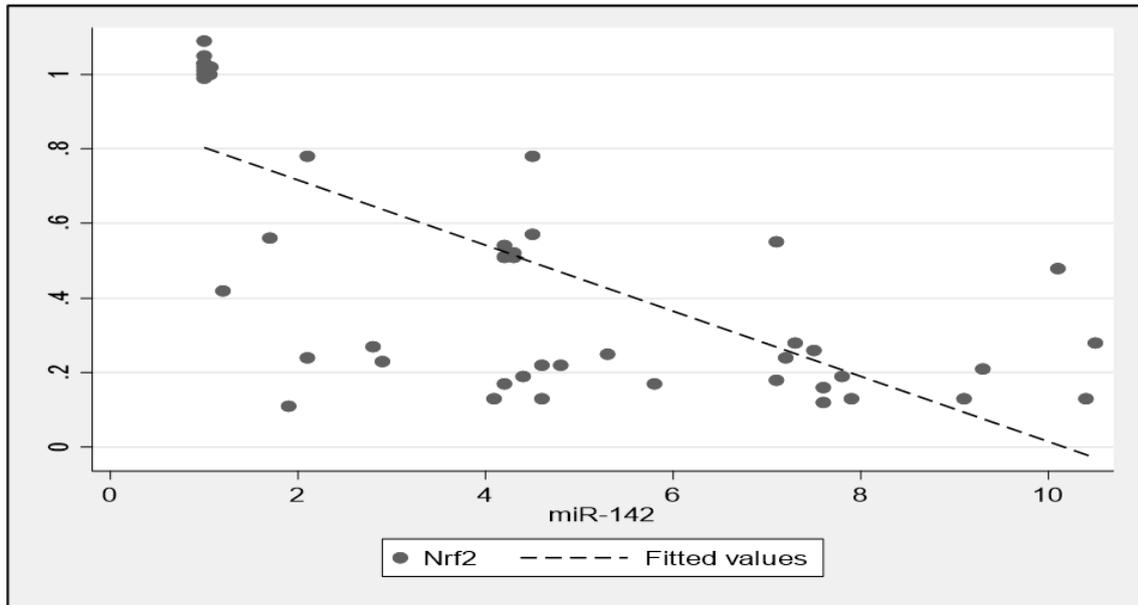


**Fig. 3:** Median (IQR) of the fold change of Nrf2 and HO-1 gene expression among the studied groups. \* Denotes a highly significant difference versus the control group (p value < 0.001).

**Correlation between the miRNA-142-5p and Nrf2:**

Figure (4) shows that there is a highly significant negative correlation between the miRNA-

142-5p and Nrf2 expression levels with (r = -75) and (p < 0.001) in COVID-19 patients.



**Fig. 4:** Correlation between the miRNA-142-5p and Nrf2

## DISCUSSION

SARS-CoV2 is a new member of Coronaviridae family which mainly attacks the human respiratory system, resulting in COVID-19. Such virus was first detected in late 2019 in the capital of China's Hubei Province, Wuhan, and subsequently, it leads to this pandemic (Zou *et al.*, 2020). The severity of the disease ranges from mild to severe, and even causes death in many cases (Karimi *et al.*, 2021). COVID-19 is a highly contagious and transmittable disease that represents the most horrible challenge to public health and medicine (Yang *et al.*, 2020).

Lately, microRNAs (miRNAs) have been detected to be one of the promising strategies for COVID-19 (Ying *et al.*, 2021). MiRNAs have the capability to downregulate the replication of the virus, modulate the progress of the viral infection, and increase the rates of survival in patients with severe COVID-19. miRNAs are involved in many pathways, decreasing the biomarkers of inflammation, formation of thrombi, and damage of the tissue to accelerate the outcome of the patient (Schultz *et al.*, 2021). miRNA-142-5p is one of the

miRNAs that was detected to be upregulated in COVID-19 patients compared with the normal control subjects (Li *et al.*, 2020).

In our study, we aimed to investigate the role of miR-142-5p in modulating the inflammatory response in COVID-19 patients through the regulation of Nrf2/Keap1 pathway. Our results suggest that miR-142-5p may be a promising therapeutic target to treat COVID-19 patients as evidenced by the different biochemical markers assessed in this study. We found that there is a highly significant increase in miRNA-142-5p gene expression levels in COVID-19 patients as compared to the control subjects ( $p < 0.001$ ), but no significant difference is seen between the ward and ICU subgroups.

In agreement with us, Li and his colleagues revealed dysregulation in the expression of miRNAs in the peripheral blood of patients with COVID-19. High-throughput sequencing analysis of miRNAs in the peripheral blood of 10 patients with COVID-19 and 4 control subjects revealed 35 miRNAs that were upregulated (including miRNA-142-5p) and 38 miRNAs which were

downregulated, where miR-183-5p and miR-16-2-3p are most astonishing in the downregulated and upregulated list, respectively (Li *et al.*, 2020).

On the contrary to our results, another study performed transcriptome sequencing of both whole blood mRNAs and noncoding RNAs for six moderate COVID-19 patients, six severe COVID-19 patients and four healthy donors, in which some miRNAs including miR-142-3p were found to be downregulated (Tang *et al.*, 2020).

It is well known that Nrf2/Keap1 pathway is protective against inflammation and oxidative stress (Lee *et al.*, 2021). Concerning the Nrf2/Keap-1/HO-1, our results showed that there is a highly significant decrease in the gene expression levels of Nrf2 and HO-1 in Covid-19 patients as compared to the control subjects. But contrary to expected, no significant difference is seen between the two patient subgroups for an unexplained reason. However, there is a highly significant increase in the Keap-1 gene expression levels in Covid-19 patients compared to the control group ( $p < 0.001$ ). In addition, a significantly increased expression is seen in the ICU patients when compared to the ward patients.

In agreement with us, a study demonstrated that there is suppression of the Nrf2 antioxidant gene expression pathway in biopsies obtained from COVID-19 patients. Moreover, it was found that dimethyl fumarate (DMF) which is clinically approved and Nrf2 agonists 4-octyl-itaconate (4-OI) cause inhibition of replication of SARS-CoV2 across cell lines through induction of a cellular antiviral program. Data suggested that the Nrf2 pathway causes restriction of replication of SARS-CoV2 as it was found that the Nrf2 antioxidant pathway is targeted by SARS-CoV2. Also, the same study, which demonstrated that the Nrf2-pathway is repressed during SARS-CoV2 infection, was encouraged by in vitro experiments where it was found that the

expression of NADPH quinone oxydoreductase 1 (NqO1) and HO-1, which are Nrf2-inducible proteins, was repressed in Vero hTMPRSS2 cells infected with SARS-CoV2 (Olagnier *et al.*, 2020). This goes with our results concerning HO-1 gene expression that is highly significantly decreased in Covid-19 patients as compared to the control subjects.

It has been very well established that Keap1 functions as a Nrf2 repressor; however, Keap1 repression has been beyond the research up till now. In accordance with our results, the presence of unrestrained Keap1 has been recognized to be damaging for cellular homeostasis. Also, recent studies focused on the role of Nrf2 as a Keap1 repressor which confirms that Keap1 and Nrf2 are mutually inhibited by each other (Kopacz *et al.*, 2020). Concerning Nrf2, it serves as a transcriptional repressor protein that restrains Keap1. Abrogation of angiogenic response is detected in endothelial cells (ECs) deficient in Nrf2. Nrf2 deficiency which is available for Keap1 leads to unrestrained Keap1, which causes stabilization of RhoGAP1 (Rho GTPase-activating protein 1), the protein that regulates the activity of the cell division cycle 42 (Cdc42), and causes potentiation of protein S-nitrosation, leading to inhibition of angiogenesis and provoking ECs premature senescence (Kopacz *et al.*, 2019, Kloska *et al.*, 2019).

Our results also elucidated a highly significant negative correlation between the miRNA-142-5p and the Nrf2 gene expression in COVID-19 patients. These findings support the previous evidences suggesting that miRNAs can directly affect the expression of Nrf2. Narasimhan and his colleagues have demonstrated that miR-142-5p forced expression downregulated the expression of Nrf2 through direct binding to the 3' UTR in SH-SY5Y neuronal cells. MiR-142-5p ectopic expression also decreased nuclear localization and transactivation of

Nrf2, independent of Keap1 (Narasimhan *et al.*, 2012). Another study aimed to investigate the association of four selected miRNAs (miR-142-5p, miR-27a, miR-128 and miR-153) with Nrf2 expression and the lipid accumulation level in vivo and in vitro models of non-alcoholic fatty liver disease (NAFLD). Such a study demonstrated that miR-142-5p and miR-27a are elevated in NAFLD, where they cause suppression of Nrf2 expression and accumulation of lipids in the hepatocytes. That study detected that miR-142-5p and miR-27a regulate the expression of Nrf2 at the post-transcriptional level and play a vital role in NAFLD. According to the data obtained from both in vivo and in vitro models of NAFLD, the increase in the expression of miR-142-5p and miR-27a, may affect the stability of Nrf2 mRNA and result in a decrease in Nrf2 levels and the levels of the antioxidant genes regulated by Nrf2, including NQO1, HO1, and Mn-SOD. It has been provided that the suppression of such miRNAs may have useful effects in ameliorating oxidative stress and lipogenesis in NAFLD through upregulation of the Nrf2 signaling pathways (Teimouri *et al.*, 2020).

Regarding the expression levels of ACE2 and NF- $\kappa$ B, our results demonstrate a highly significant upregulation in the COVID-19 cases as compared to the control ( $p < 0.001$ ), with more increased levels of ACE2 in the ICU than the ward patients, but the values are non-significant ( $p = 0.2$ ). Our data support the results of previous research which established that in COVID-19 patients, the deficiency of Nrf2 has been shown to increase the levels of ACE2, whereas the activators of Nrf2 have been shown to decrease ACE2 levels, thus suggesting that Nrf2 activation may cause a decrease in ACE2 levels, thus reducing the total docking sites for entry of the virus (Zhao *et al.*, 2018).

On the contrary, Khan and his colleagues demonstrated that there is downregulation in ACE2 protein levels in COVID-19 and SARS-CoV which causes

injury of the lungs, considering the protective role of ACE2 against tissue injury (Khan *et al.*, 2021). They found that downregulation of ACE2 enhances the phosphorylation of NF- $\kappa$ B and thus activating it. As the result of NF- $\kappa$ B activation, large quantities of many other pro-inflammatory cytokines are secreted, mainly IL-8 and IL-6 which in turn causes migration of many other immune cells and cytokines at the infection site (Dosch *et al.*, 2009). The generation of free radicals represents the main mechanism causing inflammation and tissue injury upon entry of the virus (Paracha *et al.*, 2013). Also, oxidative stress is enhanced to facilitate viral replication (Salihefendic *et al.*, 2015). This controversy could be explained by the differential expression of both the soluble circulating and the cellular ACE2 in the different stages of viral infection and the host response against entry of the virus. These explanations should be further analyzed and verified through future research on a greater number of cases.

In conclusion, the host's inflammatory response in COVID-19 patients is inflicted by the increased number of free radicals, oxidative stress leading to cytokine storm. Nrf2 activators could be a torch of hope because they have multiple facets to their mode of action. miRNA-142-5p could affect the severity of cytokine storm through modulation of Nrf2/ Keap1 axis. Targeting miRNA-142-5p could be a potential therapeutic strategy against COVID-19.

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### ARABIC SUMMARY

هل يمكن أن يكون استهداف miRNA-142-5p استراتيجية علاجية ضد COVID-19 من خلال تنشيط مسار Nrf2 / Keap1 المضاد للالتهابات؟

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مرض فيروس كورونا 19 (COVID-19) هو عدوى فيروسية شديدة الأمراض وسريعة الانتشار يحدث نتيجة الإصابة بفيروس كورونا 2 (SARS-CoV-2) المسبب لمتلازمة الأعراض التنفسية الحادة الوخيمة. عدوى كوفيد-19 لها مدى واسع من الشدة تتراوح من شكل بدون أعراض إلى شكل حاد مع ضائقة تنفسية حادة قد تؤدي إلى الوفاة. Nrf2 هو عامل النسخ الرئيسي الذي يتحكم في نظام الدفاع المضاد للأكسدة الذي يلعب دورًا مهمًا في تقليل الأعراض التي تسببها عاصفة السيتوكين في كوفيد-19. ولقد وجد أن الأحماض النووية الريبوزية متناهية الصغر (miRNAs) متورطة في تنظيم الالتهابات الفيروسية المختلفة، ومع ذلك لا يزال الارتباط بين miRNAs وكوفيد-19 قيد التحقيق. وكنا نهدف في هذه الدراسة إلى التحقيق في دور miR-142-5p في تعديل الاستجابة الالتهابية لمرضى كوفيد-19 من خلال تنظيم مسار Nrf2 / Keap1.

**الطريقة:** أجريت الدراسة على 50 شخصًا تم تقسيمهم إلى مجموعتين: 15 فردًا صحيًا و35 مريضًا بفيروس كوفيد-19 وتم سحب الدم (10 مل) من جميع الأشخاص لتقدير التعبير الجيني لـ miRNA-142-5P و ACE2 و NF-kβ و Nrf2 و Keap-1 و HO-1 بواسطة QRT-PCR.

**النتائج:** أظهرت النتائج زيادة كبيرة في مستويات التعبير الجيني miRNA-142-5p و Keap1 و ACE2 و NF-kβ وانخفاضًا كبيرًا في مستويات التعبير الجيني لـ Nrf2 و HO-1 في مرضى كوفيد-19 مقارنةً بمجموعة التحكم.

كما تم الكشف عن ارتباط سلبي كبير بين مستويات التعبير miRNA-142-5p و Nrf2. **الخلاصة:** تشير هذه البيانات إلى أن miRNA-142-5p يمكن أن تؤثر على شدة عاصفة السيتوكينات من خلال تأثيرها على محور Nrf2 / Keap1 ، ويمكن أن يكون استهداف miRNA-142-5p نهجًا علاجيًا محتملاً ضد كوفيد-19.