Clin. Lab. 2022;68:XXX-XXX


## ORIGINAL ARTICLE

# Evaluation of *MX1* Gene Promoter Methylation in Different Severities of COVID-19 Considering Patient Gender

Zohreh-al-sadat Ghoreshi [1, 2], Mojtaba Abbasi-Jorjandi [2, 3], Gholamreza Asadikaram [2, 4], Mohsen Sharifzak [2, 4], Mitra Rezazadeh-Jabalbarzi [5], Hamidreza Rashidinejad [6]

[1] *Research Center of Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran*
[2] *Department of Clinical Biochemistry, Afzalipour Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran*
[3] *Endocrinology and Metabolism Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran*
[4] *Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran*
[5] *Clinical Research Development Center of Imam Khomeini Hospital, Jiroft University of Medical Sciences, Jiroft, Iran*
[6] *Department of Cardiology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran*

## SUMMARY

***Background:*** **Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), led to a pandemic in March 2020. During a viral infection, it has been reported that epigenetic changes occur for both sides: Infected cells elicit an antiviral environmental response, which induces and initiates certain pathways for proper response to the virus, while the virus silences the expression of vital genes in the antiviral host cell. In this study, we aimed to examine the methylation level of the *MX1* gene promoter in different stages in COVID-19 patients compared to the control group.**

***Methods:*** **In total, 470 COVID-19 patients with a positive polymerase chain reaction (PCR) test (235 women and 235 men) were recruited into the study as the test group. Patients were divided based on the World Health Organization (WHO) classification into three groups: moderate, severe, and critical. Moreover, 100 healthy individuals (50 women and 50 men) were selected as the control group. Peripheral white blood cells were collected and PCR was performed using two types of primers designed for methylated and unmethylated states of the *MX1* gene. The PCR products were then loaded on agarose gel and the band intensities were calculated by ImageJ software.**

***Results*****: The results showed a decrease in the methylation of the *MX1* gene promoter in moderate and severe groups and an increase in the *MX1* gene promoter methylation in the critical group. In addition, the level of methylation was higher in men than in women.**

***Conclusions:*** **Increased methylation of the *MX1* gene in the critical group may indicate the role of SARS-CoV-2 in reducing the expression levels of this antiviral gene and thus promoting virus replication and disease progression.**
**(Clin. Lab. 2022;68:xx-xx. DOI: 10.7754/Clin.Lab.2022.220104)**

**Correspondence:**
Gholamreza Asadikaram
Neuroscience Research Center
Institute of Neuropharmacology and
Department of Clinical Biochemistry
Afzalipour Faculty of Medicine
Kerman University of Medical Sciences
Kerman
Iran
Email: Gh_asadi@kmu.ac.ir
Orcid: https://orcid.org/ 0000-0002-9100-0756u

Manuscript accepted February 8, 2022

**KEYWORDS**

MX1, COVID-19, SARS-CoV-2, epigenetic change

## INTRODUCTION

The coronavirus (CoV) family includes positive-sense viruses and coated RNAs that have been linked to many intestinal and respiratory diseases in animals and humans [1]. Three major CoV strains have caused human pandemics: Middle East respiratory syndrome-related

coronavirus (MERS-CoV) from 2002 to 2003, severe acute respiratory syndrome coronavirus 1 (SARS-CoV) in 2012, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2020 [2]. The last strain was identified in Wuhan, China, in late 2019, and is the cause of coronavirus disease 2019 (COVID-19), which has led to a global outbreak [3]. Viral infection, in contrast to intracellular pathogens, requires continuous adherence and the utilization of cell transcription and metabolism machines to ensure virus survival and proliferation [4]. For this purpose, host genome expression must be used, and its success depends on chromatin dynamics and transcriptional regulation, which are primarily driven by epigenetic mechanisms such as DNA methylation, post-translational histone modification (HPTM), and transcription factors (TFs) [5]. During a viral infection, epigenetic and transcriptional changes are reported to occur on both sides: The infected cell elicits an antiviral environmental response, which leads to the induction of certain pathways for survival and defeating the virus [6], while the virus silences the expression of the host's vital antiviral genes [7]. Few experimental studies have been conducted to unravel the epigenetic changes and markers involved in MERS-CoV and SARS-CoV infections and their pathogenesis.

A typical transcriptional repression epigenetic modification, i.e., DNA methylation, occurs when the methyl group binds to adenine or cytosine in a nucleotide with the help of DNA methyltransferases (DNMTs). Because cytosine is widely methylated at the C5 position in mammalian cells, the term C5-methylcytosine (5-mC) was introduced [8]. This type of methylated cytosine is commonly found in CpG pairs, which are specific sites in the DNA where the cytosine nucleotide is immediately followed by a guanosine nucleotide in the 3´-5´ direction [9]. Cytosine is methylated in approximately 80 - 90% of CpGs. The remaining 10 - 20% of unmethylated CpGs are usually found in high concentrations in certain areas of the DNA called "CpG islands", which are often found in gene promoters. CpG methylation is inversely related to gene expression. High levels of methylation in promoter-associated CpGs lead to low levels of gene expression [10].

Although many studies have examined virus cell receptors, little research has been conducted on effective antiviral proteins. It is well accepted that interferon type I (IFN) plays a pivotal role in preventing viral infection by inducing IFN-stimulated genes (ISGs), which act synergistically to inhibit virus replication through multiple mechanisms [11,12]. Human myxovirus (MX) resistance genes encode GTPases that are part of the antiviral response induced by type I/III IFNs [13]. Humans have two different MX proteins, MX1 and MX2, which differ significantly in viral properties and mechanisms of action. MX1 has been shown to have significant antiviral activity against RNA and DNA viruses, while MX2 activity is limited to specific viruses such as HIV [11]. MX1 has a direct effect on the viral ribonucleoprotein complex and its GTPase activity is required for its antiviral function [11,14].

Type I IFNs mediate several mechanisms that enhance host defense, including the induction of ISGs [15]. ISGs create an antiviral state in the host by restricting virus replication and disrupting the virus genome [16]. MX proteins are highly conserved in most vertebrates. The amino acid sequence of the human MX1 exhibits about 67% similarity to mouse MX1 [17]. Because MX1 is an important regulator of intracellular viral replication, its deficiency increases the susceptibility to influenza infection in mice [18]. Intracellular localization affects the antiviral activity of MX proteins. While rodent MX1 is located in the nucleus and limits the replication of the nuclear virus, human MX1 is located in the cytoplasm and inhibits virus replication by preventing the viral RNA from entering the nucleus [18]. COVID-19 patients present a distinct expression pattern for antiviral genes. The expression of the antiviral gene *MX1* is triggered by SARS-CoV-2. It is worth mentioning that MX1 can be induced by hemin, an FDA-approved drug, and therefore it can be considered a potential druggable target [11].

In this study, we sought to investigate the methylation rate of the *MX1* gene promoter at different intensities of COVID-19 in comparison with the control group and to compare the difference in methylation levels between women and men.

## MATERIALS AND METHODS

### Subjects

This is a case-control study in which patients and healthy individuals consented to enter the study.

### Sample and data collection

The participants were selected from the infectious ward of Jiroft Hospital in Kerman Province (Iran). A total of 470 patients, including 235 men and 235 women, aged between 20 and 80 years were chosen after obtaining ethical approval. COVID-19 was diagnosed via a positive SARS-CoV-2 reverse transcription-polymerase chain reaction (RT-PCR) test on nasopharyngeal and throat swabs. Moreover, 100 healthy individuals, including 50 men and 50 women, were used as the control group.

Patients under 20 years old or over 80 years old and pregnant women were excluded from the study.

The selected patients were divided into three groups according to the World Health Organization (WHO) criteria [19]: moderate, severe, and critical groups (Table 1). All patients were treated according to the hospital protocol, and 3 mL of extra blood was collected from each patient in a complete blood count (CBC) tube for the study. Whole blood was separated by centrifugation, and small aliquots of leukocytes were stored at −80°C until the measurements.

**Methylation-specific PCR (MSP)**

Using the Favorgen Kit (Cat No. FABGK 001-1), DNAs were extracted and kept at -80°C until further assessment. The MSP method is based on the reaction of DNA with sodium bisulfite, which results in the conversion of cytosine to uracil and subsequently to thymidine in the PCR steps. Methylated cytosine, on the other hand, remains unchanged. The extracted DNAs were treated with sodium bisulfite according to a method proposed by Tiwari et al. [20].

Using the University of California, Santa Cruz (UCSC) website, we found the CpG Islands of the MX1 gene promoter, and then with the help of the MethPrimer databases, we obtained the appropriate primers. The modified DNA sequence was amplified by PCR with specific primers for methylated and unmethylated DNA (Table 2). Methylated primers detect unchanged cytosines, and unmethylated primers identify thymines derived from modified cytosines in the MX1 promoter region. After PCR, the products were loaded on 1.6% agarose gel, and electrophoresis was performed. The intensity of electrophoresis bands was determined using ImageJ software.

**Statistical analysis**

All statistical analyses were carried out using SPSS software version 21 (Chicago, IL, USA). Data were analyzed by independent-samples *t*-test and one-way analysis of variance (ANOVA) test followed by Tukey's post hoc test. p-values less than 0.05 were considered significant.

## RESULTS

**Interpretation of the results for moderate, severe, and critical groups using ImageJ software based on the corresponding band intensities in agarose gel**

**Intensity of MX1 gene methylation in women with COVID-19 based on disease severity**

By comparing the level of *MX1* gene methylation between female participants in different groups, we found a decrease in the methylation level in the moderate and severe groups compared with the control group (n = 50). However, female patients in the critical group had higher methylation levels compared with women in the control group (Figure 2).

**Intensity of MX1 gene methylation in men with COVID-19 based on disease severity**

By comparing the level of *MX1* gene methylation between male participants in different groups, we found a reduction in the methylation levels in the moderate and severe groups compared with the control group (n = 50). However, male patients in the critical group had higher methylation levels compared with male participants in the control group (Figure 3).

**Methylation levels in women compared with men**

By comparing the level of methylation between women and men in different groups, we found a higher level of methylation in men in all groups (Figure 4).

**Methylation levels in all patients compared with the control group**

By comparing the percentage of methylation between all patients and the control group, we observed a decrease in the percentage of methylation in the patients compared to the control group ($p < 0.05$).

**Table 1. Patient classification.**

| | Moderate | Severe | Critical |
|---|---|---|---|
| **Male** | **106** | **96** | **33** |
| **Female** | **130** | **70** | **35** |
| **Overall** | **236** | **166** | **68** |

## DISCUSSION

COVID-19 is caused by the new coronavirus SARS-CoV-2, which has created a global pandemic and raised international concerns [21]. Many aspects of the human body's antiviral response, which is varied depending on disease severity, are still unclear.

Little is known about the epigenetic changes caused by SARS-CoV-2 infection in humans. In this study, we investigated the methylation level of the *MX1* gene promoter, which is one of the most crucial genes in the antiviral response.

The most prominent finding in the present study is an increase in the methylation rate of the *MX1* gene promoter in the critical stage in male and female patients (Figures 2 and 3).

DNA methylation is one of the most important epigenetic changes that affect gene expression [22]. DNA methylation occurs in CpG islands located in gene promoters. CpG methylation is inversely related to gene expression. High levels of methylation in promoter-associated CpGs lead to low levels of gene expression [23].

Various studies have examined the methylation levels of gene promoters involved in the antiviral response in virus-infected individuals. A study claimed that DNA methylation in MERS, rather than histone modification, plays an important role in the immune system's response to MERS-CoV [24].

According to a study on the *MX1* gene in patients with influenza, MX1 possibly interacts with the viral polymerase basic protein 2 (PB2) and nucleoprotein (NP) present in the incoming viral ribonucleoproteins (vRNPs). After this initial binding, MX1 actively disrupts the interaction between PB2 and NP in a GTPase-dependent manner, blocking the virus transcription and replication [25].

**Table 2. MSP primers.**

| MX1 Gene | Forward primer sequences (5´-3´) | Reverse primer sequences (5´-3´) | Product size (bp) |
|---|---|---|---|
| Methylated | GTTTCGGAGTACGGGTACGA | GTAAATCTAAAACTTTCCCGACG | 250 |
| Unmethylated | GTTTTGGAGTATGGGTATGA | CATAAATCTAAAACTTTCCCAACAC | 252 |

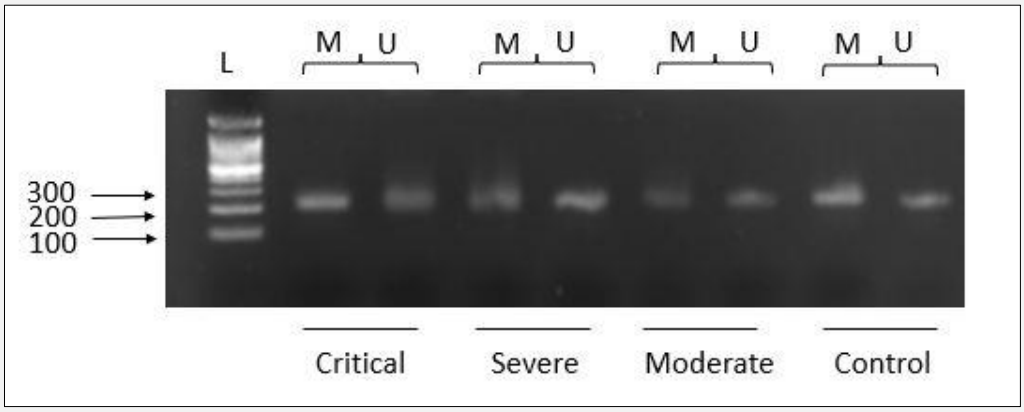



**Figure 1. The electrophoresis image of the methylation statuses of the MX1 gene.**
**M - Methylated band, U - Un-methylated band, L - Ladder. The methylated band is 250 bp and the unmethylated band is 252 bp.**

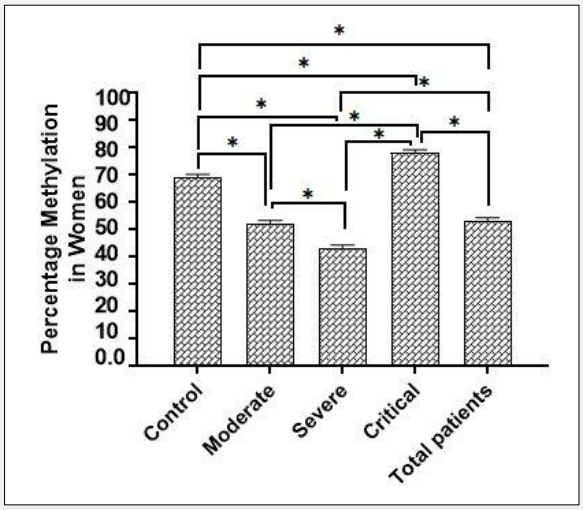



**Figure 2. The percentage of MX1 methylation in moderate, severe, and critical female patients as well as total female patients compared with healthy women in the control group. There are significant differences between the methylation states in each group (* $p < 0.05$).**

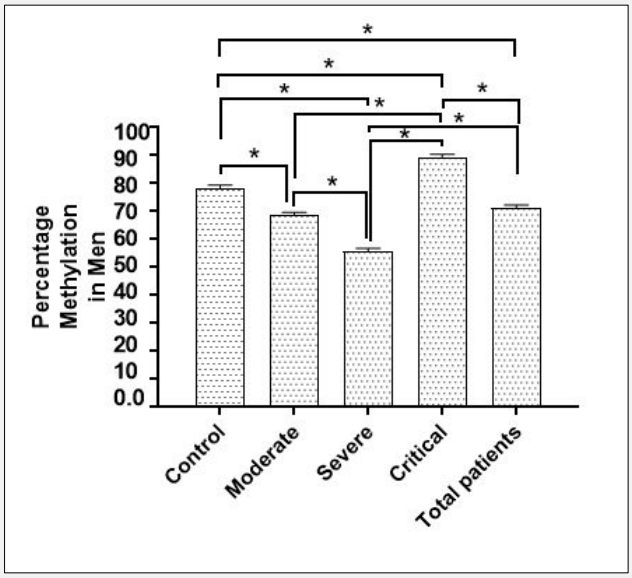



**Figure 3. The percentage of MX1 methylation in moderate, severe, and critical male patients as well as total male patients compared with healthy men in the control group. There are significant differences between the methylation levels in each group (* $p < 0.05$).**

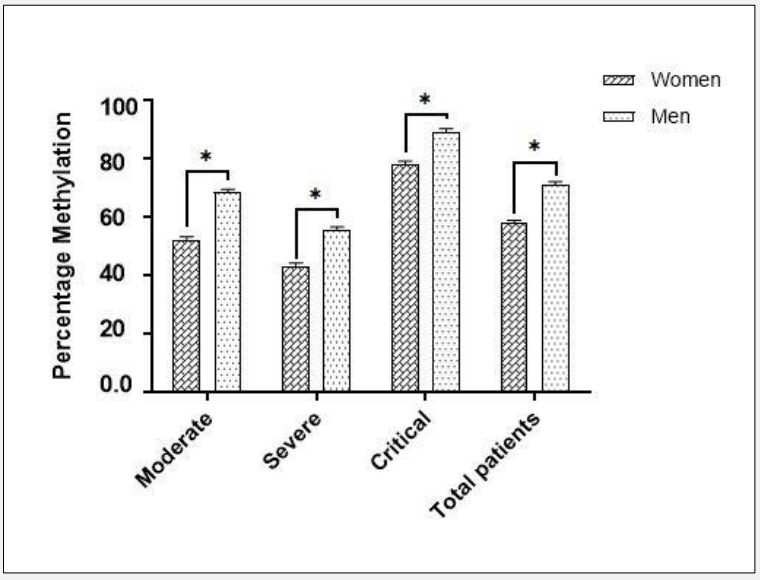



**Figure 4. By comparing the percentage of methylation between women and men in different groups, we observed a higher percentage of methylation in men in all groups (* $p < 0.05$).**

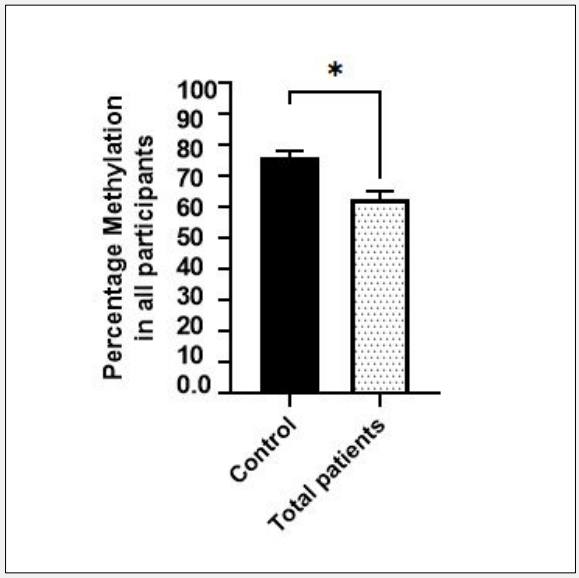
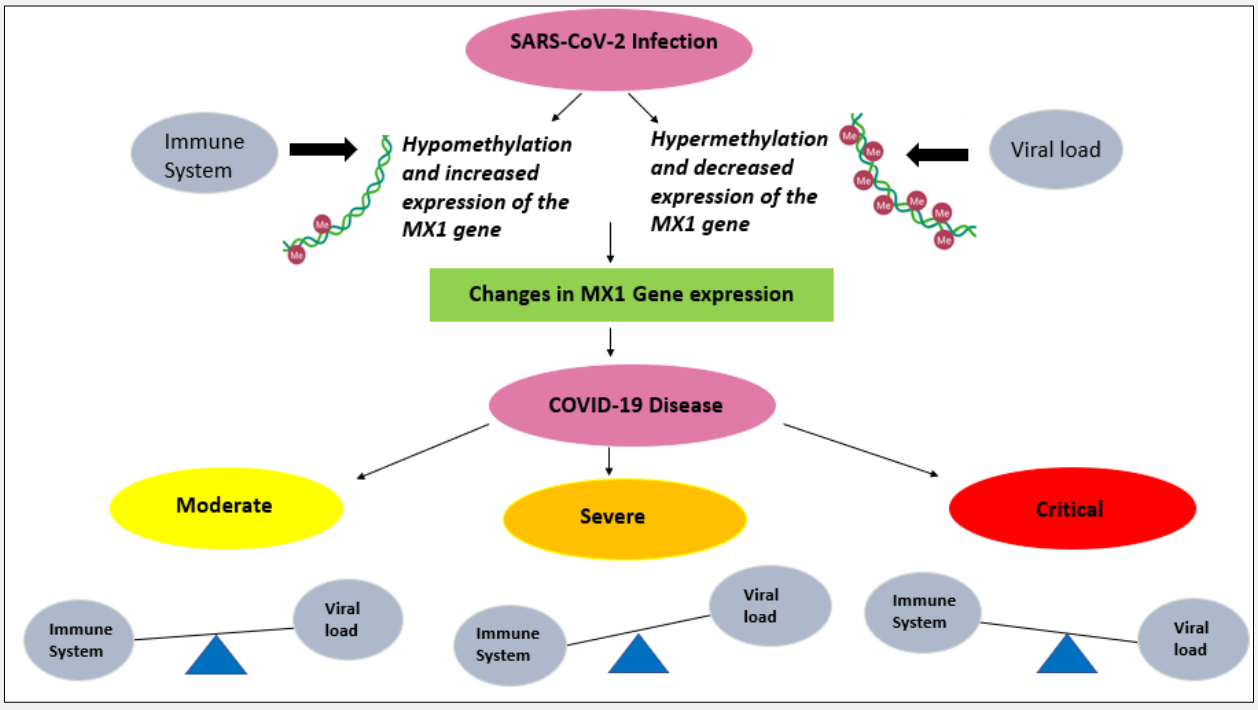



**Figure 5. The percentage of methylation in all patients compared with the control group (* p < 0.05).**




**Graphical abstract.**

A study by Marcos-Villar et al. on lung cells infected with the influenza virus indicated that the increased expression of the *MX1* gene was identified after 8 and 16 hours of infection with the virus [26]. On the other hand, a study conducted by Michael J. et al. on COVID-19 patients revealed that as the load of SARS-CoV-2 rises, the rate of the *MX1* gene methylation increases [27].

According to our results, in both moderate and severe stages of COVID-19, the methylation of the *MX1* gene promoter decreased compared to the control group (Figures 2 and 3), which could be due to the immune system attempting to raise the *MX1* gene expression in order to combat virus replication and survival.

However, in the critical group, our results showed an increase in the methylation of the *MX1* gene promoter compared to the control group (Figures 2 and 3), which could indicate the effect of the high viral load in this group on the methylation of this region and reducing its expression.

Our findings in the three groups of COVID-19 patients, including moderate, severe, and critical groups, could indicate a conflict between the immune system, which increases the *MX1* gene expression in order to combat virus replication and survival, and the virus, which increases the *MX1* promoter methylation, decreasing the expression of the *MX1* gene and finally leading to increased virus replication and survival.

It is well known that epigenetic processes in response to various infections and disease susceptibility are affected by gender differences. In women, the changes in the methylation level of the *MX1* gene promoter in the three patient groups were compared with the control group. It was found that the methylation levels in women in the moderate and severe groups were lower than the control group, whereas these levels were increased in the critical group compared to the control group (Figure 2). Therefore, it could be concluded that increased methylation along with an elevated viral load in the critical stage of COVID-19 can be effective in reducing the expression of the antiviral *MX1* gene and worsening the patient's condition.

In men, the methylation levels in moderate and severe groups declined compared to the control group, while these levels in the critical group were significantly higher compared to the control group (Figure 3). This finding suggests that the increased methylation of the *MX1* gene promoter in the critical group may be associated with the elevated viral load and the worsening of patients' clinical status at this stage.

Previous studies have reported differences in epigenetic changes between men and women for a variety of reasons including the effects of sex hormones on epigenetic changes [28] and the varied expression of DNMTs in men and women [29].

In this research, we compared the methylation rate of the *MX1* gene promoter between men and women in moderate, severe, and critical groups. Our findings revealed that the percentage of the methylation of this gene in women was lower than in men (Figure 4). Decreased methylation of the *MX1* gene promoter in women compared to men might explain why women respond better to SARS-COV-2 infection and have lower mortality rates.

Comparing the percentage of methylation in all the patients with the control group revealed a drop in the methylation percentage in the patients (Figure 5). This decrease is due to the fact that a large number of patients were in the moderate and severe groups (n = 402).

As mentioned in the introduction, MX1 can be induced by hemin, an FDA-approved drug, and therefore it can be considered a potential druggable target [11]. According to our results, the induction of *MX1* expression may be effective in boosting the immune system's response to SARS-CoV-2. Since this is the first study to examine the methylation level of *MX1* gene promoter in COVID-19 patients with different disease severities (moderate, severe, and critical), further studies are needed to elucidate various aspects of this phenomenon.

## CONCLUSION

The results of our study revealed a reduction in the methylation of the *MX1* gene promoter in moderate and severe stages of the disease, which indicates that the immune system is attempting to counteract the virus by enhancing the expression of the *MX1* gene. On the other hand, in the critical group, we found an increase in the promoter methylation of this gene compared to the control group, indicating that the high load of the virus in this group was successful in methylating the promoter of this gene and reducing its expression. The level of *MX1* gene promoter methylation in men was higher in all three groups (moderate, severe, and critical) when compared with women. These findings demonstrate the important role of epigenetic changes in managing the severity of COVID-19 and that the regulation of *MX1* gene expression level may be an important factor in controlling the severity of COVID-19.

**Acknowledgment:**
The authors acknowledge the patients' participation and specimen donation in this study.

**Source of Funds:**
This research was financially supported by Kerman University of Medical Sciences, Kerman, Iran. This research was part of a Ph.D. thesis (Ethics code: IR.KMU.REC.1400.412).

**Declaration of Interest:**
The authors declare no conflict of interest.

## References:


1. Rabi FA, Al Zoubi MS, Kasasbeh GA, Salameh DM, Al-Nasser AD. SARS-CoV-2 and coronavirus disease 2019: what we know so far. Pathogens 2020;9(3):231. (PMID: 3224508)

2. Lee PI, Hsueh PR. Emerging threats from zoonotic coronaviruses-from SARS and MERS to 2019-nCoV. J Microbiol Immunol Infect 2020;53(3):365-7. (PMID: 32035811)

3. Sohrabi C, Alsafi Z, O'neill N, et al. World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19). Int J Surg 2020;76:71-6. (PMID: 32112977)

4. Baggen J, Vanstreels E, Jansen S, Daelemans D. Cellular host factors for SARS-CoV-2 infection. Nat Microbiol 2021 Oct; 6(10):1219-32. (PMID: 34471255)

5. Muhammad JS, Saheb Sharif-Askari N, Cui Z-G, Hamad M, Halwani R. SARS-CoV-2 infection-induced promoter hypomethylation as an epigenetic modulator of heat shock protein A1L (HSPA1L) gene. Front Genet 2021;12:622271. (PMID: 33679887)

6. Marcos-Villar L, Díaz-Colunga J, Sandoval J, et al. Epigenetic control of influenza virus: role of H3K79 methylation in interferon-induced antiviral response. Sci Rep 2018;8(1):1230. (PMID: 29352168)

7. Khan MA, Islam ABMMK. SARS-CoV-2 Proteins Exploit Host's Genetic and Epigenetic Mediators for the Annexation of Key Host Signaling Pathways. Front Mol Biosci 2020;7:598583. (PMID: 33585554)

8. Jurkowska RZ, Jeltsch A. Mechanisms and biological roles of DNA methyltransferases and DNA methylation: from past achievements to future challenges. Adv Exp Med Biol 2016:1-17. (PMID: 27826832)

9. Corso-Díaz X, Jaeger C, Chaitankar V, Swaroop A. Epigenetic control of gene regulation during development and disease: A view from the retina. Prog Retin Eye Res 2018;65:1-27. (PMID: 29544768)

10. Shirvaliloo M. Epigenomics in COVID-19; the link between DNA methylation, histone modifications and SARS-CoV-2 infection. Epigenomics 2021 May;13(10):745-50. (PMID: 33876664)

11. Bizzotto J, Sanchis P, Abbate M, et al. SARS-CoV-2 infection boosts MX1 antiviral effector in COVID-19 patients. iScience 2020;23(10):101585. (PMID: 32989429)

12. Crosse KM, Monson EA, Beard MR, Helbig KJ. Interferon-stimulated genes as enhancers of antiviral innate immune signaling. J Innate Immun 2018;10(2):85-93. (PMID: 29186718)

13. Fuchs J, Hölzer M, Schilling M, et al. Evolution and antiviral specificities of interferon-induced Mx proteins of bats against Ebola, influenza, and other RNA viruses. J Virol 2017;91(15): e00361-17. (PMID: 28490593)

14. Verhelst J, Spitaels J, Nürnberger C, et al. Functional comparison of Mx1 from two different mouse species reveals the involvement of loop L4 in the antiviral activity against influenza A viruses. J Virol 2015;89(21):10879-90. (PMID: 26292322)

15. Wang W, Xu L, Su J, Peppelenbosch MP, Pan Q. Transcriptional regulation of antiviral interferon-stimulated genes. Trends Microbiol 2017;25(7):573-84. (PMID: 28139375)

16. Yang E, Li MM. All about the RNA: interferon-stimulated genes that interfere with viral RNA processes. Front Immunol 2020;11: 605024. (PMID: 33362792)

17. Das BK, Roy P, Rout AK, et al. Molecular cloning, GTP recognition mechanism and tissue-specific expression profiling of myxovirus resistance (Mx) protein in Labeo rohita (Hamilton) after Poly I: C induction. Sci Rep 2019;9(1) :3956. (PMID: 30850653)

18. Jung HE, Oh JE, Lee HK. Cell-penetrating Mx1 enhances antiviral resistance against mucosal influenza viral infection. Viruses 2019;11(2):109. (PMID: 30696001)

19. World Health Organization. COVID-19 clinical management: living guidance, 25 January 2021. World Health Organization; 2021. https://apps.who.int/iris/handle/10665/338882

20. Tiwari S, Manoj G, Prasanth K, et al. Simplified and versatile method for bisulfite-based DNA methylation analysis of small amounts of DNA. J Clin Lab Anal 2009;23(3):172-4. (PMID: 19455637)

21. Golahdooz M, Taherizadeh M, Laali A, et al. A review on Coronavirus 2019 Disease (COVID-19, SARS-CoV-2): Control and Prevention. RJMS. 2020;27(5):98-107. https://rjms.iums.ac.ir/browse.php?a_id=6150&sid=1&slc_lang=en&ftxt=0

22. Dhar GA, Saha S, Mitra P, Nag Chaudhuri R. DNA methylation and regulation of gene expression: Guardian of our health. Nucleus (Calcutta) 2021;64(3):259-70. (PMID: 34421129)

23. Anastasiadi D, Esteve-Codina A, Piferrer F. Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species. Epigenetics Chromatin 2018;11(1):37. (PMID: 29958539)

24. Menachery VD, Schäfer A, Burnum-Johnson KE, et al. MERS-CoV and H5N1 influenza virus antagonize antigen presentation by altering the epigenetic landscape. Proc Natl Acad Sci U S A 2018;115(5):E1012-E21. (PMID: 29339515)

25. Verhelst J, Parthoens E, Schepens B, Fiers W, Saelens X. Interferon-inducible protein Mx1 inhibits influenza virus by interfering with functional viral ribonucleoprotein complex assembly. J Virol 2012;86(24):13445-55. (PMID: 23015724)

26. Marcos-Villar L, Díaz-Colunga J, Sandoval J, et al. Epigenetic control of influenza virus: role of H3K79 methylation in interferon-induced antiviral response. Sci Rep 2018;8(1):1230. (PMID: 29352168)

27. Corley MJ, Pang AP, Dody K, et al. Genome-wide DNA methylation profiling of peripheral blood reveals an epigenetic signature associated with severe COVID-19. J Leukoc Biol 2021 Jul; 110(1):21-6. (PMID: 33464637)

28. Migliore L, Nicolì V, Stoccoro A, et al. Gender Specific Differences in Disease Susceptibility: The Role of Epigenetics. Biomedicines 2021;9(6):652. (PMID: 34200989)

29. Mamrut S, Avidan N, Staun-Ram E, et al. Integrative analysis of methylome and transcriptome in human blood identifies extensive sex-and immune cell-specific differentially methylated regions. Epigenetics 2015;10(10):943-57. (PMID: 26291385)