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Neutrophil Markers as Predictors of COVID-19 Severity at Hospital Admission: A Cross-sectional Study

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ABSTRACT

COVID-19 is capable of undermining self-tolerance in a host's immune system and triggering autoimmunity by hyper-activating the innate and adaptive immune systems, which has not investigated in Iranian society until now. In the innate immune system neutrophils are the predominant immune cells that protect the human body against invaders.

Here, we sought to explore 2 important variables related to neutrophil: DNA complexes with myeloperoxidase (MPO-DNA) as a reliable indicator of neutrophil extracellular traps (NETs) by MPO-DNA complex evaluation using a sandwich ELISA and the underlying role of IL-8 in (NETs) formation during COVID-19 infection.

According to our results, in COVID-19 patients, neutrophil-to-lymphocyte ratio (NLR) was significantly higher in ICU patients (14.62±11.81) compared to non-ICU patients (3.16±3.33). Elevated IL-8 levels were observed in COVID-19 patients, particularly in ICU patients. MPO-DNA levels, indicating NETosis, were significantly higher in COVID-19 patients and strongly correlated with neutrophil counts (r=0.472).

Thus, our studies suggest that neutrophils count, IL-8, and MPO-DNA can be used as powerful biomarkers in diagnosing COVID-19 severity. patients with severe COVID-19 infection are prone to heart disease because most of them develop excessive NET formation. Additionally, In COVID-19 patients, a higher MPO-DNA level may increase the risk of developing heart disease too.

Keywords: Autoimmunity; COVID-19; IL-8; Neutrophil extracellular traps; Neutrophil

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a global pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection.[1] In the initial response to a new infection, the innate immune system,

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which is prominent in the respiratory and intestinal mucosa, seeks out and eliminates potential threats by interacting with soluble mediators.[2] In COVID-19 infection, the innate immune system attempts to eliminate COVID-19, but it sometimes interacts with the body in a way that stimulates immune and non-immune cells, leading to autoinflammation.[3] Furthermore, recent research has revealed that COVID-19 components are homologous with human components, which relates to the virus's ability to induce hyper-stimulation of the immune system.[4]

In the bloodstream, neutrophils are the most abundant immune cells, accounting for approximately 50–70% of all leukocytes. They serve as the first responders to a wide variety of infectious diseases.[5] During bacterial or fungal infections, these polymorphonuclear cells (PMNs) play a protective role in the body; however, their role in viral infections remains controversial.[6] Despite their protective role during infectious diseases, these cells are sometimes implicated in causing autoimmune disorders. Neutrophils kill pathogens through several mechanisms when part of the inflammation process associated with autoimmune diseases. Neutrophils act quickly to eradicate invading pathogens, thereby functioning as a host defense system.[7] Furthermore, there is evidence that neutrophils are present in various lung diseases associated with acute respiratory distress syndrome (ARDS), as seen in infections by the influenza virus and SARS-CoV-1.[8] Neutrophil recruitment has been observed in the immune response triggered by the SARS-CoV-2. Neutrophilia has been described as an indicator of severe respiratory symptoms and poor outcomes in patients with COVID-19.[9] Indeed, neutrophil infiltration has been suggested as a significant contributor to the COVID-19 immunopathological manifestations, such as cytokine storms and impaired adaptive immunity.[10]

It is vital that these cells are activated appropriately for adequate pathogen clearance, particularly during systemic inflammation, as in sepsis. Powerful chemoattractants, such as the complement activation product C5a or the chemokine interleukin (IL)-8 (also known as C-X-C motif chemokine ligand 8, CXCL8), activate neutrophils and cause them to migrate to the inflammatory environment.[11] Infections (e.g., septic) and inflammatory (e.g., traumatic) conditions are frequently detected by IL-8 as both diagnostic and prognostic markers.[12] Neutrophils recruited to the lung express IL-8, which, in turn, activates and enhances IL-8 release from peripheral neutrophils.[13] IL-8 has a central role in activating and recruiting neutrophils during severe COVID-19 infections.[13]

In acute viral infections, neutrophils perform several important functions. They are responsible for controlling viral replication and diffusion through phagocytosis, degranulation, respiratory burst, cytokine secretion, and the release of neutrophil extracellular traps (NETs), as well as activating the adaptive immune system. NETs are characterized by double-stranded DNA bound to citrullinated histone 3, neutrophil elastase, and myeloperoxidase, with these proteins adsorbed onto the DNA web complexes. Numerous microbial components and inflammatory mediators have been shown to induce NETosis, including CXCR1 and CXCR2.[14] NETs help prevent the dissemination of pathogens through their neutralizing and killing functions; however, for small pathogens like viruses, NETs can play a double-edged role.[15] NETs provide key insights into the systematic pathogenesis of inflammation, which is related to the development and progression of autoimmune diseases.[15] According to several reports, COVID-19 infection is associated with excessive formation of NETs in patients with severe organ dysfunction, leading to unfavorable coagulopathy and immunothrombosis.[16] Furthermore, post-mortem examinations of COVID-19 patients have shown that neutrophil extravasation occurs widely in pulmonary capillaries, myocardium, and the liver.[3]

As mentioned, there is a definite relationship between the development of autoimmune reactions and the exacerbation of disease in COVID-19. However, in Iran, no study has been conducted to investigate autoimmune markers in COVID-19 patients. Therefore, this study aims to measure another autoimmune marker in Iranian patients with COVID-19, following previous research. We are investigating neutrophil-related markers, which may play an important role in causing autoimmune reactions in COVID-19 infection. Furthermore, in this study, the term 'COVID-19' will be used to refer to both the disease caused by SARS-CoV-2 and the virus itself

MATERIALS AND METHODS

This cross-sectional study involved 103 patients over 17 years old with confirmed COVID-19, diagnosed by reverse transcriptase polymerase chain reaction (RT-PCR), and 28 healthy controls. The study examined

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patients who visited the hospital for the first time with symptoms commonly associated with COVID-19, such as fever, cough, fatigue, loss of taste or smell, and difficulty breathing, and who also provided informed consent. Blood samples (5 cc) were collected from these patients on the first day of hospitalization, before any medication was administered. Samples were only collected if they were intact and accompanied by complete clinical information.

Samples were excluded from the study if they were damaged, lacked sufficient clinical information, or were from patients with autoimmune diseases, chronic viral infections such as AIDS, or those using medications that increase antinuclear antibodies, including sulfadiazine, hydralazine, or isoniazid.[17,18] Patients were classified into two groups based on the severity of their condition at admission, categorized according to the oxygen therapy required:

Non-ICU patients: Those with SpO2 levels of ≥94% in ambient air who received supportive oxygen via nasal cannula or mask.

ICU patients: Those with SpO2 levels of <94% in ambient air who required noninvasive ventilation or mechanical ventilation (intubated).[19]

Measurement of Interleukin 8

An ELISA kit for human IL-8 (sensitivity: 7.5 pg/mL) was purchased from R&D Systems (Minneapolis, Minnesota, USA). The kit accurately measures both natural and recombinant human IL-8. According to the datasheet, no significant cross-reactivity or interference was observed with other factors tested at 50 ng/mL in a mid-range recombinant human IL-8 control, indicating that the assay is highly specific to IL-8, ensuring reliable and precise measurements.

Analyses were performed according to the manufacturer's instructions. The absorbance of the plate was recorded at 450 nm with 630 nm as the reference wavelength.

Quantification of MPO-DNA Complexes

As described previously, MPO-DNA complexes were quantified.[20] First, 96-well plates were coated overnight at 4°C with anti-human MPO monoclonal antibody (clone 4A4, IgG2b, verified for ELISA use, RRID: AB_617350, no. 0400–0002, Bio-Rad, Hercules, CA), diluted to 1 μg/mL in coating buffer from the Cell Death ELISA Kit (Cell death detection ELISAPLUS, Cat. No 11-774-425-002; Roche Diagnostics Nederland B.V., Almere, The Netherlands). All reagents used in this study were obtained from the Cell Death ELISA Kit. The plates were then washed three times with 0.05% Tween/PBS, and the non-specific binding sites were blocked with 4% bovine serum albumin (MilliporeSigma, USA) in PBS (supplemented with 0.05% Tween-20) for 2 hours at room temperature. Plasma samples were diluted 1:10 in 1% BSA; 100 μL of this mixture were added to the plate and incubated at 4°C. After three washes with 0.05% Tween/PBS, the plate was incubated for one hour at room temperature with the anti-DNA antibody conjugated with peroxidase (POD) from the Cell Death Detection Kit (Roche, The Netherlands). The plate was washed five times with 0.05% Tween/PBS. Tetramethylbenzidine (TMB) substrate (Thermo Fisher, USA) was added in a dark room,by adding the stop solution, and and the plates were then read at 450 nm.

RESULTS

Demographic Characteristics

In this cross-sectional study, 131 individuals participated, with their demographic and clinical characteristics presented in Table 1. Among them, 28 were in the control group and 103 were in the COVID-19 group. The COVID-19 patients were divided into two groups: ICU (n=44, 42.71%) and non-ICU (n=59, 57.28%). The mean±SD age of the COVID-19 patients was significantly higher ($p \leq 0.000$) than that of the control group. Of the 103 patients with COVID-19, 64 (62.1%) were male and 39 (37.9%) were female. There was no significant difference in gender compared with the control group.

Among COVID-19 patients, the prevalence of hypertension was significantly higher in those admitted to the Intensive Care Unit (ICU) compared to non-ICU patients presented in figure 1 (43.2% vs. 22.2%, p=0.026). Diabetes Mellitus was more common in the ICU group (40.9%) than in the non-ICU group (24.1%), approaching significance (p=0.075). Other comorbid conditions did not show significant differences: Cancer (ICU 2.3% vs. non-ICU 1.9%, p=0.883), heart disease (ICU 27.3% vs. non-ICU 14.8%, =0.128), renal disease (ICU 13.6% vs. non-ICU 3.7%, p=0.074), Liver Disease (ICU 4.5% vs. non-ICU 0%, p=0.113), and respiratory disease (ICU 13.6% vs. non-ICU 7.4%, p=0.311).

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Table 1. Demographic characteristics of Patients

	Control Mean ± SD	ICU Patients Mean ± SD	Non-ICU Patients Mean ± SD
Number	28	44	59
Age(years)	54±12	64±14	53±15
Sex	Male 16(57.1%)	28(63.2%)	36(61%)
	Female 12(42.9%)	16(34.4%)	23(39%)

Elevated Neutrophil and Decreased Lymphocyte Count in COVID-19 Patients

Our study revealed significant differences in neutrophil and lymphocyte counts between Non-ICU and ICU COVID-19 patients. The mean neutrophil count in the non-ICU group was 384.96±177.75, whereas the ICU group had a markedly higher mean of at 909.03±466.71, with this difference being statistically significant with a (p<0.001). For lymphocyte counts, the non-ICU patients had a mean of 174.73±101.99, compared to a mean of 95.59± 68.72) in the ICU group which was also statistically significant(p<0.001), (Figure 2). Additionally, the mean neutrophil-to-lymphocyte ratio (NLR) of ICU patients was 14.62±11.81, while the non-ICU group was had a mean of 3.16±3.33. There is a positive association between NLR and disease severity in our samples.

Elevated IL-8 Levels in COVID-19 Patients

The concentration of IL-8 was measured in the serum of COVID-19 patients upon admission and in healthy controls. Serum concentrations of the cytokines studied in our study are reported in Table 2. We observed that IL-8 concentrations were significantly higher in COVID-19 patients compared to controls (p=0.004). Furthermore, serum IL-8 levels were significantly elevated in ICU COVID-19 patients compared to non-ICU survivors (p=0.001).

Increased NETs in COVID-19 Patients' Sera

Compared with serum samples from 28 healthy controls, COVID-19 samples showed higher levels of MPO-DNA complexes which are markers of NETosis (Figure 3A) (p=0.000). Additionally, MPO-DNA levels were higher in ICU patients (1.9372±1.0067) than in non-ICU patients (0.7640±0.7246) (Figure 3B).

Receiver operating characteristic (ROC) Curve Analysis

ROC curves were analyzed to determine the cutoff values for MPO-DNA complexes. The results are presented in Figure 2. The cutoff point was observed at 1.13, with a sensitivity of 84.1% and a specificity of 86.4%.

Correlation Between MPO-DNA Levels and Neutrophil Counts

Using Pearson's correlation coefficient, we explored the relationship between MPO-DNA levels and interleukin-8 levels as well as neutrophil counts. We found a strong correlation (r=0.472, p=0.001) between neutrophil count and MPO-DNA levels. However, there was no correlation (r=0.07, p=0.541) between MPO-DNA levels and IL-8 levels.

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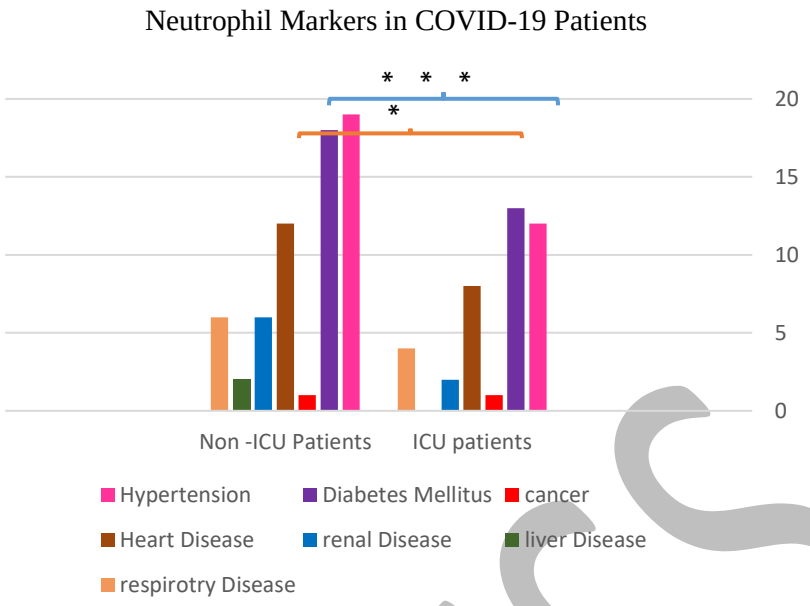

Figure 1. Prevalence of Comorbid Conditions in COVID-19 Patients: ICU versus Non-ICU Admission. This bar chart illustrates the proportion of patients with various comorbid conditions, including Hypertension, Diabetes Mellitus, Cancer, Heart Disease, Renal Disease, Liver Disease, and respiratory disease in both ICU and non-ICU groups. The Chi-square test was used to compare the prevalence of these conditions between groups. Significant differences were observed for Hypertension (p=0.026*), while Diabetes Mellitus approached significance (p=0.075). Other conditions showed trends but did not achieve statistical significance. *: Significant (p<0.01), *: Approaching Significance (p<0.1), No stars (p≥0.1): Not Significant**

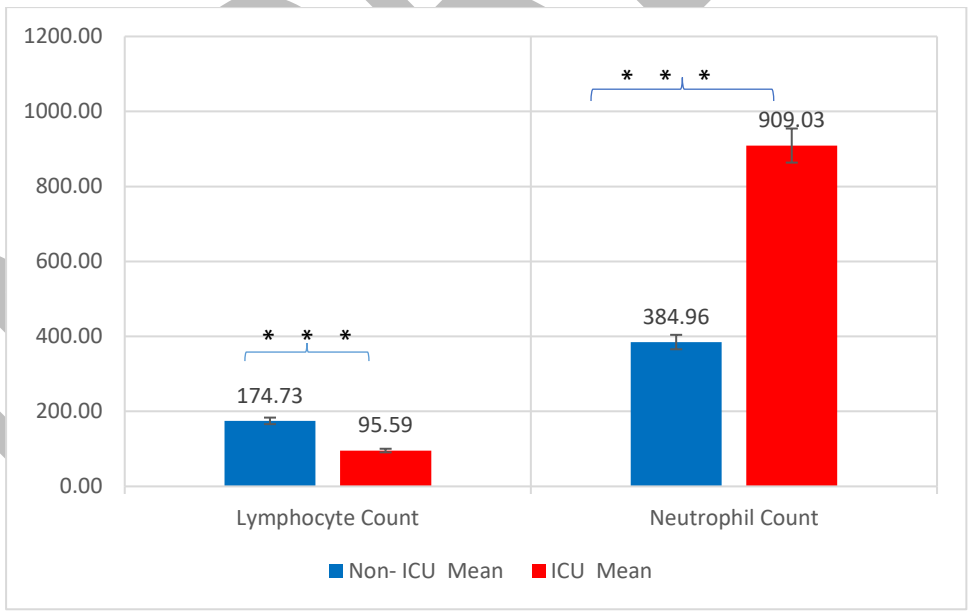

Figure 2. Neutrophil and lymphocyte count in Covid-19 patients. T-test was used to compare the results of neutrophil and lymphocyte counts between the two groups, and a significant difference was found, with a p value of<0.001. *: Significant (p<0.01)**

Table 2. IL-8 Level in COVID-19 Patients

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Group	Mean (pg/mL)	S.D	Median (pg/mL)	*p*
Control	0.13	0.10	0.08	
COVID-19	28.83	95.76	0.17	**0.004**

This table presents the serum concentrations of IL-8 measured in COVID-19 patients upon admission and in healthy controls. The comparison was performed using the T-test. The results indicate that IL-8 concentrations were significantly higher in COVID-19 patients compared to healthy controls (p=0.004). Significant differences are highlighted.
Standard Deviation; S.D

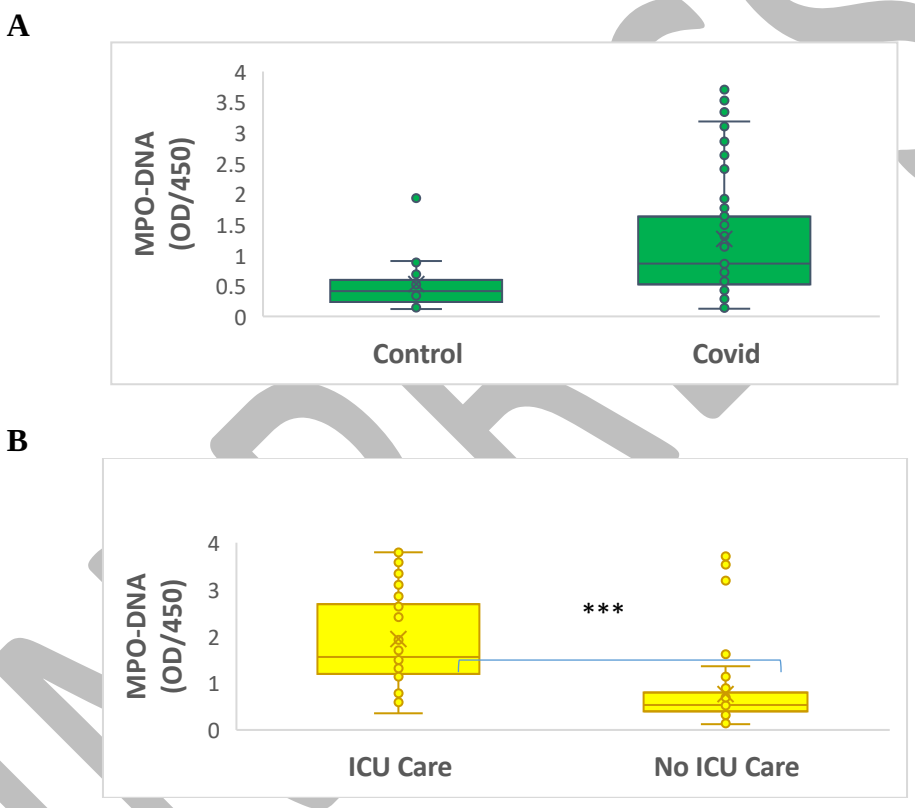

Figure 3. Elevated Levels of DNA complexes with myeloperoxidase (MPO-DNA) Complexes in COVID-19 Patients. (A) Serum levels of MPO-DNA complexes, a marker of neutrophil extracellular traps (NETs) , are significantly higher in COVID-19 patients compared to healthy controls (t-test, p=0.000). (B) Among COVID-19 patients, MPO-DNA levels are notably higher in ICU patients (mean±SD: 1.9372 ± 1.0067) compared to non-ICU patients (mean±SD: 0.7640±0.7246) (t-test, p=0.000). *: Significant (p<0.01)**

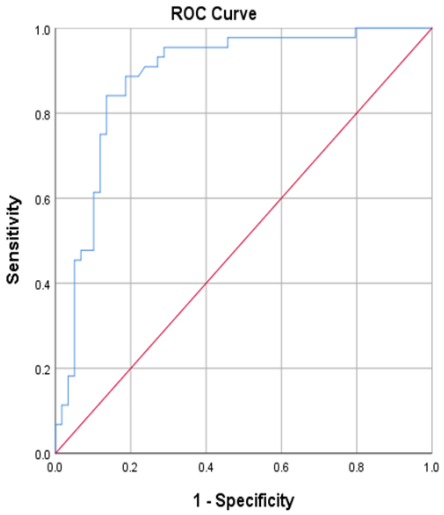

Figure 4. Receiver operating characteristic (ROC) Curve Analysis for DNA complexes with myeloperoxidase (MPO-DNA) Complexes. ROC curve analysis was performed to establish the optimal cutoff value for MPO-DNA complexes in distinguishing COVID-19 patients from controls. The analysis identified a cutoff point at 1.13, which provides a sensitivity of 84.1% and a specificity of 86.4%. The curve demonstrates the balance between sensitivity and specificity at this threshold, highlighting its effectiveness in predicting disease presence.

DISCUSSION

Severe COVID-19 is often characterized by severe pneumonitis and excessive inflammation[21,22,23] Neutrophils, as the first responders to infections, rapidly extravasate from blood vessels into inflamed tissues.[24] In severe COVID-19 cases, neutrophils adopt a degranulated, prothrombotic phenotype, leading to increased production of and responsiveness to IL-8. IL-8 is a key chemokine that facilitates neutrophil chemotaxis and significantly promotes the formation of NETs.[13,25]

Our study was designed to investigate the roles of two crucial neutrophil-related markers—IL-8 and NETs—in predicting the severity of COVID-19 infection. We hypothesized that elevated levels of these markers would correlate with more severe disease outcomes. To test this hypothesis, we compared neutrophil and lymphocyte counts, serum IL-8 levels, and MPO-DNA complexes as NETosis markers between ICU and non-ICU patients.

Our findings revealed that ICU patients had significantly higher neutrophil counts compared to non-ICU patients , with a *p*-value of <0.001. Conversely, lymphocyte counts were significantly lower in ICU patients compared to non-ICU patients . These results align with previous research linking elevated neutrophil counts and decreased lymphocyte counts to severe COVID-19 outcomes.[25,26] Elevated neutrophil counts reflect the role of neutrophils in extensive tissue damage and disease severity. Additionally, the neutrophil-to-lymphocyte ratio (NLR) was significantly higher in ICU patients compared to non-ICU patients . This supports previous studies suggesting that NLR is a reliable predictor of disease severity, with fluctuating NLR levels potentially indicating disease progression.[27,28]

Elevated levels of IL-8 in the plasma of patients with severe COVID-19 have been implicated in driving a prothrombotic neutrophil phenotype. This is supported by a strong correlation between high IL-8 levels and increased risk of respiratory failure and SARS-CoV-2-related mortality.[33,3629,30] Our study corroborates these findings, demonstrating significantly higher IL-8 levels in patients with severe COVID-19 compared to those with milder forms of the disease or healthy controls. This underscores the potential of IL-8 as a prognostic biomarker.

The formation of NETs is another critical factor contributing to COVID-19 severity.[31,32] Our study found significantly higher levels of MPO-DNA complexes in COVID-19 patients compared to healthy controls , with ICU patients showing higher MPO-DNA levels compared to non-ICU patients . These results align with existing research linking increased NET formation to

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severe COVID-19.[31,32] NETs exacerbate inflammation and may drive autoimmune responses by exposing autoantigens, potentially triggering autoimmune diseases.[33] Although our previous research did not find a direct correlation between antinuclear antibodies (ANAs) and COVID-19 severity, the role of NETs in sustained inflammation and their impact on comorbid conditions, such as cardiovascular diseases, warrant further investigation.[33]

Our findings also suggest a potential link between high NET markers and cardiovascular complications. We observed that patients with elevated MPO-DNA levels were more likely to have hypertension, suggesting that high NET marker levels might contribute to COVID-19 progression and cardiovascular issues. Excessive NET formation has been associated with a ˡhigher risk of heart disease, highlighting the importance of cardiovascular monitoring in severe COVID-19 patients, particularly those with a history of cardiovascular disease. This finding supports the need for comprehensive management strategies that address both COVID-19 severity and associated comorbid conditions.

Our study, alongside existing literature, emphasizes the potential for targeting NETs and related pathways as therapeutic strategies for high-risk COVID-19 patients.[34] NETosis is influenced by various factors, including TNF-α, IL-8, IL-1β, C5a, IL-17A, and other inflammatory mediators.[19,25] Although our study did not find a statistically significant correlation between IL-8 and MPO-DNA levels, the clinical relevance of IL-8 in neutrophil recruitment and NET formation remains significant. Anti-IL-8 therapies have shown promise in reducing neutrophil activation and NET formation, potentially mitigating acute respiratory distress syndrome (ARDS) and related microthrombosis.[35]

In conclusion, our study contributes to the understanding of neutrophil-related markers, such as IL-8 and NETs, in predicting COVID-19 severity. These markers provide valuable insights into the disease's progression and offer potential targets for therapeutic interventions aimed at improving outcomes and managing severe COVID-19 cases. Integrating these findings into clinical practice could enhance our ability to predict and manage severe COVID-19, ultimately improving patient care and outcomes.

Neutrophils play a fundamental role in COVID-19 severity. In this study, two important markers related to neutrophils were measured in COVID-19 patients. Firstly, IL-8, an important chemokine in activating neutrophils, is an easily detectable and sensitive biomarker for both mild and severe COVID-19 patients. Secondly, MPO-DNA, a crucial marker of NETosis, was significantly increased in COVID-19 patients.

STATEMENT OF ETHICS

Ethics approval and consent to participate

This study was conducted in accordance with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects, amended in 2013). The study was approved by the Ethics Committee of the Ministry of Health and Medical Education of I.R.Iran (IR.SHAHED.REC.1401.004). Written informed consent was obtained from all participants in this study.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request

AI Assistance Disclosure

Not applicable.

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