RESEARCH ARTICLE

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Post-COVID-19 Cardiovascular Disorders and the Molecular Mechanism of NET Formation

ABSTRACT

Objective: The post-COVID-19 process is not completely understood, as it affects COVID-19 survivors at all levels of disease severity, not all of whom are hospitalized. One of the long-lasting COVID-19 symptom categories, cardiovascular disorders (including acute heart failure, palpitations, hypotension, venous thromboembolic diseases, arrhythmias, myocarditis, and increased heart rate), may derive from a systemic inflammatory response to the viral infection. NETs (neutrophil extracellular traps) that fight invading viruses in extracellular cardiac spaces accumulate due to COVID-19, hyperinflammation, and cytokine storms. Our study focuses on cardiovascular disorders as COVID-19 sequelae. To determine the role of NETs in these disorders, we aimed to measure levels of PAD4, MPO, MMP-9, and H3Cit.

Methods: Forty patients with long-term cardiac complications associated with a history of COVID-19 and forty healthy people were included in this study.

Results: We found significant differences in PAD4, H3Cit, and MPO plasma levels between the post-COVID-19 and control groups (p values < 0.05). The expression levels of PAD4 mRNA were lower and MMP-9 mRNA levels were higher in the post-COVID-19 group compared with the control subjects.

Conclusions: These findings suggest that PAD4, MPO, MMP-9, and H3Cit are potential biomarkers of NET dysregulation and may cause post-COVID-19 symptoms, especially cardiovascular disorders.

Keywords: Cardiovascular Abnormalities, Citrullinated Histone H3, Neutrophil Extracellular Traps, Peptidyl Arginine Deiminases 4, Post-Acute COVID-19 Syndrome.

COVID-19 Sonrası Kardiyovasküler Bozukluklar ve NET Oluşumunun Moleküler Mekanizması

ÖZET

Amaç: Tamamı hastaneye yatırılmayan, hastalık şiddetinin her seviyesinde ki COVID-19 mağdurlarını etkilediği için, COVID-19 sonrası süreç tam olarak anlaşılamamıştır. Uzun süreli COVID-19 semptom kategorilerinden biri olan, kardiyovasküler bozukluklar (akut kalp yetmezliği, çarpıntı, hipotansiyon, venöz tromboembolik hastalıklar, aritmiler, miyokardit ve artmış kalp hızı dahil), viral enfeksiyona sistemik bir inflamatuar yanıttan kaynaklanabilir. Hücre dışı kalp boşluklarında istilacı virüslerle savaşan NET'ler (nötrofil hücre dışı tuzakları), COVID-19, hiperinflamasyon ve sitokin fırtınaları nedeniyle birikir. Çalışmamız, COVID-19 sekeli olarak kardiyovasküler bozukluklara odaklanmaktadır. NET'lerin bu bozukluklardaki rolünü belirlemek için PAD4, MPO, MMP-9 ve H3Cit düzeylerini ölçmeyi amaçladık.

Gereç ve Yöntem: COVID-19 öyküsü ile ilişkili uzun süreli kardiyak komplikasyonları olan kırk hasta ve kırk sağlıklı birey, bu çalışmaya dahil edildi.

Bulgular: COVID-19 sonrası ve kontrol grupları arasında PAD4, H3Cit ve MPO plazma seviyelerinde anlamlı farklılıklar bulduk (p< 0.05). PAD4 mRNA ekspresyon seviyeleri, kontrol deneklerine kıyasla COVID-19 sonrası grupta daha düşük ve MMP-9 mRNA seviyeleri daha yüksekti.

Sonuç: Bu bulgular, PAD4, MPO, MMP-9 ve H3Cit'in, NET düzensizliğinin potansiyel biyobelirteçleri olduğunu ve özellikle kardiyovasküler bozukluklar olmak üzere COVID-19 sonrası semptomlara neden olabileceğini düşündürmektedir.

Anahtar Kelimeler: Kardiyovasküler Anormallikler, Sitrulline Histon H3, Nötrofil Hücre Dışı Tuzakları, Peptidilarginin Deiminazlar 4, Akut Post -COVID-19 Sendromu

INTRODUCTION

Coronavirus disease 2019 (COVID-19) arises from RNA virus, called the acute severe respiratory syndrome-coronavirus-2 virus (SARS-CoV-2) and properties a wide variety of medical complications (1). Concerning the data from the World Health Organization (WHO), globally, as of 30 November 2022, there have been more than 639 million confirmed cases of COVID-19 of whom at least 6 million have died (2). Clinical course and long-term effects are still not understood of the disease which ranges from asymptomatic to mild or severe patients, some requiring intensive care have serious complications and affect different organs and systems in the body (3). It was determined that 30% of asymptomatic and 80% of cases requiring hospital care may have post-COVID conditions when all cases were evaluated (4,5). Some of the systemic manifestations, and symptoms that develop after COVID are called "long-haul" "long COVID-19", "persistent COVID-19 symptoms", or "post-COVID- syndrome" and may continue for weeks, months, or even years. One of them is cardiac manifestation which includes, tachycardia, palpitations, dysrhythmias, chest pain, and tightness (6,7).

NETs involved in NETosis, a form of cell death dependent on neutrophils, are networks of DNA surrounded by histones and granular proteins. They capture and destroy invading pathogens in the extracellular space (8,9). Possible mechanisms of myocardial disorder in Covid 19; myocarditis caused by hyperinflammation and cytokine storm, cardiac myocyte damage caused by respiratory failure and hypoxia, development of hypercoagulation, and coronary microvascular thrombosis are endothelial damage in many organs including the heart (10). The most common cause of heart attack coronary artery thrombosis is associated with NET accumulating in the cardiac extracellular spaces resulting in both systolic and diastolic dysfunction (11).

Although there is not much data on Post-Covid-19, the symptoms that occur after the healing duration are not firsthand caused by the virus, but are related to the coagulopathies and inflammatory response of the body to heal during the disease duration. Uncontrolled NET formation in coagulopathies induced by Covid 19 reasons, acute cardiac injuries, thrombosis, sepsis, respiratory failure, and heart failure (12). It has been reported that NET dysregulation also may cause post-disease symptoms, and an imbalance occurs in genetic and epigenetic factors that neutralize pathogen invasion and manage NETosis (13).

Peptidylarginine deiminases 4 (PAD4) neutrophil histones by citrullinating, myeloperoxidase (MPO) neutrophil elastase by stimulating, provide chromatin condensation through histone modification during NET formation. Studies have found that citrullinated histone H3 (H3Cit) and the MPO-DNA complex are elevated in patients with COVID-19, and they are thought to be potential biomarkers for plasma NET formation (14,15). Because of their capacity to disrupt the extracellular matrix, regulate tissue structure, and induce proteolysis, matrix metalloproteinases (MMPs) cause atherogenesis and vascular damage. Studies have shown that the amount of matrix metalloproteinases-9 (MMP-9) released during NETosis plays a role in endothelial integrity (16). One of the covid-sequelae in myocardial disorders, to determine the role of NETs, we aimed to measure the levels of PAD4, MPO, MMP-9, and H3Cit, which are involved in the molecular mechanism of NET formation.

MATERIAL AND METHODS

Study Population: Our prospective case-control study consisted of individuals between the ages of 18 and 45 who applied to the Cardiology Clinic of Kahramanmaraş Sütçü İmam University (KSU) Faculty of Medicine. Forty patients with histories of or confirmed SARS-CoV-2 infection and cardiological complaints lasting at least two months were included, along with 40 healthy individuals without histories of COVID-19 and normal sinus rhythms on an electrocardiogram (ECG) (17). Those with a history of systemic disease (diabetes, hypertension, thyroid disorder, etc.), morbid obesity, heart valve disease, left ventricular hypertrophy, electrolyte imbalance, permanent pacemaker implantation, atrial fibrillation, branch block or other intraventricular conduction defects, smoking, alcohol use, or cardiac problems were excluded. Those using drugs that affected the heart's rhythm (antiarrhythmics, antihistamines, etc.) were not included in our study.

Enzyme-linked immunosorbent assay (ELISA): Peripheral blood was collected from controls and patients. Blood samples were stored at room temperature for 30 min to allow for clotting and were then centrifuged at 2,000 × g for 15 min at 4°C. Serum was collected, mixed by inverting, and aliquoted into 0.5 mL Eppendorf tubes and stored at −80°C until further processing time. A quantitative sandwich ELISA was performed to assess serum levels of PAD4, MPO, and H3Cit (ELK Biotechnology, ELK3904, ELK1062, ELK8743, Wuhan, China) following the manufacturer's instructions. The absorbency of the reaction product was measured at 450 nm using a micro-plate reader.

Real-Time PCR: Lymphocytes were isolated from heparinized whole blood using Ficoll-Paque Plus solution as directed by the manufacturer. Separated mononuclear cells and plasma were then collected and stored at −80◦ C until use for RNA isolation. Total RNA extraction was made using TRIzol reagent (Life Technologies) on ice as described by the suppliers. Thereafter, the integrity and purity of the total RNA were measured using by spectrophotometer and agarose

gel. cDNA was synthesized using the reverse transcription system (Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit). QPCR was performed to measure human PAD4, and MMP-9 using a commercially available Taqman gene expression assay kit (Applied Biosystems). Expression data were normalized using the housekeeping gene GAPDH for each sample and fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistics: In the evaluation of the data, the conformity of the variables to the normal distribution was examined using the Shapiro-Wilk test. Statistical analyses for the ELISA and qPCR experiments were performed using the two-tailed Mann -Whitney test and independent samples t-test (Prism 8, GraphPad Software Inc., San Diego, CA). A P-value of ≤ 0.05 was considered significant.

RESULTS

The study groups consisted of forty patients who have a history of COVID-19 infection, 3 months after the onset of the disease and cardiological complaints durable at least 2 months, and forty healthy individuals without a history of COVID-19.

Plasma levels of PAD4, which supports NET formation by inducing chromatin condensation via histone citration, were higher in the post-COVID-19 group compared to the without histories of COVID-19 group, but no statistically significant difference was observed ($p \geq 0.05$, $p = 0.38$) (Figure 1A). PAD4 mRNA levels were decreased in the post-COVID-19 group compared to the control group, and there was a significant difference between the groups in terms of PAD4 mRNA levels ($p \leq 0.05$, $p = 0.001$) (Figure 2A). MMP-9 mRNA levels were also found to be high in the post-COVID-19 cases, but the results were not statistically significant ($p \geq 0.05$, $p = 0.725$) (Figure 2B). Citrulline H3 and MPO levels, which are important plasma biomolecules for NET formation, were found to be above in the post-COVID-19 group than in the control group, and this difference was statistically significant ($p \leq 0.05$, $p = 0.003$, and $p = 0.0487$) (Figures 1B, 1C).

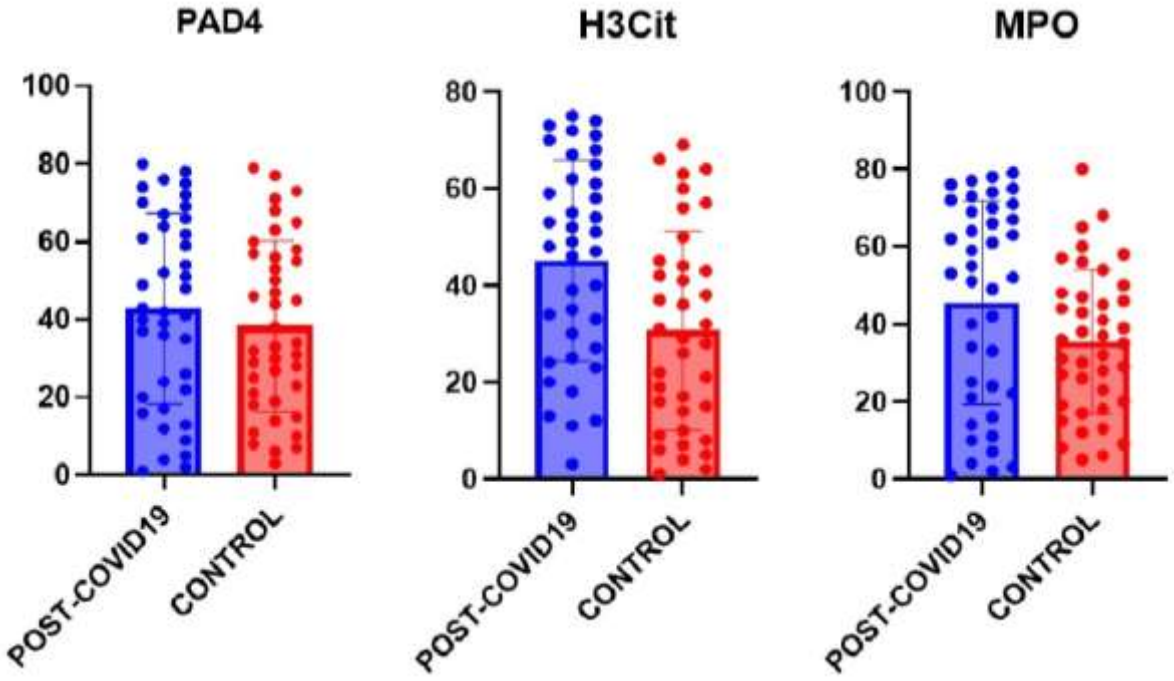

Figure 1. Plasma molecules responsible for NETs formation: **A)** PAD4 **B)** H3Cit, **C)** MPO were measured by Elisa in individuals per group. The plasma level of molecules was calculated and groups were compared by a non-parametrical test (Mann-Whitney Rank Sum Test) with GraphPad Prism software (version 8.0.2). Results were expressed as medians and interquartile ranges. * P-values were considered significant if ≤ 0.05.

DISCUSSION

To understand the pathologies mediated by NETs that develop after COVID-19, we aimed to indicate the levels of PAD4, MPO, MMP-9, and H3Cit biomolecules, which are involved in the molecular mechanism that governs disease-related NETosis, in the patient group with cardiological complaints for at least two months after COVID-19. Studies on COVID-19 sequelae and their molecular mechanisms are increasingly prevalent, but our study is the first in which these biomarkers were investigated together in this patient groupIn a study in which septic shock and critical COVID-19 cases were compared with a healthy control group in terms of NET formation, the H3, circular H3, neutrophil elastase, and MPO in the nucleosome content were analyzed. In that study, circulating nucleosome and neutrophil activation indicators were found to be above in the COVID-19 and septic shock groups (15).

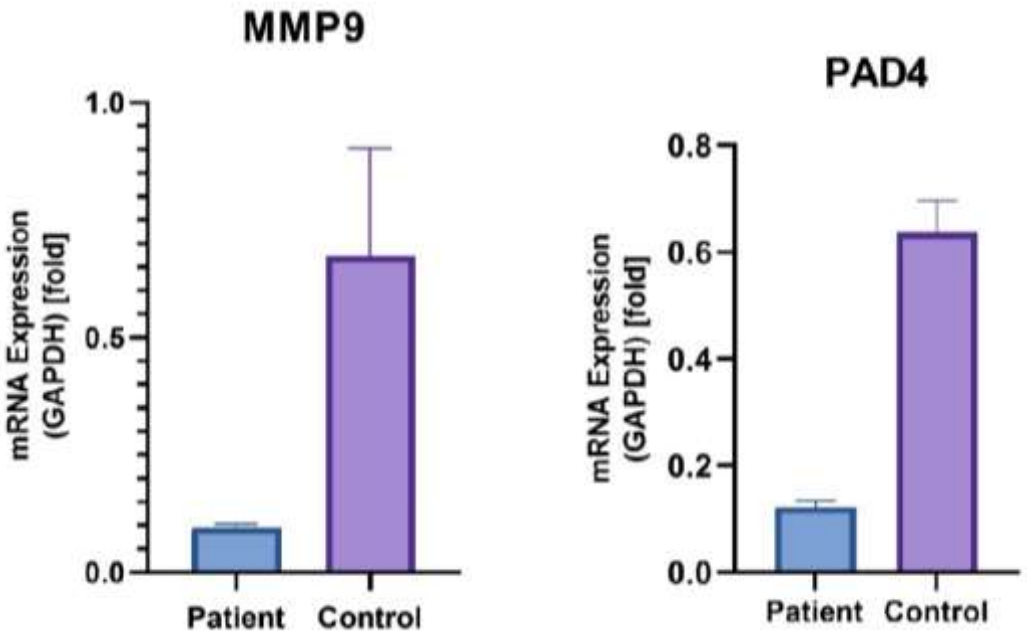

Figure 2. A) MMP-9 **B)** PAD4 were measured by real time RT-PCR in per group. The expression level was calculated as the mean ± s.d. for each group as individual data points using the relative expression (fold change over CONT) by the 2-ΔΔct method, where Ct is the threshold cycle. Groups were compared by a unpaired t test with GraphPad Prism software (version 8.0.2). Results were expressed as medians and interquartile ranges. * P-values were considered significant if ≤ 0.05.

In another study comparing hospitalized patients with a diagnosis of COVID-19 and a healthy group, the NET concentration was found to be higher in the COVID-19 group. In the same study, plasma MPO levels were found to be above in the COVID-19 group, confirming previous findings. Consistent with this research, in our study, plasma MPO levels were found to be higher in the post-COVID-19 group. At the same time, it has been determined that PAD4, which catalyzes arginine stratification and mediates NET release from neutrophils, creates a systemic increase in NETs (18). Consistent with this, in our study, PAD4 plasma levels were found to be above in the post-COVID-19 group. The increase in thromboembolic events and cardiovascular diseases following COVID-19 and its relationship to NETs and their production of long-term pathologies are still controversial (19). It has been determined that thrombotic complications occurring in COVID-19 are responsible for mortality and morbidity. Studies have shown that thrombosis in patients with COVID-19 affects both arterial and acute coronary syndrome, venous circulation, deep vein thrombosis, pulmonary embolism, and heart attack. It has been determined that NET residues, which are manifested by the presence of circular DNA, citrulline H3, and MPO-DNA complex, are abundant in the blood of patients with SARS-CoV-2, while neutrophil-platelet aggregates and neutrophil activation indicators increase in COVID-19 cases (20). In our study with patients with cardiological complaints after COVID-19, it was determined that plasma levels of PAD4, MPO, MMP-9, and H3Cit, which activate NETosis, increased. Histological studies have shown that NET increase causes vascular damage, and NET-related immuno-thrombosis is associated with organ damage in severe COVID-19 cases (21,22). NET-rich thrombi, platelets, and fibrins have been found in the lungs, hearts, and kidneys (20,23).

In a study conducted with 282 people with suspected coronary artery disease, coronary tomographic angiography and measurement of NET markers (citrulline H3, MPO-DNA complex) determined that these markers were also associated with the severity of coronary disease, prothrombotic state, and major side effects of cardiac disorders. In that study, it was reported that NET formation may contribute to the formation of atherosclerosis (24). Histological studies have demonstrated the presence of NETs in the lumen of mouse and human atherosclerotic lesions (25). In this study, in which we set out with the hypothesis that uncontrolled NET formation may lead to cardiovascular disorders after COVID-19, the biomarkers state involved in the molecular mechanism of NETosis are correlated with the results in the intersection of cardiovascular disorders, COVID-19, and NET. Our sample size for the study is limited, and we were unable to investigate other molecules involved in the same pathways as the biomarkers we examined. As the sample size was limited, the subjects in all groups could not be subdivided according to the test score. Therefore, further studies with a larger study population are needed to clarify this subject. In upcoming studies, the examination of more biomarkers is planned.

In COVID-19 patients, MMP-9 is increased, and this has been reported to stimulate platelet and neutrophil activation and NET formation in vitro (26). In another study, the neutrophil activation markers NET and MMP9 were associated with the severity of the disease in COVID-19 cases requiring intensive care (27). In this study, MMP9 mRNA levels were found to be higher in patients who had COVID-19 compared to the control group, which is consistent with the literature.

CONCLUSION

This study reveals that PAD4, MPO, MMP-9, and H3Cit appear to be potential biomarkers in NET dysregulation, which may cause certain post-COVID-19 symptoms – especially cardiovascular disorders.

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Ethical Approval: For this study, a certificate of ethical approval was obtained from the KSU Faculty of Medicine Non Interventional Clinical Research Ethics Committee with the meeting decision 11.01.2021/04 dated and numbered written informed consent was obtained from each patient before inclusion in the study.

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