Evaluation of the relationship between pentraxin 3 (PTX3) rs2305619 (281A/G) and rs1840680 (1449A/G) polymorphisms and the clinical course of COVID-19

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Abstract
Macrophage activation syndrome (MAS) is one of the main causes of morbidity and mortality in patients with coronavirus disease 2019 (COVID-19). This study aimed to investigate the relationship between the pentraxin 3 (PTX3) gene polymorphisms rs2305619 (281A/G) and rs1840680 (1449A/G) and the development of MAS in patients with COVID-19. The study included a total of 94 patients aged 18–45 who were diagnosed as having COVID-19 between June and December 2020. PTX3 281A/G and 1449A/G polymorphism frequencies were evaluated. PTX3 281A/G allele and genotype frequencies did not deviate from Hardy–Weinberg (HW) equilibrium in the MAS or non-MAS group ($\chi^2$: 0.049, df: 2, $p$ = 0.976, $\chi^2$: 0.430, df: 2, $p$ = 0.806). PTX3 1449A/G allele and genotype frequencies deviated significantly from HW equilibrium in the non-MAS group ($\chi^2$: 6.794, df: 2, $p$ = 0.033) but not in the MAS group ($\chi^2$: 2.256, df: 2, $p$ = 0.324). The AG genotype was significantly more frequent in the non-MAS group, while the AA genotype was significantly more frequent in the MAS group ($\chi^2$: 11.099, df: 2, $p$ = 0.004). Analysis of the PTX3 1449A/G polymorphism showed that individuals with the GG genotype had higher serum PTX3 levels than those with the AA and AG genotypes ($p$ = 0.001 for both). Analysis of the PTX3 1449A/G polymorphism in patients with COVID-19 showed that those with the AG genotype were relatively more protected from MAS compared with individuals with the AA genotype. In addition, lower serum PTX3 levels are observed in patients carrying the A allele.

KEYWORDS
COVID-19, macrophage activation syndrome, pentraxin 3

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) spread rapidly from its original epicenter in Wuhan, China after it appeared in December 2019. As of February 2021, more than 100 million confirmed cases have been reported worldwide, and this figure continues to rise daily. Infection is often asymptomatic or clinically mild, with typical symptoms including fever, cough, malaise, muscle and joint pain, and loss of smell and taste. A smaller proportion of patients experience a severe clinical course, particularly the older population, patients with comorbidities such as diabetes, patients with HIV or receiving long-term immunosuppressive therapy, and pregnant women.1, 2

Manifestations of severe COVID-19 include acute respiratory distress syndrome (ARDS) leading to hypoxemic respiratory failure,
and macrophage activation syndrome (MAS). Both have been attributed to an overabundance of pro-inflammatory cytokines that causes endothelial dysfunction and damage to various vital organs, primarily the lungs. The prognostic parameters most commonly used in COVID-19 include C-reactive protein (CRP), d-dimer, ferritin, leukopenia, fibrinogen, prothrombin time, and interleukin-6 (IL-6) levels.

Of the cytokines produced by active macrophages, IL-6, IL-1β, and tumor necrosis factor-α (TNF-α) are the main contributors to the development of MAS in COVID-19. IL-6 induces hepatic synthesis of CRP and serum amyloid P, which are known as short pentraxins, whereas IL-1β and TNF-α induce synthesis of pentraxin 3 (PTX3), a long pentraxin. PTX3 can be synthesized by macrophages, monocytes, leukocytes, dendritic cells, adipocytes, endothelial cells, and smooth muscle cells. Elevated PTX3 levels have been observed in bacterial, viral, and fungal lung infections, and this elevation was strongly associated with mortality. Studies of the PTX3 rs2305619 (281A/G) and rs1840680 (1449A/G) gene polymorphisms have shown that patients with the AA genotype are more susceptible to pulmonary tuberculosis and aspergillosis. In a study conducted on patients with coronary artery disease, in which macrophages play an important role in the development and progression of atheromatous plaques, no relationship between 281A/G and 1449A/G polymorphisms and coronary artery disease was detected, and plasma PTX3 levels did not differ significantly between genotypes.

PTX3 is a recently discovered molecule and its mechanism of action is not yet clear. Although the data on plasma PTX3 levels in inflammatory diseases are consistent, single-nucleotide polymorphism (SNP) studies are lacking. Therefore, this study investigated the association between the PTX3 281A/G and 1449A/G polymorphisms and the development of MAS, which is associated with elevated pro-inflammatory cytokine levels and has high mortality, in patients with COVID-19.

2 | PATIENTS AND METHODS

The study included 94 patients between the ages of 18 and 45 years with no known comorbidities who were diagnosed and treated for COVID-19 at the Erzurum Regional Training and Research Hospital between June and December 2020. Of these, 46 patients were admitted to the intensive care unit due to MAS and 48 patients were treated in the COVID-19 ward and did not develop MAS or ARDS.

2.1 | Patient selection

Patients who presented with symptoms such as fever, cough, dyspnea, malaise, and sudden loss of taste/smell and had a history of contact with a confirmed or suspected COVID-19 patient or a history of international travel in the past 2 weeks were evaluated by posterior-anterior chest X-ray. Those with suspicious lesions were further examined with high-resolution thoracic computed tomography (CT). COVID-19 diagnosis was confirmed by SARS-CoV-2 real-time polymerase chain reaction (PCR) testing of nasopharyngeal swab samples.

The patients’ history and laboratory results were reviewed for assessment of eligibility according to the following exclusion criteria: any comorbidity such as chronic obstructive pulmonary disease (COPD), diabetes, uncontrolled hypertension, coronary artery disease, and malignancy; history of infectious or inflammatory disease or invasive surgical procedures within the last month; and high fasting blood glucose. The presence of coronary artery disease, asthma, COPD, and diabetes was assessed through consultations with the cardiology, chest diseases, and internal medicine departments. Three patients with MAS were excluded due to abnormal fasting blood glucose levels.

Biochemical parameters including CRP, d-dimer, troponin-I, ferritin, liver, and kidney function tests, and hematological, coagulation, and arterial blood gas parameters were evaluated at admission and updated daily.

2.2 | Definitions and diagnosis

Fever was defined as an axillary temperature of 37.3°C or higher. Secondary bacterial infection was diagnosed in patients with clinical findings consistent with bacteremia or pneumonia and the isolation of a new pathogen in a culture of sputum or endotracheal aspirate from the lower airway. Patients with ventilator-associated or hospital-acquired pneumonia were treated as per current guidelines. ARDS was diagnosed and graded according to the Berlin 2015 diagnostic criteria. Patients with elevation in daily cardiac-specific troponin levels were evaluated by echocardiography for emerging cardiac pathologies. Coagulopathy was defined as prothrombin time 3 s longer than normal and activated partial thromboplastin time 5 s longer than normal. COVID-19 treatment was planned according to disease severity as specified in the diagnosis and treatment guidelines for adults issued by the Turkish Ministry of Health.

Patients with signs such as refractory fever, CRP and ferritin levels that remained high or continued to rise, d-dimer elevation, cytopenia manifesting as thrombocytopenia or lymphopenia, abnormal liver function tests, hypofibrinogenemia, or elevated triglyceride levels in spite of treatment were monitored for MAS. As changes in serial measurements are more important than set threshold values for laboratory findings, we established the diagnosis of MAS according to repeated follow-up measurements of clinical and laboratory parameters. If these parameters continued to deteriorate during follow-up with no apparent secondary bacterial infection, patients were treated with methylprednisolone at a dose of 250 mg/day or greater for 3 days. If no response was obtained with this treatment, 400 mg tocilizumab was administered for MAS unless contraindicated. Clinical and laboratory response was evaluated after 24 h. If an adequate response was not observed, a second 400 mg dose of tocilizumab was given.
2.3 | Biochemical analyses

After 15 min of semi-supine rest, blood samples were collected from an antecubital vein into ethylenediaminetetraacetic acid (EDTA) anticoagulant blood collection tubes. Troponin-I concentrations were measured by chemiluminescent immunoassay using an Immulite 2500 (Siemens Medical Solutions). IL-6 and PTX3 were measured by enzyme-linked immunosorbent assay (Elabscience human ELISA Kit).

2.4 | Molecular analyses

2.4.1 | DNA isolation protocol

DNA was isolated from blood collected in EDTA tubes using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s protocol. DNA quality was measured using the NanoDrop (ND-1000, Thermo Fischer Scientific).

2.4.2 | Analysis of rs2305619 and rs1840680 SNPs

Allele-specific SNP Type™ assays were performed using a Fluidigm Flex Six™ Genotyping IFC (Fluidigm Corp.). Specific target amplification (STA) was performed to increase the initial number of molecular targets. Thermal cycling was run on a Bioer Gene Pro thermal cycler at 95°C for 15 min followed by 14 cycles of 95°C for 15 s and 60°C for 4 min. SNP Type Assay mixes and sample mixes were prepared according to the manufacturer’s protocol. A dynamic array was loaded with 4 μl of each 10× assay mix and 5 μl of each sample mix, then placed in the IFC Controller HX (Fluidigm) to complete the loading process. The dynamic array was then placed in the BioMark system (Fluidigm) for thermal cycling and fluorescent image acquisition using the SNP type E Flex Six v1 protocol. Data were collected using the BioMark system’s built-in software (Figure 1). Genotyping application, ROX passive reference, and SNP type-FAM and SNP type-HEX probe types were selected.

2.5 | Statistical analysis

SPSS Statistics version 24.0 for Windows (IBM Corp.) was used for statistical analyses of the data. Comparisons of characteristics between patients with and without MAS were analyzed by χ² test for categorical variables and independent-samples t-test or Mann–Whitney U test for continuous variables, as appropriate. Pearson’s χ² test was used to evaluate differences in allele and genotype frequencies between the MAS and non-MAS groups and deviation of the observed genotype frequencies in each group from those expected according to the Hardy-Weinberg (HW) model. Independent-samples t-test was used to compare laboratory values between the groups. A p-value less than 0.05 was considered statistically significant.
The mean age was 41.7 ± 9.6 years in the MAS group and 40.5 ± 10.4 years in the non-MAS group (p > 0.05). There were 25 (54.3%) men and 21 (45.7%) women in the MAS group, and 26 (54.2%) men and 22 (45.8%) women in the non-MAS group (p > 0.05).

The laboratory results at the admission of COVID-19 patients with and without MAS are compared in Table 1. Compared with the non-MAS group, MAS patients had significantly lower white blood cell (WBC) and lymphocyte counts (p = 0.05, 0.001) and a higher neutrophil to lymphocyte ratio (NLR), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT), creatine, prothrombin time, CRP, troponin, arterial oxygen partial pressure to fractional inspired oxygen (PaO₂/FiO₂) ratio, D-dimer, ferritin, and IL-6 levels (p = 0.001, 0.03, 0.001, 0.02, 0.04, 0.02, 0.001, 0.001, 0.03, 0.001, and 0.001, respectively). PTX3 level was significantly higher in the patients with MAS compared with those without (p = 0.001).

Comparisons of the groups' PTX3 1449A/G allele and genotype frequencies with the HW equilibrium are shown in Table 2. No significant deviation was detected in the MAS group, whereas a statistically significant deviation was found in the non-MAS group (χ²: 22.56, df: 2, p = 0.032). There were no statistically significant deviations from HW equilibrium in PTX3 281A/G allele and genotype frequencies in the MAS and non-MAS groups.

Comparison of PTX3 1449 A/G genotypes between the groups is shown in Table 3. AG genotype frequency was significantly higher in the non-MAS group, whereas AA genotype frequency was significantly higher in the MAS group (χ²: 11.099, df: 2, p = 0.004). Comparison of PTX3 1449 A/G allele
frequencies also showed no significant differences between the groups ($p = 0.84, 0.53$).

When serum PTX3 levels were analyzed according to PTX3 1449A/G genotype, we found that levels of PTX3 were significantly lower in patients with the AA and AG genotypes compared to patient GG genotype ($p = 0.001, 0.001$). In the statistical comparison between patients with AA and AG genotypes, there was no significant difference in PTX3 levels ($p = 0.53$) (Figure 2). When PTX3 levels were compared between COVID-19 patients with and without the A allele, it was observed that patients without the A allele had significantly higher PTX3 levels ($p = 0.001$).

**FIGURE 2** The relationship between pentraxin 3 (PTX3) 1449A/G genotype frequency and plasma PTX3 levels

**FIGURE 3** The relationship between pentraxin 3 (PTX3) A allele frequency and plasma PTX3 levels

COVID-19 can be more deadly in older people and those with comorbidities. However, increasing case numbers have shown that the disease can also result in mortality among young patients, regardless of these risk factors. Although younger people are still more likely to experience mild disease, some do develop MAS, which greatly increases the risk of mortality. However, we still cannot predict which patients will develop MAS at the onset of this mysterious disease. Observations of severe clinical course in multiple members of the same family have suggested the role of genetic factors.

The pro-inflammatory cytokines released during the development of MAS have an important function in humoral immunity. The main problem is that abnormal synthesis cannot be adequately balanced by the anti-inflammatory system and can reach a level that requires therapeutic intervention. PTX3 is a recently characterized acute-phase reactant and evidence suggests it may have an important role in balancing inflammation. It can be synthesized by many tissues, especially endothelial, monocyte, macrophage, and dendritic cells, and is known to be a key component in innate humoral immunity. PTX3 is involved in complement system activation, which stimulates inflammation. It has roles in all three pathways of complement activation by increasing C1q synthesis in the classical pathway, interacting with factor H, which is involved in the degradation of C3b in the alternative pathway, and forming a complex with mannose-binding lectin (MBL) in the lectin pathway. In addition, PTX3 also regulates inflammation by inhibiting selection-dependent neutrophil recruitment and complement system activation. Activation via the classical pathway plays an important role in antiviral activity, while lectin pathway activation is important for antifungal action. In studies investigating PTX3 polymorphisms in pulmonary tuberculosis and aspergillosis patients, the 1449A/G polymorphism showed significant differences in both patient groups. In addition, the AA genotype was found to be a risk factor for disease...
in both studies and was associated with lower plasma PTX3 levels in patients with pulmonary aspergillosis compared to those with the AG and GG genotypes. Both studies concluded that the AA genotype, which was thought to cause low pentraxin levels, reduced antifungal and antibacterial activity. Neither study demonstrated a significant difference in the PTX3 281A/G polymorphism.9,10

Uncontrolled complement system activation is a major factor in the pathogenesis of severe COVID-19, thereby making this system a therapeutic target. A study investigating PTX3 levels in patients with COVID-19 showed that PTX3 level was positively correlated with disease severity. IPTX3 elevation in COVID-19 may be a reflection of failed negative regulation of inflammation.20,21

Based on our data in this study, we determined that NLR, AST, LDH, GGT, prothrombin time, CRP, troponin, PaO2/FiO2 ratio, d-dimer, ferritin, and IL-6 levels differed significantly between the MAS and non-MAS groups, as observed in previous studies. The differences in these laboratory values may be interpreted to be a result of the intense pro-inflammatory response, and the decrease in PaO2/FiO2 ratio a result of severe parenchymal involvement in patients who develop MAS.

The deviation from HW equilibrium observed in PTX3 1449A/G allele and genotype frequencies in the non-MAS group suggests that the AG genotype may be protective against MAS. Analysis of the PTX3 1449A/G polymorphism showed that the AG genotype was more frequent in the non-MAS group, while the AA genotype was more frequent in the MAS group. Evaluation of serum PTX3 levels based on the 1449A/G polymorphism showed that COVID-19 patients with the GG genotype had higher serum PTX3 levels compared with those with the AA and AG genotypes. In addition, PTX3 levels were found to be higher in patients without the A allele compared to carriers of the A allele. Considering these two findings together, although no statistically significant difference was observed in individuals with the GG genotype, this may have resulted in a higher number of MAS cases. In addition, the A allele may have played a role in reducing or suppressing PTX3 production. PTX3, an acute-phase protein believed to play a role in antiviral activity through complement system activation, was correlated with disease severity in our study, being found at higher levels in patients who developed MAS compared with those who did not. However, the 1449A/G polymorphism may be responsible for the varying levels among patients with MAS. The higher frequency of the AA genotype and associated low serum PTX3 level may be a factor contributing to MAS development. In another evaluation of serum PTX3 levels and polymorphism 1449A/G, no statistically significant difference was observed between COVID-19 patients with AG and AA genotypes. This can be mainly attributed to the lack of guiding analysis due to the low number of individuals with the AA genotype. The most important limitation of our study was that PTX3 1449A/G and 281A/G polymorphism levels were analyzed in a single race and in a limited population. However, the age range of the patients included in the study and ensuring they did not have any comorbidities were the main factors that limited our sample size. Larger multicenter studies are needed for our current findings to be generalized and utilized in the early diagnosis of MAS in clinical practice. As none of the patients in our study died during follow-up, it was not possible to evaluate serum PTX3 level or PTX3 1449A/G and 281A/G polymorphisms in terms of predicting mortality.

In conclusion, PTX3 is a recently characterized molecule in the long pentraxin family of acute-phase reactants that may guide treatment in the future. The clinical presentation of MAS that develops in COVID-19 can be diagnosed with follow-up, and these patients may not respond adequately to medical treatment. The results of our study on the PTX3 1449A/G polymorphism indicate that the AA genotype is more frequent among patients who develop MAS, while the AG genotype may be protective. With acquired immunity research and applications continuing at a brisk pace, early detection of polymorphisms may be life-saving for at-risk patients. Therefore, the results of our study may provide insight into the role of the newly discovered PTX3 in the future.

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CONFLICT OF INTERESTS
The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS
Ferhan Kerget, Buğra Kerget, and Çiğdem Yüce Kahraman: Conceptualization, Methodology, Software, Validation, Formal analysis. Ferhan Kerget and Buğra Kerget: Investigation, Resources, Data Curation. Ö.A: Writing - Original Draft, Writing - Review & Editing. Leyla Sağlam, Metin Akgün, and Elif Yılmazel Uçar: Visualization, Supervision, Project administration.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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