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The association of *PON1* and *NOS3* genetic variants with the severity of COVID-19

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ABSTRACT

The pandemic of coronavirus disease 2019 (COVID-19) is a global pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The aim of this study was to investigate the association of single nucleotide polymorphisms (SNPs) of paraoxonase 1(PON1) and nitric oxide synthase 3 (NOS3) genes with the severity of COVID-19. Study subjects were divided into mild and severe groups. Genotyping was conducted by allele specific real-time polymerase chain reaction (RT-PCR). Gene-gene interaction was assessed by multifactor dimensionality reduction (MDR) analysis. The results showed a significant association of both *PON1* rs662 (A575G), and NOS3 rs2070744 (T786C) with the severity of COVID-19 ($p = 0.0026$, and $p = 0.033$, respectively). *PON1* 575AG (OR = 0.30, 95 % CI [0.13–0.68]) and *NOS3* 786TC (OR = 0.35, 95 % CI [0.15–0.78]) were associated with mild COVID-19 symptoms. The resulted SNP-SNP interaction model by MDR analysis was statistically significant ($p = 0.0003$). *NOS3* 786C * *PON1* 575G haplotype was significantly associated with mild cases of COVID-19 ($p = 0.045$).

1. Introduction

Coronavirus disease 2019 (COVID-19) is a highly contagious disease, firstly reported in Wuhan, China in 2019, and then rapidly spread worldwide to be declared as a pandemic in March 2020. Around 768 million cases were confirmed globally according to the WHO COVID-19 dashboard (https://covid19.who.int/; 21 June 2023), including >6.9 million deaths. Patients experience different clinical manifestations of the disease, therefore grouped into categories such as asymptomatic, mild, moderate, or critical illness, according to the severity of their symptoms (https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/; 6 March 2023). Common features include fever, cough, shortness of breath, altered sense of smell and taste, and other gastrointestinal and cutaneous symptoms (Booth et al., 2021). In critical cases, more severe symptoms could be noticed, including acute respiratory distress syndrome (ARDS), virus-induced septic shock, and multiple organ dysfunction. The most important risk factors are older age, male gender, and existing health problems such as diabetes, hypertension, cardiovascular diseases, and cancer (Rashedi et al., 2020). Another important factor is the genetic predisposition of the host organism. For example, polymorphisms in the angiotensin-converting enzyme-2 (ACE2) gene, which encodes the cellular receptor for SARS-CoV-2, were proved to be associated with disease development and severity by affecting the virus's S protein binding to ACE2 (Glotov et al., 2021).

Oxidative stress - an imbalance between the production of free radicals and the ability of the antioxidant defense system to detoxify the resulted harmful products. Several risk factors related to COVID-19 are associated with an elevated oxidative stress (Chernyak et al., 2020). For example, the severity of COVID-19 symptoms is generally associated with the age of the patient (Wong et al., 2020). This could be explained by the disruption of redox balance and accumulation of oxidative damage. Wieczfinska and colleagues described the infection of SARS-CoV-2 as a vicious circle. Tissues are damaged due to reactive oxygen species (ROS) overproduction by the immune system cells. Prooxidant cytokines, such as TNF-α (tumor necrosis factor-alpha), are also released by the activated phagocytes. The uncontrolled inflammatory process increases oxidative stress, which in turn promotes the cytokine production by inflammatory cells. All these reactions leads eventually to a

Abbreviations: COVID-19, Coronavirus disease 2019; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; PON1, Paraoxonase 1; NOS3, Nitric oxide synthase 3; SNP, Single nucleotide polymorphism; ROS, Reactive oxygen species; OR, Odds Ratio; CI, Confidence interval; HWE, Hardy-Weinberg Equilibrium; LD, Linkage disequilibrium; MDR, Multifactor Dimensionality Reduction.

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molecular vicious circle in COVID-19 (Wieczfinska et al., 2022).

In general, the role of the redox status in the pathogenesis of most diseases, including viral infections, made it important to study the variations in the oxidant/antioxidant system in all levels, including the genetic variants of genes, encoding antioxidant enzymes.

Paraoxonase 1 (PON1) - a calcium-dependent hydrolytic enzyme, and the most popular member of serum paraoxonases family (Shunmoogam et al., 2018). It thought to possess antioxidant properties by reducing oxidized lipids in low-density lipoprotein (LDL) and high-density lipoprotein (HDL), as well as inhibiting of lipoprotein peroxidation (Grzegorzewska et al., 2021).

The endothelial nitric oxide synthase (eNOS), or NOS3, is responsible for the production of nitric oxide (NO) in the vasculature. NO is known for its function in the maintenance of regular vascular tone (Cruz-González et al., 2009). It also exerts multiple antioxidative effects, including inhibition of LDL oxidation, terminating radical chain reactions, and inhibiting peroxidases and oxidases (Förstermann et al., 2017).

Several known polymorphisms in the genes of PON1 and NOS3, such as *PON1* rs662 and *NOS3* rs2070744, have been reported to affect their enzymatic activity, which make the studying of these polymorphisms a possible way to better understand the pathogenesis of COVID-19 infection, in which the oxidative stress is considered as a "pivotal point" (Vollbracht and Kraft, 2022).

Therefore, the aim of this study was to investigate the association of polymorphisms in the genes of PON1 and NOS3, with the severity of COVID-19.

2. Materials and methods

2.1. Patients

A total of 110 COVID-19 patients were divided into 2 groups according to the severity of their symptoms (55 mild, and 55 severe cases). Age of all participants was (20–64) years old, and the male/female ratio was the same for both groups. Blood samples were collected in the period between 12.03.2021 and 08.07.2022 in "Nauka" medical center (Rostov-on-Don, Russian federation). The study was conducted in the post-COVID period (at least 2 months after recovery). Criteria for inclusion in the study: 1) - a history of SARS-CoV-2 infection confirmed by PCR; 2) - the presence of IgG antibodies to the virus (in case of mild infection). Exclusion criteria: absence of IgG antibodies to SARS-CoV-2. The studied groups were classified by following the WHO guidelines (https://www.who.int/publications/i/item/WHO-2019-nCoV-clinical-2021-2; 23 November 2021). The study was conducted with the approval of the Local Ethics Committee of the Academy of Biology and Biotechnology of the Southern Federal University. All procedures performed in studies were in accordance with the ethical standards of the institutional research committee and with the Helsinki declaration (2013) and its later amendments or comparable ethical standards. Informed consents were obtained from all study participants.

2.2. DNA extraction and genotyping

Venous blood samples were collected and stored at −20 °C. Total genomic DNA was extracted using the RNA/DNA isolation kit RIBO-prep (FBSI Central Research Institute of Epidemiology, Rospotrebnadzor, Russia). The isolated DNA was quantitatively assessed using NanoDrop (Thermo Fisher Scientific, USA), and then optimized to have a concentration of 5 ng/μl. Genotyping of SNPs *PON1* Q192R (c.575A > G) (rs662) and *NOS3* (c.-786 T > C) (rs2070744) was conducted using SNP-express kits with a SYBR green qPCR reagent (Lytech. Co. Ltd., Russian Federation) and performed on QuantStudio™5 Real-Time PCR instrument (Thermo Fisher Scientific, USA). The cycling conditions were: predenaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 93 °C for 10 s, annealing at 60 °C for 10 s, and a terminal extension step of 72 °C for 20 s. The fluorescence yield was measured to obtain the allelic amplification plot and identify individual genotypes following PCR using QuantStudio™ Design & Analysis Software. Melt curve analysis was performed after running qPCR to ensure the sensitivity and specificity of the assay. Melting was performed from 60 to 95 °C at either 1.6 or 0.15 °C/s melt rates.

2.3. Statistical analysis

Student's *t*-test was used to compare different variables between studied groups. Continuous variables were expressed as mean (M) ± standard deviation (SD). To assess the differences in allelic variants distribution between studied groups, χ2-test was used and $p \leq 0.05$ was considered statistically significant. Odds Ratios (OR), indicated with 95 % confidence interval (CI), were calculated to evaluate the risk of COVID-19 severe outcome. Hardy-Weinberg Equilibrium (HWE) was tested by Fisher's exact test χ^2 analysis for a 2 × 2 table, and calculated using an online HWE calculator for 2 alleles https://gene-calc.pl/hardy-weinberg-page. Since both studied polymorphisms are for genes closely located on the same chromosome, linkage disequilibrium (LD) was performed using SNPStats https://www.snpstats.net/start.htm. The Lewontin (D') coefficient was calculated to evaluate the possibility of recombination. $D' = 1$ indicates complete LD, thus no evidence for recombination between the two sites, while $D' = 0$ indicates no LD. In our study, $D' > 0.90$ was considered as a strong LD. For the analysis of haplotype association with COVID-19 severity risk, the most common haplotype was selected as reference. Using SNPStats, OR and 95 % CI were calculated to estimate the degree of association between haplotypes and the risk of severe COVID-19. Multifactor Dimensionality Reduction (MDR) 3.0.2 software (Computational Genetics Laboratory, Institute for Quantitative Biomedical Sciences, Dartmouth, NH, USA) was used to study the possible SNP-SNP interactions and evaluate their relation to the risk of severe cases. The best model to predict the risk of severe COVID-19 was screened based on the minimum classification error in the training set. To evaluate the predictive accuracy, 10-fold cross-validation was applied. The SNP-SNP interaction shows a percentage of the entropy risk. A positive percentage refers to synergistic interaction, while a negative percentage refers to redundancy or antagonism.

3. Results

3.1. Study subject characteristics

No difference in the gender ratio between the studied groups, with more women (67.3 %) than men (32.7 %). Patients with severe symptoms were older (51.8 ± 8.8) as compared to the mild group (42.5 ± 12.9). Furthermore, a significant difference was observed in the ceruloplasmin levels (1.32 ± 0.39 in mild cases vs. 1.5 ± 0.37 in severe cases, $p = 0.048$). However, no such difference was noticed in the total peroxidase activity (TPA) ($p = 0.37$). All mild group members had a lung CT-scan category CT-1 (Pulmonary parenchymal involvement ≤25 %), while the severe group included CT-3 (50–75 %), and CT-4 (≥ 75 %) categories. The characteristics of the study subjects are presented in Table 1.

Table 1
Characteristics of study subjects.

Characteristics	Mild ($n = 55$)	Severe (n = 55)	*P* value
Age (years)	42.50 ± 12.90	51.80 ± 8.80	0.00002
Gender (n (female %/male %))	37 (67.30 %)/18 (32.70 %)	37 (67.30 %)/18 (32.70 %)	1
Ceruloplasmin (μM/L)	1.32 ± 0.39	1.50 ± 0.37	0.048
Total Peroxidase Activity (TPA) (units/ml)	1.29 ± 1.90	1.65 ± 1.70	0.370
Lung CT scan (CT category)	CT-1	CT-3, CT-4	

3.2. Association between COVID-19 severity and the studied polymorphisms

The *PON1* rs662 genotypes were detected and their genotype distributions were consistent with the Hardy-Weinberg equilibrium (HWE) ($p > 0.05$) in both mild ($p = 0.06$) and severe ($p = 0.087$) groups. The same was for the *NOS3* rs2070744 severe group ($p = 0.99$), but not for the mild group ($p = 0.005$). The deviation from the expected values based on HWE might be resulted from the small sample size. Other factors can have an effect also, such as genetic drift, specific for the studied population. Accordingly, it could be suggested that the frequency of the studied polymorphisms could be affected by genetic and demographic parameters of Rostov region population. Amplification plots of the studied alleles are presented in Fig. 1.

Genotypes frequencies for both studied polymorphisms showed a significant association with the severity of COVID-19. Genotypes AA (*PON1* rs662) and TT (*NOS3* rs2070744) were considered reference groups for the association studies. The *PON1* rs662 AG genotype was significantly more frequent in mild cases (58.2 %) than in severe cases (27.3 %), which means that most of AG genotype carriers had a mild course of COVID-19 (OR = 0.30; 95 % CI [0.13–0.68]). On the other hand, severe cases included more AA genotype carriers (60 %), compared to 38.2 % in mild group. Studying the genotypes of *NOS3* rs2070744 showed that TC genotype was more frequent in mild group (63.6 %) compared to (40 %) in severe group, thereby suggested to be associated with a mild course of infection (OR = 0.35; 95 % CI [0.15–0.78]), while the TT genotype frequency was more in patients with severe symptoms (52,7 %) than in the mild group of patients (29.1 %).

Thus, *PON1* rs662 AG and *NOS3* rs2070744 TC genotypes were significantly associated with a mild course of COVID-19. Genotypes and alleles frequencies of *PON1* rs662, and *NOS3* rs2070744 polymorphisms in both mild and severe groups of COVID-19 patients are presented in Table 2.

3.3. Gene–gene interactions

SNP-SNP interaction analysis was performed using MDR algorithm. A two-locus model of interaction between the studied polymorphisms was established. The resulted interaction model is statistically significant (p = 0.0003, OR = 10.06; 95 % CI [2.7–37.5]), with a training balanced accuracy of 75.7 %, and cross-validation consistency of 10/10 (Shown in Table 3).

Table 2
Genotype and allele frequencies of *PON1* (rs662) and *NOS3* (rs2070744) in mild and severe groups of COVID-19 patients.

Genotype/allele	Mild (n)	Severe (n)	*P* value	OR (95 % CI)
PON1 A575G (rs662)				
AA	21 (38.2 %)	33 (60 %)		Reference
AG	32 (58.2 %)	15 (27.3 %)	**0.0026**	**0.30 (0.13–0.68)**
GG	2 (3.6 %)	7 (12.7 %)		2.23 (0.42–11.76)
AG + GG	34 (61.8 %)	22 (40 %)	**0.022**	**0.41 (0.19–0.89)**
A	74 (67.3 %)	81 (73.6 %)	0.29	
G	36 (32.7 %)	29 (26.4 %)		0.73 (0.40–1.32)
NOS3 T786C (rs2070744)				
TT	16 (29.1 %)	29 (52.7 %)		Reference
TC	35 (63.6 %)	22 (40 %)	**0.033**	**0.35 (0.15–0.78)**
CC	4 (7.3 %)	4 (7.3 %)		0.55 (0.12–2.51)
TC + CC	39 (70.9 %)	26 (47.3 %)	**0.011**	**0.37 (0.17–0.81)**
T	67 (60.9 %)	80 (72.7 %)	**0.04**	
C	43 (39.1 %)	30 (27.3 %)		**0.52 (0.27–0.98)**

OR = Odds Ratio, CI = Confidence Interval, *p < 0.05.

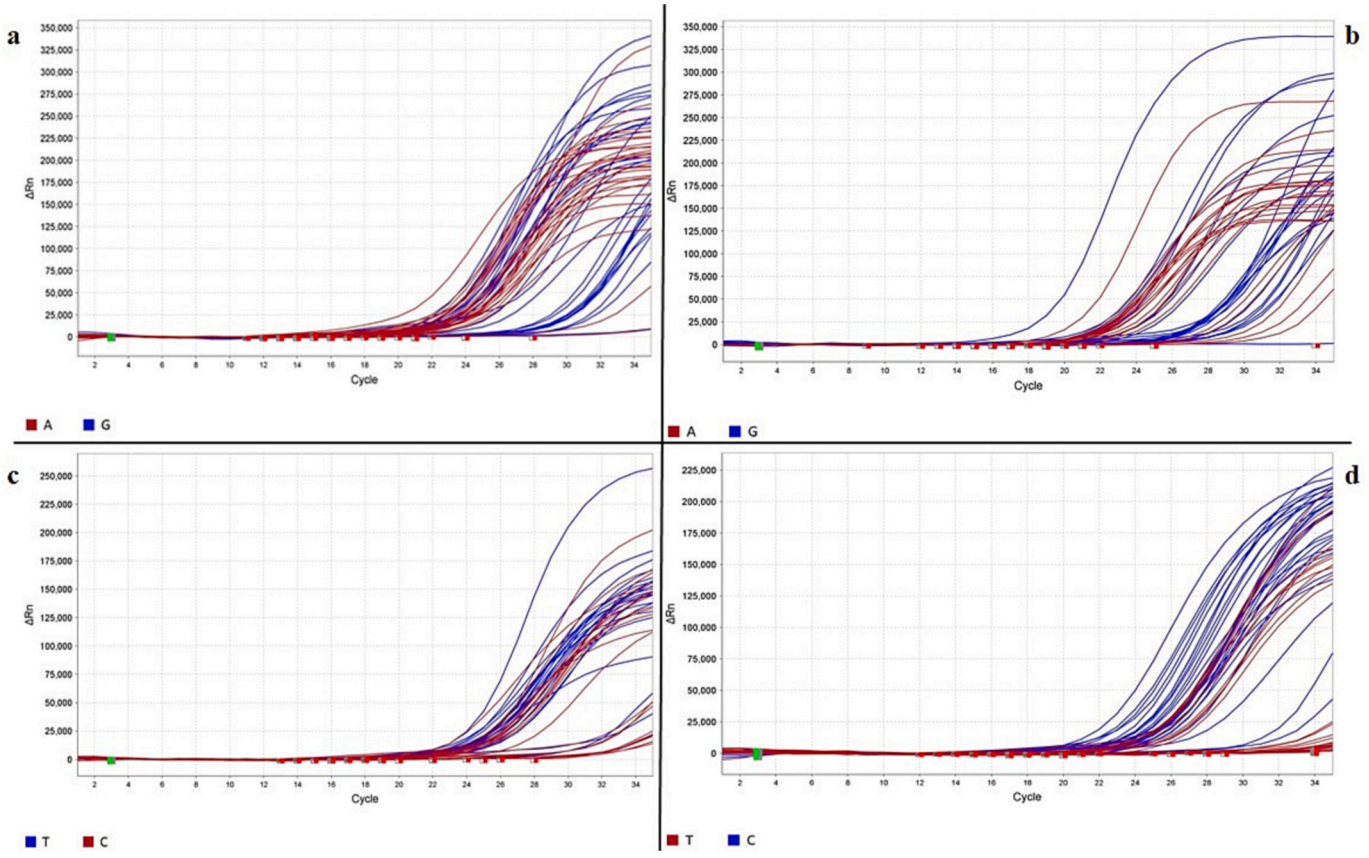

Fig. 1. Amplification plots of *PON1* rs662 and *NOS3* rs2070744 alleles. a) *PON1* rs662 in mild COVID-19 group, b) *PON1* rs662 in severe group, c) *NOS3* rs2070744 in mild group, d) *NOS3* rs2070744 in severe group.

Table 3
MDR analysis of gene-gene interactions.

Model	CV-consistency	Accuracy	Sensitivity	Specificity	OR (95 % CI)	*P value*
PON1 rs662 and *NOS3* rs2070744	10/10	76 %	76.1 %	75.8 %	10.06 (2.7–37.5)	**0.0003***

OR = Odds Ratio, CI = Confidence Interval, *$p < 0.05$.

Data-analysis indicated that individuals with homozygous *PON1* AA and *NOS3* TT genotypes have a significant 2.3-fold higher risk of severe COVID-19 outcome. However, a notable lower risk of severity (group ratio < 1) was observed in individuals with heterozygous *PON1* AG and *NOS3* TC genotypes. Graphical representation of these findings is shown in Fig. 2.

According to the Fruchterman-Rheingold graph (Fig. 3), a high level of redundancy (−1.63 %) was noticed. The independent effect of *PON1* rs662 was higher than that of *NOS3* rs2070744 (7.82 % and 4.46 %, respectively).

3.4. LD and haplotypes association with COVID-19 severity

The targeted genes *PON1* and *NOS3* are located on the same chromosome (7q21.3 and 7q36.1, respectively). Therefore, linkage disequilibrium analysis was performed to assess the correlation of the studied genetic variants. LD coefficient $D' > 0.9$ was considered as an evidence of high LD possibility. Our analysis showed no evidence for LD ($D' = 0.1532$, $p = 0.299$), which indicated a high possibility of recombination between those two sites.

Next, we performed a haplotype association test. All possible haplotypes: TA, CA, TG, and CG, were with a frequency of >1 %, where the TA was the reference haplotype. The CG haplotype was more frequent within the mild group (OR = 0.25, 95 % CI [0.06–0.96], $p = 0.045$), thus it can be suggested as associated with lower risk of severe COVID-19 outcome. Haplotype association test results are shown in Table 4.

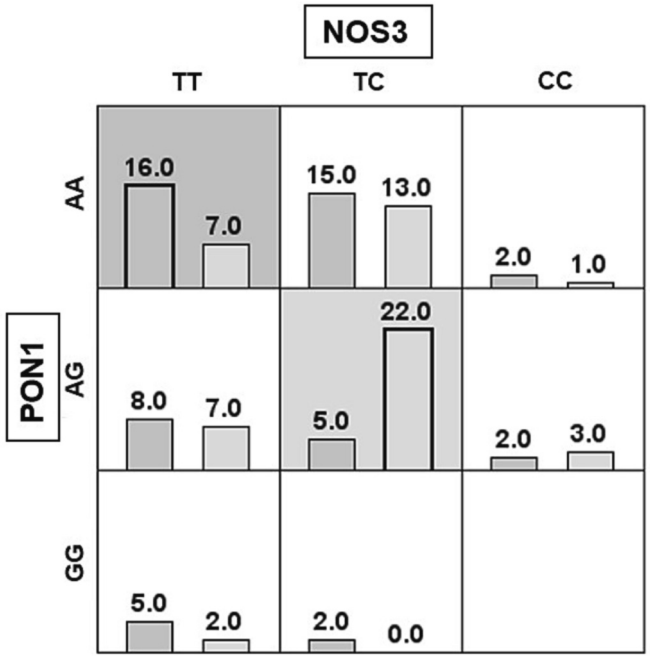

Fig. 2. Multifactor dimensionality reduction (MDR) analysis. The summary of the two-factor model (*NOS3* and *PON1*). Dark and light backgrounds were representative of high-risk and low-risk genotype combinations respectively. Patients with severe COVID-19 cases are represented by the left column, whereas the right column represents the mild cases.

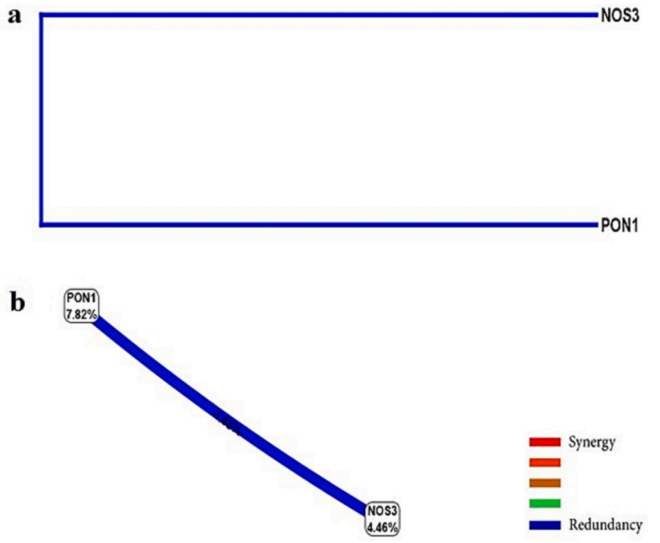

Fig. 3. Analysis of gene-gene interaction (MDR). a) Dendrogram, b) Fruchterman-Rheingold graph. Entropy values (%) in the cells indicates the independent effect of each studied polymorphism, and the values on the connecting line indicates the interaction entropy. The blue line represents a high level of redundancy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Haplotype analysis on association of *NOS3* T786C and *PON1* A575G with COVID-19 severity.

Haplotype (Alleles)	Frequencies			OR (95%CI)	*P value*
	Total	Mild	Severe		
NOS3 786T * *PON1* 575A	0.4558	0.3947	0.5170	1	
NOS3 786C * *PON1* 575A	0.2487	0.2780	0.2193	0.63 (0.24–1.67)	0.36
NOS3 786T * *PON1* 575G	0.2124	0.2144	0.2103	0.84 (0.63–1.98)	0.69
NOS3 786C * *PON1* 575G	0.0831	0.1129	0.0534	**0.25 (0.06–0.96)**	**0.045***

OR = Odds Ratio, CI = Confidence Interval, *$p < 0.05$.

4. Discussion

The COVID-19 pandemic had a serious influence on the aspects of everyone's life, including health care, environment, economy, education and many other affected fields. It even changed the landscape of science. Scientists around the globe dedicated themselves and made remarkable efforts to study the novel disease, and better understand its pathogenesis and treatment options. As of other earlier known viral infections, oxidative stress was considered as a key player in the pathogenesis of SARS-CoV-2 infection (Delgado-Roche and Mesta, 2020). Previous studies suggested that ROS overproduction and a deprived antioxidant system are crucial risk factors of several pathological conditions, including neurodegenerative, cardiovascular, and infectious diseases (Preiser, 2012).

PON1 gene is located on chromosome 7q21.3, and has 9 exons in its coding region (Vavlukis et al., 2022). Q192R rs662 (c.575A > G) is one

of the most studied *PON1* single nucleotide polymorphisms (SNPs). It has been reported to be associated with cardiovascular diseases, including coronary artery disease (Liu et al., 2014), and carotid atherosclerosis (Gnasso et al., 2002). In addition, several studies assessed the association of *PON1* rs662 with different types of cancer (Pan et al., 2019). In a recent study, the associations of *PON1* polymorphisms L55M (rs854560) and Q192R (rs662) with the prevalence and mortality of COVID-19 were investigated in different countries, and while the M55 frequency was positively correlated with both morbidity and mortality, allelic frequency of *PON1* R192 showed no significant association (Saadat, 2021).

NOS3 gene is located on chromosome 7q36.1 and has 26 exons (Rai et al., 2014). *NOS3* (c.-786 T > C) point mutation has been shown to be associated with the risk of hypertension (Hyndman et al., 2002), myocardial infarction (Nakayama et al., 2000), cancer (Zhang et al., 2014), and fetal growth restriction (Novikova et al., 2021).

In this study, the associations of *PON1* rs662 and *NOS3* rs2070744 with the COVID-19 intensity were investigated. Our results showed that the AG genotype of *PON1* rs662 was significantly associated with a mild COVID-19 course. Dominant model AG + GG was also associated with mild symptoms of the infection. This can be explained by the fact that, the polymorphism causes a glutamine/arginine replacement at position 192 of the amino acid sequence, which is linked to an increased enzymatic activity (Sikora et al., 2020). Furthermore, previous studies showed that PON1 protein levels were lower in severe COVID-19 patients, in comparison with non-severe group. The abundance of PON1 significantly decreased as the disease progressed to severe (Lee et al., 2021). *NOS3* rs2070744 TC genotype was notably more frequent in mild group. The same with the dominant TC + CC model, and the C allele. It's known that *NOS3* rs2070744 is located in the promoter, reducing its activity by about 50 %. Therefore, it leads to a decreased eNOS (NOS3) function, which causes a reduced NO synthesis (Rai et al., 2014). NO has been shown to play a protective role in cell death or tissue damage induced by oxidative stress (Wink et al., 2001). However, it was suggested that modestly increased levels of NOS3 may enhance sensitivity to oxidative stress by impairing the integrity of the cell membrane (de la Monte et al., 2003). eNOS uncoupling and NO levels disruption are related to the development of ARDS (Kellner et al., 2017). It should be mentioned that, in spite of the known protective role of NO in viral infections, it may also contribute in the immunopathology of COVID-19 (Guimarães et al., 2021).

The performed MDR analysis confirmed the results of genotype association, by showing that the carriers of *PON1* 575AA * *NOS3* 786TT had a significantly higher risk of having a severe COVID-19 course. Furthermore, *PON1* 575AG * *NOS3* 786TC interaction was associated with a lower risk of COVID-19 severity, in accordance with genotyping data presented in Table 2.

Haplotype association analysis indicated that *NOS3* 786C * *PON1* 575G haplotype was significantly associated with the mild case of COVID-19. This suggests that the studied gene variations could have a protective role against the severe complications of COVID-19 infection. Biochemical analysis of the studied enzymes, and a larger group of both severe and mild patients should be studied further to confirm our findings.

5. Conclusion

To our knowledge, this is the first study to show the association of both *PON1* rs662 and *NOS3* rs2070744 with the COVID-19 intensity, and to investigate the possible impact of SNP-SNP interaction on the severity of symptoms. The results may serve as a novel additional prognostic factor of COVID-19 infection.

CRediT authorship contribution statement

Moez A. Eid: Methodology, Formal analysis, Writing – review & editing, **Anzhela A. Aleksandrova**: Methodology, Formal analysis, **Mikhail A. Shkurat**: Formal analysis, Samples collection and diagnosis, **Tatiana P. Shkurat**: Supervision, resources, funding acquisition.

Declaration of competing interest

The authors declare no conflict of interests.

Data availability

No data was used for the research described in the article.

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