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NOS3 (rs61722009) gene variants testing in prediction of COVID-19 pneumonia severity

Liliia Fishchuk [a,*], Zoia Rossokha [b], Valeriy Pokhylko [c], Yuliia Cherniavska [c], Olha Dubitska [b], Viktoriia Vershyhora [b], Svitlana Tsvirenko [c], Serhii Kovtun [d], Nataliia Gorovenko [a]

[a] Department of Genetic Diagnostics, State Institute of Genetic and Regenerative Medicine of the National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine
[b] State Institution «Reference-centre for Molecular Diagnostic of Public Health Ministry of Ukraine», Kyiv, Ukraine
[c] Department of Pediatrics №1 with Propedeutics and Neonatology, Poltava State Medical University, Poltava, Ukraine
[d] Poltava Regional Clinical Infectious Diseases Hospital of Poltava Regional Council, Poltava, Ukraine

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ABSTRACT

Background: There is a hypothesis that a sufficient level of endothelial nitric oxide synthase is important for reliable protection against COVID-19. Theoretical ideas about the *NOS3* gene demonstrated that it can have an effect on links of the complications pathogenesis in COVID-associated pneumonia. We determined the goal – to investigate the association of the *NOS3* gene variants with the occurrence of the disease and its clinical course in patients of the intensive care unit.

Methods: The study group included 117 patients with a diagnosis of severe "viral COVID-19 pneumonia". Determination of *NOS3* gene variants was performed using the PCR method. The probability of differences in the quantitative results were determined using ANOVA or Kruskal-Wallis test (depend of normality of studied parameters).

Results: Our results indicate that the presence of the *NOS3* gene 4a allele increase the risk of complicated COVID-19-associated pneumonia ($\chi^2 = 18.84$, $p = 0.00001$, OR = 3.53 (1.95–6.39)). It was showed, that carriers of the 4aa genotype had a significantly higher ratio of SpO_2/FiO_2 on the first and second days after hospitalization ($p = 0.017$ and $p = 0.03$, respectively). Patients with the 4aa genotype also had the acid-base imbalances, as showed by indicators of base deficiency and standard bicarbonate, which were beyond the reference values. Potassium and sodium concentrations on the first and second day after hospitalization were also significantly lower in patients with 4aa genotype ($p = 0.009$ and $p = 0.048$, respectively), for whom, in the same time, the concentrations of C-reactive protein and total bilirubin were significantly higher ($p = 0.002$ and $p = 0.033$, respectively).

Conclusions: Our results confirmed that the rs61722009 variant of the *NOS3* gene is associated with an increased risk of severe COVID-19-associated pneumonia and its adverse clinical course with potential progression of kidney and liver damage, and occurrence risk of systemic inflammatory response syndrome. These results require further research for the new metabolic strategy formation, in order to prevent the severe COVID-19 associated pneumonia and its complications.

1. Introduction

The emergence and rapid spread of SARS-COV-2 has become a challenge for global medicine. The medical systems of both economically developed and low-income countries faced some problems in the provision of medical care, which were associated with a high incidence of pneumonia in COVID-19 patients and its complications. Many theories were put forward for the pathogenetic explanation of this process, among which cytokine/bradykinin storm and microthrombosis were the most important [1,2]. These theories were constantly compared with

Abbreviations: ARDS, acute respiratory distress syndrome; eNOS, endothelial nitric oxide synthase; MLV, mechanical lung ventilation; SIRS, systemic inflammatory response syndrome.

* Corresponding author. Department of Genetic Diagnostics, State Institute of Genetic and Regenerative Medicine of the National Academy of Medical Sciences of Ukraine, Vyshgorodska Street, 67, 04114, Kyiv, Ukraine.
E-mail address: medgen@ukr.net (L. Fishchuk).

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each other. Attention was immediately drawn to the population differences in morbidity and mortality, as well as to the virus strains and host genetics. Host genetics theoretically explained population differences in mortality and clinical features of the course of this infectious disease. But the first own practical studies changed our theoretical ideas about the influence of genetics in the population on the risk of developing COVID-19, its complications and mortality. Thus, previous studies refuted the influence of the *ACE* gene on the increased risk of infection and the severe course of this disease, and revealed a significant influence of variants of the *TNF-α* and *VDR* genes [3,4]. Continuing the study of the genetics of the host, we paid attention to the prospects of determining the 4a/4b variants of the *NOS3* gene (27 bp-VNTR in intron 4, rs61722009), for which the impact on mortality from COVID-19 rates was theoretically calculated, taking into account the distribution of the *NOS3* WT haplotype (T-4b-Glu) [5]. The above study found a negative correlation between COVID-19 mortality per 100000 people when compared to the percentage of *NOS3* gene WT haplotype. The authors suggested that this finding may explain the difference in global mortality among populations on different continents. It was especially striking that the death rate in India was significantly lower than in the US, despite the less access to health care resources among infected individuals. The authors associated this exceptional effect with the hypothesis that a sufficient level of endothelial nitric oxide synthase (eNOS, or nitric oxide synthase 3 – NOS3) is important for reliable protection against COVID-19 [5].

Theoretical ideas about the *NOS3* gene demonstrated that it can have an effect on all three mentioned links of the complications pathogenesis in COVID-associated pneumonia. The activity of eNOS can be regulated by several factors, one of which is bradykinin, in particular, through its action on receptors located on the membrane of endothelial cells by increasing the intracellular concentration of Ca^{2+}, which binds to calmodulin and activates the calmodulin-binding domain of eNOS [6]. On the other hand, it is known about overproduction of bradykinin during COVID-19, which in turn, leads to the development of a bradykinin storm [7]. Bradykinin storm is at the core of many disparate symptoms and problems of COVID-19, like dry cough, gustatory and olfactory dysfunctions, increases in vascular permeability and vascular leakage, fluid accumulation in tissues and organs, abnormal coagulation, impair oxygen transfer from lungs to the blood, etc. [7,8].

Taking into account the potential pathophysiological role of variants of the *NOS3* gene as a link of the bradykinin storm in the development of severe COVID-associated pneumonia, we determined the goal – to investigate the association of the *NOS3* gene polymorphism with the occurrence of the disease and its clinical course in patients of the intensive care unit.

2. Methods

2.1. Study subjects

This study included 117 patients with a complicated course of COVID-19-associate pneumonia, who were hospitalized in the intensive care unit of the Municipal enterprise "Poltava Regional Clinical Infectious Diseases Hospital of the Poltava Regional Council" (from December 2020 to December 2021).

The patients underwent a standard clinical and laboratory examination at the beginning of hospitalization and during treatment in the department, in accordance with the national protocol. All of them received respiratory support – an oxygen mask with positive expiratory pressure, and when the condition worsened, they required mechanical lung ventilation (MLV). Among the examined patients, deterioration of the condition and the need for transfer to MLV was observed in 56.4%. The clinical characteristics of the patients are shown in Table 1.

Patients had a history of the following diseases: 73.5% – cardiovascular, 17.1% – type 1 and 2 diabetes, 10.3% – oncology, 4.3% – tuberculosis.

Table 1
Clinical characteristics of patients on the first day of hospitalization.

Indicators		COVID-19 patients (n = 117)
Sex	male	65 (55.6%)
	female	52 (44.4%)
Age, years		62.6 ± 13.8
BMI, kg/m^2		31.3 ± 7.1
Axillary temperature,°C		37.6 ± 0.7
Respiratory rate, per minute		23.7 ± 2.3
SpO_2/FiO_2		130.2 ± 68.6
SOFA scale score[a], in points		2 [2–2]
Glasgow coma scale[a], in points		8 [7–10]

[a] Values are presented as the median [25th–75th percentile].

2.2. Molecular genetic studies

Isolation of DNA from leukocytes of peripheral blood was carried out using a set of reagents "Quick-DNAUniversalKit" (ZymoResearch, USA).

Determination of genotypes for the variant rs 61722009 of the *NOS3* gene was carried out using the polymerase chain reaction method according to the previously described protocols [9].

2.3. Statistical methods

SPSS v.27 software package was used for statistical analyses. To compare the frequency of genotypes among the groups we used descriptive statistics and computation of Pearson's χ2 criteria. Odds ratios (OR) with 95% confidence intervals (95% CI) were used to evaluate the size and strength of relationship between genotype and the risk of developing the disease. Studied parameters were checked for normal distribution using the Kolmogorov-Smirnov test. Then, the probability of differences in the quantitative results were determined using ANOVA or Kruskal-Wallis test followed by post-hoc analysis with the Bonferroni correction. Differences were considered significant at $p < 0.05$.

3. Results

Genotype frequencies for the rs61722009 variant of the *NOS3* gene in the group of patients with complicated course of COVID-19-associated pneumonia were determined (Table 2). Since no large-scale studies were conducted in Ukraine, the frequencies detected in the work of Tsymbaliuk et al. were used as a comparison group [10]. These frequencies were determined for unrelated healthy individuals, who lived on the territory of Ukraine and belonged to the Caucasian ethnic group. The comparison group consisted of 62 men and 40 women (average age – 54.5 ± 8.2 years).

The results of the genotypes comparison indicate that the presence of the 4a allele of the rs61722009 variant of the *NOS3* gene is associated with a 3.5-fold increase in the risk of complicated COVID-19 associated pneumonia. The presence of 4b allele (in particular, in the homozygous state) was, on the contrary, associated with a protective effect regarding the risk of developing this pathological condition.

The impact of rs61722009 variant of the *NOS3* gene on the risk of fatal consequences and deterioration of the patients with severe COVID-19 associated pneumonia was analyzed (Table 3).

As the results of the analysis showed, no statistically significant difference was found in the distribution of genotypes in subgroups of patients with and without complications. Therefore, the studied variant of the *NOS3* gene does not affect the risk of complications in patients with severe COVID-19-associated pneumonia.

If we compare the determined frequencies with those for the comparison group, then for carriers of the 4b allele in the homo- or heterozygous condition, a protective effect could be determined, regarding the risk of MLV use (for 4bb – $\chi^2 = 14.55$, $p = 0.0001$, OR = 0.24 (0.12–0.52); for 4ab – $\chi^2 = 12.95$, $p = 0.00003$, OR = 0.25 (0.11–0.55)).

For the variants of the *NOS3* gene, the relationship between the

Table 2
Comparison of genotype frequencies according to the rs 61722009 variant of the *NOS3* gene.

Gene	Genotype, allele	COVID-19 patients (n = 117)	Comparison group (n = 102)	Results of statistical analysis
NOS3 rs61722009	4bb	71 (60.7%)	88 (86.3%)	$\chi^2 = 17.94$, p = 0.00001, OR = 0.25 (0.13–0.48)
	4ba	38 (32.5%)	12 (11.8%)	$\chi^2 = 13.27$, p = 0.0003, OR = 3.61 (1.76–7.38)
	4aa	8 (6.8%)	2 (2.0%)	p > 0.05
	4b	0.77	0.92	$\chi^2 = 18.84$, p = 0.00001, OR = 0.28 (0.16–0.51)
	4a	0.23	0.08	$\chi^2 = 18.84$, p = 0.00001, OR = 3.53 (1.95–6.39)

Table 3
Effect of the rs61722009 variant of the *NOS3* gene on the risk of a complicated course of COVID-19 associated pneumonia.

Parameter (complication)		*NOS3* rs61722009			Results of the statistical analysis
		4bb	4ba	4aa	
Death	Yes	45 (66.2%)	20 (29.4%)	3 (4.4%)	p > 0.05
	No	26 (53.1%)	18 (36.7%)	5 (10.2%)	
ARDS	Yes	16 (76.2%)	4 (19.0%)	1 (4.8%)	p > 0.05
	No	55 (57.3%)	34 (35.4%)	7 (7.3%)	
SIRS	Yes	18 (69.2%)	6 (23.1%)	2 (7.7%)	p > 0.05
	No	53 (58.2%)	32 (35.2%)	6 (6.6%)	
MLV	Yes	40 (60.6%)	23 (34.8%)	3 (4.5%)	p > 0.05
	No	31 (60.8%)	15 (29.4%)	5 (9.8%)	

ARDS – acute respiratory distress syndrome.
SIRS – systemic inflammatory response syndrome.
MLV – mechanical lung ventilation.

determined genotypes and clinical and laboratory indicators in examined patients with severe COVID-19 associated pneumonia at the moment of hospitalization and during the treatment in the department was analyzed (Table 4).

As the results of our study showed, carriers of the 4aa genotype had a significantly higher ratio of SpO_2/FiO_2 on the first and second days after hospitalization. Patients with the 4aa genotype also had the acid-base imbalances, as indicated by indicators of base deficiency and standard bicarbonate, which were beyond the reference values. Potassium and sodium concentrations on the first and second day after hospitalization were also significantly lower in patients with 4aa genotype, for whom, in the same time, the concentrations of C-reactive protein and total bilirubin were significantly higher.

Table 4
Effect of the studied variant of *NOS3* gene on clinical parameters in patients with COVID-19.

Parameters	*NOS3* rs61722009			Results of the statistical analysis
	4bb	4ba	4aa	
SpO_2/FiO_2 1d	126.9 ± 65.6	116.5 ± 40.7	220.1 ± 122.3	p = 0.017
SpO_2/FiO_2 2d	157.2 ± 106.1	124.0 ± 40.7	285. ±157.9	p = 0.03
BE 1d, mmol/l	2.5 ± 3.7	5.8 ± 8.0	−8.7 ± 2.1	p = 0.037
SBC 2d, mmol/l	26.1 ± 3.1	24.4 ± 4.7	13.9 ± 2.8	p = 0.044
K 1d, mmol/l	4.6 ± 0.8	4.2 ± 0.7	3.4 ± 0.5	p = 0.009
Na 2d, mmol/l	143.3 ± 6.7	143.7 ± 6.5	136.7 ± 2.5	p = 0.048
CRP 2d, mg/l	21.9 ± 12.6	32.1 ± 15.9	74.6 ± 8.2	p = 0.002
Bilirubin total 1d, μmol/l	17.9 ± 28.4	17.6 ± 24.5	21.4 ± 12.0	p = 0.033

1d, 2d – day after hospitalization.
BE – basis excess.
SBC– standard bicarbonate.
CRP – C-reactive protein.

4. Discussion

eNOS is an enzyme that catalyzes the oxidation of L-arginine in the presence of O2 (as a co-substrate) and cofactors NADPH, 6(R)-5,6,7,8-tetrahydrobiopterin (BH4), FAD and FMN to L-citrulline NO [11]. Since the formation of NO is a tightly regulated process, pathophysiological conditions that cause a deficiency of any of these cofactors can lead to the formation of superoxide and its derivatives, and as a result, an increase in oxidative stress [11]. Therefore, the prevention of deficiency, in particular, of L-arginine BH4, may be a promising direction of therapy, regarding the regulation of NO metabolism. This enzyme is constitutive and is expressed in various types of cells, including endothelial cells, platelets, T-cells, hippocampal neurons, some epithelial cells (in particular, lung, gut), etc. [12].

eNOS activity is determined by the *NOS3* gene, which is localized on the long arm of the 7th chromosome at positions 35–36 (7q35-q36). One of the most studied variants of the *NOS3* gene is tandem repeats in intron 4. Allele variant 4b ("wild type") contains 5 tandem 27 bp-repeats, and variant 4a – 4 tandem repeats. In a study involving 413 healthy individuals, it was determined that the average level of NO in carriers of the 4aa genotype was almost 20% lower than in individuals with the 4b allele [13]. There are several explanations for the effect of the rs61722009 variant on the functioning of the *NOS3* gene. Some researchers indicate that this intronic variant can affect the transcriptional activity of the *NOS3* gene. In particular, Wang et al. (2002) demonstrated the *cis*-acting role of the 27-bp repeats in *NOS3* promoter function [14]. Another research group indicates, that the presence of this variant reduces mRNA *NOS3* expression and enzyme levels by changing histone acetylation and DNA methylation in regions adjacent to the 27-bp repeat element and core promoter [15].

It is also well known, that NO, which is produced by eNOS, is an important element of antiviral protection [16]. Therefore, variants of the *NOS3* gene that modulate NO concentration at the individual level in viral diseases are influential factors in maintaining the condition of the bronchial epithelium, endothelium of respiratory tract vessels and the immune response formation. This fact is supported by the results of our study, as it was identified an increase in the risk of COVID-19-associated pneumonia, and, contrary, – a protective effect, for different variants of this gene, depending on the number of tandem repeats.

In our study, the association of the 4a allele with the risk of severe COVID-19-associated pneumonia was determined. In contrast, in the study of Pehlivan et al. no significant difference in the distribution of genotypes for the rs61722009 variant among patients diagnosed with COVID-19 (all patients had typical COVID-19 lung involvement in their initial CT) and healthy controls was found [17].

As it was noted earlier in the Guan et al. study, a significant negative correlation was determined between variants of the *NOS3* gene and mortality from COVID-19 [5]. Our results did not confirm the existence of associations between the presence of certain genotypes of the *NOS3* gene and the risk of complications, including death, in patients with COVID-19.

The results, we obtained, turned out to be interesting, regarding a higher SpO_2/FiO_2 indicator in carriers of the 4aa genotype. After all, a high SpO_2/FiO_2 ratio indicates the lower risk of acute respiratory distress syndrome and mortality in patients with COVID-19 [18,19]. It can be assumed, based indirectly on the data of Vaporidi et al. and Liu et al. [20,21], that the presence of eNOS deficiency (it should be reminded, that 4aa genotype is associated with a decrease in eNOS activity) in patients with lung damage can be a "good" factor. The authors explain this positive effect by reducing the production of superoxide, which increases vascular dysfunction.

In patients of our study with genotype 4aa, a decrease in the levels of potassium, magnesium and acid-base balance was determined, in comparison with patients, having a different genotype. This indirectly suggests that the rs61722009 variant of the *NOS3* gene is associated with renal dysfunction in patients with severe COVID-19-associated pneumonia. It should be noted that the presence of the 4a allele may be related to kidney dysfunction and other pathological conditions. Thus, there are data on the association of the rs61722009 variant of the *NOS3* gene with the progression of nephropathy in patients with diabetes and Fabry disease, an increased risk of end-stage renal failure in patients with autosomal polycystic kidney disease [15,22,23].

Higher levels of total bilirubin and C-reactive protein in patients with the 4aa genotype, compared to patients with 4bb and 4ba genotypes, potentially can indicate the presence of liver failure. Most likely, this effect is related to the fact that carriers of the 4aa genotype have a lower NO level [13]. It is well known, that NO synthesized by eNOS in liver sinusoidal endothelial cells has protective properties against the development of liver diseases [24].

Changes in the electrolyte balance and C-reactive protein can be regarded as a manifestation of systemic inflammatory response syndrome with potential activation of disseminated intravascular coagulation in the form of microthrombosis [25,26]. In our study, in contrast to the previously published data, we did not find any influence of the investigated gene variants on the increase in mortality rates, but in carriers of the 4bb and 4ba genotypes a reduction in the need for mechanical ventilation was determined. The revealed features indicate the possibility of developing new approaches to the prevention of a severe course of COVID-19-associated pneumonia by maintaining of NO concentration and ensuring its optimal metabolism.

In conclusion, our results confirmed that the rs61722009 variant of the *NOS3* gene is associated with an increased risk of severe COVID-19 associated pneumonia and its adverse clinical course with potential progression of kidney and liver damage, and occurrence risk of systemic inflammatory response syndrome. Further research in this direction is necessary for the new metabolic strategy formation, in order to prevent the severe COVID-19 associated pneumonia and its complications.

Ethics approval and consent to participate

Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with The Code of Ethics of the World Medical Association, and has been approved by the Ethics Committee of the Ukrainian Medical Stomatological Academy (protocol No. 188 of November 25, 2020). Informed consent was obtained from all individuals included in this study.

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Declaration of competing interest

The authors declare that they have no potential conficts of interests.

Data availability

Data will be made available on request.

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