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Research Article

Laboratory and genetic predictors for severe COVID-19 infection

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Abstract

This study aims to identify laboratory and genetic markers important for COVID-19 severity to improve patient assessment and treatment. COVID-19 patients were divided into two groups based on disease severity. Clinical, laboratory (complete blood count, complete biochemical parameters - lactate dehydrogenase (LDH), serum ferritin), and genetic markers (*OAS1* rs4767027) were analyzed. A total of 61 COVID-19 patients and 48 negative controls were investigated. Group I showed more often lymphopenia – 3.16 (1.39–3.89) vs 5.61(4.21–7.98), p-0.027 and thrombocytopenia – 165 (75–256) vs 212 (198–349), p-0.031, higher LDH (621 ± 218 U/L vs 312 ± 110 U/L), p-0.014. OAS1 rs4767027 genotype and allele frequencies did not differ significantly from worldwide population frequencies. Lymphopenia and thrombocytopenia are likely associated with immune inflammation and COVID-19 severity. While increased OAS1 transcript levels are correlated with reduced risk of infection, they can contribute to NLRP3 inflammasome activation once the infection has been established.

Keywords

COVID-19, OAS1, laboratory and genetic predictors

Introduction

The COVID-19 outbreak was declared a pandemic by the WHO in February 2020 (Phelan et al. 2020; Singhal 2020; Zhu et al. 2020). COVID-19 pneumonia was associated with dyspnea, fever, dry cough, fatigue, and myalgia. Most of the patients had a mild-to-moderate clinical course, for whom standard treatment protocols proved effective for treatment. However, up to 14% of cases developed severe pneumonia with acute respiratory distress syndrome (ARDS) and multiple organ failure (Wu and McGoogan 2020; AlOtaibi et al. 2021). Fatal disease outcome was recorded in between 1 and 15% of the cases (Chen et al. 2020). The major challenge in treating severe COVID-19 patients is the rapid disease detection and accurate monitoring of symptom progression; for this reason, a major goal of current studies is the search for clinical and laboratory predictors for the severity of COVID-19. Previous reports indicate old age, obesity, cardiovascular comorbidity, and diabetes mellitus as predictors for the severe course of the disease (Zhou et al. 2020). Laboratory biomarkers revealed from investigations such as CRP,

serum ferritin, D-dimer, platelets count and mean platelets volume, and cardiac enzymes have also been reported as indicators of severe COVID (Korean Society of Infectious Diseases and Korea Centers for Disease Control and Prevention 2020). Despite numerous studies, results regarding predictive clinical parameters are controversial, in part due to the involvement of genetic and autoimmune factors.

One of the main causes of COVID-19 mortality is the cytokine storm (CS) leading to the severe symptoms of ARDS, multi-organ failure and death (Hojyo et al. 2020; Mehta et al. 2020; Ruan et al. 2020). The reason for the CS is the aberrant activation of the Nod-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome (Vitiello et al. 2021). Why the NLRP3 inflammasome responds abnormally to the virus in some people remains the biggest mystery.

One of the largest genetic studies focused on COVID-19 susceptibility and severity, consisting of 14,134 COVID-19 cases and 1.2 million controls of European ancestry, discovered a promising protective association (Zhou et al. 2021). Results from this investigation showed that increased plasma OAS1 (2'-5'-oligoadenylate synthetase 1) levels in the non-infectious state correlate to reduced risk of severe forms of the disease, of approximately 50% decrease in the odds of COVID-19 severity per standard deviation increase in OAS1 plasma levels. Furthermore, this protective association was shown to be in part due to a Neanderthal isoform of the OAS1, tagged by the T allele of rs4767027 (Zhou et al. 2021).

The interferon-induced 2'-5'-Oligoadenylate Synthetase gene encodes a protein (OAS1) that synthesizes a unique oligonucleotide second messenger, 2',5'-oligoadenylates (2–5A). 2–5A then binds to the inactive Ribonuclease-Latent (RNase L) monomer, forming an active dimer, which degrades cellular and viral RNA, thus reducing the viral replication (load) (Fig. 1). The family of OAS proteins are a part of innate immunity and are thus assumed to have a plausible biological activity against SARS-CoV-2. However, there is no direct relationship between viral load and the hyperactivation of the NLRP3 inflammasome, leading to CS and tissue injury (Freeman and Swartz 2020; Fu et al. 2020; Fung et al. 2020; Mehta et al. 2020; Zhang and Ma 2020; Zhao et al. 2020, 2021). This is also the reason why antiviral medications recommended by the WHO have partial success (Mitev 2023).

Further genetic studies have shown that the Neanderthal isoform of OAS1 in individuals of European ancestry is protective for SARSCoV2 and attenuates the risk of COVID-19 death or ventilation (Mendez et al. 2013; Zeberg and Pääbo 2021).

This study aims to determine whether standard clinical markers and comorbidities such as arterial hypertension, diabetes, dyslipidemia, ischemic heart disease, heart failure, cerebrovascular disease, chronic kidney disease, and obesity can be used to predict COVID-19 severity. A secondary aim was to establish whether the protective effect of the rs4767027 T allele can be observed in a small patient cohort and on an individual level.

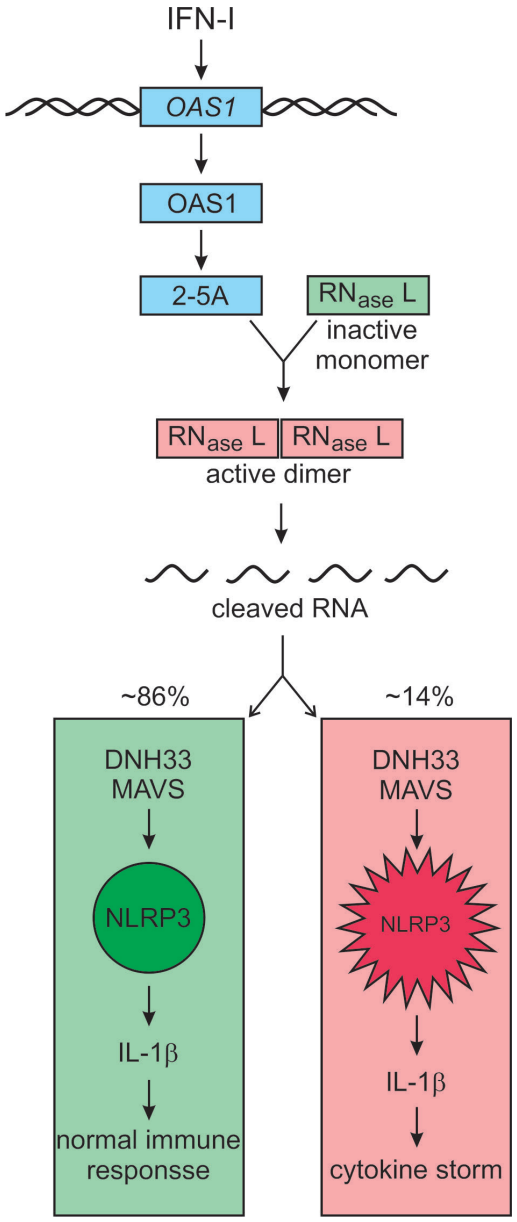

Figure 1. The OAS–RNase L pathway and the NLRP3 inflammasome activation. DsRNAs and IFN signaling activate the OAS gene. OAS1 induces the synthesis of the second messenger 2–5A from ATP. 2–5A then binds and transform the RNase L monomer to its active dimer formation. The active RNase L degrades viral RNA, thus inhibiting virus replication. Cleaved RNA, through DHX33 (DEAH-box helicase 33), which binds to cytosolic RNAs and interacts with MAVS (Mitochondrial Antiviral Signaling protein), can activate NLRP3 inflammasome. In most cases, this stimulates the normal anti-viral response, but theoretically, further stimulation of the already hyperactivated NLRP3 inflammasome would intensify the CS.

Patients and methods

This is an observational cross-sectional study. A total of 61 patients with COVID-19 and 48 controls, that have been hospitalized at the University Hospital for Respiratory Diseases "Ivan Rilski" were included in the study. Informed consent was obtained from all subjects involved.

The COVID-19 group of patients were admitted during February – April 2022 and the control group was formed after the end of the pandemic in June – July 2022. The subjects from the control group have been admitted for an exacerbation of chronic respiratory diseases. They were PCR-negative for SARSCOV2 and did not require intensive care treatment. The study was approved by the Ethics Committee.

COVID-19 patients were confirmed by real-time polymerase chain reaction. The patients were divided into two groups: group II – mild/moderate cases and group I - severe/critical cases. The severity of COVID-19 was considered: (1) mild: mild symptoms, no pneumonia on CT; (2) moderate: fever, cough, and CT pneumonia; (3) severe: respiratory distress (respiratory rate > 30/min, oxygen saturation (O2Sat) ≤ 93% at rest and/or ratio of arterial oxygen partial pressure to fractional inspired oxygen ≤ 300 mmHg (PaO2/FIO2); and (4) critical: respiratory failure receiving mechanical ventilation, shock, and/or organ failure.

All clinical symptoms including fever, cough, dyspnea, loss of smell, myalgia, hemoptysis, and diarrhea were defined. Radiological evaluation was done by chest X-ray or CT if possible and appropriate. Routine laboratory indicators: complete blood count (CBC), coagulation profile, serum biochemical tests, and arterial blood gas analysis were taken.

Blood samples for the standard laboratory parameters (blood count, coagulation, electrolytes, liver enzymes, blood glucose, creatinine, urea, uric acid, and urine) were taken immediately after hospital admission. Blood samples were collected within 4–7 days after hospitalization. Clinical and standard laboratory parameters and their association with the research biomarker were analyzed.

Genetic analysis

DNA extraction was performed via QIAamp DNA Blood Mini kit. The quality of the isolated DNA was proved by direct spectrophotometry. The following forward and reverse primers were used to amplify the region of interest containing rs4767027: 5'-GATTCACCCTTCCTCGGTC-3' and 5'-CAGCAAAAATGTCTATGCCCT-3'. PCR amplification reactions were carried out in a 25 µL volume containing 50–100 ng of DNA, 0.2 µM of each dNTP, 0.2 µM of each primer, 0.1 U Taq polymerase, and 1× Pol buffer B with 2,5 mM MgCl2. Conditions used for the PCR reaction were as follows: 5 min initial denaturation at 95 °C, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s; final extension was conducted at 72 °C for 5 min. Evaluation of the quantity and quality of the obtained amplification products was performed through visualization on agarose gel electrophoresis using a 3% agarose gel. The products were processed via Sanger sequencing using the BigDye Terminator v.3.1 sequencing kit (Applera Corporation, Norwalk, CA, USA) and electrophoretic separation on a capillary sequencer (ABI Prism 3130 Sequence Genetic Analyzer, Applied Biosystems, Woburn, MA, USA). The obtained data were automatically processed in the ABI3130 Data Collection Software v3.0 program.

Statistical methods

Kolmogorov-Smirnov and Shapiro-Wilk tests of normality were performed. Continuous variables were presented as the mean and standard deviation of normal distribution and as a median and interquartile range in case of non-normality. Continuous variables were analyzed with independent samples – t-tests or Mann-Whitney; categorical outcomes were analyzed with Chi-square or Fisher's exact tests. A comparison between the three groups regarding OAS1 allelic forms was done, applying Kruskal-Wallis tests. Post-hoc comparisons between two specific groups were analyzed with Mann-Whitney *U* tests. P values < 0.05 were considered significant.

Results

Clinico-demographic predictors

The study analyzed 61 patients proved to have COVID-19 by real-time PCR and 48 patients free from the virus. Demographic and clinical parameters of the three groups are presented in Tables 1, 2. The demographic data of both groups is similar, regarding age and gender. Comorbidities were also evenly distributed between arterial hypertension (70% vs 65%), diabetes mellitus (27% vs 13%), ischaemic heart disease (27% vs 0%), and dyslipidemia (23% vs 6%). The duration of symptoms between the two groups before admission is similar. Laboratory assessments of patients at admission are shown in Table 2. Compared to group II, group I showed more often lymphopenia – 3.16(1.39–3.89) vs 5.61(4.21–7.98), p-0.027 and thrombocytopenia – 165(75–256) vs 212(198–349), p-0.031, higher LDH (621 ± 218 U/L vs 312 ± 110 U/L), p-0.014.

Genetic parameters

The allelic distribution of OAS1 rs4767027 is presented in Table 3. In group I genetic analysis was performed in 26 of the patients and in group II in 22 of the subjects. In group I 10/26 (38.4%) were with C/C genotype presentation; 13/26 (50%) had C/T and 3/26 (12.6%) were T/T. In group II 13/22 (59%) were C/C genotype isoform; 5/22 (23%) were C/T; 4/22 (18%) had T/T genotype distribution. Statistical analyses revealed that within this specific cohort, the distributions of genotypes and alleles (C and T) in different COVID groups (Group I, II, and combined) did not significantly differ from each other or the established global allele frequencies. Chi-squared tests were performed to test for an allelic or genotype association between rs4767027 and the following clinical markers: arterial hypertension, diabetes, dyslipidemia, ischemic heart disease, heart failure, cerebrovascular disease, chronic kidney disease, and obesity. A weak association was found for the T allele and dyslipidemia (Chi-Square = 7.327, P-value = 0.026) for the combined Group I+II COVID cohort.

Table 1. Clinical and demographic characteristics of the subjects.

Demographics	Group I (30)	Group II (31)	*P-value	All Covid19 (61)	Controls (48)	**P-value
Sex						
Male, n (%)	20 (67%)	14 (45%)	0.087	34 (56%)	32 (67%)	0.054
Female, n (%)	10 (33%)	17(55%)	0.072	27 (44%)	16 (33%)	0.197
Age, years	68.1 (67–84)	67.8 (66- 81)	0.071	69.8 (66- 84)	66.9 (59–67)	0.367
Smoking history						
Current, n (%)	30 (100%)	27(87%)	0.542	57(93%)	39(81%)	0.093
Former, n (%)	–	4(13%)	0.068	4(7%)	9(19%)	0.084
Non-smoker, n(%)	–	–	–	–	–	–
Comorbidities						
Arterial hypertension, n(%)	21(70%)	20(65%)		41(67%)	23(48%)	
Coronary Artery Diseases, (%)	8(27%)	0(0%)		8(13%)	18(38%)	
Cerebrovascular disease, n (%)	3(10%)	1(3%)		4(7%)	10(21%)	
Diabetes, n(%)	8(27%)	4(13%)		12(20%)	12(25%)	
Dyslipidemia, n(%)	7(23%)	2(6%)		9(15%)	2(4%)	
Chronic kidney disease, n(%)	4(13%)	1(3%)		5(8%)	5(10%)	
Concomitant therapy						
ACEI, n(%)	10(33%)	1(3%)		11(18%)	14(29%)	
ARB, n(%)	7(23%)	6(19%)		13(21%)	5(10%)	
B-blockers, n (%)	12(40%)	6(19%)		18(30%)	18(38%)	
Ca-anatgonist, n(%)	5(17%)	0(0%)		5(8%)	12(25%)	
Diuretics, n(%)	9(30%)	1(3%)		10(16%)	26(54%)	
Aldosteron antagonist, n (%)	6(20%)	1(3%)		7(11%)	1(2%)	
Statins, n (%)	6(20%)	2(6%)		8(13%)	10(21%)	
Mean days of symptoms before admission, n(%)	6.9 (5.2–8.9)	4.5 (3.8–7.2)	0.652	5.2 (4.5–9.2)	–	–
Mean days between admission and plasma sampling, n(%)	6.4 (3–9)	2.1 (1–3)	0.029	6.9 (4.5–9.2)	–	–

*p-value-comparing milder to severe disease patients; **p- value – comparing controls to COVID19 patients.

Table 2. Clinico- laboratory parameters.

Laboratory parameters	Group I (30)	Group II (31)	*P-value	All Covid19 (61)	Controls (48)	**P-value
Complete Blood Count						
Hb, g/l	131 (91–143)	129 (97–149)	0.218	130 (94–146)	138 (96–168)	0.978
Leu × 10^9	8.15(7.89–9.49)	7.98(7.17–9.28)	0.417	8.06(7.89–9.38)	9.74(6.76–10.05)	0.821
Neu × 10^9	6.83(1.78–7.54)	7.12(2.48–7.69)	0.064	6.98(1.78–7.54)	6.64(3.98–8.93)	0.674
Eo × 10^9	1.08(0.98–1.38)	0.98(0.75–1.27)	0.089	1.03(0.88–1.33)	1.21(0.98–1.31)	0.913
Lymph × $1^0$9	3.16(1.39–3.89)	5.61(4.21–7.98)	0.027	6.93(2.81–5.89)	7.09(3.96–7.21)	0.616
Plt, × $1^0$6	165(75–256)	212(198–349)	0.031	189(136–303)	192(178–212)	0.497
Biochemistry						
Glu, mmol/l	8.56 (5.21–15.23)	9.12 (5.03–11.13)	0.059	8.93 (5.12–13.18)	6.98(4.38–9.21)	0.021
Create, mkmol/l	105(67–172)	102(87–123)	0.912	103(77–182)	98(54–126)	0.098
Urea, mmol/l	21(18–34)	19(12–29)	0.878	20(15–26)	14(12–18)	0.075
LDH UI/ml	614±163	329±113	0.039	462±185		
Liver enzymes						
ASAT, IU/l	55(28–76)	48(31–81)	0.121	52(29–78)	32(21–34)	0.032
ALAT, IU/l	49(31–68)	41(27–63)	0.817	45(28–65)	33(18–39)	0.118
GGT, IU/l	78(62–93)	64(56–72)	0.409	77(59–83)	43(29–46)	0.043
Coagulation						
Fibr, g/l	5.38(2.41–6.27)	4.16(2.11–5.18)		4.77(2.41–5.77)	4.54(3.23–6.20)	0.219
INR	1.21(0.98–1.34)	1.08(0.87–1.29)		1.16(0.96–1.31)	1.18(1.07–1.54)	0.398
aPTT	38(27–76)	32(29–68)		35(28–77)	38(27–76)	0.721
Electrolytes						
Na, mmol/l	138(130–149)	134(132–151)	0.387	136(131–150)	136(130–142)	0.985
K, mmol/l	4.12(3.89–4.89)	4.33(3.91–5.01)	0.898	4.28(3.90–4.98)	4.27(3.96–5.69)	0.873
Cl, mmol/l	103(98–114)	105(101–119)	0.613	104(100–117)	103(101–106)	0.901
Inflammatory markers						
CRP, mg/dl	165 (128–324)	108 (93–176)	0.026	187 (98–324)	34(29–63)	0.018
IL-6, U/ml	12.11(5.59–9.38)	8.64(1.84–14.13)	0.039	15.86(1.97–19.62)	4.28(0.11–1.43)	0.001
Ferritin	681±214	402±127	0.028	540±170		

*p-value when comparing mild to severe patients; **p- value when comparing controls to COVID19 patients.

Table 3. Genotype distribution of OAS1 in the subjects.

Parameters	Group I (26)	Group II (22)	*P-value	All Covid19 (48)	Controls (37)	**P-value
C/C	10(38.4%)	13(59%)	0.619	23(48%)	16(43%)	0.932
C/T	13(50%)	5 (23%)	0.830	18(38%)	13(35%)	0.350
T/T	3 (12.6%)	4(18%)	0.917	7(14%)	8(22%)	0.629

*p-value-comparing mild to severe disease patients; **p- value – comparing controls to COVID19 patients.

Discussion

The clinical presentation of COVID-19 varies from asymptomatic to multi-organ dysfunction. It is difficult to distinguish it from other respiratory diseases because of the common features – cough, fever, headache, fatigue, sore throat, and dyspnea (Huang et al. 2020a). Previous reports show that about 20 to 30% of patients with pneumonia could deteriorate to acute respiratory distress (ARDS) (Li et al. 2020b). Elderly patients with DM, hypertension (HTN), ischemic heart disease (IHD) and chronic respiratory disease suffered from severe disease. Du et al. defined that age above 65 with cardiovascular and cerebrovascular comorbidities were associated with severe COVID-19 disease (Du et al. 2020). He et al. revealed that patients with chronic kidney disease had severe COVID-19 clinical presentation (He et al. 2020). A recent meta-analysis recorded that severe COVID-19 patients are more likely to be older with different cardiovascular and respiratory comorbidities due to weak immune function (Yang et al. 2020). Other studies showed that the risk factors were male sex, smoking habits, and obesity (Huang et al. 2020b, Wang et al. 2020). Similar to other viral infections, SARS-CoV-2 infection, shows changes in the hematopoietic system: lymphocytopenia, neutrophilia, thrombocytosis, or thrombocytopenia. The total leukocyte count may show normal values, decrease, or increase during the disease course (Debuc and Smadja 2021). According to our data, the lymphocytes were decreased in the severe group rather than the mild-moderate. The same could be observed regarding the platelets – their total number decreased to thrombocytopenia in the severe forms of the disease; total leucocytic count showed insignificant differences (Li et al. 2020a, Xu et al. 2020). On the other hand, there is a controversial study that revealed elevated TLC and PLT count in severe COVID-19 (He et al. 2020). There is also elevation in ESR, CRP, LDH, ASAT, ALAT, D-dimer, fibrinogen, and creatinine levels in severe COVID-19 patients, which corresponds to the data from previous studies.

The role of platelets in inflammation in COVID-19 infection is crucial. The activated platelets cause lung injury through different mechanisms: direct damage due to released inflammatory mediators; and surface exposure of E- and P-selectin, which attract other inflammatory cells, causing inflammatory and immune responses. In consequence, the hypercoagulable state explains the formation of small thrombi in different organs and a bad prognosis. The tests of coagulation help in identifying critical patients, as well as early treatment (Frater et al. 2020; McRae et al. 2020; Salamanna et al. 2020). The study conducted by He et al. showed that platelet count was significantly lower in severe disease and was a predictor for mortality. Another retrospective study (Zhong and Peng 2021), investigated the ratio between mean platelet volume over platelet count and considered that it was higher and could be used as an independent risk factor for the severity of COVID-19 disease.

According to our study ferritin levels were not significantly different between the two groups and could not be used as an independent predictor of severe COVID-19. In contrast, recent publications show that the level of ferritin was elevated in different types of infections, as well as in COVID-19 patients (Mehta et al. 2020; Velavan and Meyer 2020). Velavan et al. and Mehta et al. showed that ferritin is an important predictor for severe COVID-19 disease due to secondary hemophagocytic lymphohistiocytosis (sHLH) and cytokine storm syndrome (Mehta et al. 2020; Velavan and Meyer 2020). The role of ferritin in immune response is associated with the significance of iron in viral replication. Iron increases the sensitivity of T-lymphocytes toward mediators, which regulates the expression of ferritin. It is assumed that the elevation of serum ferritin was associated with the pro-inflammatory cytokines (IL-6), which stimulate the production of hepcidin, thus increasing ferritin levels (Ruddell et al. 2009).

OAS1 proteins are a part of the innate immune response against RNA viruses and are usually induced by interferons. They activate latent RNase L and stimulate direct viral RNA destruction. The biological activity of OAS1 is related to different allelic forms (Lim et al. 2009; Simon-Loriere et al. 2015; Hu et al. 2016). The protective alleles of rs4767027-T (the OAS1 pQTL) and rs10774671-G (the OAS1 sQTL) are found in a Neanderthal haplotype (Bonnevie-Nielsen et al. 2005). Due to OAS1 alternative splicing, the rs10774671-G allele increases the p46 isoform of the encoded protein. The latter has a higher enzymatic activity against viruses than the p42 isoform (Zeberg and Pääbo 2021). The p46 isoform is produced during infection (Sams et al. 2016). Although further studies are needed to unravel the entire biological role of pQTL and sQTL-OAS1, the antiviral activity of the gene products is higher for the Neanderthal haplotype in comparison to the common haplotype in Europeans (Liu et al. 2017). Increased OAS1 levels of the Neanderthal haplotype in the contemporaries of European ancestry have a protective effect, according to a study of 931 proteins assessed for three COVID-19 outcomes in up to 14,134 cases and 1.2 million controls of European ancestry (Zhou et al. 2021). SARS-CoV-2 can activate the NLRP3 inflammasome directly (through SARS-CoV-2 ORF8b and N proteins) or indirectly (via diverse cellular

signaling mechanisms) (Mitev 2023). The 2',5'-OAS – RNase L pathway can also activate the NLRP3 inflammasome, which is responsible for the normal antiviral immune response (Gusho et al. 2020). The Neandertal OAS protein variant (p46) is associated with higher enzymatic activity (Bonnevie-Nielsen et al. 2005). However, as noted above, about 14% of COVID-19 patients respond with an abnormally activated NLRP3 inflammasome. In this case, further stimulation of the NLRP3 inflammasome will aggravate the already developing CS. This calls into question the use of medication for the activation of OAS1. According to our strategy, the fight against severe COVID-19 should target the inhibition of the hyper-activated NLRP3 inflammasome. Intracellular SARS-CoV-2 viral load can be successfully reduced if the TRMPSS2 inhibitor bromhexine is taken prophylactically or inhaled immediately after contact with a COVID-19 patient (Mitev 2023; Mitev et al. 2023). While reports that increased OAS1 levels in the non-infectious state are strongly associated with reduced risk of COVID-19 susceptibility, severity, and hospitalization, the strength of this correlation with the protective Neanderthal haplotype is yet unclear. Our investigation shows that there isn't a strong association that can be observed on an individual level, and the effects are not observed in a smaller patient cohort.

The results of our study, however, did not show a statistically significant difference between group I and group II patients regarding the allelic forms of the gene, encoding this protective protein. A predominance of the protective allele is observed in the mild/moderate group. This data should be interpreted with caution: 1) the sample size is not sufficient to be generalized for the whole population; 2) the genetic analysis is performed only in hospitalized patients, which deters the determination of the protective effect of the gene, if any; 3) the genetic background of the Bulgarian population is heterogeneous and cannot be classified solely as originating from European ancestry.

Conclusion

We establish that lymphopenia and thrombocytopenia are likely associated with immune inflammation and COVID-19 severity. The previously reported protective effects of rs4767027 could not be observed, possibly due to the small effect size of the protective allele and the smaller patient cohort. Based on our study, we would not recommend testing for the *OAS1* rs4767027 variant as a predictive marker for COVID-19 severity. While increased OAS1 transcript levels are correlated with reduced risk of infection, these increased levels can further contribute to NLRP3 inflammasome activation once the infection has been established. Therefore, potential treatment options might benefit more if they target the inflammasome directly.

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