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Severity of coronavirus disease 19: Profile of inflammatory markers and *ACE* (rs4646994) and *ACE2* (rs2285666) gene polymorphisms in Iraqi patients

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ABSTRACT

Susceptibility to coronavirus disease 2019 (COVID-19) and disease severity has recently been associated with inflammatory markers and genetic polymorphisms of *ACE* (angiotensin-converting enzyme) and *ACE2* genes, but the evidence has been inconclusive. This case-control study (99 COVID-19 patients and 96 controls) sought to assess the significance of age, C-reactive protein (CRP), neutrophil-to-lymphocyte ratio (NLR) and SARS-CoV-2 RT-PCR cycle threshold (Ct) in severity of COVID-19. Besides, two variants of *ACE* and *ACE2* genes (rs4646994 and rs2285666, respectively) were analyzed to determine their role in COVID-19 susceptibility and/or disease severity. Results revealed that age, CRP and NLR were significantly elevated in severe cases compared to moderate cases, while RT-PCR Ct value was significantly decreased. Allele and genotypes of both variants were not associated with COVID-19 risk, with the exception of rs2285666 *A* allele. It showed a significantly higher frequency in female patients than in female controls (probability = 0.041). In conclusion, the study indicated the role of age, CRP, NLR and SARS-CoV-2 RT-PCR Ct in susceptibility to COVID-19 severity. However, analysis of the *ACE* and *ACE2* gene variants (rs4646994 and rs2285666, respectively) showed that the two variants were not associated with the risk of developing COVID-19.

1. Introduction

Coronaviruses (CoV) are novel viruses enveloped in a lipid membrane embedded with viral surface proteins and have a single-stranded RNA genome. They are classified in the subfamily *Orthocoronaviridae* (Family: *Coronaviridae*, Order: *Nidovirales*) and have been recognized to be associated with acute respiratory infections (Ludwig and Zarbock, 2020). Human CoV were first described in nasopharyngeal secretions of patients with common colds in 1960s, but were not considered human pathogen until three outbreaks emerged; severe acute respiratory syndrome (SARS) in 2002–2003 (China), Middle East respiratory coronavirus (MERS-CoV) in 2012 (Saudi Arabia), and novel coronavirus SARS-CoV-2 in late 2019 (Wuhan, China) (Ye et al., 2020). The latter has been named coronavirus disease 2019 (COVID-19) and the World Health Organization (WHO) has declared the disease a pandemic with the infection spreading to more than 200 countries. To date (November 1, 2021), 247,618,301 cases have been reported globally with 2.03% deaths. The corresponding figures for Iraq were 2,056,401 and 1.13%, respectively (Peng, 2020; Worldometer, 2021).

SARS-CoV-2 infection may have a broad clinical spectrum, ranging from asymptomatic or mild forms to severe acute pneumonia causing severe lung injury and an increased risk of multiple organ failure and eventual death (Parasher, 2021). Several factors have been described as being associated with a higher risk of severe or fatal COVID-19. Advanced age, male gender, obesity, chronic disease (cardiovascular and diabetes) and vitamin D status are among the risk factors proposed to be associated with disease severity and higher mortality (Wolff et al., 2021). Besides, severity of COVID-19 is complicated by exacerbated inflammatory response induced by SARS-CoV-2 in the form of up-regulated expression of inflammatory markers, particularly pro-inflammatory cytokines (Darif et al., 2021). Accordingly, cytokine release syndrome, or cytokine storm, has been presented as an important contributor to COVID-19 severity and co-morbid conditions such as disseminated intravascular thrombosis (Rabaan et al., 2021).

The exaggerated inflammatory response in COVID-19 has also recently been linked to enzymes of the renin-angiotensin system (RAS)

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(Wiese et al., 2020). The RAS is viewed as a coordinated hormonal cascade involved in controlling multiple physiological functions, in particular blood pressure, but its role in pathogenesis of COVID-19 has also been recognized (Gemmati and Tisato, 2020). The two most important RAS enzymes are angiotensin-converting enzyme (ACE) and ACE2, and SARS-CoV-2 has been shown to be an ACE2-tropic virus (Abassi et al., 2020). Virus interaction with RAS in cells infected with SARS-CoV-2 can trigger dysregulated immune responses and coagulation abnormalities in COVID-19 patients (Wiese et al., 2020). ACE catalyzes angiotensin-I (Ang1) to Ang-II, the latter of which is hydrolyzed by ACE2 to Ang-1-7. Ang-II binding to angiotensin II type 1 (AT1) receptor results in vasoconstriction, inflammation and thrombosis, while increased vasodilation and reduced inflammation and thrombosis occur due to Ang-1-7 binding to AT2 receptor. Thus, ACE and ACE2 play an opposite role in hypertension and cardiovascular disease (Gómez et al., 2020). Hypertension and cardiovascular disease have been cited as significant comorbidities in COVID-19 patients, and are closely related to disease severity and mortality (Fresán et al., 2021). To understand the role of ACE2 in the development of COVID-19, its gene expression was examined in the nasal epithelium of children and adults. A positive association was found between *ACE2* gene expression and age and was independent of gender. It has been suggested that lower ACE2 expression in children compared to adults may explain the lower prevalence of COVID-19 in children (Bunyavanich et al., 2020). Further, it has been proposed that RAS, and through the opposite functions of ACE and ACE2, plays a significant role in the development of acute lung diseases, particularly acute respiratory distress syndrome (Gemmati and Tisato, 2020).

Common variants (single nucleotide polymorphisms; SNPs) in the *ACE* and *ACE2* genes have been described, and their association with the risk of various diseases has been indicated. The *ACE* gene (Gene ID: 1636) is located in the long arm of human chromosome 17 (17q23.3) and consists of 26 exons (https://www.ncbi.nlm.nih.gov/gene/1636). Insertion/deletion (I/D: rs4646994) is among the distinct genetic polymorphisms of this gene, and besides being associated with the risk of hypertension, heart disease, kidney failure, acute respiratory distress syndrome, SARS and COVID-19, it has been shown that DD genotype may influence ACE serum levels (Gómez et al., 2020). The ACE2 gene (Gene ID: Gene ID: 59272) is located in the short arm of human X chromosome (Xp22.2) and consists of 22 exons (https://www.ncbi.nlm.nih.gov/gene/59272). Several SNPs of the gene, for instance rs4240157, rs4646155, and rs4830542, have been associated with the risk of hypertension, but recent studies in COVID-19 have focused on SNP rs2285666 (Cafiero et al., 2021; Devaux et al., 2020; Gómez et al., 2020; Karakaş Çelik et al., 2021; Pouladi and Abdolahi, 2021; Singh et al., 2021). Although these studies proposed a role for SNPs rs4646994 and rs2285666 in COVID-19 susceptibility and/or disease severity, the evidence has not been conclusive as some findings have not been replicated.

This study sought to assess the significance of age, C-reactive protein (CRP), neutrophil-to-lymphocyte ratio (NLR) and SARS-CoV-2 RT-PCR cycle threshold (Ct) in severity of COVID-19. Besides, two variants of *ACE* and *ACE2* genes (rs4646994 and rs2285666, respectively) were analyzed to determine their role in COVID-19 susceptibility and/or disease severity. To the best knowledge of investigators, the second part of study has not been conducted on Iraqi patients with COVID-19.

2. Materials and methods

2.1. Populations studied

A single center case-control study was conducted on Iraqi patients with COVID-19 during the period from October to December 2020. Patients were recruited from the COVID-19 Care Units of Baghdad Teaching Hospital after obtaining approval from the Ethics Committee at the Department. Biology (University of Baghdad) and written consent to participate in the study (Reference: CSEC/0920/0017). A total of 99 cases were included (mean age ± SD: 49.1 ± 9.1 years; range: 28–70 years; 50.5% males). According to severity of COVID-19, the patients were divided into two groups, moderate and severe cases (68 and 31, respectively). Criteria established by the WHO Interim Guidance was followed to define the case as moderate (patient with symptoms of pneumonia and no signs of severe pneumonia) or severe (severe respiratory distress, respiratory rate $\geq$ 30 breaths/min or pulse oxygen saturation (SpO_2) $\leq$ 93% on resting state) (World Health Organization, 2020). According to the patient's clinical history, some had diabetes and some had hypertension (51.5 and 53.5%, respectively). A control group of 96 individuals was also included (mean age ± SD: 43.1 ± 10.3 years; range: 24–70 years; 50.0% males). They were healthy and had no respiratory infection in the past 12 months or chronic diseases (cardiovascular and diabetes). Besides, their serum tested negative for COVID-19 IgG and IgM antibodies (FREND COVID-19 IgG/IgM Duo kit, NanoEntek, South Korea).

2.2. Diagnosis of COVID-19

Nasopharyngeal swabs were obtained from patients 4–5 days after their hospitalization. The QIAamp Viral RNA Mini kit was used to isolate viral RNA (Qiagen, Germany). Real-time polymerase chain reaction (RT-PCR) analysis was performed to diagnose SARS-CoV-2 using commercial kit (DIAGNOVITAL SARS-COV-2 real time PCR kit, RTA laboratories Biological products, Turkey), and manufacturer's instructions were followed. Based on this analysis, the SARS-CoV-2 RT-PCR Ct value was recorded for each patient. In combination with molecular testing, the diagnosis was confirmed by chest computerized tomography (CT). The only patients included were those who showed a positive molecular test and had a CT scan indicating COVID-19.

2.3. Blood tests

Five milliliters of venous blood were collected and distributed into plain and ethylene-diamine-tetra-acetic-acid (EDTA) tubes (3 and 2 mL, respectively). Plain tube was left to clot and then centrifuged (15 min at 4 °C for 15 min) to collect serum. Serum was tested for CRP using electro-chemiluminescence immunoassay system (Roche Cobas Integra 400 plus, Switzerland). EDTA blood was used to count white blood cells (WBC: total, neutrophils and lymphocytes) using automated hematology analyzer (ABX Micros ES 60, Horiba, USA). The NLR was obtained by dividing the absolute neutrophil count by the absolute lymphocyte count.

2.4. Polymorphism of ACE and ACE2 genes

Two SNPs of *ACE* and *ACE2* genes were determined (rs4646994 I/D and rs2285666 G/A). Genomic DNA was isolated from EDTA blood using the gSYNC DNA extraction kit (Geneaid, Taiwan). Isolated DNA was subjected to polymerase chain reaction (PCR) analysis to detect target SNPs. For SNP rs4646994, a previous method was followed (Khan et al., 2014). Briefly, the PCR was performed in a total volume of 25 μL, with 5 μL AccuPower PCR PreMix (Bioneer, Korea), 1 μL forward primer (5'-CTGGAGACCACTCCCATCCTTTCT-3'), 1 μL reverse primer (5'-GATGTGGCCATCACATTCGTCAGAT-3'), 3 μL DNA and 15 μL deionized distilled water. The tube was transferred to thermal cycler (Eppendorf, Germany) that was programmed for the following optimized conditions: an initial denaturation cycle (94 °C for 5 min), followed by 35 cycles of denaturation (94 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 45 s), and a final extension cycle (72 °C for 5 min). The PCR products were electrophoresed in agarose gel (1.5%; 5 V/cm^2 for 55 min), and migrating bands were visualized using gel documentation system. Two bands of different molecular sizes were encountered; 490 bp (*I* allele) and 190 bp (*D* allele) (Supplementary Figure).

For SNP rs2285666, also a previous method was adopted (Wu et al.,

2017). Briefly, the PCR was performed in a total volume of 25 μL, with 5 μL AccuPower PCR PreMix (Bioneer, Korea), 1 μL forward primer (5'-CATGTGGTCAAAAGGATATCT −3′), 1 μL reverse primer (5′-AAAGTA AGGTTGGCAGACAT −3′), 3 μL DNA and 15 μL deionized distilled water. The tube was transferred to thermal cycler (Eppendorf, Germany) that was programmed for the following optimized conditions: an initial denaturation cycle (95 °C for 1 min), followed by 35 cycles of denaturation (94 °C for 30 s), annealing (50.6 °C for 30 s) and extension (72 °C for 45 s), and a final extension cycle (72 °C for 7 min). The PCR products were digested with the restriction enzyme *Alu I* for 4 h at 37 °C. Then, the digested PCR products were electrophoresed in agarose gel (1.5%; 5 V/cm^2 for 55 min), and migrating bands were visualized using gel documentation system. Allele *G* was presented with a single band with a molecular size of 466 bp, while allele *A* had two bands with molecular sizes of 281 bp and 185 bp (Supplementary Figure).

2.5. Statistical analysis

Number and percentage were used to describe categorical variables and significant differences were assessed by two-tailed Fisher exact test. Continuous variables were tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk test). Normally distributed variables were given as mean and standard deviation (SD), and significant differences were evaluated with Student *t*-test. Nonparametric variables (skewed) were expressed as median and interquartile range (IQR), and Mann-Whitney *U* test was used to determine significant differences. A goodness-of-fit Chi-squared test was used to test the departure from Hardy-Weinberg equilibrium (HWE) of *ACE* and *ACE2* genotype distributions. Strength of association between *ACE* and *ACE2* gene polymorphisms (rs4646994 and rs2285666, respectively) and risk of COVID-19 was measured by odds ratio (OR) with 95% confidence interval (CI). A probability (p) $\leq$ was considered statistically significant. These analyses were performed using IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.). G*Power software (version 3.1.9.7) was used to determine power of sample size.

3. Results

3.1. Power of sample size

The power of sample size was estimated for COVID-19 patients (N = 99) and controls (N = 96) at an α error probability of 0.05 and effect size of 0.3. The actual power (1-β error probability) was 0.66, which is less than 0.80; statistically acceptable power.

3.2. Baseline characteristics of patients

COVID-19 cases with severe disease showed a significantly higher mean age compared to moderate cases (56.7 ± 8.0 *vs.* 45.6 ± 7.2 years; $p < 0.001$). Males outnumbered females in severe disease (61.3 *vs.* 38.7%), while females outnumbered males in moderate disease (54.4 *vs.* 45.6%), but the difference was not significant between the two groups of patients ($p = 0.194$). Prevalence of diabetes and hypertension was significantly higher in severe cases than in moderate cases (87.1 *vs.* 35.3% and 93.5 *vs.* 35.3%, respectively; $p < 0.001$). Serum levels of CRP were significantly higher in severe cases compared to moderate cases (29.8 [IQR: 27.3–61.1] *vs.* 9.7 [IQR: 5.1–16.2] mg/L; $p < 0.001$). Leukocyte (3.5 ± 0.8 *vs.* 6.5 ± 1.4 *10^9/L; $p < 0.001$), neutrophil (2.4 ± 0.7 *vs.* 3.7 ± 0.8*10^9/L; $p < 0.001$) and lymphocyte (1.0 ± 0.3 *vs.* 2.6 ± 0.7*10^9/L; $p < 0.001$) counts decreased significantly in severe cases compared to moderate cases, while the NLR increased significantly (2.3 [IQR: 2.2–2.9] *vs.* 1.5 [1.2–1.6]; $p < 0.001$). Finally, SARS-CoV-2 RT-PCR Ct value showed a significantly decreased median in severe cases compared to moderate cases (28 [IQR: 25–29] *vs.* 32 [30–35]; $p < 0.001$) (Table 1).

Table 1
Baseline characteristics of COVID-19 patients.

Characteristic†		COVID-19 patients; N = 99		*p*-value
		Moderate; *N* = 68	Severe; *N* = 31	
Age; year		45.6 ± 7.2	56.7 ± 8.0	< **0.001**
Gender	Male	31 (45.6)	19 (61.3)	0.194
	Female	37 (54.4)	12 (38.7)	
Diabetes	Present	24 (35.3)	27 (87.1)	< **0.001**
	Absent	44 (64.7)	4 (12.9)	
Hypertension	Present	24 (35.3)	29 (93.5)	< **0.001**
	Absent	44 (64.7)	2 (6.5)	
C-reactive protein; mg/L		9.7 (5.1–16.2)	29.8 (27.3–61.1)	< **0.001**
White blood cells; *10^9/L		6.5 ± 1.4	3.5 ± 0.8	< **0.001**
Neutrophils; *10^9/L		3.7 ± 0.8	2.4 ± 0.7	< **0.001**
Lymphocytes; *10^9/L		2.6 ± 0.7	1.0 ± 0.3	< **0.001**
Neutrophil-to-lymphocyte ratio		1.5 (1.2–1.6)	2.3 (2.2–2.9)	< **0.001**
SARS-CoV-2 RT-PCR Ct		32 (30–35)	28 (25–29)	< **0.001**

†: Data are given as mean ± standard deviation, median and interquartile range (continuous variables) or number and percentage (categorical variables). Means were compared with Student *t*-test. Medians were compared with Mann-Whitney *U* test. Categorical variables were compared with two-tailed Fisher exact test. Significant *p*-value is indicated in bold.

3.3. ACE (rs4646994 I/D) and ACE2 (rs2285666 G/A) SNPs

Genotype frequencies of rs4646994 and rs2285666 SNPs were in good agreement with HWE in COVID-19 patients and controls, as there were no significant differences between observed and expected frequencies. When comparing patients to controls, allele and genotype frequencies of the two SNPs did not show significant variation. The rs2285666 *A* allele was an exception and showed a significantly higher frequency in female patients than in female controls (29.6 *vs.* 16.7%; OR = 2.10; 95% CI = 1.06–4.18; $p = 0.041$), while males did not show this difference (Table 2). When allele and genotype frequencies of rs4646994 and rs2285666 SNPs stratified by disease severity (moderate and severe), gender (males and females), diabetes (present and absent) and hypertension (present and absent) in COVID-19 patients, no significant differences were found in each stratum (Supplementary Tables 1 and 2).

4. Discussion

The study results demonstrated that age, CRP, NLR, diabetes and hypertension can be considered as significant risk factors in determining severe illness in COVID-19 patients, and this may help determine the risk classification for the likelihood of severe infection. In terms of age, the study showed that advanced ages paralleled the severity of COVID-19, and severe cases tended to be over the age of 50 years, while moderate cases were under 50 years. Consistent with this observation, most studies pointed out that the elderly are not only at increased risk of contracting COVID-19, but severe forms of illness and mortality are most common among these people (Farshbafnadi et al., 2021). Older people have been reported to have more difficulty breathing compared to younger people and have less frequent symptoms or signs of classic COVID-19, such as fever or shortness of breath (Kennedy et al., 2020). Instead, they are more likely to develop uncommon presentations of disease; for instance, confusion, delirium and dementia, which have also been linked to an increased risk of death (Poloni et al., 2020). Further, COVID-19 patients 60 years of age or older tend to have more severe

Table 2
Logistic regression and Hardy-Weinberg equilibrium analyses of *ACE* (rs4646994) and *ACE2* (rs2285666) gene SNPs in COVID-19 patients *versus* controls.

SNP	Allele/ genotype	Patients (N = 99)		Controls (*N* = 96)		OR (95% CI)	*p*-value
		N	%	N	%		
rs4646994	*I*	74	37.4	63	32.8	Reference	
	D	124	62.6	129	67.2	0.82 (0.54–1.24)	0.396
	II	14	14.1	8	8.3	Reference	
	ID	46	46.5	47	49.0	0.56 (0.20–1.52)	0.253
	DD	39	39.4	41	42.7	0.56 (0.21–1.56)	0.266
p-HWE		0.941		0.279			
rs2285666†							
Female	*G*	69	70.4	80	83.3	Reference	
	A	29	29.6	16	16.7	2.10 (1.06–4.18)	**0.041**
	GG	26	53.1	33	68.8	Reference	
	GA	17	34.7	14	29.2	1.98 (0.83–4.72)	0.124
	AA	6	12.2	1	2.1	1.82 (0.82–4.06)	0.142
p-HWE		0.241		0.729			
Male	*G*	35	70.0	35	72.9	Reference	
	A	15	30.0	13	27.1	1.15 (0.48–2.75)	0.825

†: Results were analyzed separately for males and females because the SNP is located on X chromosome; SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; *I*: Insertion; *D*: Deletion; OR: Odds ratio; CI: Confidence interval; *p*: Two-tailed Fisher exact probability (significant *p*-value is indicated in bold).

pneumonia with lesions of multi-lobar appearance than patients younger than 60 years of age (Liu et al., 2020).

Several factors have been described that contribute to an increased risk of COVID-19 infection and disease severity in older adults, and the immune system is one of the most important proposed factors in this context (Chen et al., 2021). A hallmark of aging is the shift of the innate and adaptive immune responses towards creating inflammatory states in the human body, and inflammatory marker genes have been shown to exhibit up-regulated expression in inflammatory cells (Bajaj et al., 2021). Besides, it has been found that aging is associated with down-regulated functions of cells of the adaptive immune system; for instance, antigen-presenting, T-cytotoxic and B cells, and this may counteract the adaptive immune system to control infection and inflammation (Zhu et al., 2021). Therefore, age-related dysregulated immune functions, which have been described as immunosenescence and inflammaging, play a major role in contributing to increased susceptibility to severe COVID-19 (Chen et al., 2021). In the current study, the WBC in general and neutrophils and lymphocytes in particular were significantly decreased in patients with severe COVID-19 compared to patients with moderate disease. Conversely, the inflammatory markers CRP and NLR were significantly elevated and indicated to be associated with COVID-19 severity. CRP is a non-specific protein produced by hepatocytes in response to infection and inflammation. Most studies agree that CRP showed up-regulated levels in serum of COVID-19 patients, but this deviation was more pronounced in severe cases and about 68% of these cases demonstrated up-regulated levels (Ali, 2020). CRP levels were also correlated with oxygen saturation, and patients with $SpO_2 \leq$ 90% were presented with markedly elevated CRP levels compared to patients with $SpO_2 >$ 90% (76.51 *vs*. 12.70 mg/L) (Xie et al., 2020). Thus, CRP has been linked to more severe disease, lung injury and worse prognosis in COVID-19 patients. NLR is an additional marker of inflammation, and it has been indicated that NLR can be used as an early warning marker for exacerbation of severe COVID-19 and can provide an objective basis for the identification and management of severe pneumonia in COVID-19 patients (Imran et al., 2021). Furthermore, the NLR has been considered as an independent predictor of poor clinical outcome and mortality in COVID-19 patients (Ciccullo et al., 2020).

In addition to the immune system, comorbidities associated with aging such as diabetes and cardiovascular disease have been shown to be associated with the risk of contracting COVID-19 and disease severity. In the current study, most severe cases already had diabetes and hypertension and their prevalence was significantly higher in patients with severe disease than in moderately ill patients. Previous studies have also shown that diabetes and hypertension are the most prevalent comorbidities among COVID-19 patients, and have been observed to increase the risk of morbidity and mortality (Barrera et al., 2020; De Almeida-Pititto et al., 2020). However, it is not clear whether the two comorbidities were independent predictors of COVID-19 severity or might have synergistic effects (Tadic and Cuspidi, 2021). To shed light on this issue, a study was conducted to evaluate the contributions of diabetes alone, hypertension alone, or their combination to the risk of severe COVID-19 infection and other clinical complications. The data obtained revealed that hypertension was not independent risk factors for respiratory failure but slightly increased the risk of severe COVID-19, and the risk associated with hypertension may be due to the confounding effects of diabetes (Sun et al., 2021). However, further recent study showed that diabetes and hypertension were the most common comorbid conditions that occurred in COVID-19 patients with an approximate prevalence (45.1 and 48.1%, respectively) and based on the Framingham risk score, most of them were classified within the high-risk group (Alshaikh et al., 2021).

This study also pointed out for the significance of SARS-CoV-2 RT-PCR Ct value in determining severity of COVID-19. A Ct value represents the number of the cycle at which the signal crosses the positivity threshold, and thus a lower Ct value indicates a higher viral load. Although the relevance of viral load to severity of COVID-19 has been controversial (Abdulrahman et al., 2021), some studies have indicated that viral load is associated with increased disease severity and mortality (Rao et al., 2020; Waudby-West et al., 2021). Besides, as in the current study, viral load of SARS-CoV-2 was associated with decreased absolute lymphocyte count and increased CRP levels (Fajnzylber et al., 2020).

Recent studies have also linked severity of COVID-19 to ACE2. Plasma ACE2 has been indicated to have potential value in predicting outcomes in COVID-19 patients, and this RAS enzyme may represent a link between disease severity and comorbidities such as cardiovascular disease (Kragstrup et al., 2021). Unfortunately, the current study did not measure ACE2 levels in plasma of COVID-19 patients, and instead focused on two variants of *ACE* and *ACE2* genes (SNPs rs4646994 and rs2285666, respectively). A comparison of COVID-19 patients and controls revealed that allele and genotype frequencies of the two SNPs showed no significant differences. Further, allele and genotype frequencies stratified by disease severity, gender, diabetes and hypertension also did not show any significant differences. The exception was allele *A* of SNP rs2285666, which showed a significantly higher frequency in female patients and scored an OR of 2.10. However, this finding should be interpreted with caution because the *p*-value was not corrected for multiple comparisons, and therefore we cannot rely on it. Similar to our findings, SNPs rs4646994 and rs2285666 were not

associated with susceptibility to COVID-19 in a cohort of Spanish patients, but the rs4646994 DD genotype showed a significantly higher frequency in severe cases than in cases of mild disease (46 *vs*. 32%; $p = 0.049$) (Gómez et al., 2020). The two polymorphisms were also not associated with COVID-19 severity in Turkish patients (Karakaş Çelik et al., 2021). However, frequency of rs4646994 II genotype was reported to be significantly increased in Czech patients with symptomatic COVID-19 compared to controls (26.2 *vs*. 21.2%; $p = 0.02$), while asymptomatic patients (SARS-CoV-2-positive) showed no significant variations compared to controls (Hubacek et al., 2021). It has also been proposed that presence of rs4646994 DD genotype with COVID-19 is likely to be associated with increased disease severity and morbidity and may impact the disease outcomes (Sarangarajan et al., 2021). In a German study, genotyping of SNP rs2285666 was performed in SARS-CoV-2-positive and SARS-CoV-2-negative patients. The results demonstrated that allele *G* and genotype GG were associated with susceptibility to COVID-19, particularly in seriously ill patients (Möhlendick et al., 2021). As presented, the association of the rs4646994 and rs2285666 polymorphisms with susceptibility to COVID-19 is inconsistent across studies around the world, and this may be related to ethnic variations between populations as these variants show some population-based differences (Sarangarajan et al., 2021; Srivastava et al., 2020). Therefore, we may encounter conflicting results but this should not underestimate the role of polymorphisms in the *ACE* and *ACE2* genes in susceptibility to COVID-19 or disease severity. This is due to the fact that the two enzymes have been implicated in causing severe lung injury and organ regression in COVID-19 patients. Besides, their receptors are key factors for the entry of SARS-CoV-2 into cells, retention of sodium and water with hypertension, and the promotion of fibrotic and inflammatory conditions that lead to cytokine release syndrome (or cytokine storm) (Cafiero et al., 2021). Thus, *ACE* and *ACE2* gene expression and polymorphism require extensive research to understand their impact on susceptibility to SARS-CoV-2 infection and disease outcome in COVID-19 patients.

Regarding hypertension and diabetes and prior to the COVID-19 pandemic, three meta-analysis studies considered the *D* allele of the rs4646994 SNP as a risk variant for developing hypertension or diabetes in various world populations including Asian, Caucasian and African populations (Oscanoa et al., 2021). In the current study, this role for the *D* allele was not confirmed and no significant differences were found in the distribution of allele or genotype frequencies of rs4646994 polymorphism between diabetic and non-diabetic or hypertensive and non-hypertensive COVID-19 patients. Consistent with our observation, the rs4646994 polymorphism was also not associated with hypertension in African-Brazilians, Caucasians-Brazilians and Peruvians (Bonfim-Silva et al., 2016; Oscanoa et al., 2020). Similarly, this polymorphism was not associated with diabetes in Brazilians and Chinese (Bonini Domingos et al., 2014; Zhou et al., 2012). Regardless of these conflicting results, we cannot rule out the need for further studies on rs4646994 polymorphism and its association with hypertension and diabetes.

The study faced limitations with the small size of patients and controls, especially patients with severe COVID-19, and a larger cohort is needed. Besides, asymptomatic cases were not investigated.

In conclusion, the study indicated the role of age, CRP, NLR and SARS-CoV-2 RT-PCR Ct in susceptibility to COVID-19 severity. However, analysis of the *ACE* and *ACE2* gene variants (rs4646994 and rs2285666, respectively) showed that the two variants were not associated with the risk of developing COVID-19.

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Declaration of Competing Interest

The authors declare that there were no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mgene.2022.101014.

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