Angiotensin-converting-enzyme insertion/deletion polymorphism, ACE activity, and COVID-19: A rather controversial hypothesis. A case-control study

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Abstract
Accumulating data has shown a contribution of the renin-angiotensin system in COVID-19 pathogenesis. The role of angiotensin-converting enzyme (ACE) insertion (I)/deletion (D) polymorphism as a risk factor in developing COVID-19 disease comes from epidemiological data and is controversially discussed. We conducted a retrospective case-control study and assessed the impact of ACE I/D genotype in COVID-19 disease prevalence and severity. In 81 COVID-19 patients explicitly characterized and 316 controls, recruited during the first wave of COVID-19 pandemic, ACE I/D genotype, and ACE activity were determined. A generalized linear model was used and Poisson regression analysis estimated the risk ratios (RRs) of alleles and genotypes for disease severity. DD patients had almost 2.0-fold increased risk (RR: 1.886, confidence limit [CL] 95%: 1.266–2.810, p = 0.0018) of developing a more severe disease when contrasted to ID and II individuals, as did D allele carriers compared to I carriers (RR: 1.372; CL 95%: 1.051–1.791; p = 0.0201). ACE activity (expressed as arbitrary units, AU/L) was lower in patients (3.62 ± 0.26) than in controls (4.65 ± 0.13) (p < 0.0001), and this reduction was observed mainly among DD patients compared to DD controls (3.97 ± 0.29 vs. 5.38 ± 0.21; p = 0.0014). Our results demonstrate that ACE DD genotype may predispose to COVID-19 increased disease severity via a mechanism associated, at least in part, with the significant fall in their ACE activity. Our findings suggest a more complex pattern of synergy between this polymorphism and ACE activity in COVID-19 patients compared to healthy individuals and set the grounds for large-scale
1 | INTRODUCTION

The renin-angiotensin system (RAS) has been at the forefront in the quest of genetic factors involved in the pathogenesis of COVID-19. The "proposed" imbalance between the angiotensin-converting enzyme 2 (ACE2) which serves as an anchor for the pathogenic coronavirus to the target cells, and the ACE in COVID-19 patients, attempted to explain, at least in part, the progression of the disease. To this direction, several studies supported a potential benefit of the use of ACE inhibitors (ACEi) and angiotensin II receptor blockers (ARBs) in COVID-19 outcome, however, their efficacy as a treatment for COVID-19 remains to be seen. It has been hypothesized that ACE gene polymorphisms that affect ACE activity may be used as a host genetic factor for COVID-19 patients triage regarding the severity of the disease and their response to ACEi and ARBs treatment.

The ACE insertion (I)/deletion (D) polymorphism (rs1799752) is a 287-bp Alu sequence in intron 16 of the ACE gene which accounts for the majority of interindividual variation in ACE activity in circulation and shows an important geographic variation. ACE activity in ACE II healthy individuals is half of that in ACE DD individuals. In the early pandemic, an extensive debate has been launched regarding the effect of the ACE I/D alleles on the prevalence and the outcome of the COVID-19 infection. First, Delanghe et al. suggested that the D allele is a confounder in the spread of COVID-19 and that with increasing the D allele there is a decrease of COVID-19 morbidity/mortality in an analysis of 33 countries in Europe, North Africa, and the Middle East. Yamamoto et al. suggested that the prevalence of the D allele in the ACE gene is integrally involved in susceptibility to SARS-CoV-2 infection and the exacerbation of COVID-19 symptoms such as pneumonia. Zheng et al. in their recent review on ACE I/D polymorphism proposed that the absence of ACE D/D genotype in patients with COVID-19 may be protective against developing severe lung injury. However, all these hypotheses were based on the analysis of data extracted from public databases regarding the ACE variant frequencies among different populations and COVID-19 incidence in these populations.

In COVID-19 patients of variable disease severity, we performed a case-control study and determined the ACE I/D genotypes and ACE activity in samples collected during the first wave of the pandemic in Greece. A cohort of blood-donors and health workers non-COVID volunteers of Greek origin were recruited at the same period and served as controls. We correlated ACE activity and ACE I/D genotypes and allelic frequencies with clinical features, disease laboratory markers, and serum anti-SARS-CoV-2S.

2 | MATERIALS AND METHODS

2.1 | Patients and controls

We conducted a single-centre retrospective case-control study. The samples had been collected in a tertiary referral hospital for COVID-19, “Attikon” University General Hospital, Athens, Greece, between 15th March 2020 and 30th June 2020 during the early pandemic in Greece. Eligible COVID-19 cases were Caucasian adult patients of Greek ancestry. No exclusion criteria regarding hospitalization requirement, the magnitude of symptoms, disease severity, and outcomes were applied. Samples from 316 sex-matched adult blood/blood product donors and volunteer healthcare workers of Greek ancestry, with no history of COVID-19-related symptoms or relevant epidemiological history within the past month, were collected and served as controls.

2.2 | Ethical statement

This study was approved by the institutional Research Bioethics Committee and was conducted according to the STrengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines. Patients’ data were collected and analyzed under strict anonymity in agreement with the Helsinki Declaration. Written informed consent had been obtained by all participants.

2.3 | Patient data and sample collection

Clinical data of patients were collected by reviewing the medical files. Routine blood tests including disease laboratory markers, namely, neutrophil to lymphocyte ratio (NLR), C-reactive protein (CRP), ferritin, d-dimers and interleukin-6 (IL-6) were retrieved by the institutional electronic system. Patients were stratified as mild, moderate, severe, and critical COVID-19 cases according to the World Health Organization (WHO) definitions for COVID-19 severity classification (Table S1) upon completion of their hospitalization (until discharge or death) or recovery from COVID-19 (for patients with mild symptoms and outpatients). The maximum Sequential Organ Failure Assessment (SOFA) Score was calculated in hospitalized patients.

Comorbid conditions were summarized based on the Charlson Comorbidity Index (CCI). In patients, ACE I/D genotype, serum ACE activity, and serum

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**KEYWORDS**

ACE, angiotensin converting enzyme, COVID-19, ACE polymorphism, ACE activity, SARS-CoV-2
| General characteristics |  |
|--------------------------|--|
| Male (n, %) | 43 (53.1%) |
| Female (n, %) | 38 (46.9%) |
| Age (years) (mean ± SD) | 65 ± 18 |
| ≥60 years old (n, %) | 51 (63%) |
| Duration of symptoms on admission (days) (mean ± SD) (N = 73) | 7.3 ± 5.4 |
| Duration of hospitalization (days) (mean ± SD) | 21 ± 17 |
| Patients required ICU admission (n, %) | 12 (14.8%) |
| ICU length of stay (days) (mean ± SD of patients admitted in ICU) (N = 11) | 20.5 ± 14.2 |
| Duration of symptom onset to resolution (days) (mean ± SD) (N = 70) | 14.8 ± 11.2 |

| Comorbidities |  |
|---------------|--|
| Diabetes (n, %) (N = 80) | 15 (18.5%) |
| Current or past smoking (n, %) (N = 78) | 18 (22.2%) |
| Cardiovascular disease (n, %) (N = 79) | 13 (16.1%) |
| Hypertension (n, %) (N = 80) | 38 (46.9%) |
| ACEi or ARB therapy (n, % of those with hypertension) | 20 (52.6%) |
| End stage renal disease (n, %) (N = 80) | 6 (7.4%) |
| Liver cirrhosis (n, %) (N = 78) | 1 (1.2%) |
| Heart failure (n, %) (N = 78) | 6 (7.4%) |
| Dyslipidemia (n, %) (N = 80) | 21 (25.9%) |
| Active cancer (n, %) (N = 78) | 8 (9.9%) |
| Autoimmune/inflammatory disease (n, %) (N = 78) | 2 (2.5%) |
| Charlson Comorbidity Index (mean ± SD) (N = 78) | 3.83 ± 3.05 |

| Symptoms (N = 78) |  |
|-------------------|--|
| Cough (n, %) | 47 (58%) |
| Fever >38°C (n, %) | 58 (71.6%) |
| Malaise/anorexia (n, %) | 44 (54.3%) |
| Myalgia (n, %) | 12 (14.8%) |
| Dyspnea (n, %) | 30 (37%) |
| GI symptoms (n, %) | 17 (21%) |
| Anosmia/ageusia (n, %) | 11 (13.6%) |
| Pharyngalgia (n, %) | 8 (9.9%) |
| CNS symptoms (n, %) | 15 (18.5%) |

| WHO COVID-19 disease severity classification |  |
|---------------------------------------------|--|
| Mild (n, %) | 8 (9.9%) |
| Moderate (n, %) | 21 (25.9%) |
| Severe (n, %) | 39 (48.1%) |
| Critical (n, %) | 13 (16.1%) |

| SOFA score (N = 78) |  |
|---------------------|--|
| Maximum SOFA (mean ± SD) (N = 78) | 3.2 ± 3.1 |
| Maximum SOFA 0–5 (n, %) (N = 78) | 65 (80.2%) |
2.4 | ACE genotyping

Investigation of the ACE I/D polymorphism in intron 16 of the ACE gene was performed by PCR in genomic DNA isolated from whole blood. A second internal PCR of the DD genotypes was performed to avoid mistyping of ID as DD genotype (details in Supporting Information Appendix).

2.5 | Determination of serum ACE activity

Serum ACE activity was determined in a subgroup of patients (n = 52) according to the manufacturer’s instructions (Sentinel Diagnostics) and analyzed on ROCHE Cobas 801 analyzer (Roche Diagnostics). The inter- and intra-assay coefficient variations were <2% and <2.6%, respectively. In a subgroup of patients (n = 10) ACE activity was measured in sequential samples (3–6 for each patient) collected at different time points of the disease course (details in Supporting Information Appendix).

2.6 | Determination of serum Anti-SARS-CoV-2S

Serum samples from patients taken on or after the 10th day of the disease course were analyzed for anti-SARS-CoV-2S antibody titer using Elecsys Anti-SARS-CoV-2S assay in Cobas 801 analyzer (Roche Diagnostics). The assay sensitivity and specificity were 98.8% and 100%, respectively. Values <0.8 U/ml are considered negative for anti-SARS-CoV-2S (details in Supporting Information Appendix).

2.7 | Statistical analysis

We estimated overall (pooled data) and per group (controls and patients) allelic and genotypic frequencies and tested for significance for differences of allelic and genotypic frequencies between groups using exact G-tests. A generalized linear model and Poisson regression analysis were used to evaluate the risk of developing a severe disease using as predictor variables the allele/genotype, the gender, and age of subjects. ACE activity was subject to multifactor analysis of variance, fitting group (controls, patients), genotype, and gender as fixed effects (factors), and age of subjects within groups as a linear covariate (details in Supporting Information Appendix).

### Table

| Outcome | | |
|---------|--|--|
| Alive and fully functioning (n, %) | 54 (66.6%) |
| Alive and functionally impaired (n, %) | 19 (14.5%) |
| In-hospital mortality (n, %) | 8 (9.9%) |

### Laboratory parameters

| Parameter | Median ± IQR |
|-----------|--------------|
| Lymphocyte count (10⁶/L) | 820 ± 700 |
| Maximum neutrophil to lymphocyte ratio | 7.00 ± 8.06 |
| Maximum CRP (mg/L) | 113.0 ± 145.3 |
| Maximum ferritin (ng/ml) | 832 ± 1074 |
| Maximum IL-6 (pg/ml) | 42.90 ± 61.35 |
| D-dimers (>1000 ng/ml) | 42 (51.9%) |

Note: Frequencies were calculated among all 81 patients.

Abbreviations: ACEi, angiotensin converting enzyme inhibitors; ARB, angiotensin II receptor blockers; CNS, central nervous system; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; ICU, intensive care unit; IL-6, interleukin 6; N, number of patients with available data (when data were not available for all patients); SOFA score, Sequential Organ Failure Assessment score; WHO, World Health Organization.

*Symptoms were not resolved in nine cases (eight deaths and one patient who was transferred to a long-term care facility with oxygen).
3 | RESULTS

3.1 | Patients' demographics and characteristics

Table 1 shows the demographics and clinical characteristics of the patients. The majority of the patients were males (n = 43, 53.1%) and their age was higher than controls (64.81 ± 1.50 vs. 41.02 ± 0.76 years; p = 0.017). Fifty-nine (72.8%) of the cases had at least one comorbidity while 24 (40.7%) had 3–6 comorbidities. Seventy-five patients (92.6%) were admitted to the hospital, whereas six were followed up on an outpatient basis. Eight patients fulfilled the definition for mild disease (including the six outpatients), while the majority of the patients (n = 60, 74%) had either moderate or severe or critical COVID-19 disease (Table 1).

Most patients (n = 73, 90.1%) were discharged from the hospital, while the in-hospital mortality was 9.9%. As shown in Table 2, disease laboratory markers (maximum NLR, maximum CRP, maximum ferritin, maximum IL-6, lower nadir lymphocyte counts, d-dimers) deteriorate with increasing COVID-19 severity class. Since laboratory data were available for only two mild cases, mild patients were not included in our analysis.

3.2 | ACE genotyping and association with clinical parameters

Results of Poisson regression analysis are shown in Table 3 and in Figure 1. In both models (allelic and genotypic), the χ² tests that deviance values followed a χ² distribution equal to residual df resulted in nonsignificant p values (allelic model: df = 774, χ² = 779.6, p = 0.4369; genotypic model: df = 384, χ² = 378.5, p = 0.5696) indicating that the specified models fitted the data reasonably well. Patients carrying the D allele had a risk ratio (RR) 1.4 (RR: 1.372; confidence limit 95%: 1.051–1.000) indicating that they were more likely to develop a more severe disease when contrasted to I allele carriers. Regarding genotype results, DD patients when contrasted to ID or to pooled ID and II individuals, had a 1.9–fold (p = 0.0018) and 1.65-fold (p = 0.0056) increased risk of developing a more severe disease, respectively, while the elevated RR for DD versus II patients was not statistically significant. With regard to the rest two predictor variables (sex and age), RR between sexes was not statistically different (1.110 and 1.092 in the allelic and genotypic models, respectively) (Table 3). Age of patients was associated with increased risk of developing a more severe disease as revealed by the estimated regression coefficients for age (0.0610 ± 0.0033, p < 0.001 and 0.0612 ± 0.0047, p < 0.001, in the allelic and genotypic model; respectively) (results not shown). The expected increase in log count for a year increase in age is about 0.06 implying that elder subjects are more likely to develop more severe disease.

As shown in Table 4, the frequency of the D allele was higher in patients than controls (0.678 vs. 0.601) and this was also the case for the DD genotypes with genotypic frequencies as high as 0.534 in patients and 0.364 in controls. Application of exact G-tests revealed that neither allelic (χ² = 4.742, df = 2, p = 0.0934) nor genotypic frequencies (χ² = 4.515, df = 2, p = 0.0045) were significantly different between controls and patients. The pooled population (controls and patients) was in Hardy Weinberg equilibrium (Fis = 0.072, p = 0.097, results not shown) as well as the controls (Fis = 0.012, p = 0.469). Patients deviated from HWE expectations (Fis = 0.347, p = 0.004) due to heterozygote deficit (21 observed vs. 32 expected homozygotes) (Table S2).

3.3 | ACE activity

Serum ACE activity (expressed as arbitrary units, AU/L) was lower in patients than in controls (3.62 ± 0.26 vs. 4.65 ± 0.13, p < 0.001) (Figure 2 top, and Table S3). ACE activity in the pooled population was highest in

| TABLE 2 | Selected laboratory parameters of patients classified by WHO COVID-19 severity criteria |
|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
|                  | Moderate | Severe | Critical | Overall p value |
| Laboratory parameters  | Median (IQR) | Median (IQR) | Median (IQR) | Median (IQR) | Median (IQR) |
| Minimum lymphocyte count (10⁶/L) | 1200 (500)  | 740 (510)  | 430 (240)  | 0.0005 |
| Maximum NLR | 4.2 (4.6)  | 6.9 (7.2)  | 19.5 (25.3)  | 0.0001 |
| Maximum CRP (mg/L) | 47.7 (89.6)  | 96.2 (92)  | 266 (53)  | <0.0001 |
| Maximum ferritin (ng/ml) | 645 (478)  | 832 (805)  | 2126 (3433)  | <0.0001 |
| Maximum IL-6 (pg/ml) | 26.2 (14.8)  | 44 (27.6)  | 177.4 (286.8)  | 0.030 |
| D-dimers >1000 ng/ml | 8 (38.1)  | 21 (56.8)  | 12 (92.3)  | 0.0066 |

Abbreviations: COVID-19, coronavirus disease 19; CRP, C-reactive protein; IL-6, interleukin 6; IQR, interquartile range; N, number of patients with available data; NLR, neutrophil to lymphocyte ratio.

a,b Medians with different letters as superscripts within the same parameter are statistically significant different p < 0.05.
Individuals (4.74 ± 0.17 AU/L), intermediate in ID (4.20 ± 0.18 AU/L), and lowest in II (3.46 ± 0.27 AU/L) (Figure 2 middle). In Figure 2 (bottom), within controls, highest (5.38 ± 0.21 AU/L), intermediate (4.68 ± 0.16 AU/L), and lowest ACE activity (3.80 ± 0.16 AU/L) was observed for DD, ID, and II genotypes, respectively. Within patients, no difference was found between the different genotypes. Importantly, ACE serum activity in DD patients was significantly lower when contrasted to DD control subjects (3.97 ± 0.29 vs. 5.38 ± 0.21 AU/L, \( p = 0.0014 \)) whereas no difference was recorded for II and ID individuals between patients and controls (Table S3). No differences were detected for ACE activity across the severity classes. Finally, the estimated intraclass correlation coefficient of ACE activity (subgroup of 10 patients) was as high as 0.79 (confidence interval 95%: 0.72–0.89) denoting relatively low variation among sequential measurements within each patient.

3.4 | Other disease laboratory parameters and anti-SARS-CoV-2S levels

Regarding other disease laboratory parameters, DD patients displayed the highest levels of inflammatory markers (NLR (7.97 ± 8.08), ferritin (907 ± 1027.0), and IL-6 (55.2 ± 77.1) (Table S4) and the lowest lymphocyte count (690 ± 650). No statistical significance could be established. Higher anti-SARS-CoV-2S levels were observed in DD patients (3.48 ± 0.61), intermediate in the ID (2.96 ± 0.89), and lower in II (1.37 ± 1.12), however, no statistical significance was observed (\( p = 0.275 \)) (Table S5). Neither the antibody levels nor disease laboratory parameters were correlated with ACE activity.

4 | DISCUSSION

We carried out a retrospective case-control study aiming to ascertain whether or not ACE I/D polymorphism may be used as a host genetic factor for COVID-19 disease severity. Our results showed that individuals with DD genotype had increased risk of developing more severe disease when contrasted with the other ACE genotypes (DI and II). However, the association between ACE D/I genotypes, ACE activity, and predisposition to COVID-19 disease and COVID-19 increased severity is not a straightforward association.

| Allele | Deviance | df | Risk ratio (Wald 95% confidence limits) | \( p \) value |
|--------|----------|----|----------------------------------------|-------------|
| D versus I | 1.372 (1.051–1.791) | 0.020 |
| Males versus females | 1.110 (0.863–1.428) | 0.416 |

Genotype

| Genotype | Deviance | df | Risk ratio (Wald 95% confidence limits) | \( p \) value |
|----------|----------|----|----------------------------------------|-------------|
| DD versus ID | 1.886 (1.266–2.810) | 0.0018 |
| DD versus II | 1.433 (0.895–2.293) | 0.1341 |
| DD versus ID and II | 1.644 (1.157–2.336) | 0.0056 |
| ID versus II | 0.760 (0.453–1.273) | 0.297 |
| Males versus females | 1.092 (0.770–1.546) | 0.622 |

Abbreviations: ACE, angiotensin-converting enzyme; GLM, generalized linear model; RR, risk ratios.

**TABLE 3** RR of patients’ ACE alleles and genotypes obtained from GLM analysis

**FIGURE 1** RR with 95% confidence limits for allele and genotype contrasts. RR, relative risks

**TABLE 4** Overall and per group allelic and relative genotypic frequencies of the ACE gene polymorphisms (n: number of subjects)

| Alleles/ genotypes | Group | Controls (95% CI) | Patients (95% CI) | Overall (95% CI) |
|---------------------|-------|------------------|------------------|------------------|
| | | D | 0.601 (0.562–0.640) | 0.678 (0.600–0.755) | 0.616 (0.581–0.650) |
| | | I | 0.399 (0.360–0.438) | 0.322 (0.245–0.400) | 0.384 (0.349–0.419) |
| | | DD | 0.364 (n = 115) | 0.534 (n = 39) | 0.396 (n = 154) |
| | | ID | 0.475 (n = 150) | 0.288 (n = 21) | 0.440 (n = 171) |
| | | II | 0.161 (n = 51) | 0.178 (n = 13) | 0.165 (n = 64) |

DD individuals (4.74 ± 0.17 AU/L), intermediate in ID (4.20 ± 0.18 AU/L), and lowest in II (3.46 ± 0.27 AU/L) (Figure 2 middle). In Figure 2 (bottom), within controls, highest (5.38 ± 0.21 AU/L), intermediate (4.68 ± 0.16 AU/L), and lowest ACE activity (3.80 ± 0.16 AU/L) was observed for DD, ID, and II genotypes, respectively. Within patients, no
The initial hypothesis, based on epidemiological data, was that ACE DD genotype may be associated with a better COVID-19 prognosis. However, the accumulation of data from additional epidemiological studies, showed that the ACE D allele was involved in the susceptibility to SARS-CoV-2 infection and the exacerbation of symptoms such as pneumonia. Gomez et al., working on case-control samples, showed that ACE DD genotype was more frequent in severely affected COVID-19 patients compared to mild patients.
this effect being dependent on their hypertensive status. This was in agreement with the findings of Itoyama et al.\textsuperscript{14} in the previous SARS endemic, who had also reported that the D allele was correlated with disease severity on the basis of hypoxia. On the other hand, recently, Celik et al.\textsuperscript{15} reported no association between ACE I/D polymorphism and the clinical course of 155 COVID-19 patients. Such findings may fairly attract some criticism on results obtained from genetic association analyses, as genetic effects along with diabetes, hypertension and other comorbidities may be confounding factors interfering with disease severity.\textsuperscript{16} The present study revealed that the link between the ACE DD genotype (or the D allele) and increased risk of developing a severe COVID-19 disease was independent of the comorbidity index (CCI). Certainly, large-scale studies are warranted to untangle the role and importance of all implicated factors. To investigate the functional role of ACE polymorphism in disease severity and aetiology, we measured serum ACE activity. The DD individuals in the control group showed higher ACE activity than ID and II subjects, as expected.\textsuperscript{17} The insertion of the intronic Alu sequence, expressed as an AluYa5 RNA, in intron 16 of the ACE gene, affects ACE mRNA expression\textsuperscript{18} and thus determines part of the interindividual variability plasma ACE levels and activity. Although this trend was not clear in the group of patients, this group showed significantly lower serum ACE activity as compared to controls. Furthermore, no differences in ACE activity were seen between the different disease severity cases, while sequential measurements of ACE activity (within each patient), showed no intra-individual variability and remained consistently low throughout the disease course. In line with our findings, Zhu et al.\textsuperscript{19} found lower ACE activity in COVID-19 patients which, however, increased after recovery. Low ACE activity has also been detected in other lung disorders such as ARDS and has been correlated with the severity of lung injury and hypoxemia.\textsuperscript{20,21} We hypothesize that the decreased ACE activity in our COVID-19 patients could be due to their hypoxic respiratory failure or to disease induced-molecules (proteolytic enzymes and natural ACE inhibitors).\textsuperscript{19}

It should be noted that the reduced ACE activity in our patient group was observed mainly among DD subjects. In an attempt to explain this finding, we refer to the modulation of ACE amino (N-) and carboxy (C-) terminal domain activities by the ACE I/D polymorphism. At the protein level, the ACE enzyme presents two functional active sites that belong to two independent catalytic domains (N- and C-) which share 60% amino acid homology and differ in substrate and inhibitor specificities and activity.\textsuperscript{22} It has been reported that in DD individuals only the C-domain converts Ang I to AngII\textsuperscript{22} while the N-activity of DD subjects is higher than that of ID (133%) and even higher (228%) than that of II.\textsuperscript{23} Based on the above, we speculate that in COVID-19 patients, disease state-induced molecules (such as proteolytic enzymes) may interfere preferentially with the catalytic C-domain of the enzyme affecting thus more the ACE activity of DD carriers than that of ID or II carriers.

It has been hypothesized that ACE2 downregulation, through binding of SARS-CoV-2S protein to ACE2, results in an imbalance in the ACE/ACE2 axis thus leading to attenuation of Ag 1–7 and augmentation of AngII.\textsuperscript{3} In line with the above hypothesis, increased levels of plasma Ang II were strongly associated with lung injury severity in COVID-19 patients.\textsuperscript{24} Given that ACE expression/activity is subject to negative feedback by Ang II,\textsuperscript{25} one may hypothesize that the increased AngII in COVID-19 may lead to decreased ACE activity. The low ACE activity, demonstrated in our study and in the study of Zhu et al.,\textsuperscript{19} supports the hypothesis of activated RAS axis in COVID-19 with increased AngII alongside with decreased ACE activity as proposed by Zhang et al.\textsuperscript{3} Taking all together, we could hypothesize that the ACE DD individuals may be more prone to interaction with disease state—induced factors compared to II and ID individuals which result in reduced ACE activity. The significant fall of ACE activity in DD patients subsequently affects the ACE/ACE2 axis, thus, leading to exaggeration of COVID-19 symptoms during the course of the disease.

In the study of Zhu et al.,\textsuperscript{19} including 120 nonsevere and 16 severe COVID-19 patients, the authors reported that baseline serum ACE activity was negatively correlated with NLR, CRP, neutrophil% and positively correlated with lymphocyte% and lymphocyte count. In our study, we find no correlations either with the above laboratory parameters or the anti-SARS-CoV-2S titers. The fact that disease laboratory parameters and ACE activity were measured at different time points of the disease course and not at baseline may explain the controversial findings.

To our knowledge, only a few studies investigating ACE I/D polymorphism in COVID-19 patients include wet-lab data, while none of them refers to associations between ACE genotypic profile and ACE activity in a well-characterized COVID-19 patient population. It should be noted that patient recruitment was conducted during early pandemic, when essentially aged people were affected and no effective treatment options were available. Hydroxychloroquine was administered to all patients as standard of care, however, this treatment has demonstrated a lack of efficacy in numerous well-designed studies.\textsuperscript{26} To this end, neither the clinical course of the disease nor the ACE activity were influenced by therapies that could potentially modify the clinical outcomes. Given that dexamethasone interferes in ACE activity,\textsuperscript{27,28} with the current guidance, whereby dexamethasone is regarded as a standard of care for patients requiring supplemental oxygen, such data are difficult, if not ethically impossible, to acquire especially among patients with severe and critical COVID-19 disease. Furthermore, this is a single-center study and a multicenter study with a larger number of patients is needed. ACE activity was assessed during the disease course and not in the recovery stage and the conclusions are based on serum ACE activity and not on ACE activity in lung tissue or in bronchoalveolar lavage fluid. To substantiate a possible role of RAS in the pathogenesis of COVID-19, ACE2 as well as AngII and Ang1–7 serum levels should be determined along with ACE activity and ACE I/D polymorphism.

In conclusion, our results showed that the D allele and D/D genotype increase the risk of developing a more severe COVID-19 disease, while in COVID-19 patients the association of ACE I/D genotypic profile with ACE activity is deviated from what is generally
observed in healthy individuals. We anticipate that our results might set the grounds for large-scale studies to assess ACE genotype-based optimized therapies using ARBs and ACE inhibitors.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Concept of work: Paraskevi Moutsatsou. Design of work: Paraskevi Moutsatsou and Anastasia Antoniadou. Literature search: Paraskevi Moutsatsou and Anna Papadopoulou. Stratification and characterization of patient and control group: Paraskevi C. Fragkou, Dimitra Dimopoulou, Anastasia Antoniadou, Argiro Tsantes, Eirini Maratou, Vasiliki Papaevangelou, Apostolos Armaganidis, and Efthyxia Polyzogopoulou. Execution of experiments (biochemical and genetic analysis): Eirini Maratou, Ioanna Kokkinopoulou, Athina Nikolaidou, Anna Papadopoulou, Christos Kroupis, and Georgios Antonakos. Statistical analysis of data: Antonis Kominakis. Writing of paper introduction, materials and methods, results, discussion: Anna Papadopoulou, Paraskevi C. Fragkou, E. Maratou, Ioanna Kokkinopoulou, Antonis Kominakis, and Paraskevi Moutsatsou. Interpretation of data: Paraskevi Moutsatsou Anna Papadopoulou, Paraskevi C. Fragkou, and Antonis Kominakis. Critically revising the paper: Anastasia Antoniadou, Vasiliki Papaevangelou, Sotirios Tsiodras, Apostolos Armaganidis, and Paraskevi Moutsatsou. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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