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ACE2, ACE, DPPIV, PREP and CAT L enzymatic activities in COVID-19: imbalance of ACE2/ACE ratio and potential RAAS dysregulation in severe cases

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Abstract

Objective and design Circulating enzymatic activity and RAAS regulation in severe cases of COVID-19 remains unclear, therefore we measured the serum activity of several proteases as potential targets to control the SARS-CoV-2 infection.

Material or subjects 152 patients with COVID-19-like symptoms were grouped according to the severity of symptoms (COVID-19 negative, mild, moderate and severe).

Methods Serum samples of COVID-19 patients and controls were subjected to biochemical analysis and enzymatic assays of ACE2, ACE, DPPIV, PREP and CAT L. One-way ANOVA and multivariate logistic regression analysis were used. Statistical significance was accepted at $p < 0.05$.

Results We detected a positive correlation among comorbidities, higher C-reactive protein (CRP) and D-dimer levels with disease severity. Enzymatic assays revealed an increase in serum ACE2 and CAT L activities in severe COVID-19 patients, while ACE, DPPIV and PREP activities were significantly reduced. Notably, analysis of ACE2/ACE activity ratio suggests a possible imbalance of ANG II/ANG(1-7) ratio, in a positive association with the disease severity.

Conclusion Our findings reveal a correlation between proteases activity and the severity of COVID-19. These enzymes together contribute to the activation of pro-inflammatory pathways, trigger a systemic activation of inflammatory mediators, leading to a RAAS dysregulation and generating a significant damage in several organs, contributing to poor outcomes of severe cases.

Keywords COVID-19 · SARS-CoV-2 · ACE2 · RAAS · Proteases

Introduction

Novel coronavirus denominated SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), identified as COVID-19, was first reported in China (December 2019) and rapidly spread across the world [1, 2]. SARS-CoV-2 presents an enveloped genome composed by a single-stranded RNA, which is packaged into a helical nucleocapsid delimited by a host-derived lipid bilayer [3]. During the infection, coronavirus in general uses the spike protein (S) to interact with specific receptors, representing a fine interplay between virus and host cells [4, 5].

ACE2 (angiotensin-converting enzyme 2) presents an essential role in COVID-19 disease, since it was identified as the main receptor to S protein [6]. ACE2 (EC 3.4.17.23) is a zinc metalloprotease (carboxypeptidase) [7] with a broad tissue distribution, expressed in heart, intestine, kidneys, lungs and other organs [8–10], contributing to essential physiological processes such as regulation of cardiovascular system and inflammation [11, 12]. Anchored ACE2 is cleaved from the membrane by different enzymes, such as ADAM17 (a disintegrin and metalloproteinase 17), MMP-14 (matrix metalloprotease 14) and TMPRSS2 (transmembrane serine protease 2), releasing into circulation the N-terminal ectodomain which contains the catalytic site, generating the soluble

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form (sACE2) [13–15]. Therefore, the proteolytic shedding is an important step to predict the differential levels of ACE2 between the cell protein and the circulating form [13].

In COVID-19, tissues and cells expressing high levels of ACE2 are potential targets to SARS-CoV-2 infection, therefore, the distribution and amount of ACE2 could be closely related to disease severity [10]. In response to the virus attachment to ACE2, the complex is internalized into the target cell, leading to ACE2 down-regulation (membrane-bound) [16]. As a result, the level of DBK (des-Arg^9-bradykinin) is increased, an ACE2 substrate, resulting in a pro-inflammatory reaction promoted by the BDKRB1 (kinin B1 receptor) activation [17]. ACE2 also reduces the generation of ANG II (Angiotensin II) by catalyzing the conversion of ANG I (Angiotensin I) to ANG(1-9) and facilitating hydrolysis of ANG II to ANG(1-7) [18]. Furthermore, reduced ACE2 levels in lung cells expose the tissue to acute inflammation [19].

Another close-related protease, ACE (angiotensin-converting enzyme), is highly expressed in endothelial lung cells [20] but might be shed from these cells, generating a plasmatic soluble form (sACE). ACE (EC 3.4.15.1) is a dipeptidyl-carboxypeptidase [21] that converts ANG I into ANG II and catalyzes BK (bradykinin) inactivation. ACE expression is altered in patients with severe forms of COVID-19 [22]. Both RAAS (renin–angiotensin–aldosterone system) and KKS (kallikrein-kinin system) pathways control vascular permeability and vasodilation, as well as inflammation, causing lung dysfunction [12].

The inflammatory response is a determining factor to predict the outcome of COVID-19 patients. DPPIV (Dipeptidyl peptidase-IV) (EC. 3.4.14.5) and PREP (prolyl endopeptidase) (EC 3.4.21.26) are serine peptidases present in a soluble form in the circulation, closely associated with inflammatory diseases [23, 24]. These multifunctional proteins are broadly expressed in numerous tissues such as gut, liver, lung and kidney [25]. The biological activity of DPPIV includes the regulation of intracellular signal transduction, cell migration and proliferation [26]; and it also functions as a cell membrane surface co-receptor for human MERS-CoV (middle east respiratory syndrome coronavirus) cell entry [27]. PREP cleaves a variety of neuropeptides and hormones [28]. Both DPPIV and PREP degrade BK, thus possibly modulating signaling pathways in COVID-19. Furthermore, recent studies correlate DPPIV and PREP to comorbidities and indicate a possible role in COVID-19 severity [29–31].

The recent discover that SARS-CoV-2 life cycle requires the activity of an endosomal cysteine protease evidences the growing importance of CAT L (cathepsin L) in studies related to COVID-19 severity [32, 33]. CAT L (EC 3.4.22.15) is a lysosomal endopeptidase primarily involved on the processing and turnover of proteins in acidic compartments. Extra-lysosomal activity of CAT L was also demonstrated, raising the possibility of a non-canonical extracellular function, in a non-acidic condition [34–36]. In COVID-19, elevated circulating levels of CAT L were detected in critical patients of ICU (Intensive Care Unit) [37].

In the present study, using a well-defined group of hospitalized COVID-19 patients and COVID-19 negative controls, we investigated whether the specific serum activities of ACE2, ACE, DPPIV, PREP and CAT L could be correlated with moderate or severe presentations of the disease. Our findings suggest correlations between the enzymatic activity of such enzymes and COVID-19 severity that could be explored in the future as biomarkers, increase the comprehension of the disease and pave the way to new therapies.

Material and methods

Ethical approval statement

The procedures performed in this work were conducted according to the project approved by the research ethics committee of the Federal University of São Paulo (CAAE 31929120.0.0000.5505).

Patients and clinical samples

A total of 152 patients with COVID-19 symptoms admitted to the São Paulo Hospital during the period between July 2020 and June 2021 were included in this study. In order to diagnose COVID-19, samples from upper respiratory tract (nasopharyngeal specimens) were collected immediately after the admission (day 0) and nucleic acid amplification test were performed by qPCR (quantitative polymerase chain reaction). The qPCR was performed according to the protocol established previously [38]. Blood samples were also collected in serum collection tubes to evaluate enzymatic activity assays.

Clinical screening

The 152 patients were grouped according to the severity of symptoms: (1) COVID-19 negative group, which includes patients with COVID-19-like symptoms with negative PCR test for COVID-19 (56 patients); (2) positive PCR test for COVID-19, presenting only mild symptoms (including fever, cough, headache, muscle pain, fatigue, nausea, diarrhea and others) but without pneumonia (31 patients); (3) positive PCR test for COVID-19 patients with moderate, respiratory tract symptoms, and chest x-ray confirming pneumonia (38 patients); (4) positive PCR test for COVID-19 patients with severe symptoms, presenting any of the following signs: respiratory distress, oxygen saturation $\leq 94\%$,

arterial blood oxygen partial pressure ≤ 300 mmHg, lung infiltrates ≥ 50%, requirement of mechanical ventilation and multiple organs dysfunction (27 patients). Patients with comorbidities (asthma, obesity, cardiac disease, diabetes and other diseases) were classified according to their clinical manifestations.

ACE2 enzymatic activity assay

ACE2 catalytic activity was examined in serum samples from patients with COVID-19 symptoms (mild, moderate and severe groups) and from COVID-19 negative group. ACE2 activity was assessed using a 75 mmol/l Tris buffer pH 7.5 containing 50 mmol/l NaCl, 10 µmol/l $ZnCl_2$ and the fluorogenic substrate Mca-Ala-Pro-Lys(Dnp)-OH (5 µmol/l). The specific activity was confirmed using 5 µmol/l DX600 (trifluoroacetate salt) as inhibitor [39]. The enzymatic activity was monitored in a microplate reader (Molecular Devices M2—San Jose, California, USA) for 1 h at 37 °C and fluorescence measured at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm.

ACE enzymatic activity assay

ACE activity was evaluate using 100 mmol/l Tris–HCl, 50 mmol/l NaCl and 10 µmol/l $ZnCl_2$, pH 7.0 assay buffer, with 10 µmol/l Abz-Phe-Arg-Lys(Dnp)-Pro-OH substrate ($\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm) at 37 °C for 30 min. The specificity of the hydrolysis was confirmed by preincubation of 10 µmol/l lisinopril with samples before the addition of substrate. The emitted fluorescence was monitored every minute in a microplate reader (Molecular Devices—San Jose, California, USA).

ACE polymorphism

To determine ACE polymorphism (I/D alleles) (rs4646994), the DNA was extracted from blood samples collected in tubes containing EDTA (ethylenediamine tetraacetic acid) using Chelex®100 resin (Sigma Aldrich—Darmstadt, Steinheim, GER). The genotypes were screened by (PCR) using sense primer (5′-GATGTGGCCATCACATTCGTCAGA T-3′) and antisense primer (5′-CTGGAGACCACTCCCATC CTTTCT-3′). Conditions for the PCR reactions were 95°C for 5 min, 35 cycles at 95°C for 45 s, 59°C for 45 s, 72°C for 45 s and a final extension at 72°C for 5 min. Homozygotes DD and II present a fragment with 190 bp and 490 bp, respectively, whereas heterozygote ID shows these 2 fragments. The amplified fragments were analyzed on a 2% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen—Waltham, Massachusetts, USA).

DPPIV enzymatic activity assay

DPPIV activity was determined according to previous published data [40]. The assays were performed on 50 mmol/l HEPES buffer, pH 7.5 with 1 mmol/l of Gly-Pro-p-nitroanilide hydrochloride substrate at 37 °C for 30 min. Absorbance was evaluated at 405 nm every minute using the direct photometric method. DPPIV inhibition was performed pre-incubating the samples for 30 min using 100 nmol/l linagliptin, a DPPIV inhibitor. Using a microplate reader, the fluorescence emission was monitored every minute (Molecular Devices—San Jose, California, USA).

PREP catalytic activity assay

The catalytic activity of PREP enzyme was monitored using the fluorogenic Z-Gly-Pro-AMC (7-amino-4-methyl coumarin) substrate ($\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm) on 50 mmol/l sodium phosphate buffer (pH 7.4), containing 2 mmol/l DTT (1,4 dithiothreitol). The KYP 2047 ((2S)-1-[[(2S)-1-(1-Oxo-4-phenylbutyl)-2-pyrrolidinyl]carbonyl]-2-pyrrolidinecarbonitrile) (5 µmol/l) inhibitor was used to confirm the hydrolysis specificity. The assays were performed at 37 °C for 1 h and the fluorescence emission was assessed every minute in a microplate reader (Molecular Devices—San Jose, California, USA).

Cathepsin L catalytic activity assay

CAT L activity was assessed using a 100 mmol/l sodium acetate buffer, pH 5.5, containing 5 mmol/l DTT and 10 µmol/l Z-Phe-Arg-AMC (7-amino-4-methyl coumarin) as fluorogenic substrate ($\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm) at 37 °C. The hydrolysis specificity was confirmed using the cysteine inhibitor 10 µmol/l E64 (*trans*-Epoxysuccinyl-L-leucylamido (4-guanidino) butane). The enzymatic activity was monitored for 1 h every minute in a microplate reader (Molecular Devices—San Jose, California, USA).

Statistical analysis

Categorical variables were represented as number (%) and compared using the Chi-square with Fisher's exact tests. Continuous variables were expressed as mean ± standard deviation (SEM) or as median (IQR -interquartile range). One-way ANOVA was used to compare more than two groups, followed by Tukey's multiple comparison test. Multivariate logistic regression analysis (relative risk) was conducted to identify risk factors, with a 95% confidence interval. All statistical analyzes were performed using GraphPad Prism software (GraphPad Prism version 6). Statistical significance was accepted at $p < 0.05$ in all analyses.

Results

Screening of patients

A total of 152 patients with COVID-19 symptoms (fever, cough, fatigue, anosmia, headache) admitted to the university hospital UNIFESP, between July 2020 and June 2021, were included in this study. The patients were screened according to age, gender, ethnicity and comorbidities (Table 1), and subdivided in four groups according with the levels of symptoms: (1) patients with COVID-19-like syndrome and qPCR negative for COVID-19; (2) patients with mild symptoms; (3) patients with moderate symptoms admitted to the infirmary; (4) patients with severe symptoms, admitted in ICU. Patients from the group 2–4 were qPCR positive for COVID-19.

Predicting severity factors in COVID-19 disease

To investigate the correlation between comorbidities and the probability to develop severe condition of disease, we performed an associative measurement in patients with COVID-19 (confirmed by PCR test). Univariate logistic regression was used to measure RR (relative risk) of experiencing severe condition in patients of mild and severe groups with comorbidities, including metabolic disease (diabetes, obesity and dyslipidemia), hypertension, heart disease, chronic kidney disease and smoking. Relative risk was significantly high in patients with comorbidities in the severe group when compared to mild group (metabolic disease: RR, 2.17; 95.0% Cl, 1.72–2.74; hypertension: RR, 3.16; 95.0% CI, 1.67–5.97; chronic kidney disease: RR, 2.4; 95.0% CI, 1.58–3.65; smoking: 1.93; 95.0% CI, 1.21–3.07) (Fig. 1a).

Posteriorly, considering the association between higher concentrations of CRP (c-reactive protein) (an earlier marker of infection and inflammation) with severity of COVID-19

Table 1 Demographics and clinical characteristics of the 152 patients with COVID-19 and controls

Characteristics (number, %)	COVID-19 Negative (n = 56)	Mild (n = 31)	Moderate (n = 38)	Severe (n = 27)
Age (years) (mean, extremes, SD)	38.6 [18–64] ± 12.1	41.5 [20–65] ± 13.9	52.1 [20–88] ± 16.9	60.8 [41–77] ± 10.0
Sex				
Male	24 (42.9)	16 (51.6)	17 (44.7)	16 (59.3)
Female	32 (57.1)	15 (48.4)	21 (55.3)	11 (40.7)
Race/Ethnicity[#]				
Afrodescendant	4 (7.1)	–	7 (18.4)	1 (3.7)
Caucasian	17 (30.4)	17 (54.8)	16 (42.1)	17 (63.0)
Pardo	12 (21.4)	9 (29.0)	12 (31.6)	9 (33.3)
Others	4 (7.1)	2 (6.5)	–	–
Not declared	19 (33.9)	3 (9.7)	3 (7.9)	–
Smoker	3 (5.4)	2 (6.5)	12 (31.6)	8 (29.6)
Comorbidities				
No	32 (73.2)	19 (67.7)	5 (13.2)	4 (14.8)
Heart diseases[a]	2 (3.6)	3 (9.7)	4 (10.5)	7 (25.9)
Hypertension	11 (19.6)	5 (16.1)	19 (50.0)	19 (70.4)
Metabolic disease[b]	4 (7.1)	2 (6.5)	22 (57.9)	20 (74.1)
Chronic Kidney Disease	1 (1.8)	1 (3.2)	10 (26.3)	10 (37.0)
Lung diseases[c]	3 (5.4)	–	3 (7.9)	3 (11.1)
Immunosuppressed[d]	1 (1.8)	1 (3.2)	12 (31.6)	7 (25.9)
Malignancy	–	–	3 (7.9)	3 (11.1)
Others[e]	2 (3.6)	1 (3.2)	13 (34.2)	4 (14.8)

[#]Race/Ethnicity was self-declared by the patients

[a]Included cardiac insufficiency, stroke

[b]Diabetes, obesity and dyslipidemia

[c]Asthma, bronchitis, tuberculosis

[d]HIV, transplanted (kidney), systemic lupus erythematosus

[e]Cirrhosis, hepatic steatosis, autoimmune hepatitis, rheumatoid arthritis, arthrosis, hypothyroidism, diverticulitis, psoriasis, epilepsy, dementia, depression

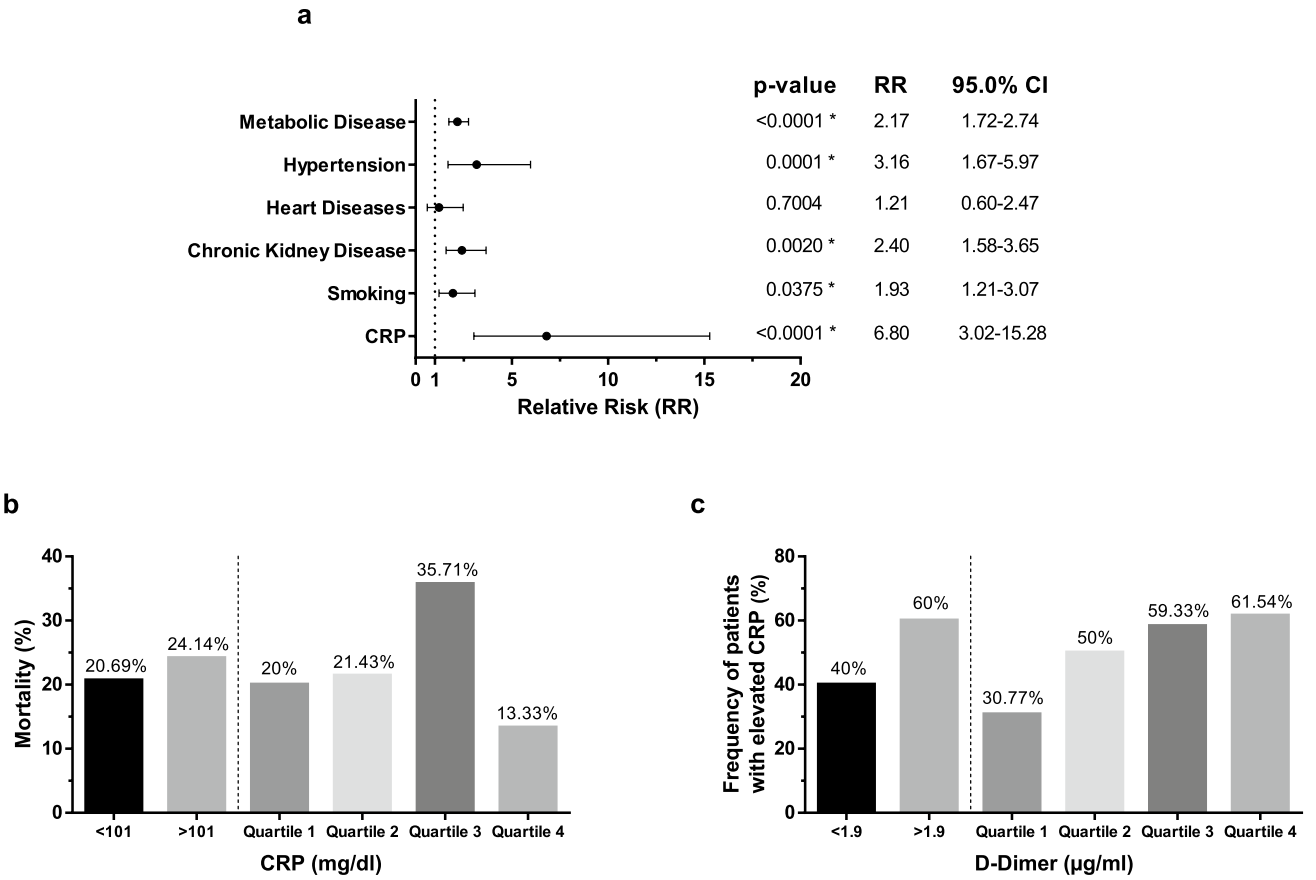

Fig. 1 Predicting severity factors in COVID-19 disease. **a** Summary plot of relative risk (RR) in patients with comorbidities (n = 56) of mild and severe groups by univariate logistic regression. **b**) Mortality rate versus CRP levels in patients of moderate and severe groups (n = 58). Median: 101 mg/dl. Quartile 1: < 54 mg/dl. Quartile 2: 54—101 mg/dl. Quartile 3: > 101 < 162 mg/dl. Quartile 4: ≥ 162 mg/dl. **c** Frequency of D-dimer concentrations in patients with increased levels of CRP (> 101 mg/dl) (n = 50). Median: 1.9 µg/ml. Quartile 1: < 1.2 µg/ml. Quartile 2: 1.2–1.9 µg/ml. Quartile 3: > 1.9 < 2.7 µg/ml. Quartile 4: ≥ 2.7 µg/ml. Statistical significance was evaluated by multivariate logistic regression analysis (relative risk), with a 95% confidence interval. An asterisk (*) denotes statistical significance ($p < 0.05$)

disease [41], we examined the RR in patients (mild and severe groups) with elevated levels of CRP. The analyses demonstrated an increase of RR in patients with high levels of CRP protein (values above 101 mg/l) (CRP: RR, 6.8; 95.0% CI, 3.02–15.28) (Fig. 1a). Furthermore, since moderate and severe groups present a higher probability to develop severe disease condition, mortality rate was evaluated in patients with CRP concentrations above of average (101 mg/l). Patients with elevated levels of CRP presented a positive correlation with mortality rate (Fig. 1b).

Another factor correlated to COVID-19 severity is D-dimer, an important coagulation marker, which is one of the products of fibrin degradation. Previous data indicate that concomitant high levels of D-dimer and CRP could be used as a prognosis for severe cases [42]. Here, we observed that the patients with elevated D-dimer and CRP levels are indicative of significant disease progression in COVID-19 (Fig. 1c).

ACE2 catalytic activity

ACE2 catalytic activity was measured in serum of patients with COVID-19 and in negative COVID-19 group using fluorogenic Mca-Ala-Pro-Lys(Dnp)-OH substrate. The analysis demonstrated increased ACE2 enzymatic activity in moderate and severe groups when compared to COVID-19 negative and mild groups (Fig. 2). The hydrolysis specificity was confirmed using DX600 inhibitor (5 µmol/l).

ACE enzymatic activity and polymorphism genotyping

Serum ACE activity was measured by a kinetic spectrophotometric assay using fluorogenic Abz-Phe-Arg-Lys(Dnp)-Pro-OH substrate. ACE catalytic activity was compared among the groups according to illness severity (Fig. 3). A significant difference in ACE activity was

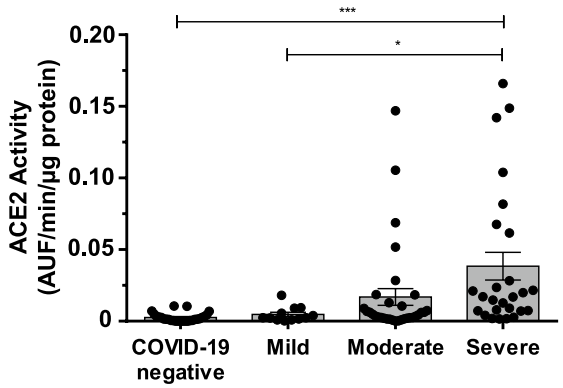

Fig. 2 ACE2 activity in serum of COVID-19 patients and controls. ACE2 enzymatic activity was measured in serum of patients with COVID-19-negative and in mild, moderate and severe groups. The patients were grouped according to symptoms levels. The assays were performed at 37 °C using the fluorescent Mca-Ala-Pro-Lys(Dnp)-OH substrate (5 µmol/l). Statistical significance was evaluated by ANOVA test. *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001

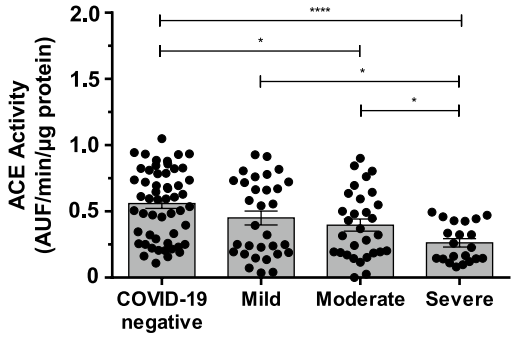

Fig. 3 ACE enzymatic activity in COVID-19 patients and controls. ACE activity was measured in the serum of patients with COVID-19 disease using the fluorogenic substrate Abz-Phe-Arg-Lys-(Dnp)-Pro-OH (10 µmol/l). Statistical significance was estimated by ANOVA test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

detected between COVID-19 negative and in patients with moderate or severe forms of the disease. The hydrolytic activity was completely inhibited by lisinopril (10 µmol/l), indicating the assay specificity. Patients treated with antihypertensive medication (ACE inhibitors) were excluded from the analysis. Therefore, our results show a decreased ACE activity in patients with more severe forms of COVID-19.

The genotype and allele frequency of the ACE polymorphism (I/D) were analyzed according to symptoms severity (Table 2). ID genotype was more prevalent (51.9% in the severe group, 50% in COVID-19 negative, 48.4% and 45.2% in moderate and mild groups, respectively), followed by DD and II genotypes in the patients. Thus, the D allele frequency was the most predominant in all the groups (Table 2). No statistical significance was observed between genotype or allele frequencies and COVID-19 severity in the analyzed groups.

Posteriorly, we compared ACE enzymatic activity among patients with and without COVID-19, according to ACE polymorphism genotype (DD or ID + II) (Fig. 4). Within the group COVID-19 negative, the DD genotype has higher ACE activity when compared with ID + II genotypes, however this difference was not found within the group of patients with COVID-19. Considering only the DD genotype, the serum ACE activity in COVID-19 patients was significantly lower when compared to COVID-19 negative, similarly observed in ID + II genotypes.

ACE2/ACE enzymatic activity ratio

Given that an imbalance in the ACE2/ACE ratio could be a risk factor for COVID-19 worsening [43, 44], the ACE2/ACE (soluble forms) ratio activity was compared between the COVID-19 (mild, moderate and severe groups) and negative groups. The results revealed an increase of the ACE2/ACE ratio only in ICU (severe patients) when compared to the other groups (Fig. 5). These data suggest that possibly an imbalance of RAAS might contribute to the worsening of symptoms and to the poor outcome of disease [43–45].

Table 2 The genotype and allele frequency of the ACE I/D polymorphism of COVID-19 negative group and patients infected with SARS-CoV-2

Frequency (number, %)	COVID-19 Negative (n = 56)	Mild (n = 31)	Moderate (n = 31)	Severe (n = 27)	p-(value)
II	10 (17.9)	8 (25.8)	5 (16.1)	3 (11.1)	0.9137[#]
ID	28 (50.0)	14 (45.2)	15 (48.4)	14 (51.9)	
DD	18 (32.1)	9 (29.0)	11 (35.5)	10 (37.0)	
I allele	48 (42.9)	30 (48.4)	25 (40.3)	20 (37.0)	0.6448
D allele	64 (57.1)	32 (51.6)	37 (59.7)	34 (63.0)	

The p value was established according to the proportions of the categorical variables analyzed by Chi-square test. p < 0.05 was considered statistically significant

[#]DD vs ID + II

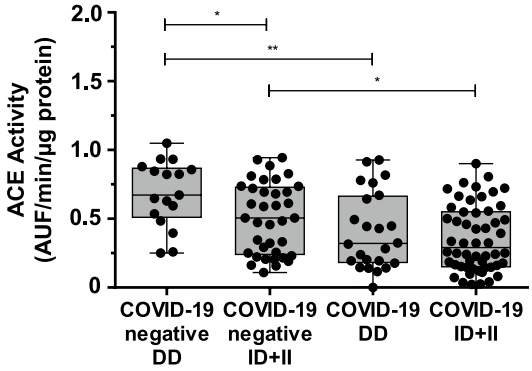

Fig. 4 Correlation of ACE activity and polymorphism. The ACE activity of patients according to genotype (DD vs ID + II) were grouped in COVID-19 negative and COVID-19 positive groups (mild, moderate and severe groups). Statistical significance was estimated by ANOVA test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

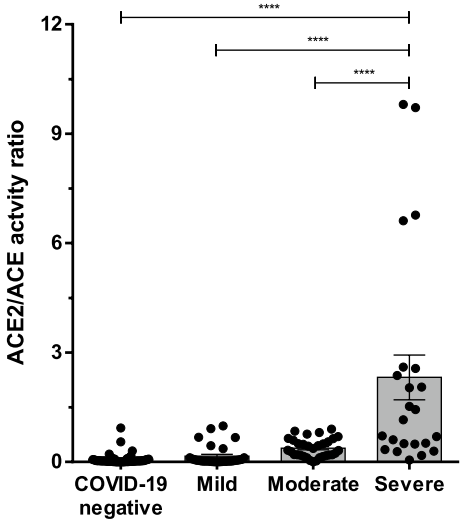

Fig. 5 Comparative ACE2/ACE ratio in COVID-19 groups and controls. The ACE2 and ACE enzymatic activities ratio was evaluated in each patient separately. The patients were grouped in COVID-19 negative and COVID-19 positive groups (mild, moderate and severe groups). Statistical significance was estimated by ANOVA test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

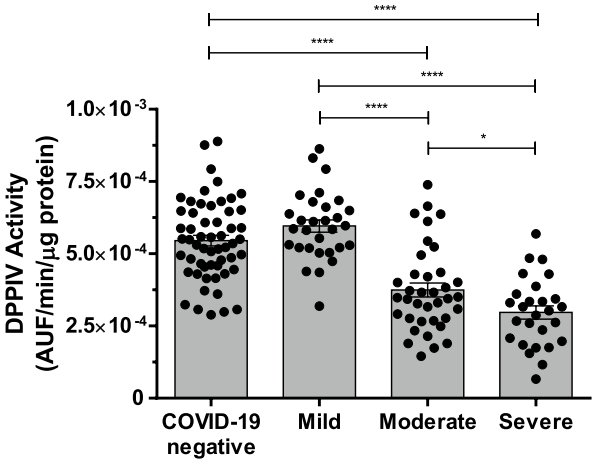

Fig. 6 DPPIV activity in COVID-19 patients and controls. DPPIV enzymatic activity was assessed in serum of COVID-19 patients using Gly-Pro-p-nitroanilide hydrochloride substrate (1 mmol/l). Statistical significance was assessed by ANOVA test. *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001

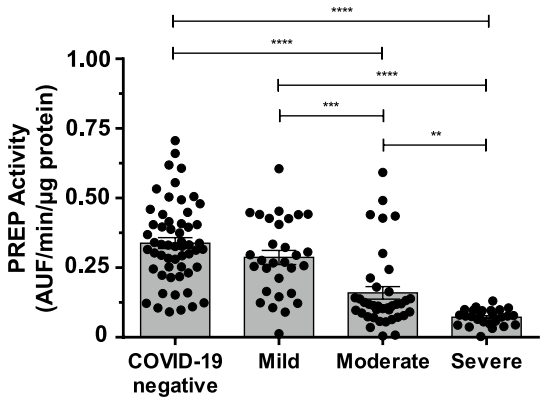

Fig. 7 Enzymatic activity of PREP (prolyl-endopeptidase). PREP catalytic activity was monitored in serum of COVID-19 patients and in negative group. The enzymatic assays were performed using fluorogenic substrate Z-Gly-Pro-AMC (5 µmol/l). Statistical significance was measured by ANOVA test. *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001

DPPIV and PREP catalytic activity assay

The activities of DPPIV and PREP enzymes were evaluated in serum of COVID-19 patients compared to negative COVID-19 group control. Our data showed that a significant decrease of DPPIV and PREP activity in patients of moderate and severe groups when compared to negative COVID-19 controls and patients with mild symptoms (Figs. 6 and 7). The hydrolysis specificity was confirmed with the complete inhibition of activity in presence of linagliptin (100 nmol/l) and KYP-2047 (5 µmol/l), DPPIV and PREP inhibitors, respectively. These data suggest a possible negative correlation between serum DPPIV and PREP activities with COVID-19 severity.

CAT L enzymatic activity in COVID-19 patients

CAT L activity was monitored in serum of COVID-19 patients and in negative COVID-19 group. The results demonstrated that patients with moderate or severe symptoms presented a significant increase of CAT L activity when compared to patients of negative COVID-19 or mild

symptomatic group (Fig. 8). The hydrolysis specificity was confirmed with complete inhibition of activity in presence of E64 inhibitor. These results suggest a possible association between increased CAT L activity and the clinical condition of the patients.

Discussion

Proteases perform essential roles in many biological processes and the inhibition of enzymatic activity has been described as an important step, regulating numerous pathways [46]. In COVID-19, some enzymes are investigated as promising targets to prevent SARS-CoV-2 spread [47, 48]. Among the signaling pathways involved in viral infection, virus entry in the host cells mediated by the spike protein, represents a critical step to disease development [49].

The S protein contains two distinct domains: S1, responsible for receptor binding to the host receptor and S2 domain, that mediates the membranes fusion with the host cells [50–52]. Specifically, the S1 RBD (receptor binding domain) of SARS-CoV-2 directly binds to PD (peptidase domain) of ACE2 [53].

The sACE2 usually circulates in low concentrations [54, 55], leading some authors to investigate the relationship between circulating ACE2 activity and COVID-19 disease severity [56–58]. The activity of circulating ACE2 is associated with disease severity and mortality [57]. Therefore, we first investigated circulating ACE2 enzymatic activity in serum of COVID-19 patients. Additionally, we examined the activity of ACE, DPPIV and PREP, enzymes involved

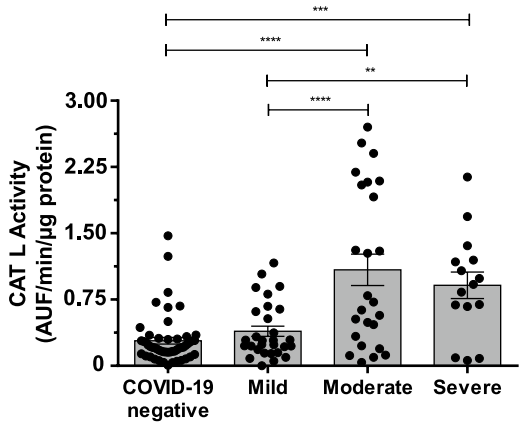

Fig. 8 CAT L activity in serum of COVID-19 patients and controls. CAT L enzymatic activity was evaluated in serum of patients with COVID-19 negative, mild, moderate and severe symptoms. These patients were grouped according to symptoms levels. The experiments were performed using the fluorescent Z-Phe-Arg-AMC substrate (100 µmol/l). Statistical significance was evaluated by ANOVA test. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

in cardiovascular and renal diseases (frequently observed in patients infected with coronavirus), and inflammatory response. The activity of CAT L was also measured due to the importance of enzymatic activation of S protein in virus entry in the host cells. We evaluated these enzymatic activities in 152 patients recruited for this study, separated in four groups: COVID-19 negative group (patients with COVID-19 symptoms with negative PCR test), mild, moderate and severe COVID-19 groups.

Regarding the comorbidities, the analyses demonstrated an increase of relative risk (RR) of experiencing severe forms of COVID-19 in patients with comorbidities, including metabolic disease (diabetes, obesity and dyslipidemia), hypertension, heart disease, chronic kidney disease and smoking comparing with patients without comorbidities. The comparison between mild and moderate groups showed the same profile observed in severe group, maintaining the increased probability of comorbidities occurrence (Fig. 1a). The analyses also showed a positive correlation between mortality rate and high levels of CRP (Fig. 1b) and D-dimer (Fig. 1c), corroborating previously published data [41, 59].

Subsequently, we evaluated ACE2 activity in the serum of COVID-19 groups. The results demonstrated elevated enzymatic activity in the moderate and severe groups when compared to mild and COVID-19 negative groups (Fig. 2). These results corroborate already published data showing the correlation between elevated levels of circulating ACE2 and COVID-19 severity [57, 60], suggesting that increased ACE2 may predispose to severe forms of COVID-19 disease [61]. The blockade of ACE2 and S protein interaction has been described as a promisor target to the development of anti-COVID-19 drugs [62–64].

Cleavage of S1 domain is an important process to expose the fusion peptide, which is a critical mechanism for membranes fusion, allowing virus entry [65]. This cleavage is acid-dependent and is accomplished by several host proteases, including cathepsins [66]. Here, we measured CAT L catalytic activity, and as observed for ACE2, moderate and severe groups presented higher activity compared to mild and negative COVID-19 groups (Fig. 8). Data from literature demonstrated that the inhibition of CAT L using E64 and K777 irreversible inhibitors (in vitro) results in reduced virus replication [37, 67], suggesting a possible proteolytic activation mechanism of spike protein by CAT L [52, 68, 69].

Contrasting to ACE2 and CAT L, our findings show decreased activity of ACE in moderate and severe groups (Fig. 3). Reduced ACE activity might cause KKS imbalance, consequently generating BK accumulation and BK storm [70]. This maintenance of inflammatory response mediated by ACE has been associated to progression of COVID-19 [61, 71]. However, the correlation between ACE and COVID-19 severity is conflicting in the literature [72–74].

Moreover, we also investigated a possible correlation between ACE polymorphism (I/D) with COVID-19 severity. Our results show that ID genotype was the most prevalent among the groups investigated. The frequency of D allele was the most predominant, however, no statistical significance was observed between genotype or allele frequencies with COVID-19 severity (Table 2). The direct association of ACE I/D polymorphism with COVID-19 severity is also conflicting in the literature data [75–78]. Posteriorly, the correlation between ACE polymorphism (I/D) with ACE enzymatic activity was also investigated, since several authors associated DD genotype with increased ACE activity [79–81]. As expected, regarding the COVID-19 negative group, the DD genotype has increased ACE activity compared to ID + II genotypes, but this difference is not observed in the COVID-19 group (Fig. 4), corroborating a previous report [78]. When comparing specifically the genotypes, the analyses demonstrated that patients with COVID-19, both DD and ID + II genotypes present lower ACE activity, indicating that decreased ACE activity is only correlated with COVID-19 severity.

RAAS dysregulation has been associated with the worsening of COVID-19 symptoms [43, 82, 83]. Reduced levels of soluble ACE and increased circulating ACE2 activity promotes higher ACE2/ACE ratio. In contrast, reduced levels of membrane ACE2 and an increase of local ACE activity results in reduced local ACE2/ACE ratio, favoring ACE axis in the system [43]. Thus, RAAS dysregulation leads to the overactivation of the AT1R (Ang II–angiotensin II type I receptor) axis, which is characterized by a prominent vasoconstriction, triggering profibrotic and proinflammatory signalization in the lungs and others organs [18, 43]. Here, increased ACE2/ACE ratio was observed only in the severe group (Fig. 5), suggesting that this imbalance could influence the worsening of symptoms and the poor outcome of the disease.

In addition to the RAAS impact in COVID-19, the inflammatory response plays a significant role in predicting the outcome of patients. Our analyses of DPPIV (Fig. 6) and PREP (Fig. 7) showed decreased activity of both peptidases in moderate and severe groups. Both PREP and ACE2 converts the pro-inflammatory ANG II into ANG(1-7), an anti-inflammatory peptide. Whereas ACE2 is more prominent in the kidney and lung, PREP is the main peptidase performing this conversion in the systemic circulation [84], suggesting a potential contribution for regulation of ANG II levels in COVID-19 [85]. The direct association between ANG II and inflammation is clearly recognized, since ANG II initiates inflammatory cascades and triggers the activation of many pro-inflammatory mediators, including ROS (reactive oxygen species), NF-κB (nuclear factor kappa B), CRP and others [86–89].

Previously published data demonstrated that in inflammatory diseases, the process of releasing DPPIV from the cell surface is inhibited, as observed in septic shock, atherosclerosis and COVID-19 [90, 91]. These findings corroborate our results, showing that patients with moderate and severe form of COVID-19 present lower activity of circulating DPPIV. These findings showing reduced circulating DPPIV in hospitalized patients with SARS-CoV-2 might help to comprehend the specific function of this enzyme in COVID-19 [92]. Accordingly, several studies investigated whether DPPIV inhibitors (DPPIVi) could affect the clinical course of COVID-19 disease [93–96]. Meta-analyses studies suggest that the use of DPPIVi in patients with COVID-19 result in a reduction of mortality and clinical improvement, mostly in patients with type 2 diabetes, while some studies showed no effects of DPPIVi [92, 95, 96]. Other data also suggest a reduction in mortality and severity of COVID-19 in patients using the antidiabetic drug metformin and/or renin-angiotensin system blockers when combined with DPPIV inhibitor, despite benefit of the DPPIVi is less pronounced when associated with these two drugs [95, 96]. However, the exact association between DPPIV, DPPIVi and coronavirus remains unclear.

Altogether, it is not surprising that the interaction of some enzymes with SARS-CoV-2 is an important regulatory factor for COVID-19 pathophysiology. Differential profile of some proteases has been reported in patients with COVID-19 and associated with clinical complications. In this context, the ACE2 has a central importance in the pathogenesis, both as coronavirus receptor and acting in the post-infection phase [48]. Computational approaches demonstrated that DPPIV also interacts with the spike protein of SARS-CoV-2, suggesting that another enzyme might play a relevant role in virus entry [48]. The viral replication and activation/cleavage of the spike protein is also dependent of proteases, such as ACE2 and CAT L [67, 69]. Mediated by CAT L, the virus achieves the cytoplasm and the infection is established, causing an extensive inflammatory response [68]. Then, the immune system presents a crucial contribution, as the cytokine storm leads to tissue damage, multiple organ failure and death [48]. Most of this damage is due to the activation of pro-inflammatory pathways, mainly pulmonary and renal, including increased levels of ANG II [84]. ACE acts directly in the conversion of ANG I to ANG II, while ACE2 and PREP convert ANG II to ANG-(1-7), suggesting a potential dysregulation in the expression/shedding control of these enzymes and the RAAS in COVID-19 patients [71, 85]. The present findings suggest a possible correlation between enzymatic activity and disease severity (Fig. 9). Taken together, our data show that COVID-19 severity impacts the activity of different proteases in the blood and therefore the knowledge about this control might contribute to a better understanding of SARS-CoV-2 infection.

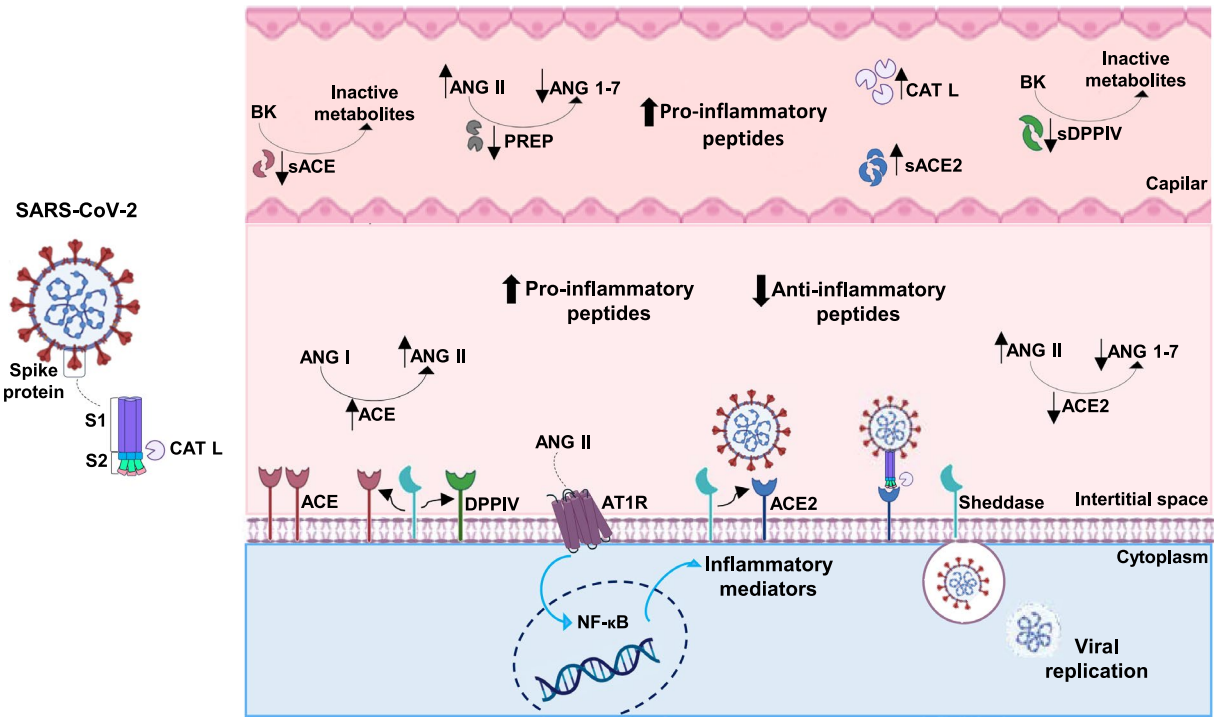

Fig. 9 Effect of imbalance ACE2/ACE ratio in COVID-19 pathogenesis of severe cases. The SARS-CoV-2 attachment to anchored-membrane ACE2 is mediated by the spike protein (S), which is proteolytic activated by CAT L, initializing the membrane fusion of virus and host cells. In response to this attachment, the virus-ACE2 complex is internalized into the target cell, leading to the down-regulation of membrane ACE2, resulting in increased levels of ANG II and reduced amount of ANG-(1–7), an anti-inflammatory peptide. The ANG II initiates inflammatory cascades mediated by AT1R, and triggers the activation of several pro-inflammatory mediators such as NF-κB, inducing the transcription of pro-inflammatory cytokines. The levels of ANG II are regulated primarily by ACE enzyme, which catalyze the conversion of ANG I to ANG II. Reduced levels of membrane ACE2 and increased of membrane ACE, results in reduced local ACE2/ACE ratio, favoring ACE axis in the RAAS system, a pro-inflammatory signaling. The circulating levels of ACE2, ACE and DPPIV are regulated by enzymatic activity of sheddases. Increased levels of soluble ACE2 and decreased of sACE contribute to the accumulation of pro-inflammatory peptides. Moreover, reduced levels of PREP and DPPIV also promotes the increased amount of pro-inflammatory peptides, including BK. These enzymes together compose a prominent activation of pro-inflammatory pathways, leading an extensive damage in different organs and contribute to a poor outcome of patients

Author contributions Conceptualization: JBP; methodology: RLN, JB, MR, GFM, DEF, JBP; formal analysis and investigation: RLN, JB, JGA, CAB, CPG, MYI; writing—original draft preparation: RLN, JB, MYI, GMK, JBP; writing—review and editing: RLN, JB, MYI, JBP; funding acquisition: MYI, GMK, JBP; resources: MYI, GMK; supervision: JBP.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interests.

Ethics approval All procedures were conducted in accordance of the principles of Helsinki Declaration. Approval was granted by the Ethics Committee of the Federal University of São Paulo (CAAE 31929120.0.0000.5505). The patients provided their written informed consent to participate in this study and for the publication of any potentially data included in this article.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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