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Decrease in Angiotensin-Converting Enzyme activity but not concentration in plasma/lungs in COVID-19 patients offers clues for diagnosis/treatment

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Although several therapeutics are used to treat coronavirus disease 2019 (COVID-19) patients, there is still no definitive metabolic marker to evaluate disease severity and recovery or a quantitative test to end quarantine. Because severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infects human cells via the angiotensin-converting-enzyme 2 (ACE2) receptor and COVID-19 is associated with renin-angiotensin system dysregulation, we evaluated soluble ACE2 (sACE2) activity in the plasma/saliva of 80 hospitalized COVID-19 patients and 27 non-COVID-19 volunteers, and levels of ACE2/Ang (1–7) in plasma or membrane (mACE2) in lung autopsy samples. sACE2 activity was markedly reduced ($p < 0.0001$) in COVID-19 plasma (n = 59) compared with controls (n = 27). Nadir sACE2 activity in early hospitalization was restored during disease recovery, irrespective of patient age, demographic variations, or comorbidity; in convalescent plasma-administered patients (n = 45), restoration was statistically higher than matched controls (n = 22, $p = 0.0021$). ACE2 activity was also substantially reduced in the saliva of COVID-19 patients compared with controls ($p = 0.0065$). There is a strong inverse correlation between sACE2 concentration and sACE2 activity and Ang (1–7) levels in participant plasmas. However, there were no difference in membrane ACE2 levels in lungs of autopsy tissues of COVID-19 (n = 800) versus other conditions (n = 300). These clinical observations suggest sACE2 activity as a potential biomarker and therapeutic target for COVID-19.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for coronavirus disease 2019 (COVID-19), infects human cells via binding of the spike protein to the angiotensin-converting enzyme 2 (ACE2) receptor.[1] ACE2 is a key enzyme involved in regulating the renin-angiotensin system (RAS) in the heart,[2] lungs,[3,4] kidneys,[5] gut,[6] and brain.[7] Given the wide distribution of the ACE2 receptor, COVID-19 patients often suffer from multisystem disease.[8,9] In healthy human lungs, ACE2 is primarily expressed in type II alveolar epithelial cells that produce surfactants to protect alveoli from collapse and possess tight junctions that limit fluid transudation. ACE2 is essential for cleavage of the vasoconstrictor angiotensin II (Ang II) to produce the anti-inflammatory, cytoprotective angiotensin 1–7 (Ang (1–7)) peptide. Ang (1–7) functions through the G-protein-coupled receptor MAS to counteract pathological effects induced by Ang II via its receptors, Ang II receptors 1 and 2 (AT1R and AT2R), including vasoconstriction, inflammation, hypercoagulation, and fibrosis.[10–13] Ang II concentration in plasma correlates with severity of COVID-19 and lung injury.[3,4] In contrast, administration of Ang (1–7) alleviates acute respiratory distress syndrome (ARDS)-related inflammation, fibrosis, and oxygenation deficiency in rats,[14] but its role in COVID-19 patients has not yet been fully characterized.

In addition to its membrane-bound form, ACE2 is also present in plasma and other biological fluids as soluble ACE2 (sACE2). Although binding of the spike protein to the cellular ACE2 receptor has been widely studied,[9,15] its interaction with sACE2 has not been fully explored. We have recently reported that SARS-CoV-2 binds directly to sACE2 and inhibits ACE2 activity.[16] We also reported that cholera toxin B subunit (CTB)-ACE2 can bind both to ACE2 receptors and GM1 coreceptors and block entry of SARS-CoV-2 in epithelial cells or debulk viral infectivity in saliva.[16]

After 2 years of the pandemic, there is still no metabolic marker or quantitative tests to end the quarantine. Most of the known biomarkers associated with COVID-19 disease severity are immune and inflammatory factors, such as interleukin (IL)-6, IL-10,[17] C-reactive protein,[18] and neutrophil activation markers (resistin,

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Table 1. Baseline characteristics of the 80 hospitalized COVID-19 patients analyzed

Characteristic	Plasma N = 59	Saliva N = 21	Total N = 80
Age(years), n (%)			
<45	9 (15.2)	5 (23.8)	14 (17.5)
45–60	22 (37.2)	7 (12.5)	29 (36.2)
61–74	11 (18.6)	5 (23.8)	16 (20)
75+	11 (18.6)	4 (19.0)	15 (18.7)
Unknown[a]	6 (10.1)		6 (7.5)
Sex, n (%)			
Female	29 (49.1)	7 (33.3)	36 (45)
Male	21 (35.5)	14 (66.6)	35 (43.7)
Unknown	9 (15.2)		9 (11.2)
Race, n (%)			
African American	33 (55.9)	10 (47.6)	43 (53.7)
White	15 (25.4)	10 (47.6)	25 (31.2)
Asian	2 (3.3)	1 (4.7)	3 (3.7)
Unknown	9 (15.2)		9 (11.2)
Ethnicity, n (%)			
Hispanic	1 (1.6)	1 (4.7)	2 (2.5)
Non-Hispanic	50 (84.7)	20 (95.2)	70 (87.5)
Unknown	8 (13.5)		8 (10)
Drugs, n (%)			
Remdesivir	25 (42.3)	5 (23.8)	30 (37.5)
Steroids	23 (38.9)	6 (28.5)	29 (36.2)
Unknown	11 (18.6)	10 (47.6)	21 (26.2)

Plasma (n = 59) and saliva (n = 21) samples were analyzed.
[a]Unknown indicates that patient data were not available.

lipocalin-2, hepatocyte growth factor, IL-8, granulocyte colony-stimulating factor,[19] and calprotectin).[20] The correlation of circulating calprotectin and COVID-19 severity was particularly significant in patients with severe pulmonary disease. Immune-related proteins seleno-protein P[21] and paraoxonase/arylesteras1 (PON1),[22] were also identified as markers for disease recovery.[23] Indeed, the Centers for Disease Control and Prevention (CDC) discourages retesting of COVID-19-positive patients for 90 days, with no other alternatives before employees return to work. This is because nucleic acid tests use PCR amplification and do not distinguish infectious from non-infectious virus particles.[24,25] Therefore, there is an urgent need to develop biomarkers that show the status of disease or recovery.

In an effort to understand the functional consequences of SARS-CoV-2 interactions with ACE2 beyond its use as a viral entry receptor, we evaluated sACE2 protein concentration and enzymatic activity along with the angiotensin cleavage product, Ang (1–7), in the plasma and saliva of individuals hospitalized with COVID-19 and in controls. Finally, we compared changes in sACE2 with membrane-associated ACE2 in lung tissue obtained at autopsy from individuals who died of COVID-19 and non-COVID-19 diseases.

RESULTS

Study participants

We analyzed plasma (n = 59) and saliva (n = 21) samples from a total of 80 severely ill hospitalized individuals with PCR-confirmed SARS-CoV-2 testing. All COVID-19 patients were confirmed to be PCR positive for SARS-CoV-2, while all controls were asymptomatic and negative for SARS-CoV-2 antibody on serology testing. Baseline characteristics of the analyzed individuals are described in Table 1. Participants' median age was 59 years, with 45% female and 44% male, and 54% identifying as African American. Saliva samples were also collected from individuals hospitalized with COVID-19, collected under institutional review board (IRB)-approved protocols (#823392, #842613). Median age of the saliva donors was 58 years, with 33% female and 66% male donors, and 47% identifying as African American. The controls were aged 25 to 69 years, clinically asymptomatic, and negative for SARS-CoV-2 antibodies on serology testing.

sACE2 activity is markedly reduced in COVID-19 patient plasma

sACE2 activity was measured in the plasma of 16 COVID-19 patients and 17 controls, collected in EDTA tubes. All COVID-19 patients were confirmed to be PCR positive for SARS-CoV-2, while all controls were asymptomatic and negative for SARS-CoV-2 antibody on serology testing. We measured the protein concentration in all samples and normalized the enzyme activity units with protein concentration. sACE2 activity was markedly reduced in plasma from COVID-19 patients compared with controls (4.86 ± 1.15 to 46.49 ± 6.43 mU/mg enzyme activity units, $p < 0.0001$) (Figure 1A). Given concerns that the EDTA may have interfered with ACE2 activity, we then validated these results in plasmas collected in acid citrate dextrose (ACD) (without EDTA) from a separate group of 43 participants with COVID-19 and 10 controls (Tables 2 and 5). We made a similar observation of suppressed sACE2 activity in COVID-19-infected individuals compared with controls (5.179 ± 0.4571 versus 37.08 ± 1.476 mU/mg enzyme activity units; $p < 0.0001$) (Figure 1B). When pooled together, the data from both collection methods demonstrate the profound effect of SARS-CoV-2 infection on sACE2 enzyme activity (4.711 ± 0.33 versus 43.0 ± 1.419 mU/mg enzyme activity units; $p < 0.0001$) (Figure 1C).

sACE2 activity is markedly reduced in COVID-19 patient saliva

sACE2 activity was analyzed in saliva from 21 COVID-19 patients (Table 3) and 20 controls (Table 4). We observed a significant reduction in ACE2 activity in COVID-19 saliva compared with controls (13,434 ± 3,741 versus 34,606 ± 7,589 ΔRFU (Relative Fluorescence Units), 70.51 ± 22.75 versus 126.8 ± 27.71 mU/mg enzyme activity units; $p = 0.0065$) (Figures 2A and 2B). Four COVID-19 patients stood out with high ACE2 activity (38,504, 43,004.5, 53,766, and 48,831 ΔRFU; 236.4, 250.159, 319.22, and 291.01 mU/mg enzyme activity units respectively). Notably, two out of the four patients were asymptomatic and all four tested PCR positive on admission with no comorbidities. Although we see a significant difference in ACE2 activity between

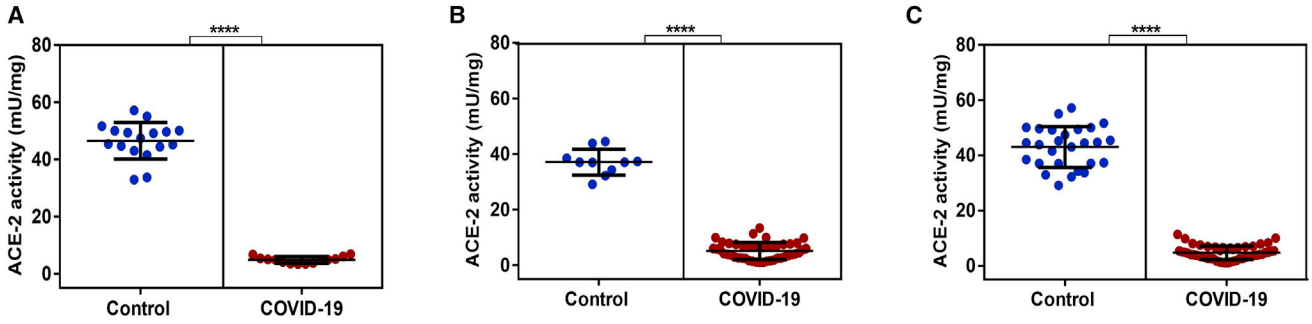

Figure 1. Kinetic reading of plasma ACE2 enzyme activity

ACE2 activity determined by cleavage of fluorogenic Mca-APK (Dnp) substrate. ACE2 enzyme activity units (mU/mg) calculated in control and COVID-19 patients. (A) In EDTA-treated samples, 17 control (blue) and 16 COVID-19 (red). (B) In Non-EDTA-treated samples, 10 control (blue) and 43 COVID-19 (red). (C) Both EDTA and non-EDTA samples combined data. Data were analyzed using the Mann-Whitney U test; ****$p < 0.0001$.

the two groups, there was variability in enzyme activity among COVID-19 patients, likely reflecting the substantial heterogeneity in disease state, even among hospitalized individuals. To assess whether the variability was due to differences in saliva turbidity, we measured the protein concentration in all samples and normalized the enzyme activity units with protein concentration. As seen in Figure 2C, where data are normalized for protein concentration, the ACE2 activity remains unchanged. Therefore, variability in the ACE2 activity in COVID-19 participants' saliva samples is unrelated to turbidity.

ACE2 enzyme activity in participants of a randomized, controlled convalescent plasma therapy study

Leveraging samples collected from participants of a randomized, controlled study of COVID-19 convalescent plasma (CCP) that found a significant benefit from CCP treatment in terms of clinical severity and 28-day mortality, we assessed the kinetics of ACE2 activity over the 60 days post CCP administration in CCP-treated (n = 45) and standard-of-care recipients (n = 22).[26] We noted that the study population had substantial baseline comorbidities, with a median of three per participant, including prevalent disease states including diabetes, hypertension, cardiovascular disease, and pulmonary disease, as well as conditions that cause immunosuppression like cancer and immunodeficiency, all of which can influence RAS activity (Table 5). In CCP-treated participants, ACE2 activity was the lowest on day 1 and steadily increased, reaching the highest value on study day 60 (6.707 ± 1.080 versus 13.79 ± 1.528 mU/mg enzyme activity units; $p = 0.0005$) (Figures 3A and 3C). In participants not receiving CCP, there was no significant difference in ACE2 enzyme activity on study day 60 compared with admission day (8,754 ± 1720 versus 10,760 ± 2,470 ΔRFU, 9.769 ± 2.197 versus 8.833 ± 2.104 mU/mg enzyme activity units; $p = 0.6857$) (Figure 3B). Thus, CCP therapy was associated with more substantial amelioration of plasma ACE2 activity than no treatment with CCP.

Inverse correlation between sACE2 concentration, sACE2 activity, and Ang (1–7) levels in COVID-19 plasmas

sACE2 and Ang (1–7) concentration was analyzed in the plasma samples obtained from 16 COVID-19 patients and 17 controls. sACE2 activity was significantly reduced in plasma from COVID-19 patients compared with controls (4.86 ± 1.15 to 46.49 ± 6.43 mU/mg enzyme activity units; $p < 0.001$) (Figure 1B). Interestingly, sACE2 protein levels were significantly higher in the COVID-19 patients' plasma than in control patients' plasma (159.9 versus 72.2 ng/mL; $p < 0.01$) (Figure 4A). The high degree of discordance between sACE2 concentration and enzymatic activity observed in COVID-19 patients indicates that ACE2 activity is regulated by mechanisms other than sACE2 abundance. Consistent with depressed sACE2 activity, the plasma levels of Ang (1–7) were significantly decreased in COVID-19 patients compared with controls (1,279 versus 3,655 pg/mL, respectively; $p < 0.001$) (Figure 4B). A positive correlation was observed between ACE2 activity and Ang (1–7) concentration in COVID-19 and control samples ($r^2 = 0.39$; $p = 0.0073$; Figure 4C). Of the COVID-19 patients, only one had received an angiotensin-converting-enzyme 1 (ACE1) inhibitor and only two had received an angiotensin receptor blocker. These individuals' values for plasma ACE2 activity and Ang (1–7) concentration were also well below the average values of the non-COVID controls. In aggregate, these data demonstrate a significant dysregulation of the angiotensin system in patients with severe SARS-CoV-2 infection and an inverse relationship between plasma sACE2 levels and enzymatic activity.

Impact of SARS-COV-2 infection on lung tissue-associated ACE2

Although downregulated cellular ACE2 expression caused by SARS-COV infection has been reported, how it is modulated by SARS-COV-2 infection *in vivo* is still unknown. To assess this, ACE2 protein quantity in lung autopsy tissues from individuals succumbing to COVID-19 compared with those dying of unrelated causes was analyzed. The demographic data and clinical presentation along with the medical history of this cohort of 16 patients consisting of eight COVID-19 and eight non-COVID-19 patients are shown in Table 6. Immunohistochemistry (IHC) for ACE2 protein was performed in lung tissue from controls and COVID-19 patients (Figures 5A–5C). Despite evidence of more extensive parenchymal pathology in the COVID-19 and heterogeneity within and between cases, there was no quantitative differences in ACE2 levels between

Table 2. Demographic and medical history data of COVID-19 patients

	Demographic			Drugs at baseline		
Subject ID	Age	Sex	Race	Cm_ Remdesivir	Cm_steroids	Viral copies (log10)/mL
NCT04397757						
#001	78	F	Caucasian	No	no	4.32
#007	92	F	African American	No	no	6.42
#013	66	F	African American	No	yes	3.18
#018	66	M	African American	Yes	no	2.65
#020	48	M	African American	Yes	yes	4.16
#028	61	M	Caucasian	Yes	yes	2.53
#029	48	F	African American	No	no	2.98
#034	48	F	African American	Yes	yes	2.58
#037	69	M	Caucasian	Yes	no	2.28
#055	73	F	African American	Yes	yes	4.76
#041	75	M	Asian	Yes	yes	2.85
#051	63	M	African American	Yes	yes	3.43
#053	83	M	Caucasian	Yes	yes	5.73
#022	77	M	African American	NA	yes	3.98
#027	82	M	African American	Yes	yes	8.22
#066	30	F	Caucasian	Yes	yes	8.78
#033	59	F	African American	Yes	yes	4.39
#036	56	F	Caucasian	Yes	yes	4.83
#025	56	F	African American	Yes	yes	4.26
#006	51	F	African American	Yes	yes	2.26
#009	55	F	African American	Yes	no	3.64
#011	62	F	African American	No	no	NA
#012	42	F	Caucasian	No	no	NA
#013	41	M	Caucasian	No	no	NA
#13	80	M	African American	No	yes	NA
#72	27	F	African American	No	no	NA
#62	29	M	Caucasian	No	no	NA
#67	83	M	Caucasian	Yes	yes	NA
#6	74	M	African American	Yes	yes	NA
#47	51	M	African American	No	n	NA
#7	83	F	Asian	Yes	yes	NA

The plasma ACE2 enzyme activities for this cohort of 32 patients are illustrated in Figure 1B. M, male; F, female; NA, not available.

lung tissue samples from COVID-19 patients versus controls (Figure 5C).

DISCUSSION

Published literature on ACE2 concentration is unclear because of interchangeable use of ACE2 concentration and activity. The concentration of ACE2 in plasma ranges from about 16 pg/mL[27] to near 15 ng/mL[28] without purification in healthy individuals, indicating the lack of a validated and standardized sACE2 quantification method. However, it is substantially increased in heart failure, hypertension, valvular heart disease, obesity, diabetes mellitus, and other pathological conditions.[10] In healthy individuals, ACE2 is mostly membrane bound, but it is shed extracellularly under pathological conditions by the action of membrane-bound proteases like disintegrin and metalloprotease domain 17 (ADAM-17). Therefore, increase in ACE2 concentration (often misinterpreted as activity) in individuals with comorbidities has been widely reported. This is because ACE2 activity cannot be measured until ACE2 inhibitors are removed,[29–32] which is a complex, laborious process as opposed to ACE2 simple direct measurement using ELISA. ACE2 catalytic activity in human plasma is masked by an endogenous inhibitor and methods to remove inhibitor were developed in 2008,[33] rendering

Table 3. Demographic data and viral load information of COVID-19 patients

Subject ID	Age	Sex	Race	N1 copies per μL
IRB #823392				
#380	58	F	Caucasian	3.38×10^4
#385	59	M	African American	5.90×10^4
#425	65	M	Caucasian	4.77×10^3
#428	52	F	African American	5.32×10^3
#463	42	F	African American	1.35×10^4
#472	68	M	Caucasian	2.49×10^4
#476	53	F	Caucasian	1.82×10^4
#464	42	M	Caucasian	2.09×10^4
#485	52	F	African American	5.62×10^4
#472	68	M	Caucasian	3.20×10^4
#13	80	M	African American	NA
#72	27	F	African American	NA
#62	29	M	Caucasian	NA
#67	83	M	Caucasian	NA
#6	74	M	African American	NA
#47	51	M	African American	NA
#7	83	F	Asian	NA
#1	40	M	Caucasian	NA
#16	63	M	African American	NA
#17	77	M	African American	NA
#35	46	M	Hispanic Latino/white	NA

Table 4. Demographic data and viral load information of control patients

Subject ID	Age	Sex	Race	Average copy number
HSAL001	62	M	white	0.00E+00
HSAL002	27	M	white	0.00E+00
HSAL003	69	M	white	1.33×10^7
HSAL004	44	F	white	0.00E+00
HSAL005	30	F	white	4.60×10^3
HSAL008	25	F	white	9.00×10^6
HSAL012	29	M	white	0.00E+00
HSAL013	25	M	Asian	0.00E+00
HSAL014	27	F	white	0.00E+00
HSAL015	40	M	white	0.00E+00
HSAL016	43	F	Asian	0.00E+00
HSAL017	26	M	white	6.36×10^1
HSAL018	31	F	white	9.81×10^8
HSAL019	29	M	white	5.44×10^7
HSAL020	29	M	white	2.96×10^6
HSAL021	62	M	white	0.00E+00
HSAL022	25	M	white	0.00E+00
HSAL023	28	M	white	3.16×10^8
HSAL024	32	M	Asian	2.39×10^2
HSAL025	31	F	white	0.00E+00

The saliva ACE2 activity for this cohort of 20 patients are in Figure 2.

most previous studies on ACE2 activity unreliable. Therefore, in this study, we measure ACE2 activity in control and COVID-19 plasma after removal of endogenous inhibitors, and quantify ACE2 and its product Ang (1–7) levels in the same plasma by performing ELISAs.

In addition to exploring several lines of ACE2 activity measurements and ACE2 concentration and enzyme product Ang (1–7) concentration, we also paid close attention to the impact of components used in plasma collection tubes. One cohort of sample collection used EDTA, whereas another cohort used ACD. Although we anticipated that EDTA could potentially interfere with ACE2 assays due to chelation of zinc metal ions, we did not observe a notable impact on sACE2 activity in these samples. Because control cohorts are not admitted to the hospital, ACE2 activity levels are not comparable, but at least it is safe to interpret that the concentrations of EDTA used in sample collection tubes are insufficient to interfere with ACE2 activity measurements. While elevated levels of circulating angiotensin II have been reported in COVID-19 patients, there is only one report on serum ACE2 activity and plasma Ang (1–7) levels in COVID-19 patients. ACE2 activity was reported in COVID-19 plasma after removal of inhibitors, but unfortunately authors missed evaluating samples during peak SARS-CoV-2 infection (1–14 days) where viral load is the highest,[34] but they reported activity on days 33, 63, and 114.[30] Furthermore, Patel et al.[30] did not measure concentration of ACE2 or Ang (1–7) in COVID-19 plasma or normalize ACE2 activity to protein concentration after removal of ACE2 endogenous inhibitors. Reindl-Schwaighofer et al.[35] used the conversion assay to measure ACE2 activity, reporting an increase in ACE2 activity in late (days 9–11) severe COVID cases compared with early (0–3 days) cases. This finding agrees with our analyses on the CCP-treated patients, with lower ACE2 activity on days 1–3 and steady increase during the recovery phase reaching levels at par with control samples by day 60 (6.707 ± 1.080 versus 13.79 ± 1.528 mU/mg enzyme activity units; p = 0.0005; Figures 4A and 4B). In our studies, ACE2 activity and Ang (1–7) levels were markedly lower in COVID-19 plasma samples than in controls, despite higher sACE2 protein levels. This suggests a functional inhibition of ACE2 activity in COVID-19 patients. When SARS-CoV-2 binds to cell-surface ACE2 receptors, they are internalized, which may result in decreased ACE2 cleavage of circulating Ang II. Plasma ACE2 may include both active and inactive forms, possibly due to binding of the spike protein.[16,36]

In the current analysis, plasma sACE2 enzyme activity did not align with its concentration but was correlated with low Ang (1–7) concentration. We observed a moderate correlation between ACE2 activity and Ang (1–7) concentration in a combined group of controls and patients ($R^2 = 0.3923$) compared with either control ($R^2 = 0.0061$) or patient group ($R^2 = 0.1786$) alone. The reason may be that the inhibitory effect of COVID-19 infection on sACE2 activity and Ang (1–7) production is prominent compared with inhibitory effect of other factors

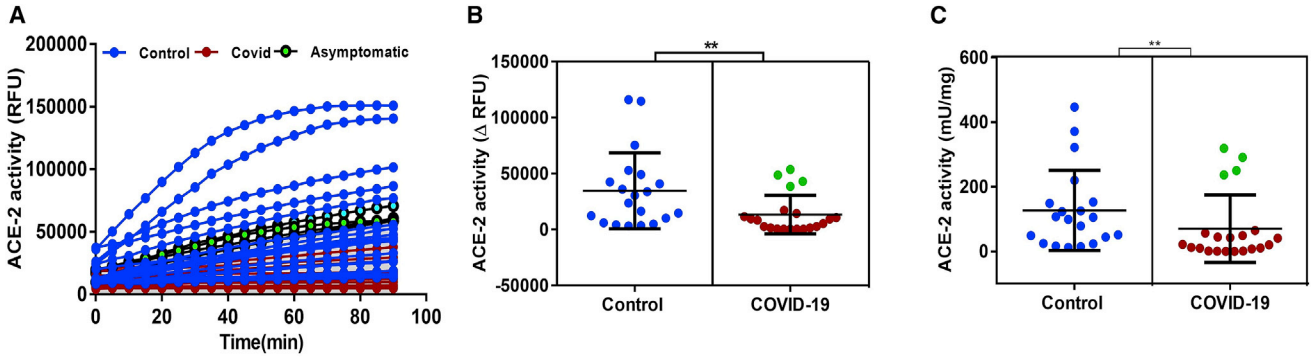

Figure 2. Kinetic reading of saliva ACE2 enzyme activity

(A and B) ACE2 activity determined by cleavage of fluorogenic Mca-APK (Dnp) substrate in 20 control and 21 COVID-19 (red) samples. ΔRFU was calculated by subtracting data of time point 0 min from data of time point 90 min. Data were analyzed using the Mann-Whitney U test; **$p < 0.05$ (0.0065). (C) ACE2 enzyme activity units (mU/mg) calculated in control and COVID-19 patients. Data were analyzed using the Mann-Whitney U test; **$p < 0.05$ (0.0099).

in control individuals. Indeed, it has been shown that spike protein is increased in plasma of COVID-19 patients or even vaccinated patients after booster injections.[37–39] Additionally, it has been reported that spike protein from SARS-CoV-2 binds and inhibits ACE2 activity.[16,36,40] In addition, Ang (1–7) is not exclusively generated by ACE2 but through other enzymes as well.[41,42] Although Ang (1–7) level in human plasma is diverse, ranging from pg/mL to ng/mL,[43–45] significantly reduced Ang (1–7) was observed in COVID-19 patients compared with controls. These findings point toward dysregulated RAS metabolic pathways as the cause for the suppressed enzyme activity.

Notably, convalescent plasma treatment was associated with improved recovery of sACE2 activity in COVID-19 patients, although the levels were still lower than in the control group. While studies of the effects of COVID-19 convalescent plasma are mixed, several recent studies and relevant subgroup analyses suggest that administration of high-titer plasma early in disease has clinical benefit.[46–51] Similarly, we see that administration of CCP early in hospitalization was associated with more substantial improvement in ACE2 activity, perhaps suggesting healthier RAS metabolic pathways. Among the remaining 38 hospitalized individuals, sACE2 activity was significantly reduced in COVID-19 patients compared with controls ($p < 0.0001$). Therefore, we posit that plasma ACE2 could serve as a metabolic biomarker for COVID-19 disease severity and recovery, with the potential to assess treatment strategies, especially those associated with RAS pathway dysregulation.

In addition to changes in plasma ACE2 activity, we also observe a significant reduction in ACE2 activity in the saliva of COVID-19 patients. The binding of the spike protein to sACE2 in saliva[16] inhibits ACE2 activity. Among volunteers, sACE2 activity in saliva is higher than that in plasma. The total protein concentration in saliva is 0.5–2.0 mg/mL[52] and that in plasma is 60–80 mg/mL.[53] The fact that saliva has approximately 3% of the total protein concentration of plasma, could explain the high sACE2 activity observed in this biofluid, when enzyme units are reported based on protein concentration. Although we observed a significant reduction in ACE2 activity in COVID-19 saliva, there is significant variability among control samples and this could pose problems for using saliva sACE2 activity as a reliable biomarker. Patient data on control subjects are very limited, especially related to their behavior during sample collection. For example, nicotine alters the homeostasis of the RAS by upregulating the detrimental angiotensin-converting enzyme/Ang II/Ang II type 1 receptor axis and downregulating the compensatory ACE2/Ang (1–7)/Mas receptor axis, contributing to the development of cardiovascular pulmonary diseases.[54] Therefore, saliva samples collected from subjects smoking cigarettes could have very low levels of ACE2 activity.

The SARS-CoV-2 interaction with ACE2 receptors with subsequent membrane fusion and internalization of the virus downregulates these receptors, resulting in increased lung injury owing to unopposed action of Ang II.[3,4] Binding of Ang II to its type I (AT1) receptor cleaves the membrane-bound ACE2, releasing the active form into the circulation, with loss of catalytic activity of the remaining part of the enzyme anchored to the membrane.[55] Lung is the primary location of SARS-CoV-2, and COVID-19-related deaths are highly correlated with breathing failure or lung damage.[42,56–58] Therefore, lung tissues in autopsy samples of COVID-19 patients were examined and compared with autopsy samples from other diseased patients. Although we observed no difference in lung ACE2 concentration between control and COVID-19 patients, we cannot exclude a difference in tissue ACE2 function because immunostaining is unable to distinguish between active and inactive ACE2.

Conceivably, therapeutic delivery of ACE2/Ang (1–7) could be employed to restore a more favorable balance of Ang II and Ang (1–7) in patients with COVID-19 disease. Indeed, soluble rhACE2 (Recombinant Human Angiotensin-Converting Enzyme 2) has been used previously to treat ARDS, lung injury, hypertension, and SARS-CoV infection, acting as a "decoy" to compete with membrane-bound ACE2 receptors for binding to spike protein.[40,59] Furthermore, several laboratories have used sACE2 for diagnosis

Table 5. Demographic and medical history data of CCP and non-CCP-treated patients

CCP treated							
	Demographic data			Drugs			
Subject ID	Age	Sex	Race	Cm_Remdesivir	Cm_Steroids	Viral copies(log10)/mL	Comorbidities
#001	78	F	Caucasian	no	no	4.32	CVD, CAD, HTN
#007	92	F	African American	no	no	6.42	DMII, CKD, HTN
#013	66	F	African American	no	yes	3.18	CAD, CHF, HTN
#018	66	M	African American	yes	no	2.65	CAD, CHF, HTN
#020	48	M	African American	yes	yes	4.16	OBE, HTN
#028	61	M	Caucasian	yes	yes	2.53	CKD, CAD, HTN
#029	48	F	African American	no	no	2.98	DMII, OBE, ID, HTN
#034	48	F	African American	yes	yes	2.58	OBE, ID, HTN
#037	69	M	Caucasian	yes	no	2.28	RESP, HTN
#055	73	F	African American	yes	yes	4.76	DMII, OBE, HTN
Non-CCP treated							
#005	87	F	African American	no	no	4.54	
#011	58	F	African American	no	no	6.74	
#014	64	M	African American	no	yes	4.42	
#015	50	F	Caucasian		yes	3.23	
#024	78	F	African American	yes	yes	5.50	
#025	56	F	African American	yes	yes	4.25	
#030	62	M	African American	yes	yes	5.77	
#033	59	F	African American	yes	yes	4.39	
#036	56	F	Caucasian	yes	yes	4.82	
#006	51	F	African American	yes	yes	2.25	
#038	53	M	Caucasian	yes	yes	0.69	

The plasma ACE2 activities for the CCP-treated patients are in Figures 3A and 3C, and those for the non-CCP-treated patients are in Figure 3B. DMII, diabetes mellitus II; CVD, cardiovascular disease; CAD, coronary artery disease; CHF, coronary heart failure); HTN, hypertension; CKD, chronic kidney disease; OBE, obesity; ID, immunodeficiency; RESP, respiratory.

or viral trap proteins to prevent SARS-CoV-2 infection or transmission.[16,36,60] Recently, delivery of soluble rhACE2 significantly suppressed SARS-CoV-2 virial load in the engineered organoid, demonstrating soluble rhACE2 has naturalizing function against SARS-CoV-2.[61] Therapeutic administration of soluble rhACE2 has been reported to successfully treat a female RT-PCR-confirmed SARS-CoV-2 patient with symptoms when admitted to hospital.[62] In contrast to exogenously delivered truncated (transmembrane-deleted) sACE2,[55,62] full-length ACE2 accumulates in the lungs at 10-fold higher concentrations than in the plasma upon oral delivery of bioencapsulated plant cells,[63] offering yet another approach to treat COVID-19 patients.[64,65] This approach is affordable because ACE2/Ang (1–7) in freeze-dried plant cells is stable for several months/years when stored at ambient temperature.[63] In addition, CTB-ACE2 with efficient binding to both GM1 and ACE2 receptors could effectively block binding of the spike protein and viral entry into human cells, especially via oral epithelial cells that are enriched with both receptors.[66] Therefore, CTB-ACE2 chewing gum was quite successful in debulking SARS-CoV-2 in saliva and blocking entry into human cells.[16]

Although we evaluated more than 100 plasma and saliva samples of 80 hospitalized COVID-19 patients and 27 volunteers, we note several limitations to this study. Although we studied a single clinical entity, hospitalized COVID-19 patients, our numbers are relatively small for this trial (21 treated patients) and there is substantial heterogeneity within this population. Further, we have limited data on the clinical courses of all these individuals, and even fewer on the controls used as comparators. Despite these limitations, results suggest that COVID-19 results in marked reductions in ACE2 activity in plasma and saliva but not concentration, and this may serve as a relevant biomarker of disease severity and recovery.

Conclusions

Defining the mechanisms of RAS dysregulation in patients with COVID-19 disease is important to improving diagnosis and treatment of individuals infected with SARS-CoV-2. sACE2 activity and the plasma levels of Ang (1–7) are significantly depressed in COVID-19 patients compared with non-COVID controls, despite increased sACE2 protein abundance in COVID-19 plasma. The decrease in ACE2 activity in COVID-19 plasma observed is independent of the

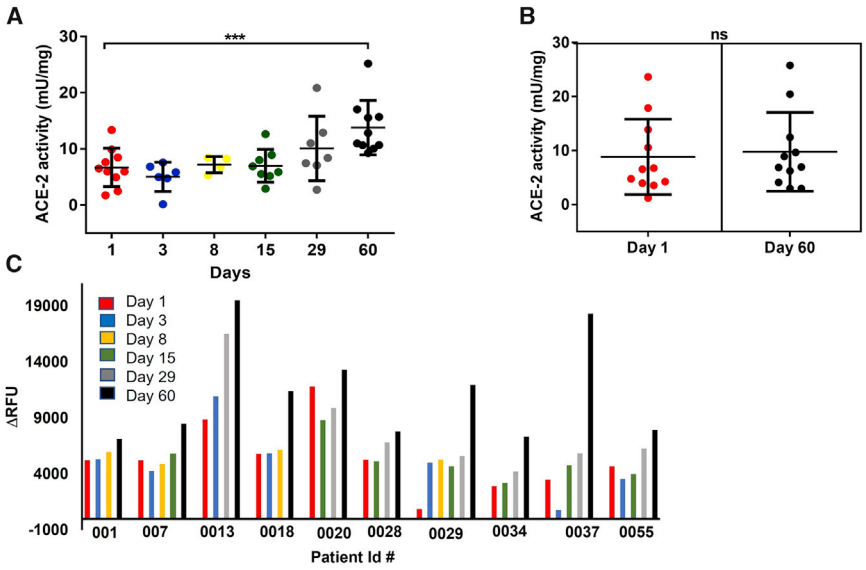

Figure 3. ACE2 enzyme activity in patients treated with convalescent plasma

(A and C) ACE2 activity measured at all timepoints: days 1, 3, 8, 15, 29, and 60 in the convalescent plasma group of 10 patients (n = 45). The plasma ACE2 activity was significantly higher on day 60 compared with day 1 in convalescent plasma-treated patients (***p = 0.0005), Mann-Whitney U test). Unavailable samples #001 (days 15 and 29), #007 (day 29), #013 (days 8 and 15), #018 (days 15 and 29), #020 (days 3 and 8), #028 (days 3 and 8), #034 (days 3 and 8), #037 (day 8), and #055 (day 8). (B) ACE2 activity measured on days 1 and 60 in 11 non-CCP-treated patients (n = 22). No significant difference was detected (p = 0.6857).

method or sample collection (EDTA or ACD) and was also observed in saliva, where no chemical reagent is used in sample collection. Convalescent plasma treatment statistically increased ACE2 activity during the 60-day recovery period compared with standard of care in hospitalized patients. We observed no significant difference in lung ACE2 immunostaining between COVID and non-COVID patients, after evaluation of 800 COVID-19 and 300 non-COVID images of lung tissue sections. These findings demonstrate the importance of examining ACE2 activity, rather than ACE2 protein concentrations, as a marker of RAS dysregulation in patients with COVID-19 disease.

MATERIALS AND METHODS

Study participants

We analyzed plasma (n = 59) and saliva (n = 21) samples from a total of 80 severely ill hospitalized COVID-19 patients. All COVID-19 patients were confirmed to be PCR positive for SARS-CoV-2, while all controls were asymptomatic and negative for SARS-CoV-2 antibody on serology testing. Samples for the plasma analyses were collected from individuals hospitalized with COVID-19 within the University of Pennsylvania Health System. Forty participants were enrolled in a randomized controlled clinical trial of convalescent plasma in individuals hospitalized with COVID-19 pneumonia (Clinicaltrials.gov NCT04397757),[26] out of which we analyzed n = 67 samples from 21 patients, seven participants enrolled in a clinical study of hospitalized COVID-19 participants, and the remaining 16 were obtained from the University of Pennsylvania BioBank under IRB-approved protocols (#808346, 813913, 817977).

Sample collection and preparation

Plasma samples obtained from the University of Pennsylvania BioBank were collected in EDTA (16 COVID-19 and 17 controls). Samples were stored at 4°C for 48 h prior to processing. Tubes were centrifuged for 10 min at 1,200 rpm and plasma collected was stored at −80°C until assayed. Plasma samples for ACE2 activity

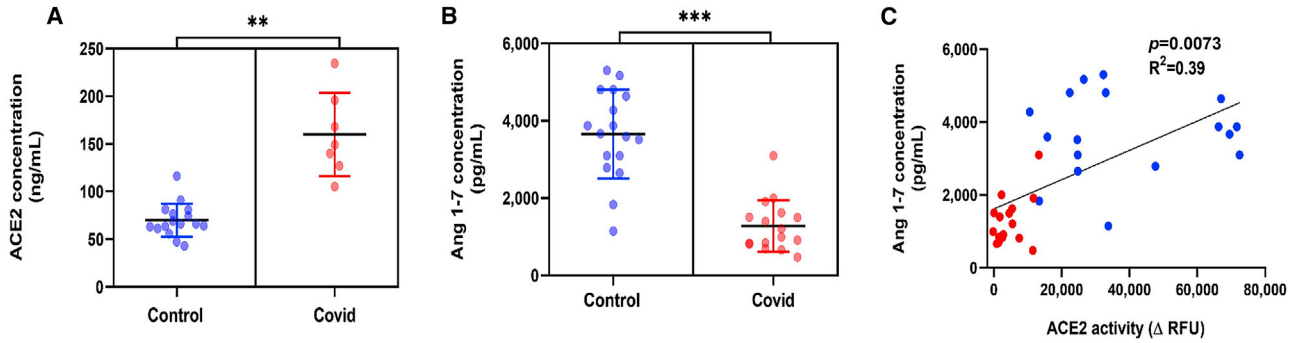

Figure 4. Measurement of ACE2 and Ang (1–7) concentration in plasma and correlation of ACE2 enzyme activity with Ang (1–7) levels

(A) ACE2 concentration determined by ELISA in a subset of seven COVID-19 and 16 control plasma samples. The level of ACE2 is significantly higher in COVID than control (**p < 0.01, Mann-Whitney U test). (B) Ang (1–7) concentration level was quantified in 17 control (blue) and 16 COVID (red) prepared samples by ELISA (***p < 0.001, Mann-Whitney U test). (C) Ang (1–7) concentration (pg/mL) in all 17 control (blue) and 16 COVID (red) plasma samples positively correlated with ACE2 activity (ΔRFU). Each dot represents a sample with both ACE2 activity and Ang (1–7) concentration data. The Pearson correlation was performed for correlation analysis. A linear regression line was formulated, and its R square value indicates the coefficient of determination (p = 0.0073).

Table 6. Demographic and medical history data of a cohort of 16 patients with COVID-19 (n = 8) enrolled for the immunohistochemistry experiments on autopsy lung sections shown in Figure 5

COVID-19							
Patient ID #	Age	Race/Sex	Comorbidities	Cause of death	Presentation	Blood type	Intubated
145	73	W	COPD	respiratory failure	unknown; died in nursing home	unknown	no
124	63	W	therapy-related myeloproliferative disorder s/p treatment for breast cancer	diffuse alveolar damage; ARDS	fever, cough on presentation for chemotherapy	B+	yes
119	61	W	asthma; prior cerebrovascular accident	CNS hemorrhage, bacterial pneumonia, renal failure	1-week fever, shortness of breath, cough	A+	yes
140	72	B	dementia; hypertension, diabetes, coronary artery disease	renal and respiratory failure	fever, hypoxia, shortness of breath; transfer from nursing home	A+	yes
143	74	B	hypertension	multisystem organ failure; shock	weight loss, cough, fatigue, for weeks, acute kidney injury	B+	yes
182	94	W	COPD, coronary artery disease, osteoarthritis, hypertension, hypothyroidism	acute kidney injury; acute respiratory failure	1-week shortness of breath, hemoptysis	A+	yes
163	85	unknown	prior cerebrovascular accident with aphasia, diabetes mellitus, hypertension, chronic kidney disease, aortic regurgitation	acute respiratory failure	1-week cough, shortness of breath, fever, weakness, joint pain (wife hospitalized with COVID-19)	A+	yes
92	50	B	JAK2 mutation with polycythemia vera	CNS hemorrhage	had been hospitalized for COVD-19 1 week prior to second admission with severe abdominal pain and portal vein thrombosis	O+	no
Non-COVID-19							
19-19	63	W/F	cardiac transplant	GI bleed			
19-61	62	W/F	chronic lung disease/diffuse alveolar damage	bronchopneumonia associated with chronic lung disease			
20-25	68	W/F	diffuse alveolar damage	diffuse alveolar damage			
20-2	59	B/F	pulmonary edema	congestive heart failure			
20-18	79	unknown/M	diffuse alveolar damage and COPD	sepsis; bronchopneumonia/COPD			
20-19	73	B/M	aspiration pneumonia	aspiration pneumonia			
20-20	69	B/F	metastasis breast cancer	metastasis breast cancer			
20-21	57	W/M	Sarcoidosis	sarcoidosis			

s/p, status post; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal.

assay were prepared by anion exchange to remove endogenous inhibitors as described previously.[33,67] The resulting eluates were used for downstream assays or stored at −80°C until activity assay. Blood samples from COVID-19 participants from the convalescent plasma trial were collected longitudinally starting at baseline day 1 and through study days 3, 8, 15, 29, and 60, including outpatient visits after hospital discharge, as described in the primary trial manuscript.[26] Plasma samples were collected in ACD tubes and processed within 6 h of collection by centrifugation for 10 min at 1,200 rpm and plasma thus collected was stored at −80°C until assayed. Saliva specimens from COVID-19 patients and controls were collected under IRB-approved protocols (#823392 and #842613, respectively).

ACE2 catalytic activity assay in plasma and saliva

ACE2 enzyme activity assay was performed using plasma and saliva of SARS-CoV-19 infected patients and controls. Catalytic activity of plasma and saliva ACE2 was assessed as described previously.[16,33,65] Briefly, ACE2 activity was determined by preparing the samples in ACE2 buffer (50 μmol/L $ZnCl_2$, 75 mmol/L Tris HCl, pH 7.5, and 1 mol/L NaCl) followed by incubation with 20 μmol/L fluorogenic Mca-APK(Dnp) ACE2 substrate (R&D Systems, Minneapolis, MN). Kinetic reading of relative fluorescence was recorded for 90 min at 5-min intervals at 28°C with optic position top and gain extended, with excitation at 340 nm and emission at 405 nm. The relative fluorescence unit at each time point was plotted. ΔRFU was calculated by subtracting data of time point 0 min from the data of time point 90 min. Total soluble protein in the remaining plasma and saliva samples was determined by Bradford assay following the standard Daniell laboratory's protocol. sACE2 enzyme activity units were normalized to the protein concentration of the patient sample. The final ACE2 enzyme activity was calculated as pmol/min/mg (mU/mg) = Δpmol/min/mg total protein.

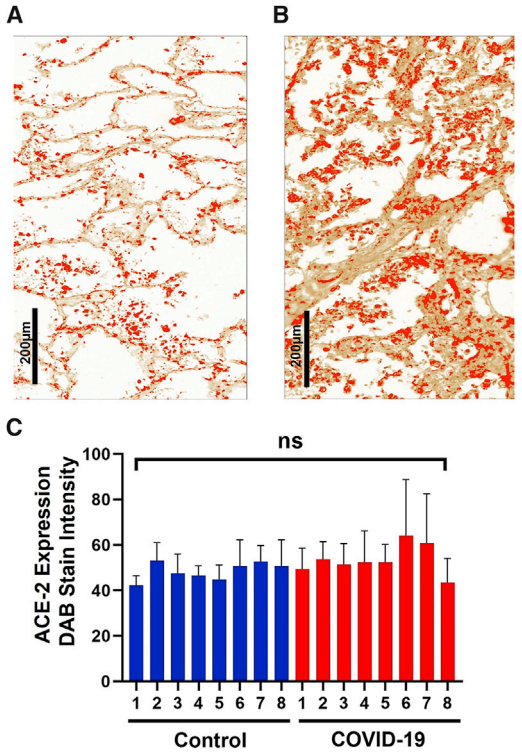

Figure 5. IHC autopsy lung tissue sections stained for ACE2 (red)

Representative image of deconvoluted DAB-stained (IHC Plugin; ImageJ software) control (A) or COVID-19-infected lung tissue (#6, B). (C) Quantitation of ACE2 staining. No statistical significance was observed (p = 0.088; Mann-Whitney test). A total of 800 images from COVID-19 lung ACE-stained 200-μm sections covered 80%–90% of the area. For controls #1 to #4, 100 images were analyzed, and for #5 to #8, 200 images were analyzed.

ACE2 and Ang (1–7) ELISA assays

The concentration of sACE2 in plasma from controls and patients infected with COVID-19 was determined by ELISA assay.[68] Ang (1–7) concentrations were measured using a competitive ELISA kit (Cloud-Clone Corp., TX, USA), as described previously.[63,69,70] Purified plasma samples of COVID-19 patients and control individuals were measured for both sACE2 and Ang (1–7) protein concentration. For sACE2 estimation, a 96-well plate was coated with 100 μL of samples prepared at 1:2 dilution in carbonate/bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) overnight at 4°C. The plate was washed with phosphate-buffered saline (1× PBS) containing 0.05% Tween 20 (PBST) and then blocked with 3% BSA in PBST for 2.5 h at 37°C. This was followed by washing the plate thrice with 200 μL of PBST buffer and incubating with 100 μL of primary ACE2 polyclonal Goat immunoglobulin G (IgG) (R&D Systems, Minneapolis), with a dilution of 1:5,000 in PBST containing 1% BSA for 2 h at 37°C. After washing the plate thrice with 200 μL of PBST buffer, the plate was incubated with 100 μL of secondary rabbit (horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody (Bio-Rad, CA, USA) at dilution of 1:5,000 in PBST containing 1% BSA for 1 h at 37°C. The plate was washed thrice with 200 μL of PBST and twice with 1× PBS buffer. One-hundred microliters of substrate 3,30,5,50-tetramethylbenzidine (TMB) was added to the reaction and covered with aluminum foil until a blue color developed. This reaction was then stopped by adding 100 μL of 2 M H_2SO_4 to the reaction, and the absorbance was read at 450 nm. ACE2 concentration was calculated using the standard curve generated by the rhACE2 (R&D Systems, Minneapolis) and reported in units of ng/mL. For plasma Ang (1–7) measurement, samples were prepared at 1:4 dilution with 1× PBS buffer. All reagents provided in the kit were brought to room temperature and reconstituted following manufacturer's instructions (standard, standard diluent, detection reagent A diluted with assay diluent A, and detection reagent B diluted with assay diluent B to the working concentration of 1:100; wash solution 30× diluted to 1× with deionized water for desired volume; TMB substrate, stop solution). Then 50 μL of sample was added in the pre-determined wells followed by 50 μL of detection reagent A. The plate was covered by a plate sealer and incubated for 1 h at 37°C. The solution was aspirated and washed with 350 μL of 1× wash solution thrice. Then 100 μL of detection reagent B was added to each well and incubated for 30 min at 37°C after covering it with the plate sealer. The plate was washed in a similar fashion five times. Then 90 μL of TMB substrate was added to the wells and incubated for 15 min while protecting it from light, and 50 μL of stop solution was added to the wells and absorbance was read at 450 nm. The Ang (1–7) thus estimated is reported in units of pg/mL. SARS-CoV-2 levels in saliva were measured by qRT-PCR as previously described.[71]

IHC of autopsy lung tissue sections

Five-micrometer-thick sections of formalin-fixed paraffin-embedded lung tissue from the eight confirmed SARS-CoV-2-infected patients and eight control patients without SARS-CoV-2 were stained using antibody against ACE2 (Goat polyclonal, R&D system, AF933), at 1:400 dilution. Staining was performed on a Leica Bond-IIITM instrument using the Bond Polymer Refine Detection System (Leica Microsystems DS9800). Heat-induced epitope retrieval was done for 20 min with ER2 solution (Leica Microsystems AR9640). The IHC images were stained with DAB (3,3'-Diaminobenzidine), counterstained with hematoxylin, and analyzed using the IHC plugin installed in the ImageJ/Fiji software. The step-by-step quantitation of the ACE2-stained images was performed as described previously.[72] On selecting the H DAB vector option, the IHC-stained image was deconvoluted and split into two images. The DAB-stained image that represents our primary antibody of interest was selected for thresholding. Once the thresholding limit was set, it was applied across all images. The "mean gray value" function was used, which represents the quantified DAB signal. With the aim of attaining 80%–90% area coverage, a total of 800 images (200 μm) from the whole-slide images of eight COVID-19 cases and 300 images from non-COVID subjects were analyzed.

Statistical analysis

Data are presented as mean ± SD. Statistical significance was determined using Mann-Whitney U test and correlation analysis was

carried out using Pearson correlation. $p < 0.05$ was considered as statistically significant.

DATA AVAILABILITY

Data for this manuscript are all included in six tables or five composite figures and no supplementary data are presented or required for interpretation or understanding.

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AUTHOR CONTRIBUTIONS

H.D. designed the experiments on plasma/saliva sample evaluation, interaction of ACE2, and spike protein; interpreted data; developed and managed collaborations; and wrote most of this manuscript/edited several versions. S.K.N. performed ACE2 assays in plasma and saliva (Figures 1B, 2, and 3), quantified ACE2 staining in lung autopsy samples (Figure 5), and wrote several sections of this manuscript. Y.S. performed Ang (1–7) and ACE2 assays, contributed to Figures 1A and 4, and wrote corresponding sections of this manuscript. R.G.C. directed collection of saliva samples and reviewed/edited this manuscript. K.J.B. directed the CCP clinical trial, interpreted data, and wrote and edited several versions of this manuscript. P.A.S. and G.H.C. organized, and Q.I. checked, the clinical trial data. K.L. directed plasma and saliva collection with assistance from D.G. P.W. prepared, stained, and scanned all lung sections. K.T.M. coordinated autopsy sample collection and reviewed histologic and clinical data on the SARS-COV-2 autopsies. S.K.N. quantified data. D.J.R. directed collection of plasma samples with J.W. and D.J.R. K.B.M. reviewed/edited this manuscript.

DECLARATION OF INTERESTS

Authors declare no competing interests.

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