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Plasma ACE2 species are differentially altered in COVID-19 patients

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Abstract

Studies are needed to identify useful biomarkers to assess the severity and prognosis of COVID-19 disease, caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2) virus. Here, we examine the levels of various plasma species of the SARS-CoV-2 host receptor, the angiotensin-converting enzyme 2 (ACE2), in patients

Abbreviations: ACE2, angiotensin-converting enzyme 2; ADAM17, a sheddase belonging to the "a disintegrin and metalloprotease" family; Ang, angiotensin; ICU, intensive care unit; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2, novel SARS-CoV virus which causes COVID-19.

María-Salud García-Ayllón and Oscar Moreno-Pérez contributed equally to this study.

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at different phases of the infection. Human plasma ACE2 species were characterized by immunoprecipitation and western blotting employing antibodies against the ectodomain and the C-terminal domain, using a recombinant human ACE2 protein as control. In addition, changes in the cleaved and full-length ACE2 species were also examined in serum samples derived from humanized K18-hACE2 mice challenged with a lethal dose of SARS-CoV-2. ACE2 immunoreactivity was present in human plasma as several molecular mass species that probably comprise truncated (70 and 75 kDa) and full-length forms (95, 100, 130, and 170 kDa). COVID-19 patients in the acute phase of infection (n = 46) had significantly decreased levels of ACE2 full-length species, while a truncated 70-kDa form was marginally higher compared with non-disease controls (n = 26). Levels of ACE2 full-length species were in the normal range in patients after a recovery period with an interval of 58-70 days (n = 29), while the 70-kDa species decreased. Levels of the truncated ACE2 species served to discriminate between individuals infected by SARS-CoV-2 and those infected with influenza A virus (n = 17). In conclusion, specific plasma ACE2 species are altered in patients with COVID-19 and these changes normalize during the recovery phase. Alterations in ACE2 species following SARS-CoV-2 infection warrant further investigation regarding their potential usefulness as biomarkers for the disease process and to asses efficacy during vaccination.

KEYWORDS
ACE2, biomarker, COVID-19, plasma, SARS-CoV-2

1 | INTRODUCTION

Angiotensin-converting enzyme 2 (ACE2) is the host receptor for the severe acute respiratory syndrome coronavirus (SARS-CoV), as well that for the novel coronavirus SARS-CoV-2 that causes COVID-19.[1,2] ACE2 is a ubiquitous glycoprotein abundantly expressed in humans, particularly in the lung epithelia and oral and nasal mucosa, providing a possible entry route for SARS-CoVs. Gastrointestinal tract, kidneys, adrenal glands, heart, testicles, liver, and brain are some examples of extra-pulmonary tissues with confirmed ACE2 expression and potential invasion by SARS-CoV-2.[3]

The human *ACE2* gene is localized on the X chromosome and encodes an 805 amino acid-long type I transmembrane glycoprotein with an apparent molecular mass of ~100-130 kDa. This glycoprotein comprises a large N-terminal ectodomain (amino acids 18-740), which includes the zinc metalloprotease active site, a membrane anchor transmembrane domain (741-763), and a short hydrophobic intracellular C-terminus (764-805).[4]

ACE2 possesses multiple physiological functions. The major ACE2 function that has been described so far is based on its carboxypeptidase activity, which catalyzes the conversion of angiotensin I (Ang I) to generate Ang 1-9, and Ang II into Ang 1-7, peptides which induce vasodilation and have anti-fibrotic, anti-proliferative, and anti-inflammatory effects.[5] ACE2 is able to cleave other vasoactive peptides, but ACE2 also acts as a partner for amino acid transporters.[6] Some of these biological functions of ACE2 are exerted by soluble species. Indeed, catalytically active, soluble forms of ACE2 are cleaved from the membrane by the tumor necrosis factor-α-converting enzyme (TACE), also known as ADAM17, a sheddase belonging to the "a disintegrin and metalloprotease" family.[7] See Figure 1A for a schematic representation of the ACE2 protein.

Plasma ACE2 levels have been found to be increased in several inflammatory processes, including renal and cardiovascular disease, as well in diabetes and several others pathological conditions,[8,9] including acute lung injury,[10] and elevated plasma ACE2 levels have been suggested to be associated with increased risk of severe COVID-19 disease. However, the relationship between changes in levels of circulating ACE2 and susceptibility to SARS-CoV-2 infection is controversial. Indeed, to date, little is known about changes in plasma ACE2 levels following SARS-CoV-2 infection. A few studies have reported that ACE2 levels in SARS-CoV-2-positive patients are unchanged compared with negative patients presenting similar symptoms,[11-14] although increased blood ACE2 activity has been reported in critically ill COVID-19 patients compared with healthy controls[15] and

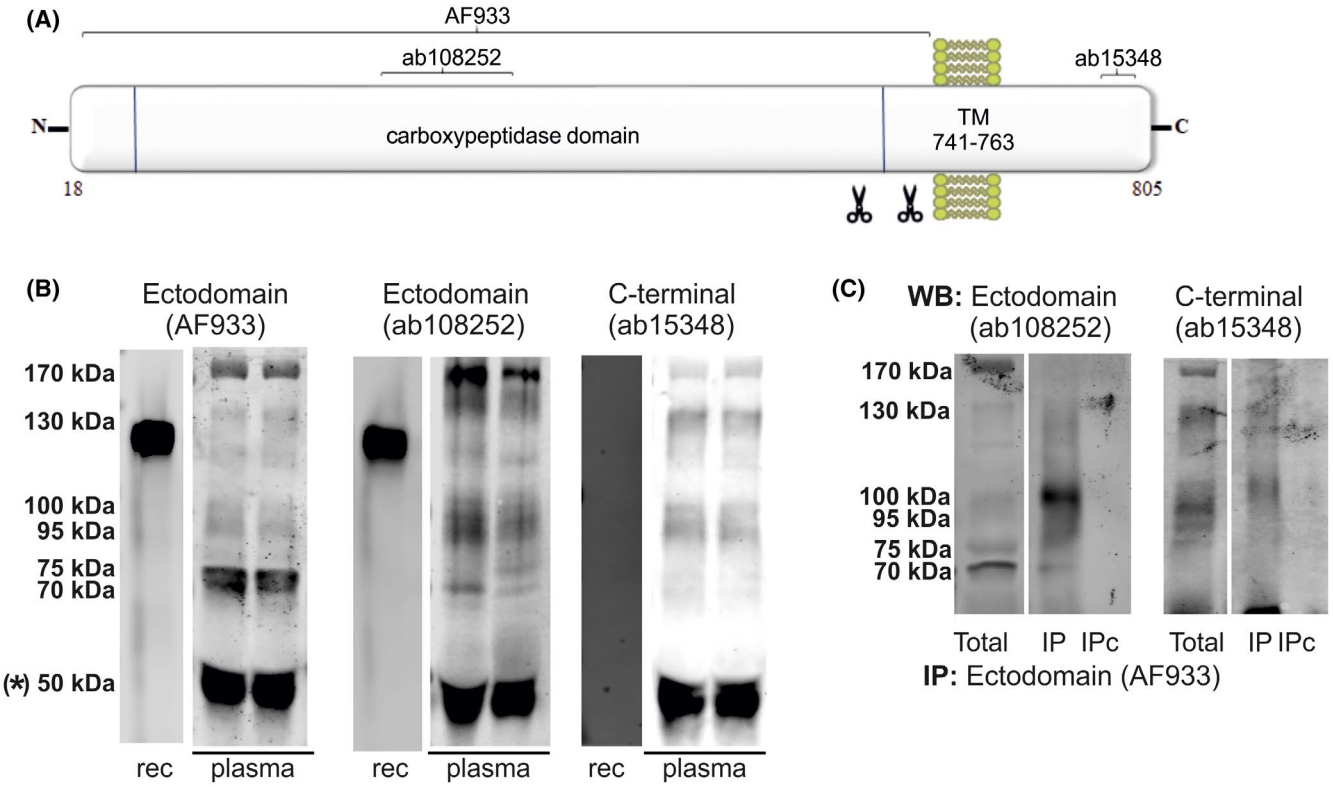


FIGURE 1 Different ACE2 species are present in human plasma. A, Schematic representation of ACE2 as a transmembrane type I protein and of the epitopes recognized by the antibodies used in this study (not drawn to scale). The carboxypeptidase and the transmembrane (TM) domains are represented. SARS-CoV-2 S-protein binds to the carboxypeptidase domain. The sites of ACE2 shedding with ADAM17 are also indicated. The resulting cleaved ACE2 species retain carboxypeptidase activity and are recognized by the AF933 and ab108252 antibodies, but not by the ab15348 antibody. B, Plasma samples from non-infected individuals were immunoblotted with the AF933 (ectodomain), the ab108252 (ectodomain), and the ab15348 (C-terminus) ACE2 antibodies. The ab15348 only recognize the full-length ACE2 which retains the C-terminal domain. A recombinant human ACE2 protein, lacking the TM and C-terminal domains (Gln18-Ser740) was used as a control. (*) Unspecific ~50-kDa band. C, Plasma samples were immunoprecipitated with the AF933 (ectodomain) antibody, and immunoprecipitated proteins (IP) were immunoblotted with either ab108252 (ectodomain) or ab15348 (C-terminus) antibodies (Total: plasma sample prior immunoprecipitation). The same plasma samples were incubated, in parallel, with a non-specific goat IgG which was used for the negative controls (IP control: IPc)

a case-report has suggested that elevated plasma ACE2 enzymatic activity may occur during the infection.[16] Interestingly, elevated plasma ACE2 enzymatic activity levels were also found in recovered COVID-19 patients compared with non-disease controls.[17] All these studies were performed by measuring ACE2 by enzymatic assay or by enzyme-linked immunosorbent assay (ELISA), approaches that are not able to distinguish changes in the levels of individual plasma ACE2 species.

The existence of circulating full-length forms of the ACE2 receptor cannot be discounted, since several transmembrane proteins that retain their transmembrane and intracellular domains have been found in human biological fluids.[18] Indeed, the presence of soluble full-length ACE2 has been suggested in human plasma[19] and urine.[20,21]

Tissue expression of ACE2 is markedly downregulated in acute lung injury of mice caused by infection with SARS-CoV,[1] although SARS-CoV-binding can also induce shedding of ACE2,[22,23] possibly resulting in increased levels of circulating cleaved fragments of ACE2. Thus, shed/truncated and full-length ACE2 species can co-exist in plasma and may be differentially affected during SARS-CoV-2 infection. Moreover, forms of ACE2 expressed by different tissues may vary in molecular mass due to different glycosylation, resulting in a complex mixture of circulating species that necessitate biochemical methods for determination of changes in individual species during COVID-19.

In this study, we aimed to characterize and to determine the levels of ACE2 in plasma using western blotting, a technique that allows for both the separation and quantification of individual ACE2 species. We sought to assess whether some of these species could constitute a biomarker of disease in patients infected by SARS-CoV-2. We also analyzed whether plasma levels of the ACE2 species are differentially affected in COVID-19 compared with non-disease subjects and if levels are restored in patients after a recovery period. The levels of plasma ACE2 species were also analyzed in patients infected by influenza A virus that uses a different host receptor

but can cause similar complications to those of SARS-CoV-2 infection.[24] In addition, the ACE2 levels were also examined in serum of susceptible mice after SARS-CoV-2 infection.

2 | METHODS

2.1 | Human plasma samples

Samples and data from patients included in this study were provided by the BioBank ISABIAL, integrated with the Spanish National Biobanks Network and with the Valencian Biobanking Network, and were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees (HGUA-ISABIAL ethics committee approved the study with expedient no. 200144 and 200145). Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes at the Hospital General Universitario de Alicante (HGUA) (Spain). Plasma was separated from whole blood by centrifugation at 3000 *g* for 15 min at 4°C, then aliquoted and frozen at −80°C until use. Fifty-nine patients with a positive reverse transcription polymerase chain reaction (RT-PCR) test for SARS-CoV-2 on nasopharyngeal swabs were included (24 females/35 males; mean age ± SE [range]: 64 ± 2 [21-89] years). All cases were hospitalized 7-9 days after the occurrence of symptoms. Forty-six plasma samples were collected during the following 1-3 days after hospitalization ("COVID-19 acute phase group"). Twenty-nine plasma samples were collected after recovery from COVID-19 with an interval of 63 ± 1 (58-70) days from the onset of symptoms. In the "recovered" group, six patients had been treated with glucocorticoids, five patients had received the IL-6-receptor inhibitor tocilizumab, and six patients had been treated with both therapies. Seventeen of the recovered samples were patients who were also in the "COVID-19 acute phase group"; thus, two samples were accessible, acute phase, and recovered. In this subset of recovered subjects, with samples at acute phase and recovered, three patients had been treated with glucocorticoids, three with tocilizumab, and four with both therapies.

Forty-eight patients infected with SARS-CoV-2 suffered a moderate presentation of COVID-19 (WHO ordinary scale 3-5), but 11 were considered as severe since they suffered from respiratory failure requiring invasive mechanical ventilation and/or were treated at intensive care unit (ICU) (WHO ordinary scale ≥6); one of the patients in the severe group was deceased. For clinical and demographic details, and hematological parameters of the COVID-19 patients see Table 1.

Two additional groups were also analyzed, one group of 17 subjects (nine females/eight males; 61 ± 4 [34-85] years) with pneumonia by influenza A virus and another of 26 non-disease controls (14 females/12 males; 66 ± 3 [34-85] years). For the "influenza A group," samples were also taken at acute phase prior to specific hospital treatment. All the samples from control and influenza A groups were obtained at the HGUA prior the COVID-19 pandemic.

2.2 | Human samples from alternative sources: CSF, urine saliva, and tissues from colon, liver, and brain

Small pieces of adjacent non-tumor tissue from colon and liver were obtained from the Hospital General Universitario of Alicante. Frozen brain cortical samples from non-demented control cases were from the Biobanco en Red de la Región de Murcia (Biobanc-Mur, Murcia, Spain) and cerebrospinal fluid (CSF) from the BioBank ISABIAL, and all were used in previous independent studies. First morning urine and saliva were collected from volunteers without any signs of disease and the fluids centrifuged at 3000 *g* for 15 min at 4°C to remove debris. All tissue and fluid samples were stored at −80°C until use. Tissues stored at −80°C were thawed gradually at 4°C and then homogenized in ice-cold extraction buffer supplemented with a cocktail of proteinase inhibitors (cat# P834; Sigma Aldrich): 50-mM Tris-HCl [pH 7.4]; 500-mM NaCl; 0.5% Triton X-100; and 0.5% Nonidet P-40. The homogenates were sonicated and centrifuged at 100 000 *g* and 4°C for 1 h, and the supernatant fraction was collected, aliquoted and frozen at −80°C until use.

2.3 | Plasma samples from K18-hACE2 mice

Female transgenic humanized K18-hACE2 mice (10 weeks old), expressing human ACE2, were obtained from the Jackson Laboratory (034860-B6. Cg-Tg[K18-ACE2]2Prlmn/J, genetic background C57BL/6J × SJL/JF2). Groups of K18-hACE2 (n = 3) mice were challenged with a lethal dose of 1×10^5 plaque-forming units (PFU) of SARS-CoV-2 (MAD6 strain) by the intranasal route, and submandibular blood collection was performed 4 days after virus challenge. Non-infected mice were used as controls. All K18-hACE2 mice challenged with SARS-CoV-2 died within 6 days post-challenge.[25] The animal study was approved by the Ethical Committee of Animal Experimentation (CEEA) of the CNB (Madrid, Spain) and by the Division of Animal Protection of the Comunidad de Madrid (PROEX 169.4/20) and was performed in the biosafety level 3 (BSL-3) facilities at the Centro de Investigación en Sanidad Animal (CISA)-Instituto Nacional De Investigaciones Agrarias (INIA) (Madrid, Spain).

2.4 | Immunoprecipitation of plasma ACE2

Immunoprecipitation was performed at 4°C by incubating 25 μL of plasma diluted in 150-μL phosphate-saline buffer

TABLE 1 Demographic, clinical data and laboratory parameters of patients with COVID-19

		COVID-19 patients			
		Sample at acute phase of infection		Sample at recovery [cases with twice sampling]	
				Moderate (n = 26)	Severe (n = 3)
		Moderate (n = 36)	Severe (n = 10)	[15]	[2]
Demographic and clinical data	Age (years)	64 ± 3	64 ± 5	58 ± 3	57 ± 13
				[54 ± 5]	[52 ± 22]
	Female/male	25/11	7/3	9/17	2/1
				[4/11]	[1/1]
	Hospitalization (days at ICU)	9 ± 1 (0)	17 ± 4 (12 ± 5)	8 ± 1 (0)	19 ± 6 (8 ± 6)
				[7 ± 1 (0)]	[22 ± 8 (8 ± 6)]
	Diabetes (%)	7 (19%)	3 (30%)	2 (8%)	1 (33%)
				[1 (7%)]	[0]
	Hypertension (%)	12 (33%)	3 (30%)	8 (31%)	1 (33%)
				[3 (15%)]	[0]
	Obesity (%)	16 (44%)	3 (30%)	8 (53%)	1 (33%)
				[7 (15%)]	[0]
Hemaetological parameters	Lymphocytes (per mm^3)	1233 ± 92	838 ± 148	1232 ± 113	1080 ± 237
				[1343 ± 143]	[845 ± 55]
	Troponin T (ng/L)	17.3 ± 4.3	13.6 ± 2.7	8.6 ± 1.1	11.0 ± 6.0
				[9.5 ± 1.7]	[5]
	CRP (mg/dL)	5.9 ± 1.1	11.3 ± 3.1	8.2 ± 1.9	18.9 ± 5.7
				[5.6 ± 2.0]	[15.1 ± 7.2]
	BNP (pg/mL)	962 ± 409	382 ± 201	169 ± 81	514 ± 463
				[169 ± 128]	[51]
	LDH (U/L)	263 ± 19	270 ± 23	283 ± 20	503 ± 326
				[255 ± 23]	[177]
	D-dimer (mg/mL)	2.88 ± 1.23	0.73 ± 0.10	0.93 ± 0.36	1.18 ± 0.12
				[1.20 ± 0.60]	[1.29]
	IL-6 (pg/mL)	48 ± 13	182 ± 76	52 ± 18	444 ± 206
				[52 ± 29]	[444 ± 206]
	Ferritin (mg/L)	1059 ± 191	923 ± 228	1205 ± 259	1231 ± 580
				[1097 ± 359]	[925 ± 853]
	PCT (ng/mL)	0.38 ± 0.28	0.45 ± 0.37	0.11 ± 0.02	0.13 ± 0.03
				[0.10 ± 0.03]	[0.12 ± 0.04]

Note: Values for the all of the samples taken at recovery (63 ± 1) days are shown as well, as the values for the subset of 17 patients for whom there are also plasma samples at acute phase. Antecedents of diabetes, hypertension, and obesity were considered. Lymphocytes and plasma markers for heart damage and inflammation such as C-reactive protein (CRP), troponin, B-type natriuretic peptide (BNP), lactate dehydrogenase (LDH), D-dimer, IL-6, and ferritin as well as the marker of bacterial infection procalcitonin (PCT) were determined.

with 25-μL of PureProteomeTM NHS Flexibind magnetic beads (Millipore) coupled to an anti-ACE2 antibody, AF933 (R&D Sytems; polyclonal goat) raised against the ectodomain (amino acid residues 18-740) of human ACE2, or alternatively as a control, beads were coupled to a non-specific goat IgG (Sigma-Aldrich). Previously, diluted plasma was pre-cleared by incubating the plasma with magnetic beads without coupled antibody. Antibody coupling was performed following indications. After overnight incubation, precipitated proteins were eluted with SDS-PAGE loading buffer by heating the beads for 5 min at 95°C. Then, fractions were analyzed by western blotting,

as described below, using a different ectodomain-specific antibody ab108252 (Abcam; rabbit monoclonal, immunogen: synthetic peptide within human ACE2 amino acid residues 200-300; 1:200 dilution), and a C-terminus antibody ab15348 (Abcam; rabbit polyclonal; immunogen: synthetic peptide corresponding to human ACE2 amino acid residues 788-805).

2.5 | Detection of ACE2 by quantitative fluorescent western blotting

ACE2 species were detected by fluorescent-based imaging after SDS-PAGE and western blotting. This technique provides a wider linear dynamic range than chemiluminescent detection,[26] including a greater upper linear range of detection.[27] Samples were heated in electrophoresis sample buffer for 7 min at 70°C to ensure virus inactivation (dilution ratio 1:10). Plasma samples (0.4 µL loaded) were resolved on 7.5% SDS-PAGE gels (Mini-PROTEAN TGX Precast Gels; Bio-Rad) and transferred to 0.2-µm nitrocellulose membranes (Bio-Rad). Then, the membrane was blocked with Odyssey Blocking Buffer (PBS) and incubated with the anti-ectodomain AF933 antibody (1:200 dilution) or alternatively with the anti-C-terminus ab15348 antibody (1:500 dilution). Finally, blots were washed and incubated with the appropriate conjugated secondary antibodies (IRDye 800CW donkey anti-goat and IRDye 800CW goat anti-rabbit, LI-COR Biosciences) and imaged on an Odyssey Clx Infrared Imaging System (LI-COR Biosciences). For quantitative analysis, all blots were resolved with the AF933 antibody. A control plasma sample was used to normalize the immunoreactive signal between blots. All samples were analyzed at least in duplicate. Band intensities were analyzed using LI-COR software (Image Studio Lite). To estimate the relative ratio of ACE2 species for each sample, the immunoreactivity was considered for each of the bands (see Results section). Recombinant human ACE2 protein (Gln18-Ser740; R&D Sytems) and overexpressed pcDNA3.1-hACE2 (a gift from Fang Li;[28] Addgene plasmid# 145033) were used as positive controls.

2.6 | Statistical analysis

All data were analyzed using SigmaStat (Version 3.5; SPSS Inc). The Kolmogorov-Smirnov test was used to analyze the distribution of each variable. ANOVA was used for parametric variables and the Kruskal-Wallis test for non-parametric variables for comparison between groups. A Student's *t* test for parametric variables and a Mann-Whitney *U* test for non-parametric variables were employed for comparison between two groups, and for determining *P* values. For correlations, the Rho Spearman test was used. The results are presented as means ± SEM.

3 | RESULTS

3.1 | Characterization of full-length and cleaved ACE2 species in human plasma

Since the presence of ACE2 full-length species in biological fluids has been suspected, but not demonstrated, we analyzed non-infected (control) human plasma samples by western blotting using either a polyclonal goat antibody (AF933) that recognizes the ectodomain of ACE2, a rabbit monoclonal antibody (ab108252) that also recognizes the ACE2 ectodomain, or a rabbit polyclonal antibody (ab15348) raised against the C-terminus of human ACE2 (Figure 1A,B). Analysis of the western blots revealed a complex banding pattern, confirming the existence of soluble/circulating full-length ACE2 species of ~95 and 100 kDa, as well as ~130- and 170-kDa species, all of which were immunoreactive to both the ectodomain and C-terminal antibodies (Figure 1B). Two additional bands of ~70 and 75 kDa were uniquely immunoreactive to the ectodomain antibodies but were not recognized by the C-terminal ab15348 antibody, thus probably represent C-terminally truncated shedded species (Figure 1B). A smaller ~50-kDa band was attributed to a nonspecific reactivity, since it was immunoreactive to both the anti-ectodomain ab108252 (aa 200-300) and the anti-C-terminal antibody (aa 788-805). Therefore this band was not further considered. The molecular mass of ~50 kDa is similar to that expected for the shorter soluble ACE2 splicing variant species that lacks part of the ectodomain, transmembrane, and C-terminus[29]; thus, it should not be reactive to a C-terminal antibody. The molecular mass of ~50 kDa is also similar to a recently identified short ACE2 variant encoding a protein isoform which includes the C-terminal epitope,[30] but this alternative variant lacks a part of the ectodomain and should not be recognized by the ab108252 antibody.[30] A recombinant human ACE2 protein (Gln18-Ser740) showed, as expected, a single ~130-kDa band that could be detected with anti-ectodomain antibodies, but not with the anti-C-terminal antibody (Figure 1B).

The identity of full-length and cleaved ACE2 species in human plasma was confirmed by immunoprecipitation using the AF933 antibody followed by western blotting using the ectodomain antibody, ab108252, or the C-terminal ab15348 (Figure 1C). The results confirmed the existence of diverse species of full-length ACE2 in human plasma, possibly derived from different tissues, and that the 70- and 75-kDa species lack the C-terminus.

To examine whether different tissues express different ACE2 species, we performed a comparative analysis

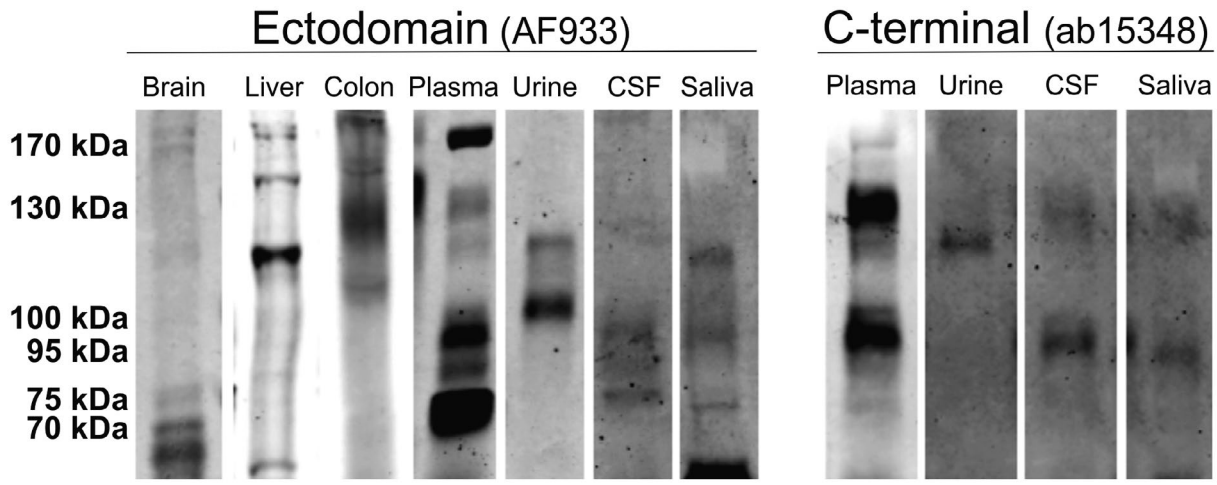


FIGURE 2 Different ACE2 species are present in human plasma, brain, liver, colon, urine, CSF, and saliva. Representative immunoblot with the ectodomain AF933 antibody of tissue extracts from brain (frontal cortex), liver, and colon. Urine, CSF, and saliva, together with plasma, were also immunoblotted with the AF933 and the ab15348 (C-terminus) antibodies. Western blots for different antibodies were performed individually, to avoid re-using blots. Samples were resolved in the same gels but are shown separately to optimize contrast for defining discrete bands. Colon was the tissue extract displaying highest immunoreactivity, followed by liver and then brain (equal protein was loaded in each lane), whereas plasma was the fluid with highest immunoreactivity, followed by urine, saliva and CSF which all displayed weaker immunoreactivities

of ACE2 from different human tissues and fluids obtained from non-diseased subjects, available from previous independent studies (Figure 2). The ACE2 banding pattern obtained with the ectodomain AF933 antibody revealed a cell type-dependent banding pattern, reflecting differences in the molecular mass of ACE2 species from colon, liver, and brain extract. Weaker bands were observed for the liver and brain extracts, as expected due to their lower ACE2 expression.[31] As expected, the ACE2 in plasma did not match the pattern of any single tissue, probably due to the diverse cellular origin of the plasma forms. Nonetheless, some of the ACE2 species detected in human plasma matched species present in the analyzed tissues. The plasma ACE2 species were similar in apparent molecular mass to those present (in different proportions) in urine, saliva and CSF. Similar to plasma, we were able to distinguish ACE2 species in the other fluids attributable to their being full-length forms. Based on their lack of immunoreactivity with the C-terminal ab15348 antibody, the ~70- to 75-kDa species observed in saliva and CSF were likely to be cleaved forms missing the C-terminus. Urine displayed cleaved species at ~100 kDa, while full-length species were of ~130 kDa.

3.2 | Plasma ACE2 species are altered in humanized mice after SARS-CoV-2 infection

Next, we analyzed the ACE2 banding pattern in K18-hACE2 mice, a transgenic mouse model with the human ACE2, susceptible for SARS-CoV-2 infection. The promoter, the human cytokeratin 18 (K18) gene, confers efficient transgene expression in airway epithelial cells (but not in alveolar epithelia), as well as in epithelia of other internal organs, including the liver, kidney, and gastrointestinal tract.[32] Therefore, in K18-hACE2 mice, tissue distribution of hACE2 is expressed similarly, with only small differences, with respect to the endogenous mACE2.[33] Thus, to define the impact of SARS-CoV-2 infection over the levels of serum ACE2, K18-hACE2 mice were challenged with 1×10^5 PFU of SARS-CoV-2 by the intranasal route and serum samples obtained at Day 4 post-infection (uninfected mice served as control group). Challenged K18-hACE2 mice died at 6 days post infection.[25] Notably, several plasma ACE2 species were increased at Day 4 post challenge, compared to controls. Forms which changed included several cleaved species and particularly species retaining the C-terminus, identified as full-length forms (Figure 3).

3.3 | Levels of plasma ACE2 species in COVID-19 acute phase patients

Next, we analyzed human plasma samples by quantitative fluorescent western blotting with the ectodomain AF933 antibody, which detected all ACE2 species, both full-length and cleaved forms. The levels of the circulating full-length ACE2 species (95-, 100-, 130-, and 170-kDa species) were lower in COVID-19 patients than in controls, whereas the cleaved 75-kDa ACE2 species was not affected and the 70-kDa species displayed a non-significant trend towards a small increase (~19%, $P = .2$) (Figure 4A-C). When the sum of the immunoreactivities of all plasma ACE2 species was

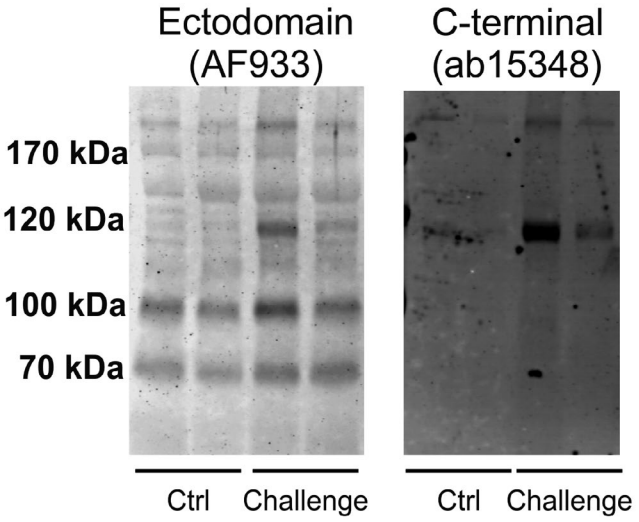


FIGURE 3 Increased levels of ACE2 in plasma from the K18-hACE2 mice. Transgenic humanized K18-hACE2 mice, expressing human ACE2, were challenged with a lethal dose of SARS-CoV-2 (MAD6 strain) by the intranasal route. Plasma ACE2 was analyzed in the control non-infected group and in SARS-CoV-2 challenged mice by western blotting with the ectodomain AF933 and the C-terminus ab15348 antibodies, in samples collected 4 days after virus challenge (the mice died at 6-day post-challenge)[25]

taken into account, we found that the levels were not significantly different between groups. Next, we calculated the ratio of the 70-kDa cleaved species to the full-length species (represented by the 95- and 100-kDa species). This ratio was found to discriminate between diseased COVID-19 and control subjects (Figure 5A), suggesting that tissue ACE2 binding with SARS-CoV-2 results in increased shedding (maybe also co-existing with downregulation).

In a subset of more severely affected COVID-19 cases, the mean levels of most of the full-length ACE2 species (and in particular, the 95-kDa form) were lower than for the moderate cases, but this did not reach statistical significance ($P = .1$) (Figure 4C). We were not able to find differences in levels of ACE2 species when individuals were grouped according to antecedents of hypertension, obesity, or diabetes, a condition more represented in the severe COVID-19 cases (Table 1). Only the 95-kDa ACE2 species displayed a significant correlation with the abnormal coagulation function marker D-dimer ($r = .31$; $P = .045$). Similarly, the mean level of the 95-kDa species was correlated with the level of the C-reactive protein ($r = .26$; $P = .08$), another inflammatory marker, and with B-type natriuretic peptide ($r = .30$; $P = .07$), a marker for heart damage, although these were not quite statistically significant.

No correlation was observed between the level of any plasma ACE2 species and age in either the COVID-19 group or in the control group. Only minor differences were observed when the COVID-19-affected subjects were sub-grouped by gender, with ~50% higher levels of the cleaved 75-kDa ACE2 species in male that than in female ($P = .027$; figure not shown).

3.4 | Changes in levels of plasma ACE2 after a recovery period

The global analysis of ACE2 species in infected SARS-CoV2 cases after recovery demonstrated that the levels of the 130-kDa ACE2 were restored, and the other full-length species showed a similar trend (Figure 4A-C). However, the mean level of the 70-kDa species was lower in the recovered COVID-19 group than in acute phase, a difference that was almost statistically significant also as compared with controls ($P = .052$) (Figure 4B). The different trend of 70-kDa cleaved ACE2 and the full-length species also resulted in significant differences in the 70-kDa/(95 + 100)-kDa ratio between COVID-19 patients at acute phase and recovered subjects (Figure 5A).

The 70- and 75-kDa ACE2 cleaved species did not show a similar trend in COVID-19 patients at acute phase and recovered subjects, resulting in significantly different 70-kDa/75-kDa ratios (Figure 5B).

The subset of patients who had been tested twice, once at hospitalization and then following recovery, were of particular interest since they probably represent reliably the differences at recovery. In comparison with levels at acute phase, there was a large (50%-197%) increase in all ACE2 full-length species in plasma from recovered subjects, while the 70-kDa band was 16% decreased (Figure 6A). The ratio of the full-length 95- and 100-kDa species to the 70-kDa cleaved species was 5.4 times higher in the recovered subjects (Figure 6B). The details about clinical and demographic data, hospitalization period, and specific treatment of the patients tested twice for plasma ACE2 levels are indicated in Figure 6B.

3.5 | Levels of plasma ACE2 species in influenza A virus

In the influenza A individuals, plasma ACE2 levels were lower compared to controls, but this was only significant for the 95-kDa species ($P = .040$; Figure 4C); mean level of the 100-kDa band was lower, but this was not quite statistically significant ($P = .076$). Similarly, the mean level of the 170-kDa species was lower, but this was not quite statistically significant ($P = .077$) (Figure 4C). Interestingly, while there was a positive correlation between the 95-, 100-, 130-, and 170-kDa full-length species in the control group ($r = .65$; $P < .001$) and COVID-19 groups, this correlation was not observed for the influenza A group ($r = .45$; $P =$

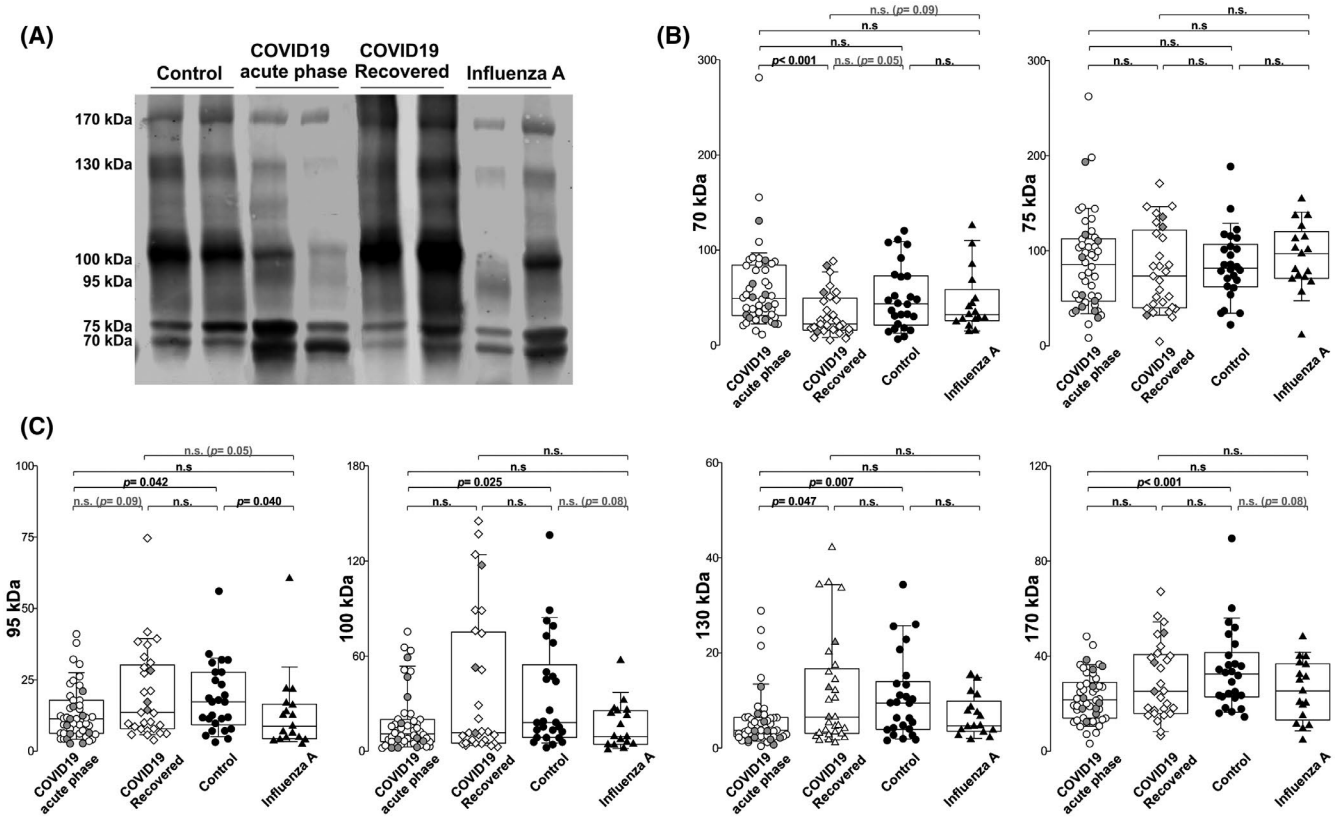


FIGURE 4 Levels of ACE2 species in plasma from individuals affected by COVID-19 or influenza A and from healthy controls. A, Representative western blots of COVID-19 at acute phase, at recovery (interval of 58-70 days between hospital admission and recovery), healthy control and influenza A plasma resolved with the AF933 antibody. B, The densitometric quantification of the cleaved 70- and 75-kDa species, (C) as well the full-length 95-, 100-, 130-, and 170-kDa species are shown. Box and scatter plots of the levels of the indicated species of ACE2 are represented (COVID-19 patients: open circles, n = 46; recover COVID-19 patients: open triangles, n = 29 [patients that were categorized as severe COVID-19 patients are represented in grey]; influenza A patients [open diamond; n = 17]; and controls [Ctrl; closed circles; n = 26]). The bars within the box plot represent the median abundance for the given group. *P* values are shown; n.s.: non-significant, the non-significant $P < .1$ are indicated

.37). This suggests that while in the affected COVID-19 individuals, plasma ACE2 full-length species from diverse cellular origins were similarly affected, this was not the case for influenza A patients. The difference between 70- and 75-kDa ACE2 cleaved species also reflect differences between COVID 19 and influenza A patients, reflected in differences in the 70-kDa/75-kDa ratio (Figure 5B).

4 | DISCUSSION

We have identified several ACE2 species in plasma by western blotting. Full-length species probably reflect tissue ACE2, and cleaved species possibly originate from shedding after interaction with ligands,[34] including the SARS-CoV-2. We have found that plasma full-length and cleaved ACE2 species were affected differentially after infection with SARS-CoV-2. The immunoreactive bands attributable to full-length species were decreased in plasma from affected COVID-19 patients, whereas the mean level of cleaved 70-kDa species was marginally higher than the mean level of control patients, although this difference was non-significant. As a result of the different trends, the ratio between full-length and cleaved species was able to discriminate among COVID-19 affected and control patients. Levels of the ACE2 full-length species returned to the normal range in patients who had recovered from the disease, while levels of the truncated 70-kDa species decreased. Levels of the truncated ACE2 species also served to discriminate between subjects infected by SARS-CoV-2 and influenza A viruses.

The presence of several full-length forms of ACE2 in biological fluid is intriguing, and the mechanism and regulation of shedding of these forms from the membrane have not been fully elucidated. Moreover, most of the previous studies that measured ACE2 levels in biological fluids used enzymatic assays or ELISA, obtaining values which probably represented all of the ACE2 species. As such, these studies would not have distinguished between cleaved and full-length species. Thus, specific analysis of the occurrence of circulating full-length ACE2 and the mechanisms by which the

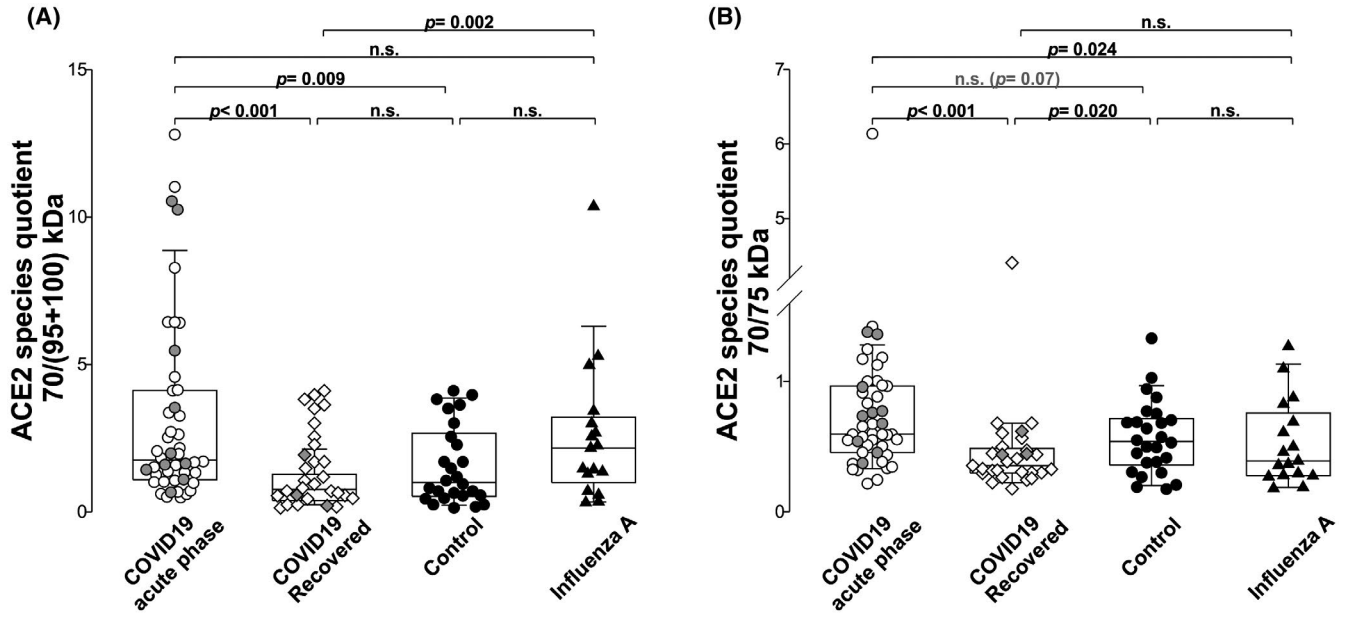


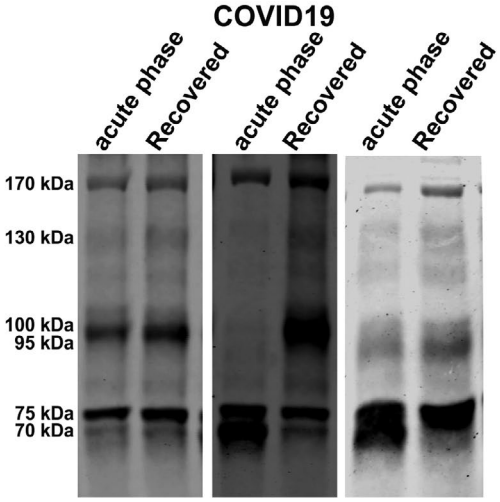
FIGURE 5 Ratios of ACE2 plasma species vary during COVID-19. The relation between several species, for each sample (as determined in Figure 2), are represented by (A) the graph of the quotient obtained by dividing the level of immunoreactivity of the 70-kDa band by the level of immunoreactivity of the 95- and 100-kDa species (70/(95 + 100) kDa); and by (B) the graph of the quotient obtained by dividing the level of immunoreactivity of the 70-kDa band by the level of immunoreactivity of the 75-kDa species (70/75 kDa). As in Figure 2, data are presented in box and scatter plots for COVID-19 patients at acute phase (open circles: moderate cases; grey circles: severe cases), recover COVID-19 patients (open triangles: moderate cases; grey triangles: severe cases); influenza A patients (open diamond); and controls (Ctrl; closed circles). The bars within the box plot represent the median abundance for the given group. *P* values are shown; n.s.: non-significant (the non-significant $P < .1$ are indicated in grey)

membrane-associated protein is shed into the circulation as a full-length species have not merited much attention.

Previous studies using western blotting have reported the presence of several immunoreactive bands ranging between 50 and 130 kDa.[19-21,35] One of these studies has indicated that a urinary ~70-kDa ACE2 in mice is a fragment of 90-kDa membrane-bound renal ACE2, being also a faint 90-kDa immunoreactive band in urine samples.[36] Similarly, in human urine, ACE2 bands were detected at ~100 and 130 kDa, the latter of which is identical in size to that of the full-length, membrane-bound recombinant protein.[20] Here, using ectodomain and C-terminus antibodies for western blotting, we examined the assumption that the full-length membrane-bound form can be released into biological fluids. We demonstrated that there are full-length ACE2 species in human plasma, urine, CSF, and saliva. A previous study on human plasma showed, in addition to a ~90-kDa ACE2 immunoreactive band, a band at ~130-kDa matching membrane-bound recombinant protein.[19] Since ACE2 is a ubiquitous protein, it could be assumed that plasma ACE2 can originate from several tissues. We have analyzed the ACE2 banding pattern from a few human tissues available in our lab, including colon, liver, and brain. Most likely, several other tissues also contribute to the plasma pool of ACE2 and even that some of the tissues examined in the present study do not contribute, or very poorly contribute, to the pool of plasma ACE2. Whatever the case, analysis of ACE2 in these tissues is important to demonstrate the diverse patterns of membrane-bound ACE2 species, exhibiting differences in molecular mass probably related to glycosylation. Since human ACE2 is a highly glycosylated protein,[37] the ACE2 that is synthesized by different tissues can exhibit differences in molecular mass due to differences in glycosylation. A recent study revealed ACE2 immunoreactive bands of ~130 kDa in human kidney, while a slightly lower size was present in colon, probably indicating differences in glycosylation.[31] Hence, the distinct full-length species present in human plasma could represent species which originate from diverse cell sources. Furthermore, ACE2 species with a large molecular mass were present in the analyzed tissues, but only plasma exhibited clear evidence of a 170-kDa form. The nature of this species, which could be a highly glycosylated form or a multimer that is stable during SDS-PAGE, requires further study. Indeed, it is known that ACE2 can form oligomeric species.[38] Understanding the identity and cellular origin of the diverse species of full-length ACE2 may be of great value for understanding ACE2 species changes can be used as a read-out of disease progression.

Proteolytic shedding from the cell surface is an important mechanism regulating the expression and function of membrane proteins. In fact, endocytosis and cleavage of

(A)



(B)

		Recovered COVID-19 patients							
		% of the levels of ACE2 species (in kDa) respect values at diagnosis						Quotients ACE2 species diagnosis-recovery	
Treatment	**Case data**	**70**	**75**	**95**	**100**	**130**	**170**	**70/(95+100)**	**70/75**
TCZ/Cort	M; 73y; 30 (14)d	35	13	38	39	76	39	1.99-1.93	0.373-0.444
TCZ/Cort	F; 30y; 13 (2)d	-72	-12	445	2365	263	103	10.54-0.21	1,379-0.438
TCZ/Cort	F; 68y; 11d; Ob	12	48	106	154	327	54	0.60-0.27	0.435-0.328
TCZ/Cort	M; 62y; 6d	-57	-28	35	31	4	105	4.14-1.33	0.592-0.354
TCZ	M; 64y; 15d; Ob	20	41	31	73	8	12	0.51-0.39	0.368-0.315
TCZ	M; 21y; 11d; Ob	-48	-48	38	41	-20	3	3.25-1.22	0.319-0.318
TCZ	M; 56y; 4d	-87	-58	19	-39	15	8	6.44-0.90	1.420-0.439
Cort	M; 28y; 8d	-21	4	82	65	21	35	0.68-0.31	0.588-0.448
Cort	M 80 y, HT, 7d	-33	95	79	14	74	96	1.82-0.81	0.882-0.305
Cort	M; 66y; 2d; Ob	-64	-71	-33	-55	-15	15	1.01-0.64	0.215-0.263
-	M; 46y; 46d	-8	58	40	97	-24	-9	0.98-0.57	0.383-0.223
-	F; 74y; HT; 7d	45	19	66	40	56	22	0.61-0.60	0.554-0.679
-	F; 24y; 6d	-5	-42	-19	-44	62	-18	1.68-2.30	0.343-0.565
-	M; 30y; 4d; Ob	-32	14	56	128	32	50	4.11-1.50	0.503-0.301
-	M; 68y; 4d; Ob	35	29	85	40	41	82	1.02-0.81	0.244-0.256
-	M; 74y; 2d, HT, DM, Ob	49	146	542	503	3404	170	1.97-0.48	1.125-0.679
-	F; 55y; 1d	-36	12	102	666	731	82	1.59-0.24	0.673-0.384
	% / fold change	**-16**	**13**	**101**	**242**	**297**	**50**	**5.4**	**1.6**

FIGURE 6 Change in ACE2 plasma species in COVID-19 patients after recovery. For 17 COVID-19 affected subjects, plasma ACE2 species were measured at two time-points, the first at acute phase and the second, after 58-70 days, when patients had recovered (recovery). A, Representative ACE2 immunoblots for samples at acute phase and after recovery from the same subject. B, Some clinical and demographic data are indicated for each patient: Age (in year: y), gender (female [F]; male [M]), number of days requiring hospitalization (including ICU for severe cases): H (in days: d); antecedent of diabetes (DM), hypertension (HT), obesity (Ob). Treatment with glucocorticoids (Cort) or tocilizumab (TCZ) are also indicated. For more demographic, clinical data and laboratory parameters, see Table 1. The change for each species, in each patient, was calculated between the two time-points and is represented as percentage of changes with respect to the baseline. The 70/(95 + 100)-kDa and 70/75-kDa quotients estimated at acute phase and at recovery are also represented. The last row shows the average of the percentages and the mean of the fold-change in the ACE2 quotients between acute phase and recovery

transmembrane receptors, with generation of soluble ectodomain fragments, occurs canonically in response to ligand binding. Indeed, it has been proposed that ACE2 plays a role in cell-cell interactions, possibly acting to fine-tune integrin signaling, while cleaved ACE2 is capable of suppressing integrin signaling. Hence, the expression and cleavage of ACE2 at the membrane may influence this function.[39]

ACE2 is cleaved from the plasma membrane through regulated shedding mediated by ADAM17, resulting in at least two large fragments with a molecular mass ~25 kDa less than the original full-length species, even though only one of the cleaved fragments appears regulable by stimulating ADAM17 activity with PMA.[7] The original report indicated that the two ACE2 fragments released by cleavage represent

different glycoforms,[7] but later analysis has indicated that shedding of ACE2 fragments also occurs at alternative cleavage sites, Met706 and between Arg577 and Lys596.[40] In the latter study, inhibition of ADAM17 had no effect on constitutive shedding of ACE2, as determined by an increase in ACE2 enzymatic activity.[40] However, this study did not examine differences in generated fragments. In addition to ADAM17, another member of the same family, ADAM10, has been also implicated in the regulated, but not in the constitutive/basal, release of ACE2 from airway epithelia.[10] Interestingly, we found changes related to COVID-19 infection only with one of the cleaved fragments lacking the C-terminal, the 70-kDa, but not in the 75-kDa species. We were not able to determine whether both species represent fragments which originate from the same cell type by two different mechanisms, or if they have different cellular origins. Indeed, the presence in plasma of additional cleaved species is plausible since we identified ACE2 cleaved forms of ~100 kDa in urine.

The interpretation of changes in tissue and circulating ACE2 in the context of SARS-CoV-2 infection and COVID-19 progression remains unclear.[41,42] ACE2 may play a protective role in the pathogenesis of acute lung injury caused by several viruses,[43-45] as well as in other conditions such as acid aspiration and sepsis.[46] However conversely, high surface localization of ACE2 may impact on SARS-CoV-2 infectivity. In this context, assuming that plasma levels of ACE2 match those on the cell surface, elevated plasma ACE2 may promote SARS-CoV-2 infection.

Our data indicating the presence of decreased full-length ACE2 in COVID-19 plasma suggest that full-length plasma ACE2 could serve to monitor levels of tissue ACE2. Most of the samples analyzed in this study were obtained from subjects suffering a moderate presentation of COVID-19. But, interestingly in plasma from K18-hACE2 mice challenged with a lethal dose of SARS-CoV-2, a large increase in a full-length form was also observed. Whether this increase in the animal model reflects cell death or tissue increased ACE2 expression remains to be determined and could serve to decipher the significance of the changes detected in moderate COVID-19 subjects. Moreover, the balance between ACE2 in plasma and the levels in tissues is uncertain, since the contribution of different tissues/cells and mechanism of by which full-length ACE2 is transported to the plasma are yet to be determined. It has been suggested that lysis following cell death or exosomes may lead to the release of membrane-bound ACE2 into plasma,[19] but this possibility remains to be elucidated. Recently, it has been reported that full-length ACE2 can be released in extracellular vesicles from cultured cells[47]; thus, an exosome release mechanism cannot be discounted.

Regarding the cleaved/truncated ACE2 species, whether SARS-CoV2 infection drives receptor proteolytic processing is not known, but K18-hACE2 mice challenged with a lethal dose of SARS-CoV-2 displayed noticeable increases in ACE2 species identified as truncated forms lacking the C-terminus domain. Like other coronaviruses, SARS-CoV2 entry is mediated by the spike (S) protein,[48] using ACE2 for entry and the serine protease TMPRSS2 for S-protein priming.[49] SARS-CoV S-protein induces ACE2 endocytosis and efficient shedding,[23,50,51] while S-protein from other human coronaviruses such as NL63 does not.[52] The SARS-CoV-induced shedding of ACE2 also appears to occur via ADAM17,[22] but the relevance of ACE2 cleavage for SARS-CoV-2 infection has not been determined yet. Whatever the case, when SARS-CoV binds to its receptor, the abundance of ACE2 on the cell surface is significantly reduced,[53] and this can be attributed, at least in part, to enhanced shedding.[10] Thus, circulating cleaved species can be expected to increase during COVID-19 infection.

Serial analysis of plasma ACE2 species, ie, at different time points during the infection, could be also informative as a read-out of the progression of the disease as well as recovery. Our results indicated that full-length species of ACE2 are restored in COVID-19 recovered patients, but, intriguingly, the cleaved 70-kDa ACE2 species was decreased, resulting in a substantial change ratio of cleaved and full-length species, following recovery. These changes may involve tissue upregulation of ACE2 but could also reflect that equilibrium of physiological ACE2 ligands, such as angiotensins, is not yet fully restored.

Changes in plasma ACE2 species, such as a decrease in full-length and an increase in cleaved ACE2 species would mirror tissue changes during viral infection in moderate COVID-19 patients. However, recent evidence points to a different scenario. Data show that the ACE2 gene is more highly expressed in SARS-CoV-2 infected than in normal tissues,[54,55] including in cells from bronchoalveolar lavage fluid of COVID-19 patients.[56] Thus, we cannot discard that changes in gene expression and ACE2 cleavage may be differently regulated over time after infection and in a tissue-dependent manner. Interestingly, in a recent study, ACE2 enzymatic activity was increased in plasma in severe COVID-19 patients, from early to late stages of the disease.[57] The increase in ACE2 activity was associated with increased interleukin-6 levels, supporting a link with inflammation. However, immunomodulatory treatment does not appear to impact ACE2 levels.[57] Different dynamics in full-length and cleaved ACE2 species may overlap during disease progression hindering the interpretation of the progression of virus infection through ACE2 assessment by ELISA or enzymatic assay.

For an appropriate understanding of changes in specific ACE2 species, the receptor-binding, internalization, and processing need to be investigated. Despite the fact that it is likely that the S-protein of the SARS-CoV-2 induces endocytosis and shedding of the ACE2 receptor, similarly to that demonstrated in SARS-CoV-infected cells, TMPRSS2,

as well other type II transmembrane serine proteases (TTSPs) can also cleave ACE2.[58,59] In fact, it has been proposed that TMPRSS2 and ADAM17 will compete for ACE2 cleavage resulting in different ACE2 cleavage products exhibiting differential fates.[59]

Interestingly, infection by other types of viruses could also cause subtle differences in circulating ACE2. In our study, we included analysis of plasma samples from influenza A virus infected subjects. Currently, the identity of the host receptor for several influenza A subtypes remains to be determined, but it is generally accepted that hemagglutinin from influenza A viruses shares specificity for receptors with α-2,3-linked sialic acid that have a linear presentation.[60] Increasing evidence indicates that ACE2 could mediate the severe acute lung injury induced by influenza A H7N9[43] and H5N1 viruses[44] and that virus infection downregulates ACE2 expression in lungs of infected animal models. In this regard, it has been previously demonstrated in cellular models that ACE2 protein levels are significantly downregulated after infection with H1N1 or H5N1 viruses, even though ACE2 was not necessary for viral replication.[61] Indeed, it has been suggested that the decrease in ACE2 protein was due to degradation via the proteasome pathway rather than to ACE2 shedding, which was not affected by ADAM17 knockdown. Moreover, ACE2 cleavage regulated by influenza neuraminidase appears different from the ADAM17-induced proteolytic cleavage of ACE2. Thus, shift in electrophoretic mobility suggests a direct cleavage by virus neuraminidase enzyme rather than host cell sheddases.[61] SARS-CoV and SARS-CoV-2 do not have hemagglutinin and neuraminidase activity.[62] In this context, we have found only subtle changes in levels of cleaved ACE2 species that serve to distinguish changes in plasma ACE2 from SARS-CoV-2-infected patients and those infected by influenza A virus. A more detailed analysis may reveal different fates in ACE2 truncated species that could be informative of the nature of the infection.

4.1 | Limitations of the study

Even if ELISA is a desirable approach for quantitative analysis of altered levels of a biomarker, the ELISA method does not easily detect subtle changes in specific ACE2 species. Thus, we used quantitative fluorescent western blotting, a more sensitive technology than the more widely used chemiluminescent methods. However, this approach still has limitations. For example, limited numbers of samples can be analyzed at one time, and there is limited precision for quantitative analysis compared with other techniques. Nonetheless, we believe that this approach, with a greater upper linear range of detection compared to chemiluminescent methods, allows us to assess the distinct plasma ACE2 species, providing an important read-out for following the evolution of COVID-19. A complete characterization of the distinct ACE2 species present in plasma will serve for developing more applicable kits for analysis. Our data indicate that further study of the plasma ACE2 species during COVID-19 could be of importance as it may be used as a biomarker of progression and therefore may have utility in clinical trials.

A potential limitation of the use of ACE2 as a biomarker for COVID-19 might be the broad range of plasma ACE2 levels existing within the general population, since ACE2 expression and shedding can be potentially altered in many common conditions such as hypertension, heart disease, diabetes, or obesity, among many others. In fact, an association between plasma ACE2 levels and age or gender is also possible. Earliest animal studies suggested the potential existence of age- and gender-related differences in ACE2 expression in lung[63] and kidney.[64] Moreover, sex hormones are also known to modulate the expression of ACE2.[65] Studies of the human secretome have shown that the average concentration of ACE2 protein in the plasma of males is higher than that in females.[66] These differences have been confirmed in different human cohorts.[67-71] However, similarly to our study, others have failed to demonstrate clear differences in ACE2 expression between males and females or between younger and older persons in any tissue[72] or plasma.[73] Nevertheless, pre-existing conditions, age, gender, and many other factors, such as smoking,[74] could potentially influence plasma ACE2. Indeed, nicotine exposure increases ACE2 expression, which in turn leads to increased competence for SARS-CoV-2 replication and a cytopathic effect.[75] The upregulation of expression of tissue ACE2 induced by compounds such as candesartan and captopril are particularly pronounced in rats with metabolic syndrome (obesity, increased blood pressure and hyperglycemia) and in aged rats.[23] In summary, many pathophysiological conditions, drugs, and lifestyle and environmental factors can modulate ACE2, generating a large range in ACE2 basal levels, thereby hindering the assignment of cut-offs needed to establish plasma ACE2 as a biomarker.

This was an observational, retrospective, single-center study undertaken under difficult circumstances, and we regard the obtained results scientifically interpretable and important to report. Further, the collection of data was not systematized in advance. Efforts were undertaken to capture and revise data by a clinical team with experience in COVID-19. As this is a hospital-based cohort, conclusions might not be applicable to outpatients with COVID-19.

5 | CONCLUSIONS

In conclusion, our data suggest that the depletion of plasma ACE2 full-length species could reflect tissue infection

occurring during COVID-19, while the levels of a truncated 70-kDa form appeared marginally higher in the acute phase of infection and could serve as a read-out of active SARS-CoV-2 cellular infection. Except for the 70-kDa ACE2 species, levels are almost completely normalized after COVID-19 recovery.

If our findings are confirmed in studies with serial samples in patients with COVID-19, the analysis of these plasma ACE2 species could serve as a sensitive biomarker to monitor the disease progression and recovery, as well as to follow the evolution of SARS-CoV-2 vaccination. Finally, levels of truncated ACE2 species could also serve to distinguish between infection by SAR-CoV and by other viruses with different host receptor. Our present findings provide sufficient evidence to justify further studies focusing on the possibility of monitoring the diverse circulating species of ACE2 to evaluate the infection, progression, and response to therapy in COVID-19 patients.

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CONFLICT OF INTEREST

All the authors declare that they have no competing interest.

AUTHOR CONTRIBUTIONS

M. S. García-Ayllón, O. Moreno-Pérez, J. M. Ramos-Rincón, J. García-Arriaza, G. Brinkmalm, H. Zetterberg, M. Esteban, E. Merino, and J. Sáez-Valero were involved with the conception, design, and interpretation of data. M. S. García-Ayllón, J. García-Arriaza, M. A. Cortés-Gómez, G. Brinkmalm, and J. Sáez-Valero performed the experiments. J. Sáez-Valero, O. Moreno-Pérez, J. García-Arriaza, and M. S. García-Ayllón were involved with data analysis. O. Moreno-Pérez, J. M. Ramos-Rincón, M. Andrés, J. M. León-Ramírez, V. Boix, J. Gil, and E. Merino were involved in the collection of the clinical material. J. Sáez-Valero provided general overall supervision of the study and acquired funding. All authors contributed to the drafting and critical revision of the manuscript and have given final approval of the version to be published.

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