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Original Article

ORIGINAL ARTICLE

Urinary angiotensin-converting enzyme 2 and metabolomics in COVID-19-mediated kidney injury

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ABSTRACT

Background. Angiotensin-converting enzyme 2 (ACE2), the receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is highly expressed in the kidneys. Beyond serving as a crucial endogenous regulator of the renin–angiotensin system, ACE2 also possess a unique function to facilitate amino acid absorption. Our observational study sought to explore the relationship between urine ACE2 (uACE2) and renal outcomes in coronavirus disease 2019 (COVID-19).

Methods. In a cohort of 104 patients with COVID-19 without acute kidney injury (AKI), 43 patients with COVID-19-mediated AKI and 36 non-COVID-19 controls, we measured uACE2, urine tumour necrosis factor receptors I and II (uTNF-RI and uTNF-RII) and neutrophil gelatinase-associated lipocalin (uNGAL). We also assessed ACE2 staining in autopsy kidney samples and generated a propensity score–matched subgroup of patients to perform a targeted urine metabolomic study to describe the characteristic signature of COVID-19.

Results. uACE2 is increased in patients with COVID-19 and further increased in those that developed AKI. After adjusting uACE2 levels for age, sex and previous comorbidities, increased uACE2 was independently associated with a >3-fold higher risk of developing AKI [odds ratio 3.05 (95% confidence interval 1.23–7.58), $P = .017$]. Increased uACE2 corresponded to a tubular loss of ACE2 in kidney sections and strongly correlated with uTNF-RI and uTNF-RII. Urine quantitative metabolome analysis revealed an increased excretion of essential amino acids in patients with COVID-19, including leucine, isoleucine, tryptophan and phenylalanine. Additionally, a strong correlation was observed between urine amino acids and uACE2.

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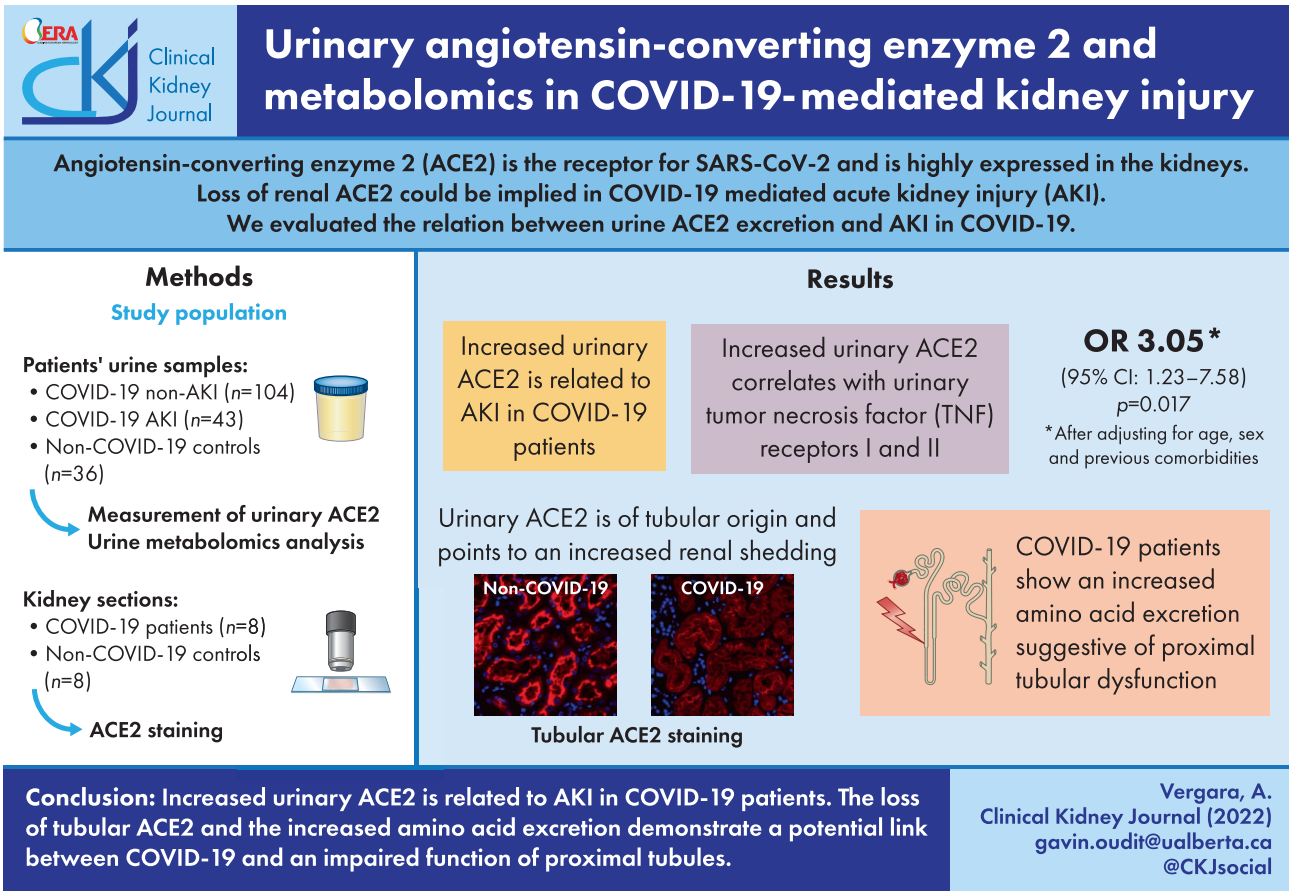
Conclusions. Elevated uACE2 is related to AKI in patients with COVID-19. The loss of tubular ACE2 during SARS-CoV-2 infection demonstrates a potential link between aminoaciduria and proximal tubular injury.

LAY SUMMARY

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) uses angiotensin-converting enzyme 2 (ACE2) as a receptor to enter host cells. Upon entry, the virus reduces membrane ACE2 expression through internalization or activation of a disintegrin and metalloproteinase 17 (ADAM17)-mediated shedding. We hypothesized that SARS-CoV-2-mediated loss of ACE2 drives kidney injury in coronavirus disease 2019 (COVID-19) patients. We demonstrated that urinary ACE2 (uACE2) is elevated in COVID-19 without acute kidney injury (AKI) and further increases in COVID-19 with AKI correlating with the loss of tubular ACE2 seen in autopsied kidney samples from COVID-19 patients. Moreover, higher uACE2 is independently associated with a greater incidence of AKI after accounting for other clinical risk factors, including age, sex or previous comorbidities. The uACE2 is of kidney origin and the urine metabolomic analysis revealed that loss of uACE2 is linked to increased urinary amino acid excretion. Therefore, increased uACE2 during SARS-CoV-2 infection represents a potential link between urine amino acid loss and kidney injury in patients with COVID-19.

GRAPHICAL ABSTRACT



Keywords: acute kidney injury, angiotensin-converting enzyme 2, COVID-19, metabolomics, renin–angiotensin system

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), uses angiotensin-converting enzyme 2 (ACE2) as the receptor to enter host cells [1]. Local ACE2 expression in tissues such as lung, gut or kidney is essential to counterbalance angiotensin II (AngII)-mediated deleterious effects [2–4]. We and others have demonstrated that SARS-CoV infection downregulates ACE2 expression at the cell membrane [5, 6]. Importantly, SARS-CoV and SARS-CoV-2 stimulate a disintegrin and metalloproteinase

17 (ADAM17) [7, 8]. ADAM17 is a metalloproteinase that mediates the ectodomain shedding of multiple cellular membrane molecules. ACE2 is a substrate of ADAM17, along with tumour necrosis factor (TNF)-α, TNF receptor I (TNF-RI) and TNF-RII. Therefore SARS-CoV-2-mediated activation of ADAM17, and the consequent ACE2 shedding, represents a potential specific injury mechanism triggered by the virus, especially for patients with obesity, diabetes or cardiovascular disease (CVD) [2, 9]. Indeed, persistent elevation in plasma ACE2 during COVID-19 is related to increased mortality and acute myocardial injury [10].

ACE2 is highly expressed in the kidney [11, 12]. Moreover, the proteases necessary for SARS-CoV-2 spike protein priming and internalization, namely transmembrane serine protease 2 and cathepsin L, are also present in the renal tissue [13]. Therefore the kidney represents a potential target for SARS-CoV-2 [14]. Acute kidney injury (AKI) is also a frequent finding in COVID-19, with a prevalence ranging between 25% and 45% among hospitalized patients, but its pathophysiology remains to be elucidated [15, 16]. Indirect mechanisms common to other causes of AKI, such as volume depletion, hypotension or exposure to nephrotoxic agents, can partly explain some of these cases [17]. However, the high prevalence of AKI [18] and the renal tropism of SARS-CoV-2 [19, 20] suggest that there are specific mechanisms for COVID-19-related AKI that need to be unravelled. Considering that ACE2 is highly expressed in proximal tubular cells and podocytes [11, 12], it is plausible that the loss of kidney ACE2 could be related to an increased risk of AKI in the COVID-19 setting. However, plasma ACE2 was not reflective of the incidence of AKI in COVID-19 patients [10] and, in this context, urinary ACE2 (uACE2) may serve as a better indicator of renal-specific pathophysiology.

Based on these observations, we hypothesized that uACE2, arising from ADAM17-mediated shedding of kidney ACE2, could be linked to renal outcomes in COVID-19. Therefore the present exploratory study aimed to evaluate if biomarkers such as ACE2, TNF-RI and TNF-RII are increased in the urine of patients with COVID-19 who developed AKI. To complement this work, we provided tissue staining in autopsy kidney sections and performed targeted mass spectrometry (MS)-based analysis of the urine metabolome to identify if COVID-19 infection led to a differential urine signature arising from its effects on kidney function.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. A more detailed explanation of the methods can be found in the Supplemental Methods.

Study participants

Patients in the current prospective observational study were participants of the COVID-19 Surveillance Collaboration (CoCollab) study that enrolled 296 hospitalized COVID-19 patients between 1 July 2020 and 30 June 2021. The enrolled patients were $\geq$18 years of age and had a laboratory-confirmed COVID-19 diagnosis based on a positive SARS-CoV-2 real-time polymerase chain reaction assay from nasopharyngeal swabs or lower respiratory tract samples. Patients who had previously received SARS-CoV-2 vaccination(s) or categorized as stage 5 chronic kidney disease (CKD) were excluded from the present study. In total, 147 patients with plasma and urine samples collected after hospital admission were included in the analysis. Due to work overload during the COVID-19 pandemic, urine samples were not available from 137 patients (Supplementary Table S1 and Fig. S1). The CoCollab study was also responsible for collecting samples from non-COVID-19 individuals during the same time frame, from which 36 age- and sex-matched participants without cardiovascular risk factors were selected as controls for comparison (Supplementary Table S2). Non-COVID-19 controls were recruited through different forms of advertisement, such as posters or newsletters. Samples from both COVID-19 patients and non-COVID-19 controls were processed and stored at the Canadian Biosample Repository (CBSR) located at the University of Alberta.

This study was conducted following the ethical principles of the Declaration of Helsinki and approved by the University of Alberta Health Research Ethics Board (Pro00100319 and Pro00100207). Written informed consent was obtained from all participants enrolled. A waiver of consent was granted for participants from intensive care units (ICUs), followed by a regained capacity consent signed whenever possible.

Outcome assessment

Demographics, comorbidities, previous renal function, medications, symptomatology, vital signs and laboratory results of participants were collected through the review of electronic medical records. AKI was defined based on changes in serum creatinine according to the Kidney Disease: Improving Global Outcomes guidelines classification system [21]. The definition criteria of other outcomes of interest (albuminuria, CKD, CVD, acute respiratory distress syndrome, acute myocardial infarction or pneumonia) are shown in the Supplemental Methods.

Sample collection and laboratory measurements

Morning spot urine and blood samples were collected by health personnel and transported to the CBSR (see sample processing in Supplemental Methods). Glomerular filtration rate (GFR) was estimated using the 2021 Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [22]. Urinary ACE2, TNF-RI, TNF-RII and neutrophil gelatinase-associated lipocalin (NGAL), along with their plasma counterparts, were measured using commercially available human enzyme-linked immunosorbent assay kits (DY933-05, DRT100, DRT200 and DY1757-05; R&D Systems, Minneapolis, MN, USA).

Kidney samples and immunofluorescence studies

Paraffin-embedded kidney samples from COVID-19 patients were obtained from a collaboration with the National Institute for Infectious Diseases “Lazzaro Spallanzani”, Rome, Italy. Renal tissue was collected from autopsies performed on eight COVID-19 patients following procedures previously described [23]. Non-COVID-19 kidney samples were obtained from the non-neoplastic portions of nephrectomies performed at the University of Alberta Hospital. Both studies were approved by the National Institute for Infectious Diseases “Lazzaro Spallanzani” Ethics Committee (9/2020) and University of Alberta Health Research Ethics Board (Pro00100319). ACE2 staining in kidney sections is described in the Supplemental Methods. Fluorescence intensity was quantified separately in tubular and glomerular compartments. Intensity data were adjusted to controls and expressed as relative fluorescence intensity (RFI).

Urine metabolomics

A nearest neighbour propensity score-matching strategy was employed to select 28 COVID-19 patients with AKI and 30 COVID-19 patients without AKI from the initial cohort for subsequent urine metabolome analysis (Supplementary Figs. S1 and S2). Patients were matched by age, sex, obesity, hypertension, diabetes and CVD. Importantly, patients with previous CKD were excluded to avoid bias linked to previous renal dysfunction. Twenty-four age- and sex-matched non-COVID-19 controls were also selected for this analysis. Urine samples from these patients were analysed with a targeted MS-based quantitative metabolomics approach using a custom assay that combines high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) coupled with to a tandem mass spectrometer (QTRAP 5500; AB Sciex, Framingham, MA, USA) (see Supplemental Methods). Metabolomics data have been deposited to the EMBL-EBI MetaboLights database [24] with the identifier MTBLS4331.

Statistical analysis

Data analysis was performed using Stata version 15.1 (StataCorp, College Station, TX, USA). Descriptive statistics are shown as number and percentage for categorical data. Continuous variables are described as median and interquartile range (IQR). Comparisons of two independent groups were performed with the chi-squared and Fisher's exact tests for categorical data, while the Mann–Whitney's U test and Student's *t*-test were employed for continuous data. A non-parametric Spearman rank-order correlation test was used to evaluate the association between two continuous variables. The receiver operating characteristics (ROC) curve's nearest (0,1) value was used to identify the cut-off point with the highest AKI discrimination power in each biomarker. These cut-off values were then employed to transform the urine biomarkers into dichotomous variables. The dichotomous biomarkers were then tested using univariable and multivariable logistic regression analysis for association with AKI and albuminuria. Multivariable regression model 1 adjusted the biomarkers for age and sex, while model 2 further adjusted them for smoking and previous comorbidities (hypertension, diabetes, CVD and CKD). A two-sided *P*-value $<.05$ was considered significant.

Regarding urine metabolome analysis, data were first normalized by urine creatinine. Log_2 fold-changes were calculated using the means of the groups compared. Statistical analysis was done by performing pairwise comparisons for each metabolite using the Mann–Whitney's U test. All *P*-values were corrected to control the false discovery rate (FDR) at 5% using the Benjamini–Hochberg method. Metabolite set enrichment analysis, pathway analysis and urine disease signature evaluation were done using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) [25].

RESULTS

Baseline characteristics of the COVID-19 cohort

A total of 147 patients hospitalized with COVID-19 were enrolled in the present study from July 2020 to June 2021. The cohort's median age was 61 years (IQR 50–71) and 80 patients (54.4%) were men. The median time elapsed between COVID-19 diagnosis and sample collection was 12 days (IQR 5–18). The three most common comorbidities were hypertension (43.5%), obesity (39.5%) and diabetes (38.8%) (Table 1). Among the included patients, 43 (29.3%) developed AKI. Most AKI cases were classified as stage 1 and 30 (88.2%) patients recovered kidney function to baseline values among the patients who had follow-up data 4 months after a COVID-19 diagnosis (see the distribution of AKI stages in Supplementary Tables S2 and S3). Patients who developed AKI were more likely to be men with pre-established CVD or kidney disease, accompanied by lower haemoglobin values. Moreover, albuminuria was also increased in COVID-19 patients with AKI compared with those without [1.0 mg/mmol (IQR 0.5–4.7) versus 2.6 (1.1–28.8)]. Urine was collected after the AKI event and the time elapsed from the AKI diagnosis to plasma and urine collection was 8 days (IQR 2–13).

Evaluation of urine ACE2, TNF-RI and TNF-RII in patients with COVID-19

uACE2 was higher in COVID-19 patients without AKI compared with non-COVID-19 controls [3.3 ng/ml (IQR 1.7–6.2) versus 1.3 (IQR 0.7–2.6)], and values were greater still in patients with COVID-19 and AKI than in those without AKI [5.3 ng/ml (IQR 2.8–10.1) versus 3.3 (1.7–6.2)] (Fig. 1A). The uACE2:creatinine ratio was also higher in patients with COVID-19 and AKI versus the non-AKI group (Supplementary Fig. S3). The finding represents a 4.2-fold median increase in uACE2 in COVID-19 patients who develop AKI compared with controls, and it is also seen in patients without previous cardiovascular risk factors (Supplementary Fig. S4). Urinary TNF receptor I (uTNF-RI) and II (uTNF-RII) showed a distribution similar to uACE2 (Fig. 1B, C). uTNF-RI and uTNF-RII were increased in COVID-19 patients without AKI compared with non-COVID-19 controls [3.4 ng/ml (IQR 1.6–6.9) versus 1.1 (0.6–2.1) and 4.9 ng/ml (IQR 2.2–8.4) versus 1.8 (0.8–4.0), respectively] and further increased in COVID-19 patients who developed AKI [8.1 ng/ml (IQR 3.5–15.6) versus 3.4 (1.6–6.9) and 7.1 ng/ml (IQR 4.4–11.5) versus 4.9 (2.2–8.4), respectively]. Furthermore, we assessed a biomarker of renal tubular damage: urinary NGAL (uNGAL) [26]. uNGAL was elevated in COVID-19 patients without AKI compared with non-COVID-19 controls [15.5 ng/ml (IQR 7.3–35.7) versus 7.2 (2.4–15.3)], but the latter biomarker was not effective in differentiating AKI patients within the COVID-19 cohort (Fig. 1D).

Urine ACE2 and TNF-RI are increased in COVID-19 patients who developed AKI

Before applying the regression models, we transformed urine biomarkers into dichotomous variables using the best cut-off points for discriminating patients with COVID-19 and AKI. In the present cohort, the uACE2 nearest (0,1) cut-off value was 3.6 ng/ml, whereas the nearest (0,1) cut-off values for uTNF-RI and uTNF-RII were 6.7 ng/ml and 6.9 ng/ml, respectively. Urine ACE2 $\geq$3.6 ng/ml was associated with a 2.8-fold increased risk [95% confidence interval (CI) 1.3–6.0] of having developed AKI during COVID-19 (Table 2). Urine TNF-RI $\geq$6.7 ng/ml and urine TNF-RII $\geq$6.9 ng/ml were also associated with a 4.0-fold (95% CI 1.9–8.4) and 2.5-fold (95% CI 1.2–5.1) increased risk of AKI, respectively. However, after adjustment for age, sex and comorbidities in model 2, only uACE2 and uTNF-RI remained significantly associated with the incidence of AKI (Table 2). In line with the latter result, after performing a propensity score matching between

Table 1: Baseline characteristics of the COVID-19 patients.

Characteristics	Entire cohort ($N = 147$)	Non-AKI patients ($n = 104$)	AKI patients ($n = 43$)	P-value
Time from COVID-19 diagnosis to urine collection (days), median (IQR)	12 (5–18)	11 (5–18)	13 (5–18)	.630
Age (years), median (IQR)	61 (50–71)	60 (49–71)	62 (53–74)	.456
Male, *n* (%)	80 (54.4)	49 (47.1)	31 (72.1)	.006
Active smoker, *n* (%)	57 (38.8)	36 (34.6)	21 (48.8)	.107
Medical history, *n* (%)				
Obesity	58 (39.5)	38 (36.5)	20 (46.5)	.260
Hypertension	64 (43.5)	37 (35.6)	27 (62.8)	.002
Diabetes	57 (38.8)	35 (33.7)	22 (51.2)	.047
CVD[a]	20 (13.6)	7 (6.7)	13 (30.2)	<.001
Atrial arrythmia	8 (5.4)	4 (3.6)	4 (9.3)	.233
Chronic obstructive pulmonary disease	19 (12.9)	12 (11.5)	7 (16.3)	.436
CKD	28 (19.1)	13 (12.5)	15 (34.9)	.002
Stage 1–2	2 (1.4)	0 (0.0)	2 (6.7)	.060
Stage 3a	12 (8.2)	7 (7.1)	5 (15.2)	.177
Stage 3b	13 (8.8)	6 (6.2)	7 (20.0)	.041
Stage 4	1 (0.7)	0 (0.0)	1 (3.5)	.242
Chronic medications, *n* (%)				
ACEi or ARBs	43 (29.3)	24 (23.1)	19 (44.2)	.010
β-blockers	19 (12.9)	8 (7.7)	11 (25.6)	.003
Statins	50 (34.0)	29 (27.9)	21 (48.8)	.015
Clinical presentation				
Fever	53 (36.1)	37 (35.6)	16 (37.2)	.739
Gastrointestinal symptoms[b]	53 (36.1)	34 (32.7)	19 (44.2)	.423
Respiratory symptoms[c]	111 (75.5)	75 (72.1)	36 (83.7)	.756
Dyspnoea	89 (60.5)	61 (58.7)	28 (65.1)	.892
X-ray-confirmed pneumonia	93 (63.3)	65 (62.5)	28 (65.1)	.517
Admission to ICU, *n* (%)	25 (17.0)	11 (10.6)	14 (32.6)	.001
COVID-19 complications, *n* (%)				
ARDS	13 (8.8)	7 (6.7)	6 (14.0)	.161
Shock	3 (2.0)	2 (1.9)	1 (2.3)	.999
AMI	10 (6.8)	4 (3.9)	6 (14.0)	.064
Mortality	13 (8.8)	10 (9.6)	3 (7.0)	.608
Laboratory tests on admission, median (IQR)				
Haemoglobin (g/L)	128 (114–139)	131 (117–141)	119 (107–136)	.019
WBC ($\times 10^9$/L)	8.5 (6.3–11.2)	8.3 (6.3–10.7)	9.5 (7.9–12.2)	.152
Lymphocytes ($\times 10^9$/L)	0.9 (0.6–1.2)	1.0 (0.7–1.3)	0.7 (0.5–0.9)	.001
Platelets ($\times 10^9$/L)	255 (206–344)	261 (217–347)	247 (185–337)	.503
GFR (ml/min/1.73 m^2)[d]	81 (56–102)	92 (74–105)	55 (28–71)	<.001
C-reactive protein (mg/L)	70 (35–123)	61 (30–105)	89 (53–140)	.035
Ferritin (µg/L)	447 (169–761)	392 (161–689)	633 (336–1839)	.069
D-dimer (mg/L)	1.2 (0.7–1.7)	0.9 (0.7–1.7)	1.3 (0.9–2.2)	.082
Lactate dehydrogenase (UI/L)	298 (237–348)	288 (238–339)	301 (237–353)	.446
UACR (mg/mmol)	1.3 (0.6–5.4)	1.0 (0.5–4.7)	2.6 (1.1–28.8)	.002
Received treatments, *n* (%)				
IMV	22 (15.0)	10 (9.6)	12 (27.9)	.005
Corticosteroids	110 (74.8)	74 (71.2)	36 (83.7)	.110
Antibiotics	93 (63.3)	58 (55.8)	35 (81.4)	.003

[a]Patients with a previous history of myocardial infarction, coronary artery disease or heart failure.
[b]Gastrointestinal symptoms include diarrhoea and nausea.
[c]Respiratory symptoms include cough and dyspnoea.
[d]The CKD-EPI equation was used to estimate GFR.
ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; ARDS: acute respiratory distress syndrome; AMI: acute myocardial infarction; WBC: white blood cell count; UACR: urinary albumin:creatinine ratio; IMV: invasive mechanical ventilation.

AKI and non-AKI patients, only uACE2 and uTNF-RI remained significantly different between the two groups (Supplementary Fig. S5).

We also assessed if urine biomarkers were able to discriminate patients with albuminuria, which acts as a surrogate marker of kidney injury. Urine TNF-RI ≥6.7 ng/ml and TNF-RII ≥6.9 ng/ml were related to a 4.7-fold (95% CI 1.9–8.4) and 2.5-fold (95% CI 1.2–5.0) increased risk of presenting albuminuria during COVID-19, respectively (Table 2). uNGAL ≥19.8 ng/ml was related as well to a 2.5-fold (95% CI 1.2–5.0) increased risk of albuminuria. However, uACE2 was not linked to albuminuria in any regression analyses performed. In the multivariable regression analysis, only uTNF-RI remained significantly associated with albuminuria in model 2.

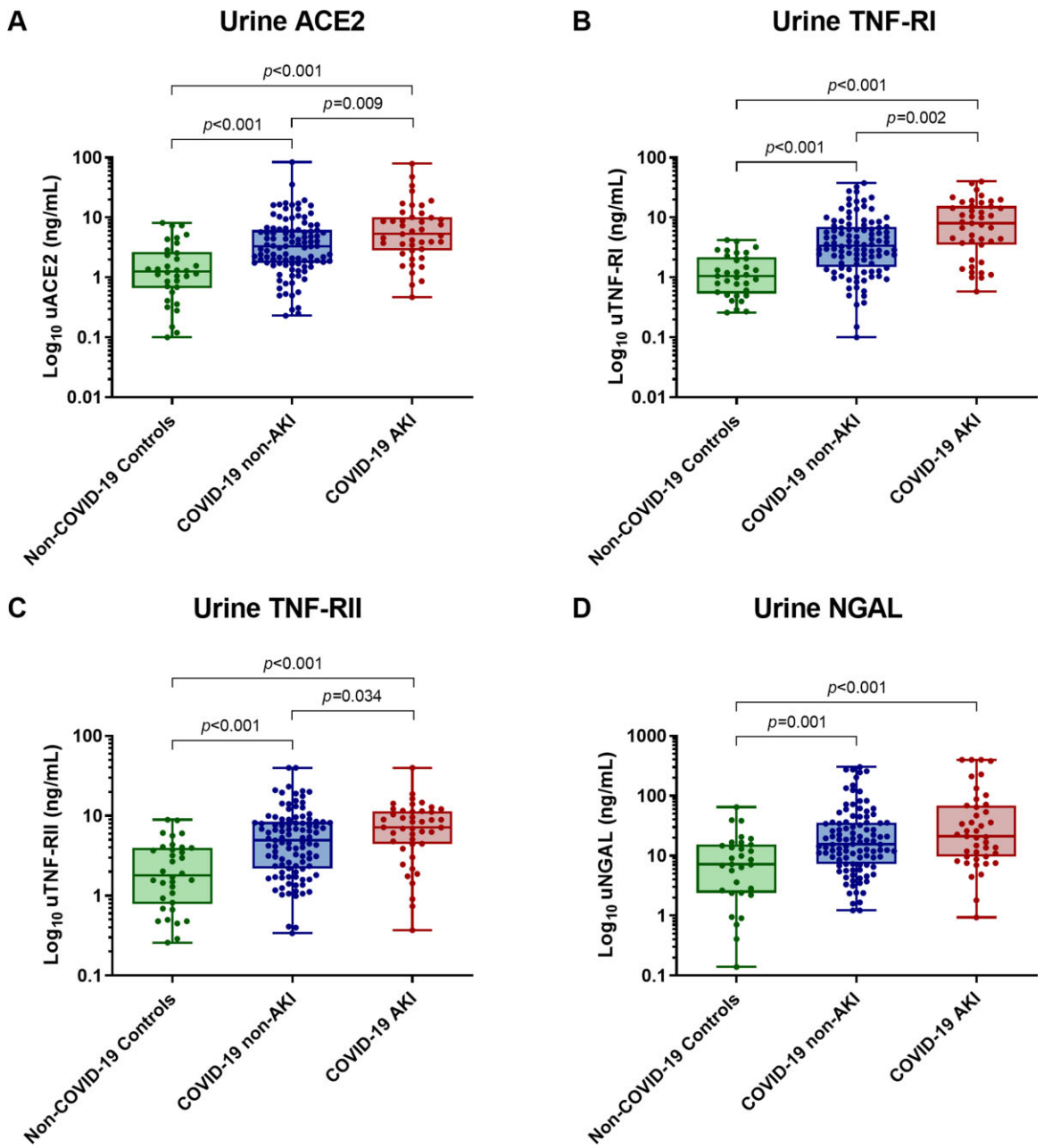


Figure 1: Urine biomarkers in COVID-19 patients with and without AKI compared with age- and sex-matched non-COVID-19 controls. The measured urine biomarkers were **(A)** uACE2, **(B)** uTNF-RI, **(C)** uTNF-RII and **(D)** uNGAL. Data are displayed in box and whisker plots. Non-COVID-19 controls, $n = 36$; COVID-19 non-AKI, $n = 104$; COVID-19 AKI, $n = 43$.

Urine ACE2 is of renal origin and correlates with uTNF-RI, uTNF-RII and NGAL levels

After establishing the relation of uACE2 and uTNF-RI with AKI, we analysed the correlation between plasma and urine ACE2 levels in COVID-19 patients. The Spearman's test showed a lack of association between plasma ACE2 (pACE2) and uACE2 collected on the same day [$\rho = -0.05$ (95% CI −0.23–0.14)] (Fig. 2A). Additionally, we did not see a correlation between uACE2 and pACE2, even in patients with overt albuminuria (>30 mg/mmol) (Supplementary Fig. S6). We further assessed if uACE2 correlated with uTNF-RI, uTNF-RII and NGAL. In this case, a moderate correlation was observed between uACE2 and uTNF-RI and uTNF-RII [$\rho = 0.58$ (95% CI 0.44–0.71) and $\rho = 0.54$ (95% CI 0.40–0.68), respectively] (Fig. 2B, C). Levels of the tubular injury marker NGAL also correlated with uACE2 (Fig. 2D). Moreover, uTNF-RI and uTNF-RII demonstrated a strong correlation among themselves (Fig. 2E).

Post-mortem renal pathology analysis reveals downregulation of tubular ACE2 in COVID-19

The increased shedding of tubular ACE2 was also confirmed by analysing post mortem renal sections of COVID-19 patients. Compared with non-COVID-19 sections, immunofluorescence

Table 2: Univariable and multivariable logistic regression models for uACE2, uTNF-RI, uTNF-RII and uNGAL biomarkers.

Outcome	Univariable, OR (95% CI)	*P*-value	Model 1, OR[a] (95% CI)	*P*-value	Model 2, OR[b] (95% CI)	*P*-value
AKI						
uACE2	2.80 (1.31–5.96)*	.008	2.34 (1.09–5.18)*	.029	3.05 (1.23–7.58)*	.017
uTNF-RI	3.96 (1.88–8.37)*	<.001	3.41 (1.57–7.39)*	.002	2.74 (1.18–6.38)*	.019
uTNF-RII	2.48 (1.20–5.13)*	.015	2.03 (0.95–4.34)	.069	2.26 (0.97–5.26)	.060
uNGAL	1.72 (0.84–3.53)	.137	2.28 (1.03–5.06)*	.042	1.90 (0.79–4.59)	.152
Albuminuria[c]						
uACE2	0.92 (0.46–1.83)	.815	0.76 (0.36–1.59)	.462	0.59 (0.26–1.35)	.214
uTNF-RI	4.73 (2.26–9.88)*	<.001	4.10 (1.90–8.84)*	<.001	3.46 (1.52–7.86)*	.003
uTNF-RII	2.47 (1.22–4.99)*	.012	1.99 (0.94–4.19)	.070	1.86 (0.83–4.15)	.132
uNGAL	2.49 (1.23–5.04)*	.011	2.64 (1.22–5.70)*	.013	1.97 (0.86–4.49)	.108

The odds ratio (OR) of presenting AKI or albuminuria for patients with uACE2, uTNF-RI, uTNF-RII and uNGAL above the cut-off points. Cut-off points: 3.6 ng/ml for uACE2, 6.7 ng/ml for uTNF-RI, 6.9 ng/ml for uTNF-RII and 19.8 ng/ml for uNGAL.

[a]Multivariate logistic regression model 1 was adjusted for age and sex.

[b]Multivariable logistic regression model 2 was adjusted for age, sex, active smoking and comorbidities related to AKI (hypertension, diabetes, CVD and CKD).

[c]Albuminuria was defined as a urinary albumin:creatine ratio ≥3 mg/mmol.

*Independent association of the biomarker with the outcome ($P < .05$).

staining revealed a 2-fold reduction of ACE2 tubular expression in COVID-19 patients (Fig. 3A, B and Supplementary Fig. S7). Conversely, despite a slight decrease in ACE2 staining in the glomeruli, no differences were present between non-COVID-19 and COVID-19 patients (Fig. 3C, D). Thus the uACE2 identified in COVID-19 patients is of tubular origin. Interestingly, a change in ACE2 staining pattern is observed in injured tubular cells, where the enzyme's expression is no longer limited to the apical membrane (Fig. 3E).

Urine metabolomic analysis of COVID-19 patients shows increased aminoaciduria

To identify a specific urine profile in patients with COVID-19, we performed an exploratory targeted MS-based metabolomics analysis in a subgroup of 28 COVID-19 patients with AKI, 30 COVID-19 patients without AKI and 24 non-COVID-19 controls selected from the main cohort (Supplementary Table S4 and Fig. S1). From the 250 metabolites that could be maximally detected, we identified 189. However, two were excluded because <5% of patients had values over the lower limit of detection (Supplementary Fig. S2). Most of the changes in the urine metabolome were due to COVID-19 and we did not identify significant differences between patients with AKI and patients without AKI (Supplementary Figs. S8 and S9). Therefore, comparisons were performed between controls and the whole COVID-19 patient cohort. The analysis revealed 18 substantially increased metabolites and 13 decreased metabolites in the urine of COVID-19 patients. Among the most significantly increased compounds, we identified several essential amino acids: lysine, threonine, leucine, isoleucine, phenylalanine, tryptophan and their metabolites (Supplementary Table S5). Moreover, most of the proteogenic amino acids identified through the metabolomic analysis were increased in the urine of COVID-19 patients, except for glycine (Fig. 4A). Thus, when we performed the enrichment analysis, the increased metabolites in COVID-19 patients were linked to amino acid metabolism pathways (Fig. 4B and Supplementary Table S6). Notably, tryptophan metabolism, and specifically the kynurerine–quinolinate pathway, was significantly represented (Fig. 4C). It is worth mentioning that amino acid excretion was slightly higher in the COVID-19 AKI group (Fig. 4A), showing a continuous increase throughout the three groups similar to that observed in uACE2. Moreover, certain amino acid metabolites such as indolelactic acid or hydroxiphenylpiruvic acid were higher in the AKI group (Fig. 4C and Supplementary S9) and a strong correlation was identified in urine between ACE2 and leucine, isoleucine, tryptophan, phenylalanine and valine.

DISCUSSION

Tissue ACE2 is crucial to counterbalance AngII-mediated deleterious effects [2, 4], and its local downregulation during SARS-CoV-2 infection may be a specific injury mechanism in multiple organs including the lungs, heart, gut or kidneys [2, 10]. In this context, AKI is a frequent complication during COVID-19 that worsens disease prognosis and contributes to the CVD burden in these patients [15]. The present study shows that uACE2 is elevated in COVID-19 patients with preserved GFR and further increased in those who developed AKI during SARS-CoV-2 infection, displaying a continuous increase in uACE2 levels throughout the three groups. Moreover, the increase in uACE2 correlates with uTNF-RI and uTNF-RII. All three proteins are substrates of ADAM17, which suggest that SARS-CoV-2 may increase the activity of the metalloproteinase in the kidney. Additionally, COVID-19 patients showed increased excretion of amino acids in the urine, which is suggestive of proximal tubular dysfunction or injury. Tubular ACE2 loss, which participates in amino acid transport [27], could be linked to both impaired amino acid reabsorption and acute tubular injury, becoming a specific mechanism that explains the high prevalence of AKI in COVID-19 patients.

SARS-CoVs promote ADAM17 metalloproteinase activity, an effect that is not shared by other human coronaviruses such as HNL63-CoV (an α-coronavirus that recognizes ACE2 and causes the common cold) [7]. ACE2 counterbalances the classical renin–angiotensin system (RAS) pathway [4, 28]. The downregulation of tissue ACE2 enhanced by the infection would increase the local exposure to AngII-mediated pro-inflammatory effects, which would lead to augmented organ injury [3, 4]. Moreover, the action of AngII on angiotensin II type 1 receptor stimulates ADAM17 [29], activating a positive feedback loop leading to progressive injury. In this context, an upward trend of soluble plasma ACE2 during SARS-CoV-2 infection was associated with an almost 4-times greater risk of mortality and incidence of acute myocardial injury after adjusting for comorbidities and established disease markers [10]. Other studies have shown similar results, relating

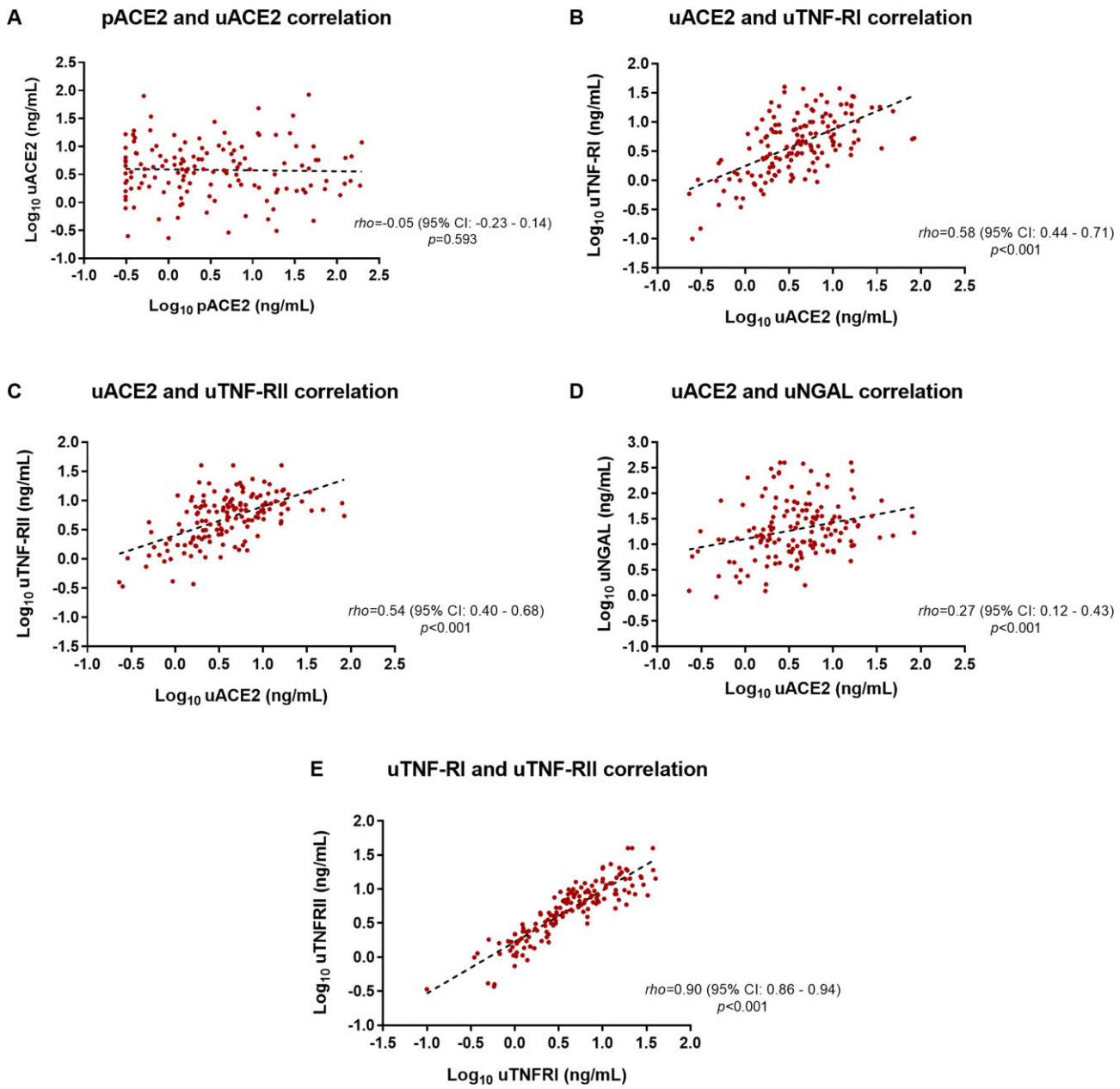


Figure 2: Correlation in COVID-19 patients of uACE2 levels with pACE2, uTNF-RI, uTNF-RII and uNGAL. **(A)** Correlation analysis of uACE2 and pACE2 shows no relation between both concentrations. In contrast, uACE2 shows moderate correlation with **(B)** uTNF-RI, **(C)** uTNF-RII and **(D)** uNGAL. **(E)** uTNF-RI and uTNF-RII concentrations also show a strong correlation. Log_{10} transformed data are displayed in scatter plots. Spearman's ρ coefficient obtained from untransformed data analysis is shown for each correlation study next to the graph. Only COVID-19 patients were included in the correlation analyses ($n = 147$).

both increased plasma ACE2 [30] and TNF-RI [31] to worsened COVID-19 prognosis. In addition, a recent study that infected human organoids with SARS-CoV-2 revealed that the virus directly infects the kidney using ACE2 as the entry receptor and the infection increased TNF-α in proximal tubular cells along with other pro-inflammatory and profibrotic factors such as transforming growth factor β1 [14]. In fact, collagen I protein expression and fibrosis were higher in infected organoids, which helps explain the increased renal tubulointerstitial fibrosis observed in COVID-19 patients. Here we demonstrate that uACE2 probably reflects an increased renal ACE2 shedding linked to worsened renal outcomes during the infection. Elevated uACE2 in COVID-19 patients was related to a 3-fold increased risk for AKI after adjusting for previous comorbidities, including CKD.

The prevalence of AKI in our cohort was 29.3%, which is line with previous series that described a prevalence of 25–45% [15, 16, 32]. AKI was more frequent in males, patients with previous comorbidities such as hypertension, diabetes or cardiovascular disease or patients with severe COVID-19 that required ICU admission or mechanical ventilation. However, age, which has been described as an independent risk factor for AKI in COVID-19 [15, 16, 33], was not higher in the AKI group. The latter finding may be ascribed to the small number of patients who had urine collected.

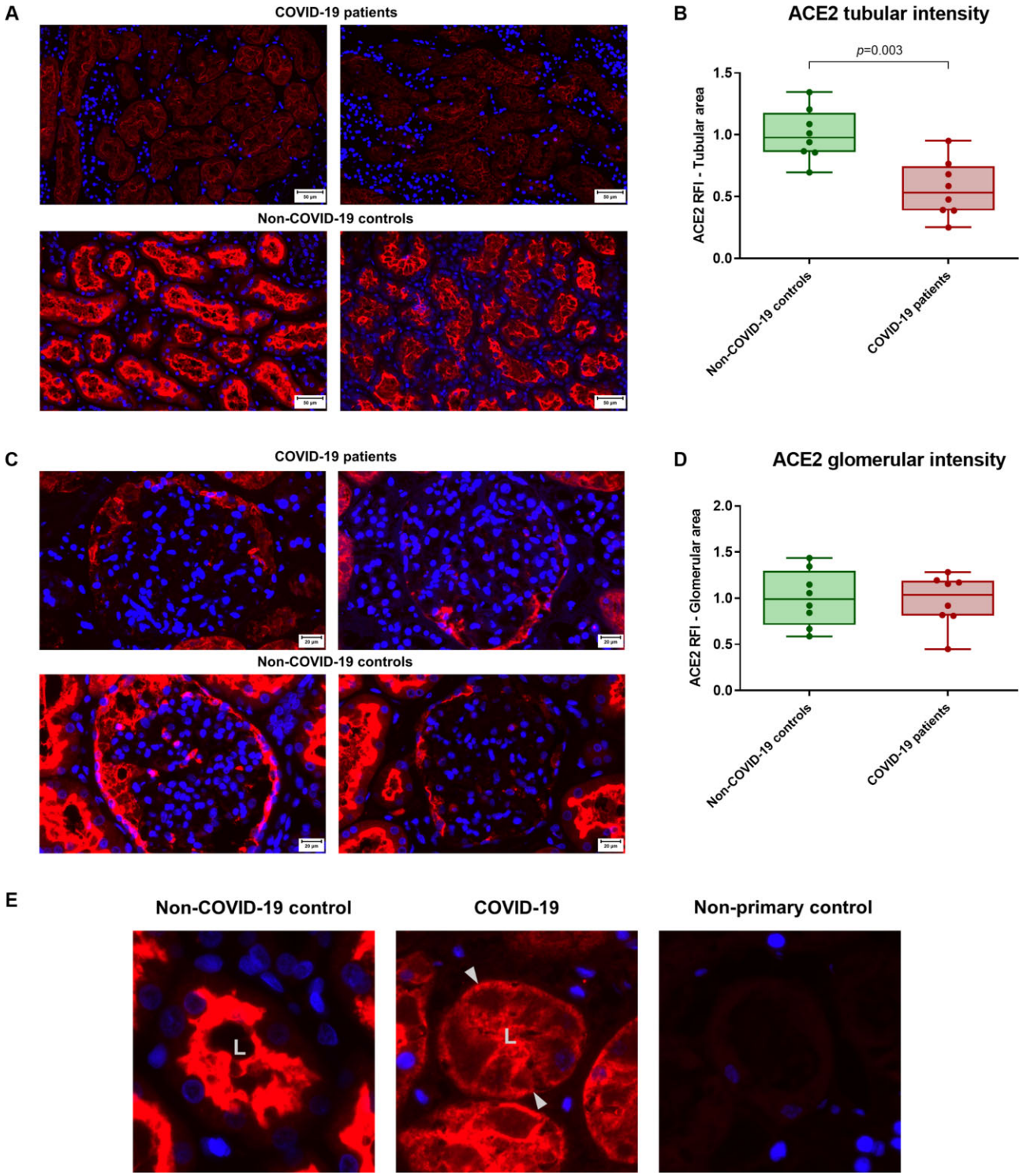


Figure 3: Kidney ACE2 analysis by immunofluorescence in COVID-19 patients and non-COVID-19 controls. Expression of ACE2 was evaluated and quantified in the cortical tubular and glomerular compartments. Differences were only observed in the tubular compartment. Immunofluorescence quantification is shown as the RFI adjusted to non-COVID-19 controls. **(A)** Representative microphotographs of tubular ACE2 staining (200× magnification). **(B)** Quantification of ACE2 tubular staining in COVID-19 and non-COVID-19 kidney samples. **(C)** Representative microphotographs of glomerular ACE2 staining (400× magnification). **(D)** Quantification of ACE2 glomerular staining in COVID-19 and non-COVID-19 kidney samples. **(E)** Proximal tubule microphotographs (400× magnification) show that in the setting of acute kidney injury, tubular cells lose their polarity and ACE2 is identified in the basolateral membrane. L: tubular lumen; arrows: ACE2 localized in the basolateral membrane.

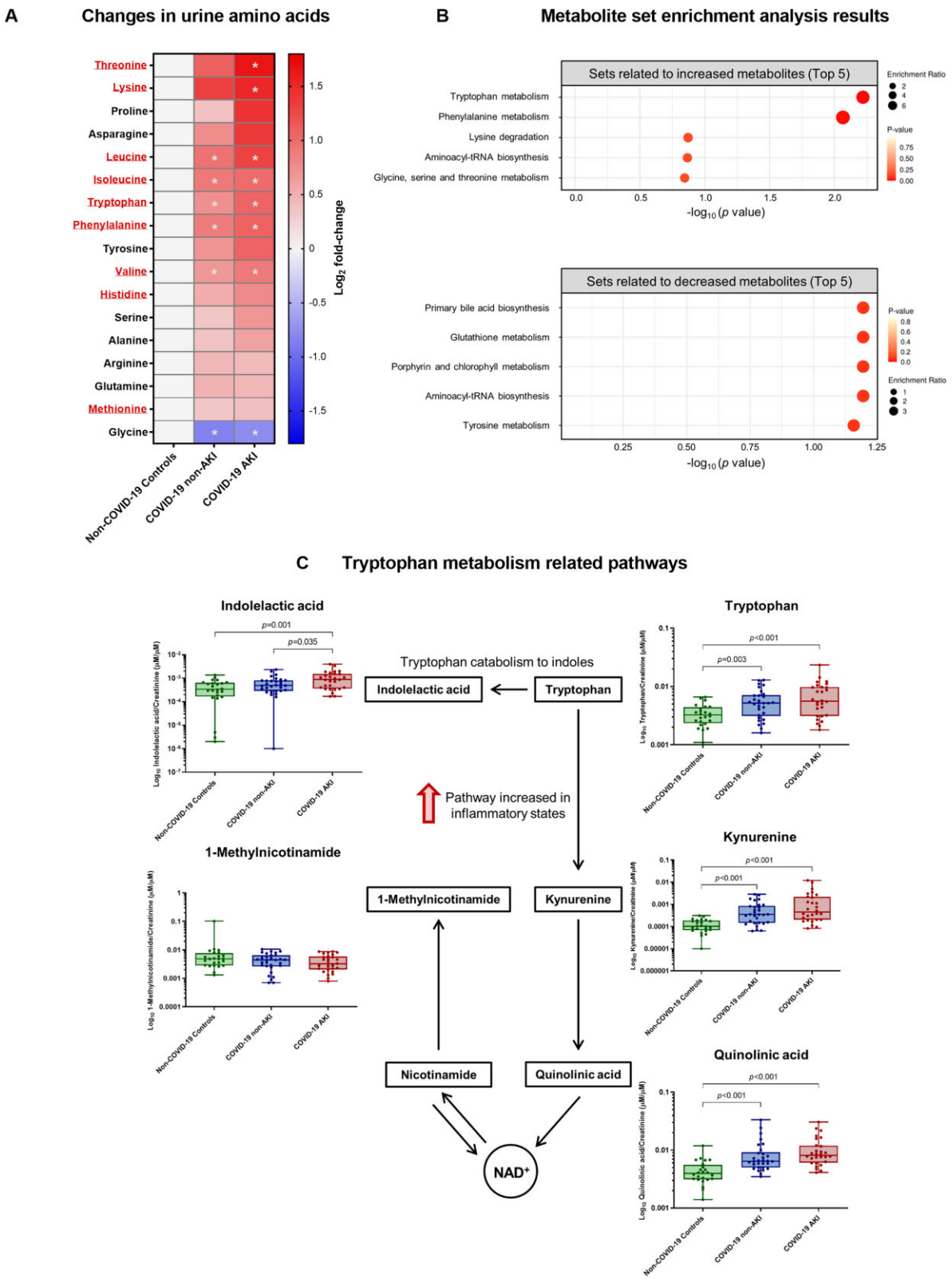


Figure 4: Quantitative urine metabolomic profile of COVID-19. Amino acid excretion was a significant change observed in COVID-19 patients' urine. **(A)** Heatmap displays an increase in excretion of proteogenic amino acids that was already observed in non-AKI COVID-19 patients and further increased in COVID-19 patients with AKI. Lysine, threonine, leucine, isoleucine, phenylalanine and tryptophan (all of them essential amino acids) were the most significantly increased, while glycine was the only amino acid that decreased. Log_2 fold-change values were previously adjusted to urine creatinine. **(B)** Metabolite set enrichment analysis revealed that the increased metabolites were related to amino acid metabolism pathways. **(C)** In fact, the tryptophan catabolism through the kynurerine–quinolinate pathway was highly represented in urine of COVID-19 patients. *The heatmap highlights the amino acids that presented a log_2 fold-change ≤ -0.75 or ≥ 0.75 and a significant corrected P-value after adjusting for a 5% FDR. Essential amino acids are displayed in red.

The measured uACE2 is of kidney origin and shows no correlation with pACE2 collected on the same day. Previous observational studies already demonstrated an absence of correlation between uACE2 and pACE2 [34]. Moreover, the immunofluorescence imaging performed in this study suggests that the majority of uACE2 comes from tubular cells, where a 2-fold decrease in ACE2 expression is observed in the COVID-19 setting. These findings are consistent with previous pathological studies that showed a dominant acute tubular injury in COVID-19 patients with AKI or incomplete Fanconi syndrome [20, 35] and is further supported by an increase in tubular proteinuria in these patients [36]. Recently a study in mice susceptible to SARS-CoV-2 infection also demonstrated significant proximal tubular injury in addition to lung injury in non-treated mice [37].

The concept that ACE2 exerts local protective effects was evidenced by studies assessing the enzyme's relation with CVD [28, 38, 39]. In the kidney, treatment with recombinant ACE2 showed protective effects [40], whereas the loss of ACE2 was detrimental in various renal diseases such as acute ischaemia or diabetic nephropathy [41]. In human diabetic kidney disease, there is a reduction in kidney ACE2 expression and a corresponding increase in uACE2 [42, 43]. Interestingly, urine ADAM17 is also increased in diabetic patients with albuminuria [43]. Studies in rodents revealed that both in acute and chronic models, ADAM17 activation increases kidney injury [44, 45], while the absence of the metalloproteinase is protective in chronic models [46]. However, the acute inhibition of ADAM17 in infectious diseases is more controversial, as the enzyme's shedding of TNF-RI has been shown to limit an excessive inflammatory response [47].

The urine metabolomic analysis performed in this study also revealed an increased excretion of essential amino acids in the urine of patients with COVID-19, which was more prominent in those who developed AKI and further supports previous findings that describe incomplete Fanconi syndrome in these patients [35]. The heightened excretion of essential amino acids in urine is often indicative of tubular kidney damage [36, 48] and, if persistent, can lead to immune dysfunction [49]. In addition to RAS regulation, ACE2 participates in amino acid reabsorption in the enterocytes and renal proximal tubular cells, where it interacts with the broad neutral amino acid transporter 1, although collectrin can replace the ACE2 scaffolding function in the kidney [27, 50, 51]. Among other neutral amino acids, tryptophan is an essential amino acid affected by the loss of ACE2 [52], leading to lower serum levels of tryptophan in COVID-19 patients [53]. Our urine metabolome study revealed higher tryptophan excretion in urine, which has not been described in ischaemic renal injury [54] and could be linked to the loss of ACE2. In addition, there was an evident increase of the kynurerine–quinolinate pathway, which is activated in acute and chronic pro-inflammatory states to produce nicotinamide adenine dinucleotide in immune cells like macrophages [55].

Due to the complexity associated with COVID-19 management, urine output could not be recorded in every patient. Therefore, AKI diagnosis and classification were based on only serum creatinine values. This classification may be adequate to discriminate patients with moderate–severe kidney injury or reduced clearance, but it may miss patients with subclinical tubular injury. The latter bias may partly explain why uNGAL or amino acid excretion is also increased in the COVID-19 non-AKI group along with uACE2, and is further supported by a recent observational study showing that even COVID-19 patients who do not develop AKI during the acute infection have an increased risk of GFR decline or ESKD during follow-up [56]. In addition, urine samples were collected after the AKI diagnosis. Thus the predictive value of uACE2 cannot be addressed and future research that includes prespecified kidney protocols and non-COVID AKI controls should validate the findings presented in this exploratory study.

In conclusion, we found that uACE2 is augmented in COVID-19 patients along with uTNF-RI and uTNF-RII, and these levels are further increased in COVID-19 patients who developed AKI. Specifically, uACE2 in SARS-CoV-2-mediated renal injuries is of tubular origin and can discriminate COVID-19 patients who developed AKI even after adjusting for age, sex and previous cardiovascular comorbidities. Moreover, COVID-19 is characterized by an increased excretion of essential amino acids in the urine, with the downregulation of tubular ACE2 being a plausible mechanistic link between impaired amino acid reabsorption and proximal tubular injury observed during SARS-CoV-2 infection.

SUPPLEMENTARY DATA

Supplementary data are available at *ckj* online.

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AUTHORS' CONTRIBUTIONS

A.V., K.W. and G.Y.O. were responsible for the conceptualization and design. A.V., K.W., D.C., M.G., J.R., R.M., F.N. and B.C. were responsible for sample collection and the acquisition of data. A.V., K.W., M.G., D.S.W. and G.Y.O. were responsible for the analysis and interpretation of data. A.V., K.W., M.G. and G.Y.O. were responsible for original draft preparation. F.N., B.C., J.W.S., M.J.S., D.S.W. and G.Y.O. were responsible for review and editing of the final manuscript. G.Y.O. was responsible for funding and resource acquisition.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request. In addition, metabolomics data have been deposited to the EMBL-EBI MetaboLights database with the identifier MTBLS4331 (see Methods section).

CONFLICT OF INTEREST STATEMENT

M.J.S. is the Editor-in-Chief of *CKJ*. The other authors declare no conflicts of interest. The manuscript or portions of it has not been published in any other journal.

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