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

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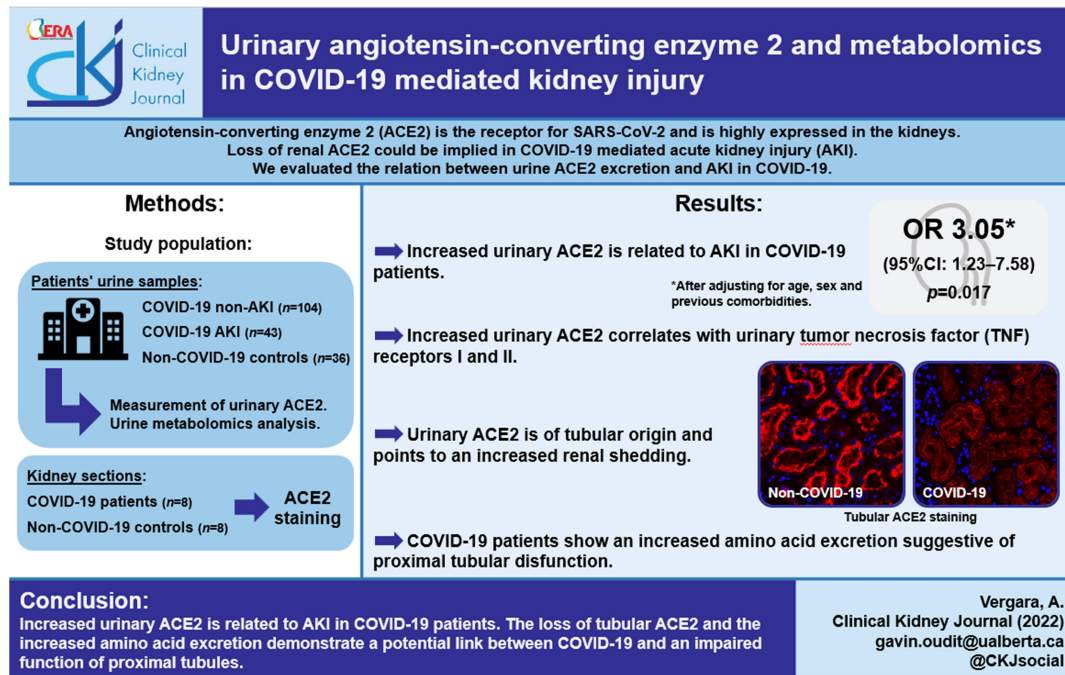[7]Department of Nephrology, Vall d'Hebron University Hospital, Barcelona, Spain

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RUNNING HEAD

Urinary ACE2 and metabolomics in COVID-19 AKI.

GRAPHICAL ABSTRACT

ABSTRACT

Background. Angiotensin-converting enzyme 2 (ACE2), the receptor for 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 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 tumor necrosis factor receptors I and II (uTNF-RI and uTNF-RII), 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 over 3-fold higher risk of developing AKI (OR 3.05, 95%CI:1.23–7.58,p=0.017). 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.

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.

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

LAY SUMMARY

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 COVID-19 patients. We demonstrated that urinary ACE2 (uACE2) is elevated in COVID-19 without AKI and further increases in COVID-19 with AKI correlating with 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.

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 (Ang II) mediated deleterious effects(2–4). We and others have demonstrated that SARS-CoV infection downregulated ACE2 expression at the cell membrane(5,6). Importantly, SARS-CoV and SARS-CoV-2 stimulate 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 TNF-α, 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 (TMPRSS2) 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 unraveled. 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 that 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 Methods can be found in the Supplemental Methods.

Study Participants

Patients in the current prospective observational study were participants of the CoCollab study (COVID-19 Surveillance Collaboration) that enrolled 296 hospitalized COVID-19 patients between July 1, 2020 and June 30, 2021. The enrolled patients were 18 years of age or older 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 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 COVID-19 pandemic, urine samples were not available from 137 patients (Table S1 and Figure S1). The CoCollab study was also responsible for collecting samples from non-COVID-19 individuals during the same timeframe, from which, 36 age and sex-matched participants without cardiovascular risk factors were selected as controls for comparison (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 by 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, 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 KDIGO guidelines classification system(21). The definition criteria of other outcomes of interest (albuminuria, CKD, CVD, ARDS, AMI 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). GFR was estimated using the 2021 CKD-EPI equation(22). Urinary ACE2, TNF-RI, TNF-RII and NGAL, along with their plasma counterparts were measured using commercially available human ELISA kits from R&D Systems (DY933-05, DRT100, DRT200, and DY1757-05, R&D Systems, MN, US).

Kidney samples and immunofluorescence studies

Paraffin-embedded kidney samples of COVID-19 patients were obtained from a collaboration with the National Institute for Infectious Diseases Lazzaro Spallanzani-IRCCS, Rome, Italy. Renal tissue was collected from autopsies performed on 8 COVID-19 patients following the 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-IRCCS 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 neighbor 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 (Figure 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 analyzed with a targeted MS-based quantitative metabolomics approach using a custom assay that combines liquid chromatography (Agilent HPLC system (Agilent, CA, US)) coupled to a tandem mass spectrometer (QTRAP 5500, AB Sciex, CA, US) (LC-MS/MS) (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 with Stata (V15.1, StataCorp LLC, TX, US). Descriptive statistics are shown as *n* (%) for categorical data. Continuous variables are described as median and

interquartile range (IQR). Comparisons of two independent groups were performed with chi-squared test and Fisher's exact test for categorical data, while Mann-Whitney's U test and Student's t-test were employed for continuous data. Non-parametric Spearman rank-order correlation test was used to evaluate the association between two continuous variables. The receiver operating characteristic (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 <0.05 was considered significant.

Regarding urine metabolome analysis, data was 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 comparison for each metabolite using Mann-Whitney's U test. All p values were corrected to control the false discovery rate (FDR) at 5% using Benjamini-Hochberg method. Metabolite set enrichment analysis, pathway analysis and urine disease signature evaluation were done using MetaboAnalyst 5.0(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 that had follow-up data 4 months after COVID-19 diagnosis (see the distribution of AKI stages in Table S2 and S3). Patients who developed AKI were more likely to be men with pre-established cardiovascular or kidney disease, accompanied by lower hemoglobin values. Moreover, albuminuria was also increased in COVID-19 patients with AKI compared to those without (1.0mg/mmol [IQR:0.5–4.7] vs 2.6mg/mmol [IQR:1.1–28.8]). Urine was collected after the AKI event, and the time elapsed from 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

Urinary ACE2 (uACE2) was higher in COVID-19 patients without AKI compared to non-COVID-19 controls (3.3ng/mL [IQR:1.7–6.2] vs 1.3ng/mL [IQR:0.7–2.6]), and values were greater still in patients with COVID-19 and AKI than in those without AKI (5.3ng/mL [IQR:2.8–10.1] vs 3.3ng/mL [IQR:1.7–6.2]) (Figure 1A). The uACE2-to-creatinine ratio was also higher in patients with COVID-19 and AKI vs the non-AKI group (Figure S3). The finding represents a 4.2-fold median increase in uACE2 in COVID-19 patients that develop AKI compared to controls, and it is also seen in patients without previous cardiovascular risk factors (Figure S4). Urinary TNF receptors I (uTNF-RI) and II (uTNF-RII) showed a distribution similar to uACE2 (Figure 1B-C). uTNF-RI and uTNF-RII were increased in COVID-19 patients without AKI than in non-COVID-19 controls (3.4ng/mL [IQR:1.6–6.9] vs 1.1ng/mL [IQR:0.6–2.1] and 4.9ng/mL [IQR:2.2–8.4] vs 1.8ng/mL [IQR:0.8–4.0], respectively) and further increased in COVID-19 patients that developed AKI (8.1ng/mL [IQR:3.5–15.6] vs 3.4ng/mL [IQR:1.6–6.9] and 7.1ng/mL [IQR:4.4–11.5] vs 4.9ng/mL [IQR:2.2–8.4], respectively). Furthermore, we assessed a biomarker of renal tubular damage: the urinary neutrophil gelatinase-associated lipocalin (uNGAL)(26). uNGAL was elevated in COVID-19 patients without AKI compared to non-COVID-19 controls (15.5ng/mL [IQR:7.3–35.7] vs 7.2ng/mL [IQR:2.4–15.3]), but the latter biomarker was not effective in differentiating AKI patients within the COVID-19 cohort (Figure 1D).

Urine ACE2 and TNF-RI are increased in COVID-19 patients that developed acute kidney injury

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, uACE2 nearest (0,1) cut-off value was 3.6ng/mL, whereas the nearest (0,1) cut-off values for uTNF-RI and uTNF-RII were 6.7ng/mL and 6.9ng/mL, respectively. Urine ACE2 ≥3.6ng/mL was associated with a 2.8-fold increased risk (95%CI:1.3–6.0) of having developed AKI during COVID-19 (Table 2). Urine TNF-RI ≥6.7ng/mL and urine TNF-RII ≥6.9ng/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 AKI and non-AKI patients, only uACE2 and uTNF-RI remained significantly different between the two groups (Figure 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 ≥6.7ng/mL and TNF-RII ≥6.9ng/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 ≥19.8ng/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.

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 analyzed the correlation between plasma and urine ACE2 levels in COVID-19 patients. The Spearman's test showed a lack of association between pACE2 and uACE2 collected on the same day (*rho*=-0.05, 95%CI:-0.23–0.14) (Figure 2A). Additionally, we did not see a correlation between uACE2 and pACE2, even in patients with overt albuminuria (>30mg/mmol) (Figure 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) (Figure 2B-C). Levels of the tubular injury marker NGAL also correlated with uACE2 (Figure 2D). Moreover, uTNF-RI and uTNF-RII demonstrated a strong correlation among themselves (Figure 2E).

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

The increased shedding of tubular ACE2 was also confirmed by analyzing postmortem renal sections of COVID-19 patients. Compared to non-COVID-19 sections, immunofluorescence staining revealed a 2-fold reduction of ACE2 tubular expression in COVID-19 patients (Figure 3A-B and Figure S7). Conversely, despite a slight decrease in ACE2 staining in the glomeruli, no differences were present between non-COVID-19 and COVID-19 patients (Figure 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 (Figure 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 (Table S4 and Figure S1). From the 250 metabolites that could be maximally detected, we identified 189 metabolites. However, two were excluded because less than 5% of patients had values over the lower limit of detection (Figure 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 (Figure 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, and tryptophan, and their metabolites (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 (Figure 4A). Thus, when we performed the enrichment analysis, the increased metabolites in COVID-19 patients were linked to amino acid metabolism pathways (Figure 4B and Table S6). Notably, tryptophan metabolism, and specifically the kynurerine-quinolinate pathway, was significantly represented (Figure 4C). It is worth mentioning that amino acid excretion was slightly higher in the COVID-19 AKI group (Figure 4A), showing a continuous increase throughout the 3 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 (Figure 4C and 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 Ang II-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 contribute towards 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 that developed AKI during SARS-CoV-2 infection, displaying a continuous increase in uACE2 levels throughout the 3 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 the impaired amino acid reabsorption and acute tubular injury, becoming a specific mechanism that explains the high prevalence of AKI in COVID-19 patients.

SARS coronaviruses 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 a common cold)(7). ACE2 counterbalances the classical RAS pathway(4,28). The downregulation of tissue ACE2 enhanced by the infection would increase the local exposure to Ang II-mediated proinflammatory effects, which would lead to an augmented organ injury(3,4). Moreover, Ang II action on AT_1R 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 patients to comorbidities and

established disease markers(10). Other studies have also shown similar results, relating 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 that the infection increased TNF-α in proximal tubular cells along with other proinflammatory and profibrotic factors such as TGF-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 chronic kidney disease.

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 that had urine collected.

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 a 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 ACE2 was detrimental in various renal diseases like acute ischemia 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 that 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 (B^0AT1), 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 a higher tryptophan excretion in urine, which has not been described in ischemic 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 proinflammatory states to produce NAD^+ 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 only on 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 are 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 that 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 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 urine ACE2 is augmented in COVID-19 patients along with uTNF-RI and uTNF-RII, and these levels are further increased in COVID-19 patients that developed AKI. Specifically, uACE2 in SARS-CoV-2-mediated renal injuries is of tubular origin and can discriminate COVID-19 patients that 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 MATERIAL

The following material is included as supplemental: Table S1 (Baseline characteristics of COVID-19 patients that had urine and plasma collected vs patients with plasma samples only (excluded from the study)), Table S2 (Baseline characteristics of non-COVID-19 controls and COVID-19 patients according to AKI stage), Table S3 (Distribution of the COVID-19 cohort and outcomes according to AKI stages), Table S4 (Baseline characteristics of patients included in the urine metabolomic study), Table S5 (Hit and candidate metabolites when comparing

COVID-19 patients to non-COVID-19 controls), Table S6 (Pathways linked to increased and decreased metabolites when comparing COVID-19 patients with non-COVID-19 controls), Figure S1 (Study flow diagram), Figure S2 (Metabolites detected in urine of COVID-19 and non-COVID-19 patients), Figure S3 (Urine biomarkers adjusted to creatinine in COVID-19 patients with and without AKI), Figure S4 (Urine angiotensin-converting enzyme 2 (uACE2) values in COVID-19 patients and non-COVID-19 controls without previous cardiovascular risk factors (CVRF)), Figure S5 (Urine angiotensin-converting enzyme 2 (uACE2) values in nearest neighbor propensity score matched COVID-19 non-AKI and AKI patients), Figure S6 (Correlation between plasma and urine ACE2 in COVID-19 patients with albuminuria >30mg/mmol), Figure S7 (Tubular angiotensin-converting enzyme 2 (ACE2) staining measurement by immunofluorescence), Figure S8 (Principal component analysis of urine metabolites and distribution of non-COVID-19 controls, COVID-19 non-AKI patients and COVID-19 AKI patients according to principal components (PC) 1 and 2.), Figure S9 (Volcano plots showing the distribution of metabolites when comparing COVID-19 patients vs non-COVID-19 controls and non-AKI COVID-19 patients vs AKI COVID-19 patients).

AUTHORS' CONTRIBUTIONS

Conceptualization and design, A.V., K.W., and G.Y.O. Sample collection and acquisition of data, A.V., K.W., D.C., M.G., J.R., R.M., F.N., and B.C. Analysis and interpretation of data, A.V., K.W., M.G., D.S.W., and G.Y.O. Original draft preparation, A.V., K.W., M.G. and G.Y.O. Review and editing of the final manuscript, F.N., B.C., J.W.S., M.J.S., D.S.W, and G.Y.O. Funding and resources acquisition: G.Y.O.

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CONFLICT OF INTEREST STATEMENT

M.J.S. is the Editor-in-Chief of CKJ. The other authors have no disclosures to make. The manuscript, or part of it, has neither been published in any other journal.

FUNDING

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).

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

	Entire cohort (*n*=147)	Non-AKI patients (*n*=104)	AKI patients (*n*=43)	*p* value
Days from COVID-19 diagnosis to urine collection	12 (IQR: 5–18)	11 (IQR: 5–18)	13 (IQR: 5–18)	0.630
Age	61 (IQR: 50–71)	60 (IQR: 49–71)	62 (IQR: 53–74)	0.456
Male sex	80 (54.4%)	49 (47.1%)	31 (72.1%)	**0.006**
Active smoker	57 (38.8%)	36 (34.6%)	21 (48.8%)	0.107
Medical history:				
-Obesity	58 (39.5%)	38 (36.5%)	20 (46.5%)	0.260
-Hypertension	64 (43.5%)	37 (35.6%)	27 (62.8%)	**0.002**
-Diabetes	57 (38.8%)	35 (33.7%)	22 (51.2%)	**0.047**
-Cardiovascular disease[a]	20 (13.6%)	7 (6.7%)	13 (30.2%)	**<0.001**
-Atrial arrythmia	8 (5.4%)	4 (3.6%)	4 (9.3%)	0.233
-Chronic obstructive pulmonary disease	19 (12.9%)	12 (11.5%)	7 (16.3%)	0.436
-Chronic kidney disease:	28 (19.1%)	13 (12.5%)	15 (34.9%)	**0.002**
-CKD stage 1-2	2 (1.4%)	0 (0.0%)	2 (6.7%)	0.060
-CKD stage 3a	12 (8.2%)	7 (7.1%)	5 (15.2%)	0.177
-CKD stage 3b	13 (8.8%)	6 (6.2%)	7 (20.0%)	**0.041**
-CKD stage 4	1 (0.7%)	0 (0.0%)	1 (3.5%)	0.242
Chronic medications:				
-ACEi or ARBs	43 (29.3%)	24 (23.1%)	19 (44.2%)	**0.010**
-β-blockers	19 (12.9%)	8 (7.7%)	11 (25.6%)	**0.003**
-Statins	50 (34.0%)	29 (27.9%)	21 (48.8%)	**0.015**
Clinical presentation:				
-Fever	53 (36.1%)	37 (35.6%)	16 (37.2%)	0.739
-Gastrointestinal symptoms[b]	53 (36.1%)	34 (32.7%)	19 (44.2%)	0.423
-Respiratory symptoms[c]	111 (75.5%)	75 (72.1%)	36 (83.7%)	0.756
-Dyspnea	89 (60.5%)	61 (58.7%)	28 (65.1%)	0.892
-X-ray confirmed pneumonia	93 (63.3%)	65 (62.5%)	28 (65.1%)	0.517
Admission to ICU	25 (17.0%)	11 (10.6%)	14 (32.6%)	**0.001**
COVID-19 Complications:				
-ARDS	13 (8.8%)	7 (6.7%)	6 (14.0%)	0.161
-Shock	3 (2.0%)	2 (1.9%)	1 (2.3%)	0.999
-AMI	10 (6.8%)	4 (3.9%)	6 (14.0%)	0.064
-Mortality	13 (8.8%)	10 (9.6%)	3 (7.0%)	0.608
Laboratory tests on admission:				
-Hemoglobin (g/L)	128 (IQR: 114–139)	131 (IQR: 117–141)	119 (IQR: 107–136)	**0.019**
-WBC (10^9/L)	8.5 (IQR: 6.3–11.2)	8.3 (IQR: 6.3–10.7)	9.5 (IQR: 7.9–12.2)	0.152
-Lymphocytes (10^9/L)	0.9 (IQR: 0.6–1.2)	1.0 (IQR: 0.7–1.3)	0.7 (IQR: 0.5–0.9)	**0.001**
-Platelets (10^9/L)	255 (IQR: 206–344)	261 (IQR: 217–347)	247 (IQR: 185–337)	0.503
-GFR (mL/min/1.73m^2)[d]	81 (IQR: 56–102)	92 (IQR: 74–105)	55 (IQR: 28–71)	**<0.001**
-C-reactive protein (mg/L)	70 (IQR: 35–123)	61 (IQR: 30–105)	89 (IQR: 53–140)	**0.035**
-Ferritin (µg/L)	447 (IQR: 169–761)	392 (IQR: 161–689)	633 (IQR: 336–1839)	0.069
-D-Dimer (mg/L)	1.2 (IQR: 0.7–1.7)	0.9 (IQR: 0.7–1.7)	1.3 (0.9–2.2)	0.082
-Lactate dehydrogenase (UI/L)	298 (IQR: 237–348)	288 (IQR: 238–339)	301 (237–353)	0.446
-UACR (mg/mmol)	1.3 (IQR: 0.6–5.4)	1.0 (IQR: 0.5–4.7)	2.6 (IQR: 1.1–28.8)	**0.002**
Received treatments:				
-IMV	22 (15.0%)	10 (9.6%)	12 (27.9%)	**0.005**
-Corticosteroids	110 (74.8%)	74 (71.2%)	36 (83.7%)	0.110
-Antibiotics	93 (63.3%)	58 (55.8%)	35 (81.4%)	**0.003**

[a]Patients with previous history of myocardial infarction, coronary artery disease or heart failure. [b]Gastrointestinal symptoms include diarrhea and nausea. [c]Respiratory symptoms include cough and dyspnea. [d]CKD-EPI estimated GFR. ACEi: angiotensin-converting enzyme inhibitors. ARB: angiotensin receptor blocker. ICU: intensive care unit. ARDS: acute respiratory distress syndrome. AMI: acute myocardial infarction. WBC: white blood cell count. UACR: urinary albumin-to-creatinine ratio. IMV: invasive mechanical ventilation.

Table 2: Univariable and multivariable logistic regression models for uACE2, uTNFRI, uTNFRII 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
Acute kidney injury						
uACE2	2.80 (1.31–5.96)*	0.008	2.34 (1.09–5.18)*	0.029	3.05 (1.23–7.58)*	0.017
uTNFRI	3.96 (1.88–8.37)*	<0.001	3.41 (1.57–7.39)*	0.002	2.74 (1.18–6.38)*	0.019
uTNFRII	2.48 (1.20–5.13)*	0.015	2.03 (0.95–4.34)	0.069	2.26 (0.97–5.26)	0.060
uNGAL	1.72 (0.84–3.53)	0.137	2.28 (1.03–5.06)*	0.042	1.90 (0.79–4.59)	0.152
Albuminuria[c]						
uACE2	0.92 (0.46–1.83)	0.815	0.76 (0.36–1.59)	0.462	0.59 (0.26–1.35)	0.214
uTNFRI	4.73 (2.26–9.88)*	<0.001	4.10 (1.90–8.84)*	<0.001	3.46 (1.52–7.86)*	0.003
uTNFRII	2.47 (1.22–4.99)*	0.012	1.99 (0.94–4.19)	0.070	1.86 (0.83–4.15)	0.132
uNGAL	2.49 (1.23–5.04)*	0.011	2.64 (1.22–5.70)*	0.013	1.97 (0.86–4.49)	0.108

Odds ratio (OR) of presenting AKI or albuminuria for patients with uACE2, uTNFRI, uTNFRII and uNGAL above cut-off points. Cut-off points: 3.6 ng/mL for urine ACE2 (uACE2), 6.7 ng/mL for urine TNF receptor I (uTNFRI), 6.9 ng/mL for urine TNF receptor II (uTNFRII), and 19.8 ng/mL for urine NGAL (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, cardiovascular disease, and chronic kidney disease). [c]Albuminuria was defined as a urinary albumin-to-creatine ratio ≥3mg/mmol. *Independent association of the biomarker with the outcome (p<0.05).
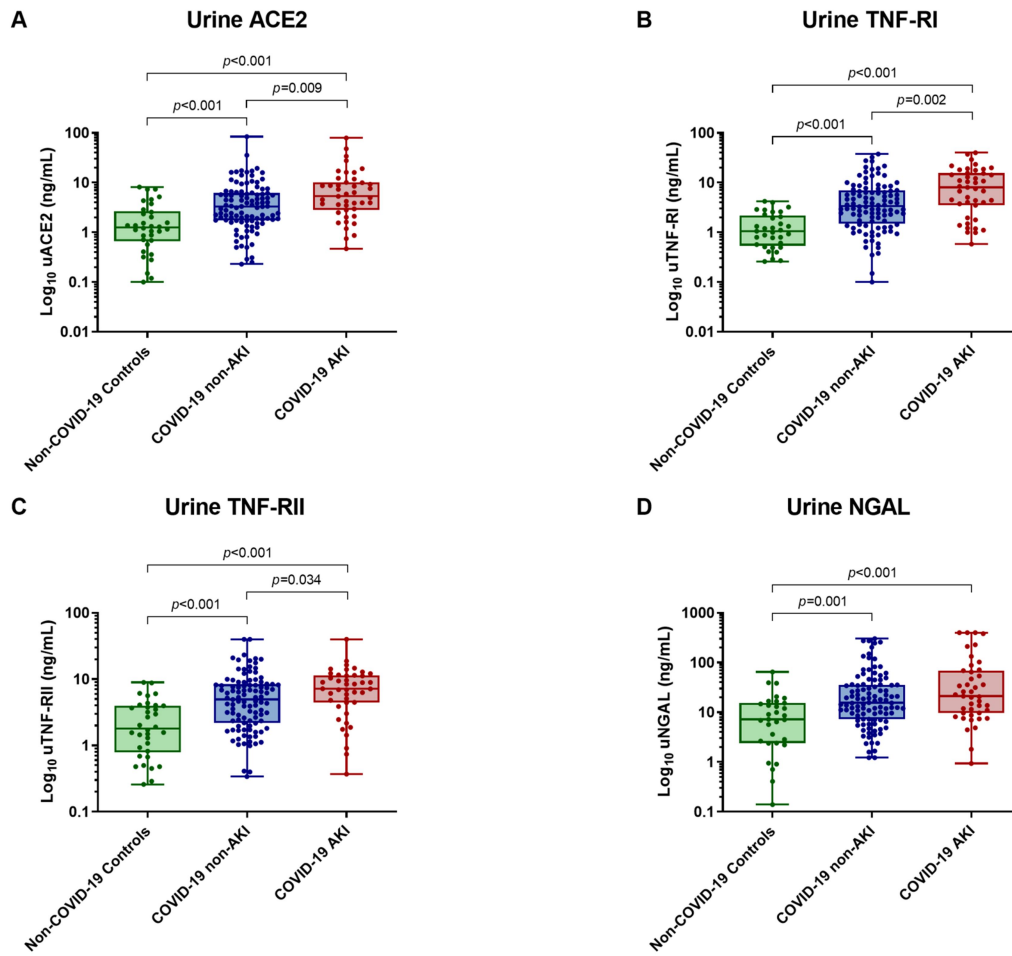
Figure 1. Urine biomarkers in COVID-19 patients with and without AKI compared with age and sex matched non-COVID-19 controls. The measured urine biomarkers were: (**A**) urine angiotensin-converting enzyme 2 (uACE2), (**B**) tumor necrosis factor receptor I (uTNF-RI), (**C**) tumor necrosis factor receptor II (uTNF-RII), and (**D**) neutrophil gelatinase-associated lipocalin (uNGAL). Data is displayed in box and whisker plots. Non-COVID-19 controls *n*=36. COVID-19 non-AKI *n*=104. COVID-19 AKI *n*=43.
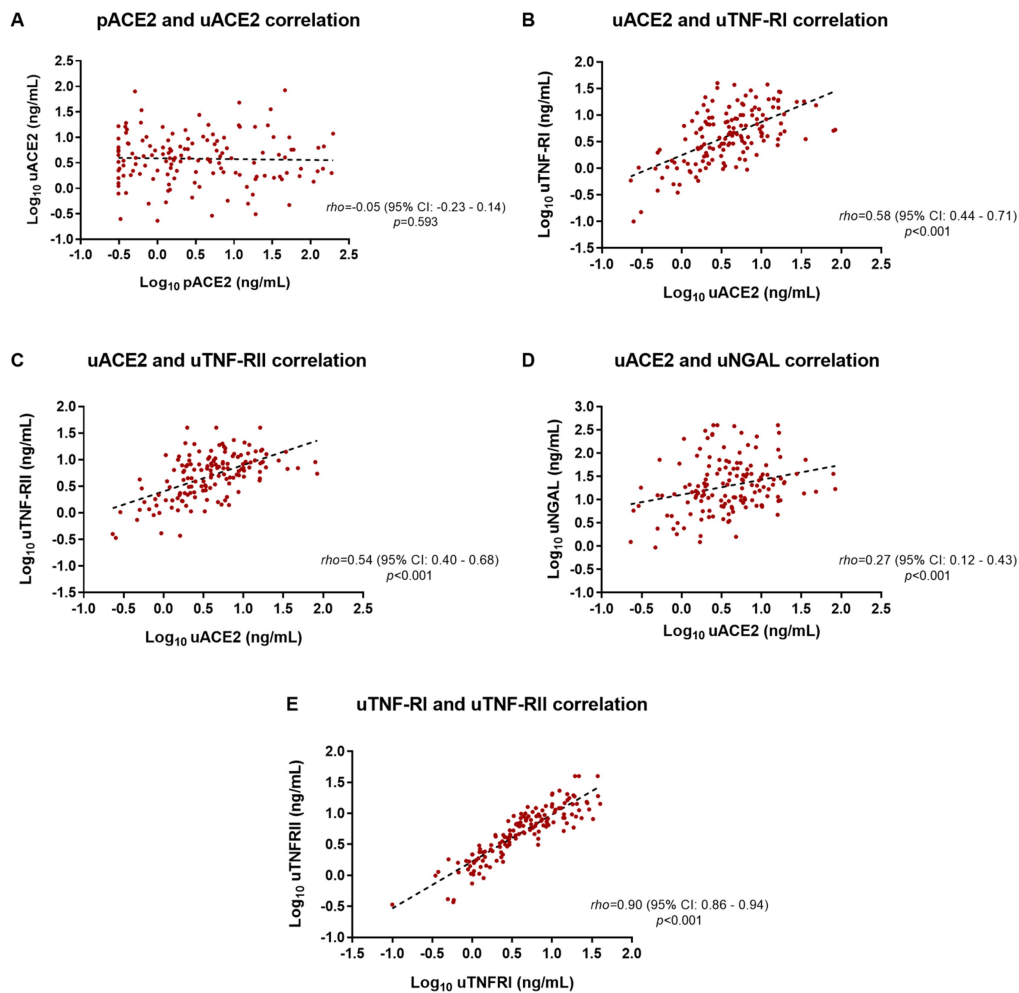
Figure 2. Correlation in COVID-19 patients of urine ACE2 (uACE2) levels with plasma ACE2 (pACE2), urine TNF receptors I and II (uTNF-RI and uTNF-RII), and neutrophil gelatinase-associated lipocalin (uNGAL). (**A**) Correlation analysis of uACE2 and pACE2 shows no relation between both concentrations. In contrast, uACE2 shows moderate correlation with (**B**) uTNF-RI, (**C**) uTNF-RII, and (**D**) uNGAL. (**E**) Urine TNF-RI and TNF-RII concentrations also show a strong correlation. Log_{10} transformed data is displayed in scatter plots. Spearman *rho* 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).
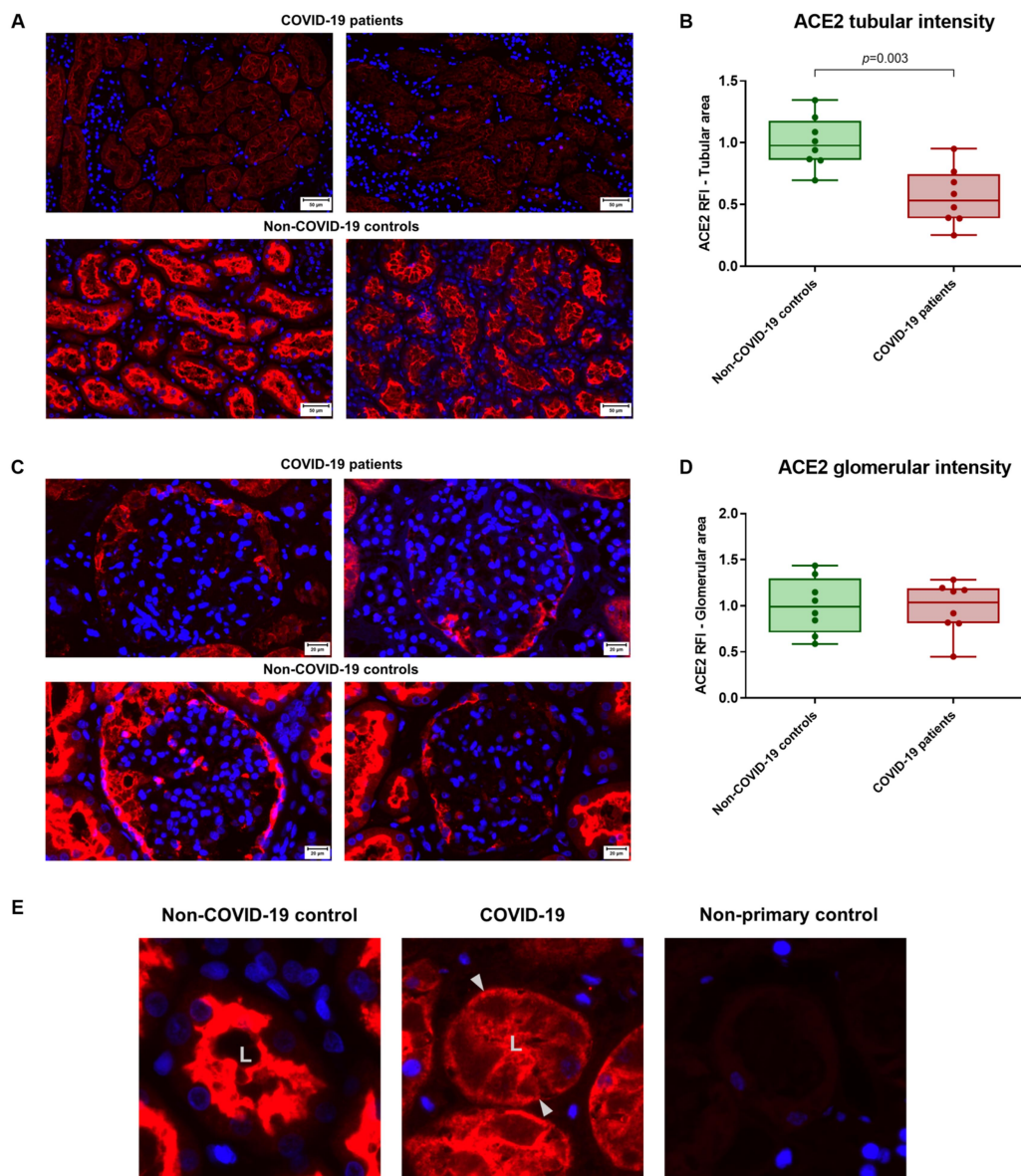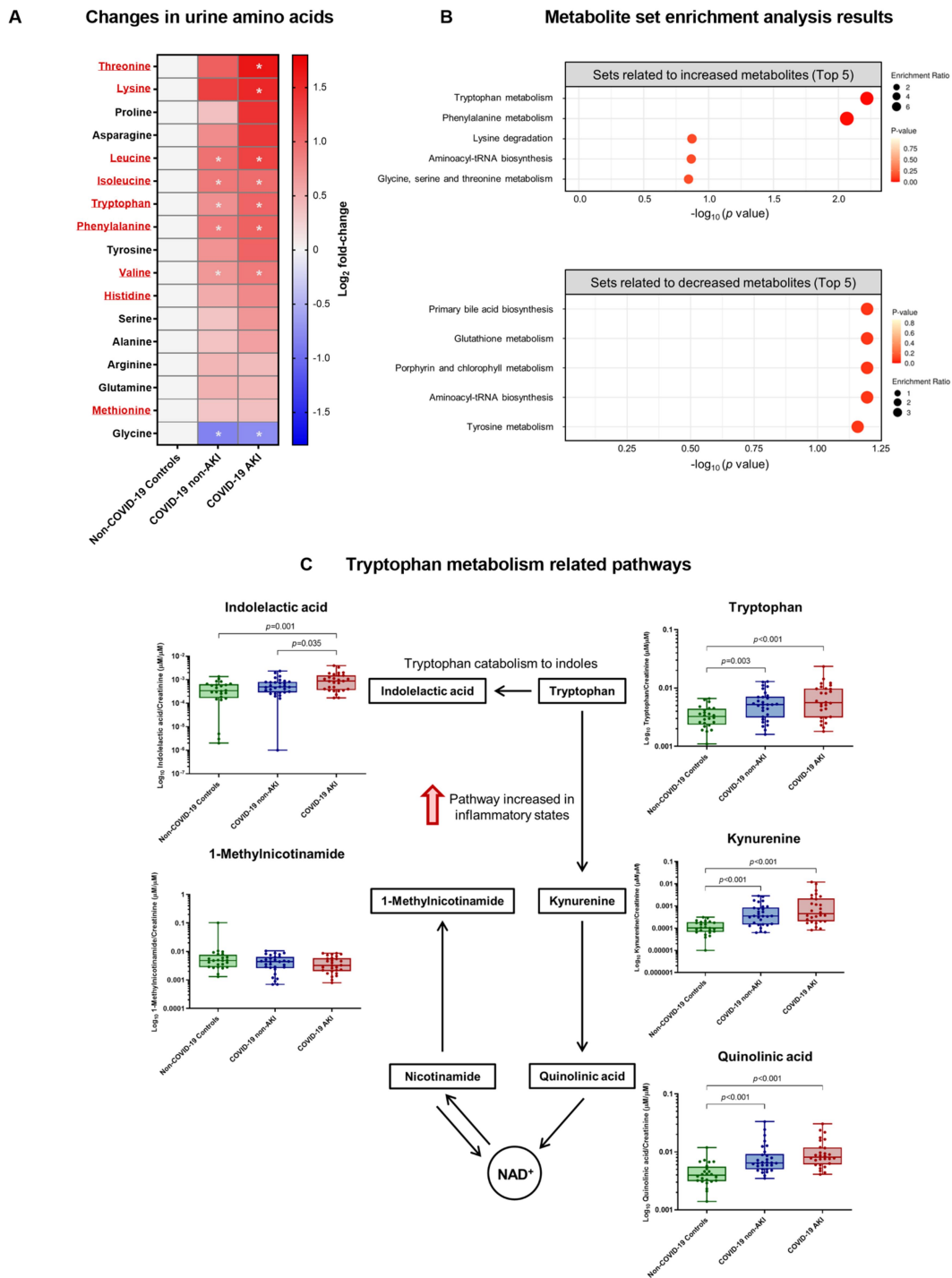
Figure 3. Kidney angiotensin-converting enzyme expression (ACE2) analysis by immunofluorescence in COVID-19 patients and non-COVID-19 controls. Expression of ACE2 was evaluated and quantified in the cortical tubular and glomerular compartments. Differences were only observed in the tubular compartment. Immunofluorescence quantification is shown as relative fluorescence intensity (RFI) adjusted to non-COVID-19 controls. (**A**) Representative microphotographs of tubular ACE2 staining (200x magnification). (**B**) Quantification of ACE2 tubular staining in COVID-19 and non-COVID-19 kidney samples. (**C**) Representative microphotographs of glomerular ACE2 staining (400x magnification). (**D**) Quantification of ACE2 glomerular staining in COVID-19 and non-COVID-19 kidney samples. (**E**) Proximal tubules microphotographs (400x magnification) show that in the setting of acute

kidney injury, tubular cells loss 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 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 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. In fact, the tryptophan catabolism through the kynurerine-quinolinate pathway was highly represented in urine of COVID-19 patients (**C**). *In 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 color.