Reduced urinary levels of angiotensin-converting enzyme 2 activity predict acute kidney injury in critically ill patients

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www.doi.org/10.51893/2020.4.oa7
Published online first 7 December 2020

ABSTRACT

Objective: Angiotensin-converting enzyme 2 activity reflects non-classical renin–angiotensin system upregulation. We assessed the association of urinary angiotensin-converting enzyme 2 (uACE2) activity with acute kidney injury (AKI).

Design, setting and participants: A prospective observational study in which we measured uACE2 activity in 105 critically ill patients at risk of AKI. We report AKI stage 2 or 3 at 12 hours of urine collection (AKI_{12h}) and AKI stage 2 or 3 at any time during intensive care unit stay in patients free from any stage of AKI at inclusion (AKI_{ICU}). AKI prediction was assessed using area under the receiver-operating characteristics curve (AUROC) and net reclassification indices (NRIs).

Main outcome measure: AKI stage 2 or 3 at 12 hours of urine collection.

Results: Within 12 hours of inclusion, 32 of 105 patients (30%) had developed AKI_{12h}. Corrected uACE2 activity was significantly higher in patients without AKI_{12h} compared with those with AKI_{12h} (median [interquartile range], 13 [6–24] *v* 7 [4–10] pmol/min/mL per mmol/L of urine creatinine; $P < 0.01$). A 10-unit increase in uACE2 was associated with a 28% decrease in AKI_{12h} risk (odds ratio [95% CI], 0.72 [0.46–0.97]). During intensive care unit admission, 39 of 76 patients (51%) developed AKI_{ICU}. uACE2 had an AUROC for the prediction of AKI_{12h} of 0.68 (95% CI, 0.57–0.79), and correctly reclassified 28% of patients (positive NRI) to AKI_{12h}. Patients with uACE2 > 8.7 pmol/min/mL per mmol/L of urine creatinine had a significantly lower risk of AKI_{ICU} on log-rank analysis (52% *v* 84%; $P < 0.01$).

Conclusions: Higher uACE2 activity was associated with a decreased risk of AKI stage 2 or 3. Our findings support future evaluations of the role of the non-classical renin–angiotensin system during AKI.

Crit Care Resusc 2020; 22 (4): 344-354

Acute kidney injury (AKI) is common in patients admitted to the intensive care unit (ICU), yet our understanding of its pathophysiology is poor.[1] AKI increases the risk of death of critically ill patients, proportionally to its severity, and has both short term and long-lasting consequences on patients' quality of life.[2-4] Suspected mechanisms leading to an acute fall in renal function include a combination of intrarenal macrovascular and microvascular mismatch associated with inflammation and metabolic stress.[1,5-7]

The non-classical renin–angiotensin system (RAS) is a recently identified endocrine cascade, with regulating effects on renal function and haemodynamics.[8,9] The pivotal enzyme of the non-classical RAS is angiotensin-converting enzyme 2 (ACE2).[10-12] The major role of this transmembrane monocarboxypeptidase is to convert angiotensin II into angiotensin.[1-7] ACE2 is highly expressed in the heart and kidneys, and is also present in the lungs and brain.[9,13,14] ACE2 is also the receptor for cell entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[15] In the kidney, ACE2 induces vasodilation and nitric oxide release, and increases renal blood flow, glomerular filtration, natriuresis and diuresis, while downregulating inflammatory and profibrogenic pathways.[16-20] Thus, increased expression of ACE2 in the kidney could be associated with decreased risk of AKI.

Because ACE2 is expressed mainly in proximal tubular epithelial cells, and can be cleaved from the cell surface into the urine, quantification of ACE2 activity levels in human urine may contribute to our understanding of the role played by the intrarenal non-classical RAS in critically ill patients at risk of AKI.[21] Consequently, we evaluated the association of urinary ACE2 (uACE2) activity with AKI, and performance of uACE2 activity in predicting AKI, in a general ICU population. We hypothesised that increased uACE2 activity would be associated with a lower risk of AKI in the acute care setting.

Methods

This single-centre prospective, observational, exploratory study was approved by the Austin Health Human Research Ethics Committee (Melbourne, Australia, approval number LNR/18/Austin/151 for patients and LNRSSA/18/Austin/315 for healthy volunteers), who waived the need for informed consent for the collection of urine in the critically ill population. The study protocol and report met the STROBE criteria for observational studies and the STARD

recommendations for reporting diagnostic accuracy studies (Online Appendix, methods).[22,23]

Study cohort

We prospectively enrolled a convenience sample of all consecutive adult patients admitted to the Department of Intensive Care at Austin Hospital, within 48 hours of their admission, and presenting one of the following inclusion criteria in the preceding 6 hours: cardiovascular sepsis-related organ failure assessment (SOFA) score ≥ 1, respiratory SOFA ≥ 2, increase in serum creatinine level > 8 µmol/L between two creatinine measurements performed during the 6-hour period preceding enrolment, or a urine output < 0.5 mL/kg/h over 4 hours during the same 6-hour block.[24]

The first two inclusion criteria were those used in the Sapphire study, a landmark study on AKI risk prediction.[25] The other two were selected to target patients at risk of AKI; they were derived from the AKI stage 1 definition of the Kidney Disease: Improving Global Outcomes (KDIGO) recommendations, to reflect a potentially small yet significant change in renal function over a limited period,[26] and have been recently validated.[27]

We excluded patients with anuria, known stage 2 or 3 AKI at the time of enrolment (including those on renal replacement therapy [RRT]), stage 4 or 5 chronic kidney disease (including renal transplant recipients and those on maintenance dialysis), history of urinary tract surgery, expected length of stay < 48 hours, and patients undergoing end-of-life care.

Urine collection and handling

Immediately after inclusion, we collected a 10 mL spot urine sample from the indwelling vesical catheter. Urine collection was performed on weekdays, between 8:00 am and 12:00 pm. Samples were then centrifuged at 1500 × g for 10 minutes, and 0.5 mL of urine supernatant was aliquoted and stored at –80°C. No adjuvant was added to the urine sample before storage.

Quantification of uACE2 activity

Urine ACE2 activity was measured using a sensitive quenched fluorescent substrate-based assay,[21] with modifications. Urine was incubated in triplicate with an ACE2-specific quenched fluorescent substrate (QFS): (7-methoxycoumarin-4-yl)-acetyl-Ala-Pro-Lys(2,4-dinitrophenyl) (Auspep, Melbourne, Vic, Australia), with or without an ACE2 inhibitor mix. Assays were performed with 50 µM of QFS, in a final volume of 200 µL per well, with ACE2 assay buffer (100 mM Tris, 1 M NaCl, pH 6.5). Reactions were performed at 37°C for 200 minutes with continuous monitoring of liberated fluorescence using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). Cleavage of the QFS was attributed to ACE2 by the use of a specific inhibitor mix containing the ACE2 inhibitor MLN-4760 (MSD, Sydney, NSW, Australia) at a final concentration of 1 µM, 10 µM Z-pro (Bachem, Bubendorf, Switzerland) and 10 mM EDTA (Sigma-Aldrich, Sydney, NSW, Australia). A protease inhibitor cocktail was also added comprising 0.0412 M of N-ethylmaleimide, 840 U/mL of Aprotinin, 2.1 UM of Leupeptin and 0.3 µM of Pepstatin A. The rate of substrate cleavage was determined by comparison with a standard curve of the free fluorophore 4-amino-methoxycoumarin (Sigma-Aldrich) and expressed as pmol of substrate cleaved/min/mL of urine. The intra-assay and inter-assay coefficients of variation were 5.7% and 9.4%, respectively. The investigators measuring uACE2 activity were blinded to the clinical primary outcome adjudication.

To adjust uACE2 activity to varying urine flow rate and density, we normalised uACE2 levels to urine creatinine levels (uCr); hence, all presented uACE2 data were uCr-corrected unless otherwise specified.[28,29] To do so, we measured uCr levels (in mmol/L) on the same urine spot sample (cobas 8000 analyser, Roche Diagnostics, Indianapolis, Ind, USA). Corrected uACE2 activity was hence expressed in pmol/min/mL per mmol/L of uCr.

Primary and secondary outcomes

For the present study, we defined the primary outcome as the occurrence of AKI stage 2 or 3 (following the KDIGO guidelines staging system) at 12 hours of urine collection.[26] To do so, we recorded 12-hour cumulative urine output after urine collection and used the serum creatinine value measured closest to the 12-hour time point (using a 2-hour bilateral time window). Adjudication of the primary outcome was performed before uACE2 results were made available to investigators. If adjudication of the primary outcome was impossible, the data were excluded from the analysis.

Secondary renal outcomes were any AKI stage at 12 hours (KDIGO stage 1 to 3), RRT during the index ICU admission, AKI stage 2 or 3 on the day following urine collection (at 24 hours), and highest AKI stage during the index ICU admission up to 7 days after inclusion or death. Elapsed time between urine collection and the highest AKI stage was also recorded.

Assessment of acute renal function

Urine output was normalised to patient weight. Serum creatinine concentration was measured with our point-of-care blood gas analyser (Radiometer ABL800, Radiometer Medical ApS, Copenhagen, Denmark).[30] If this measurement was missing, we used that measured by the Austin Health Department of Pathology.

Premorbid serum creatinine level was assessed using all available data present in the electronic medical record, and

corresponded to the lowest serum creatinine level measured between 365 days and 7 days before ICU admission, and closest to the latter. If this information was unavailable, we retrospectively estimated it by reporting the lowest stable serum creatinine level recorded during the index admission. Premorbid estimated glomerular filtration rate was systematically re-estimated using the modified diet in renal disease formula, to avoid bias relating to differing estimation methods used in result reports.[31]

Other patient characteristics

We recorded patient demographics, comorbidities, category and origin of ICU admission, severity of illness as assessed by APACHE (Acute Physiology and Chronic Health Evaluation) III and SOFA scores, characteristics of organ failure and support, premorbid treatment with ACE inhibitors or angiotensin receptor blockers, and treatment with diuretics before urine collection.[24,32]

Healthy volunteer study

To compare uACE2 activity to that of a non-critically ill population, we collected 10 mL of urine from 10 healthy volunteers between 8:00 am and 12:00 pm, and analysed these samples according to the protocol used for patient samples.

Statistical analysis

We analysed data using the R software (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria), with the survival, pROC and nricens packages.[33-35] A *P* value below 0.05 was considered statistically significant. Unless stated otherwise, continuous variables are expressed as median with interquartile range (IQR) and compared between groups using the Wilcoxon–Mann–Whitney test, and categorical variables are expressed as count with percentage and compared between groups using the Fisher exact test. No imputation for missing data was performed. A convenience sample size of at least 100 uACE2 measurements was chosen, as sample size calculation was unfeasible due to the absence of previous reference uACE2 data in this population.

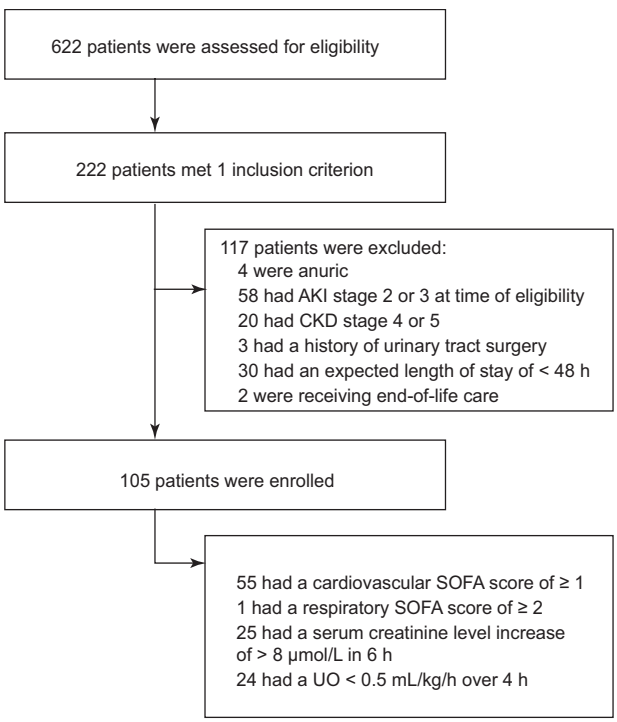
We assessed the association of uACE2 activity with AKI risk, using generalised linear models. We then performed a predetermined sensitivity analysis, after exclusion of oliguric (non-anuric) patients at time of urine collection (defined as a urine output of < 0.5 mL/kg/h over 6 hours). Adjustment for AKI risk was performed using the following predefined variables: age, premorbid serum creatinine levels and APACHE III score (the reference model). Model variables were selected following the Sapphire study, which studied the predictive performance of AKI biomarkers, using an AKI reference model.[25] The calibration of the reference model was assessed using the Pearson -χ^2 test. Time to AKI stage 2 or 3 during ICU stay was analysed using the log-rank test, after dichotomising the study population based on the observed median uACE2 value.

To assess the biomarker's performance to predict AKI, we pre-specified the computation of the area under the receiver-operating characteristics curve (AUROC) of uACE2 activity corrected for uCr for the detection of the primary outcome. A similar method was applied to the analysis of secondary outcomes and to the assessment of the adjusted AKI risk models. We completed this analysis by calculating the net reclassification index (NRI) of uACE2, with the aim of reclassifying patients into three risk categories of the primary outcome (low < 5%, medium 5% to < 30%, high ≥ 30%), using the same variables as described for the reference risk model.[36] For exploratory purposes, we also determined the optimal cut-off value of uACE2, using the Youden criterion. From this, we computed the sensitivity, specificity and other relevant parameters of diagnostic accuracy.

Results

Between February 2018 and October 2018, we enrolled 105 critically ill patients (Figure 1). Inclusion (ie, time of urine

Figure 1. Study flow chart



AKI = acute kidney injury. CKD = chronic kidney disease. SOFA = Sepsis-related Organ Failure Assessment.

Table 1. Patient characteristics based on absence or presence of AKI stage 2 or 3 at 12 hours*

	Whole cohort (*N* = 105)	AKI– at 12 h (*n* = 73)	AKI+ at 12 h (*n* = 32)	*P*
Demographics				
Sex (male)	56 (53%)	37 (51%)	19 (59%)	0.52
Age (years)	64 (51–74)	64 (52–74)	64 (51–73)	0.85
Comorbidities				
Hypertension	53 (50%)	35 (48%)	18 (56%)	0.53
Diabetes	22 (21%)	14 (19%)	8 (25%)	0.60
Peripheral vascular disease	12 (11%)	7 (10%)	5 (16%)	0.51
Ischaemic heart disease	25 (24%)	20 (27%)	5 (16%)	0.22
Chronic heart disease	10 (10%)	8 (11%)	2 (6%)	0.72
Cerebrovascular disease	8 (8%)	6 (8%)	2 (6%)	> 0.99
Chronic obstructive pulmonary disease	16 (15%)	12 (16%)	4 (12%)	0.77
Chronic liver disease	15 (14%)	9 (12%)	6 (19%)	0.38
Use of ACE inhibitors	15 (14%)	12 (16%)	3 (9%)	0.55
Premorbid renal function				
Serum creatinine (μmol/L)	76 (57–89)	69 (54–86)	86 (73–100)	< 0.01
eGFR (mL/min/1.73m^2)	90 (64–90)	90 (74–90)	80 (56–90)	< 0.01
ICU admission category				
Elective surgery	27 (26%)	18 (25%)	9 (28%)	0.01
Emergent surgery	31 (30%)	16 (22%)	15 (47%)	–
Medical	47 (45%)	39 (53%)	8 (25%)	–
ICU admission subgroup				
Cardiac surgery	16 (15%)	11 (15%)	5 (16%)	0.13
Neurologic admission	14 (13%)	9 (12%)	5 (16%)	0.13
Severity of disease				
APACHE III score	47 (40–64)	47 (38–64)	51 (42–61)	0.39
SOFA score	6 (4–8)	6 (5–8)	6 (4–9)	0.79
Vasopressor support	69 (66%)	48 (66%)	21 (66%)	> 0.99
Mechanical ventilation	73 (70%)	52 (71%)	21 (66%)	0.65
Sepsis	32 (30%)	25 (34%)	7 (22%)	0.25
Blood results at inclusion†				
Highest white cell count (x 10^9/L)	13.1 (10.2–16.9)	13.3 (10.2–15.7)	13 (10.5–17.6)	0.65
Highest lactate (mmol/L)	2.6 (1.6–4.4)	2.6 (1.6–4.2)	2.5 (1.8–4.5)	0.86
Renal function and related characteristics at inclusion				
Serum creatinine (μmol/L)	87 (71–122)	80 (67–110)	102 (81–158)	< 0.01
Estimated baseline creatinine	45 (43%)	29 (40%)	16 (50%)	0.39
Urine output (mL/kg/h)‡	0.7 (0.4–1.0)	0.8 (0.5–1.1)	0.4 (0.3–0.7)	< 0.01
Fluid balance (mL/kg/h)‡	0 (–0.8 to 0.6)	–0.2 (–0.8 to 0.6)	0.1 (–0.6 to 0.6)	0.52
Diuretic use§	13 (12%)	12 (16%)	1 (3%)	0.10
Prior use of ACE inhibitors¶	15 (14%)	12 (16%)	3 (9%)	0.54
Prior use of ARBs¶	21 (20%)	12 (16%)	9 (28%)	0.19

(*continues*)

Table 1. Patient characteristics based on absence or presence of AKI stage 2 or 3 at 12 hours* (*continued*)

	Whole cohort (*N* = 105)	AKI– at 12 h (*n* = 73)	AKI+ at 12 h (*n* = 32)	*P*
ICU-related outcomes				
Length of stay (day)	3 (2–6)	3 (2–6)	4 (2–6)	0.56
Duration of mechanical ventilation (h)	26 (10–69)	25 (11–64)	29 (6–96.5)	0.99
Duration of vasopressor support (h)	18 (2–39)	20 (8–41)	12.5 (0–35)	0.14
ICU mortality	11 (10%)	9 (12%)	2 (6%)	0.50

ACE = angiotensin-converting enzyme. AKI = acute kidney injury. AKI– = AKI stage 0 or 1 at 12 h of urine collection. AKI+ = AKI stage 2 or 3 at 12 h of urine collection. APACHE = Acute Physiology and Chronic Health Evaluation. ARB = angiotensin receptor blocker. eGFR = estimated glomerular filtration rate. ICU = intensive care unit. SOFA = sepsis-related organ failure assessment. * Data are median (interquartile range) or count (percentage). † Measured in the 24 h preceding inclusion. ‡ Recorded over the 6 h preceding inclusion. § Administered within the 6 h preceding inclusion. ¶ Defined as the prescription of ACE inhibitors or ARBs in the most recent history.

collection) occurred at a median of 14 (IQR, 10–17] hours of ICU admission. The patients' characteristics are summarised in Table 1. Co-existence of inclusion criteria is shown in the Online Appendix (table S1). Adjudication of the primary outcome was possible for all 105 included patients.

Corrected uACE2 activity in ICU patients and healthy volunteers

The median corrected uACE2 activity for the study cohort was 8.7 (IQR, 5.4–19.1) pmol/min/mL per mmol/L of uCr (*N* = 105), which was similar to that for the healthy volunteers (Online Appendix, table S2). No difference in uACE2 activity was observed based on study inclusion criteria (Online Appendix, figure S1). uACE2 activity was similar in patients on ACE inhibitors (7.8 [6.1–20.3] pmol/min/mL per mmol/L of uCr) to those treated with angiotensin receptor blockers (8.0 [6.1–14.5] pmol/min/mL per mmol/L of uCr) and those not treated with either of these classes of RAS inhibitors (9.4 [5.4–19.7] pmol/min/mL per mmol/L of uCr) ($P = 0.98$, Kruskall–Wallis test across the three groups).

AKI incidence and severity

Within 12 hours of inclusion, 32 of 105 patients (30%) had developed AKI stage 2 or 3. Their premorbid renal function and renal function at time of inclusion significantly differed from patients who did not develop the primary outcome (Table 1). The characteristics of the primary and secondary outcomes are shown in Table 2. Oliguria was the main AKI defining criterion; seven of 25 patients with oliguria-defined AKI showed a subsequent increase in serum creatinine level greater than 1.5 times more than the baseline value during their ICU stay.

Corrected uACE2 activity in patients with AKI

Corrected uACE2 activity in patients who developed the primary outcome was significantly lower compared with that for those who did not develop the primary outcome, and comparable to measurements in healthy volunteers (Figure 2, panel A). For patients without AKI stage 2 or 3 at 12 h versus those with AKI stage 2 or 3 at 12 h, the median [interquartile range] corrected uACE2 activity values were 13 [6–24] and 7 [4–10] pmol/min/mL per mmol/L of urine creatinine, respectively ($P < 0.01$). uACE2 levels based on AKI status for each inclusion criterion are shown in the Online Appendix (table S3).

Association of corrected uACE2 activity with risk of severe AKI

On unadjusted analysis, higher uACE2 activity was associated with a 28% decrease in risk of AKI stage 2 or 3 at 12 hours (OR, 0.72 [95% CI, 0.46–0.97] for each 10 pmol/min/mL per mmol/L of uCr increase in concentration) (Figure 3). This persisted after adjustment for the predefined covariates (Table 3). The significant association between uACE2 levels and the primary outcome remained in patients without oliguria at inclusion (Online Appendix, figure S2).

In the subgroup of patients without any degree of AKI before inclusion, 39 of 76 (51%) developed AKI stage 2 or 3 at any time during ICU admission, at a median time of 13 (IQR, 10–52) hours after inclusion. Those with uACE2 activity > 8.7 pmol/min/mL per mmol/L of uCr experienced a lower time-weighted risk of AKI stage 2 or 3 during ICU admission (Figure 4).

Performance of uACE2 as a predictor of risk of AKI

In isolation, uACE2 showed a poor, yet significant, discriminative ability to predict AKI stage 2 or 3 at 12 hours,

Table 2. Analysis of primary outcome and secondary renal outcomes for AKI– and AKI+ patients*

	AKI– (*n* = 73)	AKI+ (*n* = 32)	*P*
Primary outcome (AKI stage 2 or 3 at 12 h)			
AKI stage†			–
Stage 1	18 (25%)	0	
Stage 2	0	30 (94%)	
Stage 3	0	2 (6%)	
Renal function indices at 12 h			
Serum creatinine (μmol/L)	80.0 (64.0–102.2)	109.0 (87.0–187.5)	< 0.01
Urine output (mL/kg/h)	0.9 (0.7–1.4)	0.4 (0.3–0.5)	< 0.01
AKI defining criterion†			–
Serum creatinine alone	–	2 (6%)	
Urine output alone	–	25 (78%)	
Combination of both	–	5 (16%)	
Secondary renal outcomes			
Renal replacement therapy	3 (4%)	4 (12%)	0.20
AKI stage 2 or 3 at 24 h	4 (5%)	17 (53%)	< 0.01
AKI stage 2 or 3 at any time	7 (10%)	32 (100%)	< 0.01
Time to highest AKI stage (h)	2.2 (–4.9 to 59.2)	12.1 (11.0–13.1)	0.11

AKI = acute kidney injury. AKI– = AKI stage 0 or 1 at 12 h of urine collection. AKI+ = AKI stage 2 or 3 at 12 h of urine collection. KDIGO = Kidney Disease: Improving Global Outcomes. * Data are median (interquartile range) or count (percentage). † According to KDIGO guidelines.

Table 3. Adjusted predictive performance of uACE2 activity in critically ill patients for the primary outcome and other renal outcomes, for an increase in uACE2 level of 10 pmol/min/mL per mmol/L of uCr

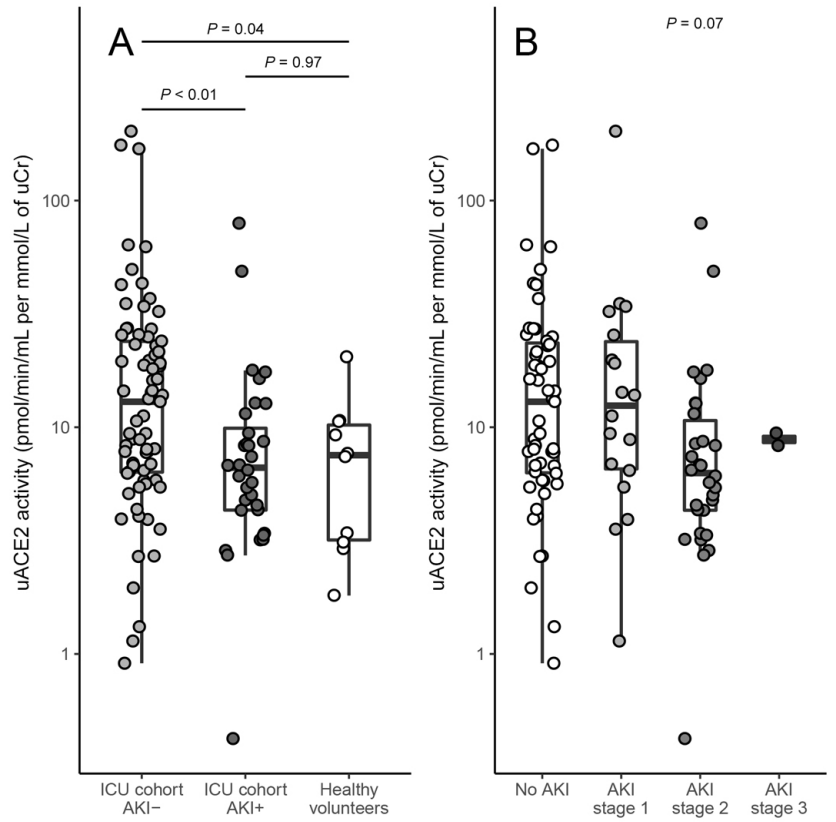
Outcome	Patients/ total	Adjusted OR (95% CI)*	AUROC (95% CI)	NRI+†	NRI–†
Primary outcome					
AKI stage 2 or 3 at 12 h	32/105	0.65 (0.40–0.90)	0.75 (0.65–0.84)	0.28	0.03
Other renal outcomes					
AKI (any stage) at 12 h	50/105	0.84 (0.69–1.00)	0.80 (0.71–0.88)	–	–
AKI stage 2 or 3 at 24 h	25/105	1.05 (0.89–1.22)	0.70 (0.57–0.84)	–	–
AKI stage 2 or 3 at any time	39/105	0.93 (0.77–1.08)	0.74 (0.64–0.84)	–	–

AKI = acute kidney injury. APACHE = Acute Physiology and Chronic Health Evaluation. AUROC = area under the receiver operator characteristics curve. NRI = net reclassification index. OR = odds ratio. uACE2 = urinary angiotensin-converting enzyme type 2. uCr = urinary creatinine. * Adjusted for age, premorbid serum creatinine levels and APACHE III score, except for the OR for AKI stage 2 or 3 at 24 h, which was adjusted for premorbid serum creatinine levels and APACHE III score only, owing to a limited number of events. Renal replacement therapy is not presented due to the limited number of cases (< 10). There were no missing data for the following variables that were included in the model: severe AKI at 12 h, uACE2 measurements, age, premorbid serum creatinine levels, and APACHE III score. The reference model has an AUROC of 0.71 (95% CI, 0.61–0.80), with no significant difference compared with the same model to which was added uACE2 activity ($P = 0.36$). Calibration was acceptable ($P = 0.51$, Pearson-χ^2 test). No significant interaction between independent terms of the reference model was identified. † To reclassify patients into three risk categories (low < 5%, medium 5% to < 30%, and high ≥ 30%), no NRI was computed if no statistical difference existed between the reference model and the model including uACE2 levels.

with an AUROC of 0.68 (95% CI, 0.57–0.79). Discriminative performance became fair after exclusion of patients who were oliguric at time of urine collection (AUROC, 0.71 [95% CI, 0.57–0.86]).

The multivariate model (using uACE2 activity, age, premorbid serum creatinine level and APACHE III score) had an AUROC of 0.75 (95% CI, 0.65–0.84) to predict AKI stage 2 or 3 at 12 hours (fair discriminative ability). uACE2

Figure 2. Levels of uACE2 in critically ill patients with AKI*



AKI = acute kidney injury. AKI– = AKI stage 0 or 1 at 12 h of urine collection. AKI+ = AKI stage 2 or 3 at 12 h of urine collection. ICU = intensive care unit. uACE2 = urinary angiotensin-converting enzyme type 2. uCr = urinary creatinine. * Panel A compares uACE2 activity in ICU patients with AKI stage 0 or 1 at 12 h of urine collection, ICU patients with AKI stage 2 or 3 at 12 h of urine collection and healthy volunteers, with *P* values shown for between-group differences. uACE2 activity was significantly higher in ICU patients without AKI stage 2 or 3 at 12 h of urine collection, compared with the other two groups. Panel B shows uACE2 levels based on AKI stages (no AKI, $n = 55$; AKI stage 1, $n = 18$; AKI stage 2, $n = 30$; AKI stage 3, $n = 2$), with the *P* value representing significance of the association between uACE2 activity with AKI stage. In both figures, the y-axis was log-transformed to improve readability, and individual data and boxplots (representing median values, interquartile ranges, minimum and maximum values) are shown.

levels correctly reclassified 28% of patients to a higher risk category of the primary outcome (positive NRI) (Table 3).

After determining an optimal cut-off point, we found that a uACE2 activity below 12.8 pmol/min/mL per mmol/L of uCr had a sensitivity of 81% (95% CI, 64–93%) and a specificity of 51% (95% CI, 39–63%) to predict the primary outcome (see Online Appendix, table S4, for cross-tabulation and other parameters of diagnostic accuracy).

Discussion

Main findings

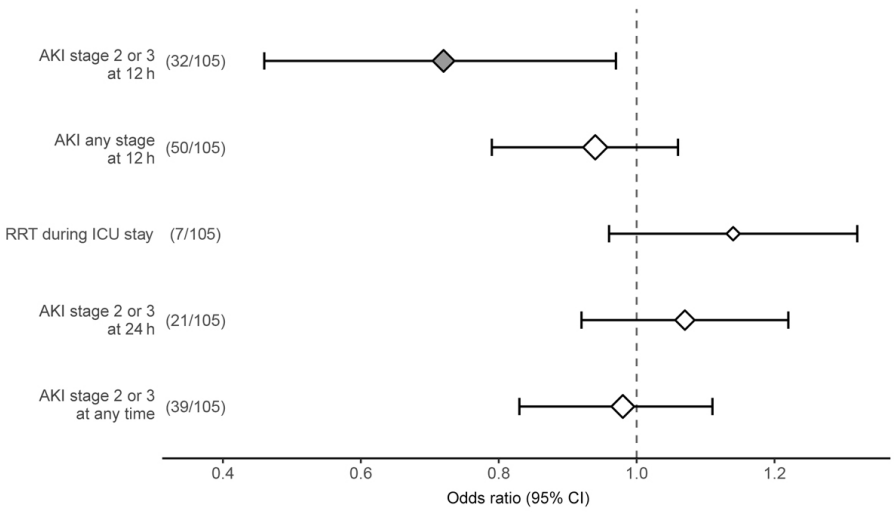
In a prospective cohort of ICU patients, we measured uACE2 activity levels and assessed their association with the development of severe AKI. We found that patients who developed severe AKI had significantly lower uACE2 activity compared with those without severe AKI. Moreover, we found that their uACE2 levels were comparable to those of healthy volunteers, and were half the levels seen in those who did not develop severe AKI. Finally, uACE2 showed potential as a biomarker for predicting early severe AKI in ICU patients, demonstrating high sensitivity yet low specificity for the primary outcome.

Relationship to previous studies

This study is the first and only report on uACE2 measurements in critically ill patients at risk of AKI. It adds a novel line of evidence regarding the role of the RAS in critical illness, and renal failure more specifically. Our results suggest that the non-classical RAS may play a role in ICU patients at risk of severe AKI. It is plausible that the non-classical RAS counterbalances the systemic and local effects of the classical RAS on inflammation and intrarenal haemodynamics, by increasing the generation of angiotensin (1–7) and (1–9).[37] Indeed, our findings are congruent with experimental evidence showing that inhibited generation of pivotal peptides of the non-classical cascade increases kidney injury in ACE2 knock-out mice after experimental ischemia–reperfusion.[38]

To put our results into further physiological perspective, increases in urinary excretion of renin and angiotensinogen were observed in patients with severe AKI, while classical RAS overexpression (angiotensin II and angiotensinogen) was demonstrated in renal biopsy specimens of patients with proven acute tubular necrosis.[39-41] On the other hand, we can hypothesise that non-classical pathway activation inducing an increased angiotensin$^{1\text{-}7}$ response may protect from AKI, as suggested by preclinical research.[16,20] This is indeed suggested by our data, as patients experiencing a

Figure 3. Association between uACE2 levels and study outcomes*



AKI = acute kidney injury. ICU = intensive care unit. RRT = renal replacement therapy. uACE2 = urinary angiotensin-converting enzyme type 2. uCr = urinary creatinine. * The figure shows the unadjusted odds ratios (with 95% CIs) for the primary outcome (AKI stage 2 or 3 at 12 h) and the secondary renal outcomes, for an increase in uACE2 level of 10 pmol/min/mL per mmol/L of uCr. The size of each diamond is proportional to the number of events (indicated in parentheses).

critical insult who did not develop AKI showed increased excretion of uACE2, and there was a trend in lower uACE2 levels being associated with the most severe cases. Conversely, those who did develop AKI showed levels comparable to those of healthy volunteers, suggesting their inability to activate this potentially protective endocrine response in the face of a renal insult. Also, increased angiotensin II levels, by increasing tubular sodium reabsorption and reducing renal blood flow, may have decreased diuresis, which would explain the higher incidence of oliguria-defined AKI in our cohort.

These findings highlight the potential importance of classical/non-classical RAS balance in the pathogenesis of AKI. However, our study did not analyse other markers of classical or non-classical pathways, such as angiotensin II or angiotensin-(1–7) levels in blood or urine, and this limits our ability to draw solid conclusions.

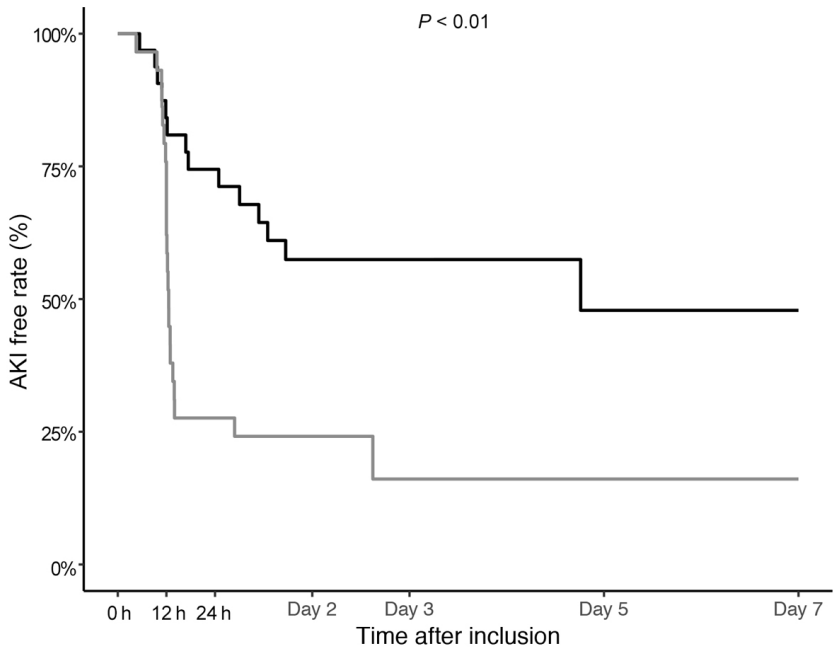
From a clinical point of view, a recent post-hoc analysis of a large randomised controlled trial of angiotensin II administration in patients with vasodilatory shock and severe AKI showed that drug-induced classical RAS upregulation accelerated RRT weaning rates.[42] While this may appear to challenge our hypothesis, it is biologically plausible that systemic angiotensin II infusion may have increased perfusion pressure and, after metabolism by ACE2, generated much higher levels of angiotensin-(1–7) and angiotensin-(1–9). Angiotensin-(1–7), the pivotal peptide of the non-classical cascade, decreases renal blood flow, increases natriuresis, and inhibits proinflammatory and fibrosis pathways in experimental models of AKI.[10,18-20] Hence, we hypothesise that in patients with upregulated ACE2 activity, the increase in angiotensin II bioavailability (through intravenous administration) would explain the benefits on RRT weaning observed in the post-hoc analysis of the Angiotensin II for the Treatment of High-Output Shock (ATHOS-3) trial.[43] However, the trial only reported markers of the classical pathway activation.

Finally, neither ACE inhibitor use nor angiotensin receptor blocker use affected uACE2 levels in our cohort, which is in line with experimental data showing their inability to inhibit ACE2.[44]

Implications of study findings

Our results imply that non-classical RAS upregulation may play a role in the development of severe AKI in ICU patients. Our findings generate new pathophysiological hypotheses regarding the development of AKI, that are congruent with the preclinical evidence of the effects of non-classical RAS upregulation on renal blood flow, natriuresis, inflammatory processes and profibrotic processes.[18,20,45,46] Based on our preliminary results, the ability of uACE2 to predict AKI should be explored further. Moreover, these preliminary findings imply that additional efforts should be made to understand how classical and non-classical RAS interact during AKI pathogenesis. Finally, based on the experimental and clinical experience in acute lung injury, our data imply that the effect of recombinant human ACE2 on AKI should be evaluated at the preclinical stage first.[47,48] This is especially relevant in the context of the SARS-CoV-2 pandemic, because SARS-CoV-2 causes ACE2-mediated viral disease. Indeed, critically ill patients infected with SARS-CoV-2 have high incidence of severe AKI, which we hypothesise could, to some level, be related to deficiency of ACE2 activity induced by the virus.[49]

Figure 4. Log-rank analysis of risk of AKI based on uACE2 levels*



AKI = acute kidney injury. ICU = intensive care unit. uACE2 = urinary angiotensin-converting enzyme type 2. uCr = urinary creatinine. * The Kaplan–Meier graph shows the AKI-free (non-stage 2 or 3) rate over 7 days in all patients who had not developed AKI (any stage) before urine collection (n = 76). We divided those patients into two groups, based on the median value of uACE2 levels in the cohort (8.7 pmol/min/mL per mmol/L of uCr). The P value represents the difference in AKI incidence, using the log-rank method, until censor date (ICU discharge, Day 7 after urine collection or death). Patients who had uACE2 levels below the median uACE2 value had a significantly higher risk of developing AKI stage 2 or 3 during ICU stay (grey line) compared with those who had uACE2 levels above the median (black line).

Strengths and limitations

Our study has several strengths. It is the first to report uACE2 activity levels in a general population of critically ill patients, and to compare their findings with those for healthy volunteers. We enrolled a large number of critically ill patients for whom there was a high incidence of severe AKI — a reflection of our study population's severity of illness. Also, although a majority of identified AKI episodes were classified using isolated oliguria in our study, low urine output is known to be associated with worse clinical outcomes in ICU patients, even if transient or in the absence of a creatinine increase.[50-52]

Another strength was the fact that uACE2 activity was measured by an investigator who was blinded to the clinical context and all investigators were unaware of the uACE2 results at the time of AKI adjudication. Also, uACE2 activity was corrected for urine creatinine concentration, similar to the methods used to quantify urinary angiotensinogen.[39] This correcting method is recommended when assessing urine biomarkers in the context of acute fluctuation of glomerular filtration rates and urine flow, although it may have led to overestimation of uACE2 sensitivity.[28,29] Finally, we used adequate and validated statistical methods, including NRIs and multivariate models, to assess the predictive performance of uACE2.

However, some limitations should be acknowledged. First, this was a single-centre study, which limits the external validity of the findings. However, our ICU has all the characteristics of a tertiary teaching centre, including a broad spectrum of both medical and postoperative patients. Also, we observed an incidence of severe AKI similar to that reported in other large studies.[3]

Second, this was an observational study, limiting any inference on causality. Moreover, the absence of association with the predefined secondary outcomes mandates cautious interpretation of our findings. However, our results on the association of uACE2 activity with severe and early AKI are in line with what would be expected based on previous work and our current understanding of the RAS. Furthermore, we showed a significant association of uACE2 with the incidence of severe AKI in our time-dependent analysis, suggesting the importance of timing when evaluating a potential biomarker in relation to a clinical process. We also acknowledge that uACE2 as a biomarker showed discrimination of limited performance in our population, although its sensitivity was high. We believe that this is at least partly due to our study's casemix — that is, of a general ICU population.

Third, it is plausible that uACE2 levels may have been affected by increased glomerular filtration rates and/or plasma protein leakage, and may not have reflected intrarenal production. However, ACE2 filtration through the glomerular membrane seems unlikely, as its molecular weight is about 120 kDa — twice that of albumin.[53] Also, this has been highlighted as a potential source of bias in patients with chronic kidney disease[46] and was accounted for by correcting for urinary creatinine levels. Apart from urine concentration and glomerular filtration, uACE2 could be affected by urine output in this population of patients

with both oliguric and non-oliguric AKI, owing to effects of circadian rhythm in our cohort of healthy volunteers. However, we confirmed our findings when assessing uACE2 activity in patients without oliguria at time of inclusion.

Fourth, we found that our study, although significant for its primary outcome, had potentially low statistical power. The risk of such a situation is to overestimate the effect size of our results. Testing the hypothesis that the effect size was half of what we observed, there was a 6% risk that the sign (direction) of the effect was wrong (a type S error) and that its magnitude could be four times lower (type M error).[54]

Finally, using NRI values to assess the performance of a biomarker may depend strongly on the quality of the reference model. In our study, the reference model was extrinsically valid, and its performance confirmed, using recommended methods.

Conclusions

In this exploratory study of patients at high risk of AKI, higher uACE2 activity was associated with a decreased risk of developing severe AKI and showed significant, yet limited, performance for predicting the incidence of severe AKI. Our hypothesis-generating study warrants further investigations to explore the complex roles of the classical and non-classical RAS in the pathogenesis of AKI.

Acknowledgements: We thank Leah Peck, Helen Young and all Austin Health ICU staff for the help and support provided for this project. The ACE2 assay costs were supported by a National Health and Medical Research Council Program Grant (APP1055214) awarded to Louise Burrell.

Competing interests

None declared.

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