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Early View

Research letter

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Increased blood Angiotensin Converting Enzyme 2 activity in critically ill COVID-19 patients

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Contributions
DvL, MK, KS, HvdH, JP and PP conceptualized the study and drafted the manuscript. DvL and MK performed data quality control and assurance, transformation, and data analysis. All authors critically revised the manuscript. The authors read and approved the final manuscript.

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Running head
Increased blood ACE2 activity in COVID-19 ICU patients

Introduction

Pharmacological blockade of the renin-angiotensin-aldosterone system (RAAS) with angiotensin-converting enzyme (ACE)-inhibitors and angiotensin receptor blockers (ARBs) are cornerstone treatments in several cardiovascular disease entities (1). The RAAS is a central regulator of blood pressure, consisting of two counterregulatory pathways, commonly described as classical and non-classical, respectively (1). The main effect of classical RAAS activation is the generation of angiotensin II by angiotensin converting enzyme (ACE) (1). In contrast, non-classical RAAS activation results in cleavage of angiotensin II by angiotensin converting enzyme-2 (ACE2) to form angiotensin 1-7, which directly counteracts the effects of classical RAAS activation (1).

As early as March 2020, observational studies reported associations between hypertension and Coronavirus-19 (COVID-19) disease outcome (2). Concerns about the risk of ACE-inhibitors and ARBs were quickly raised, as these drugs were implied to upregulate ACE2, the functional receptor for SARS-coronavirus 2 (SARS-CoV-2), thereby putatively increasing SARS-CoV-2 virulence (2). However, a recent large observational study mitigated these concerns by demonstrating neither increased susceptibility for COVID-19, nor impaired outcome of COVID-19 patients on ACE-inhibitor/ARB therapy (3). Other observational data even suggest improved outcome of COVID-19 patients on ACE-inhibitor/ARB therapy (4), a finding currently being investigated in a randomized controlled trial (ClinicalTrials.gov NCT04311177).

Although observational studies highlight ACE2 as both friend and foe in COVID-19, there is a paucity of actual data on angiotensin metabolism in COVID-19. This is highly warranted to determine a possible causative relationship between ACE2 and COVID-19 severity.

Methods

The study was carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent. All patients or legal representatives were informed about the study details and could decline to participate. The study was conducted in accordance with the declaration of Helsinki, including current revisions, and Good Clinical Practice guidelines.

We obtained blood samples from COVID-19 patients (n=10) admitted to the intensive care unit because of respiratory failure requiring invasive mechanical ventilation and from age-matched healthy controls without history of cardiovascular disease or medication (n=5). Blood was centrifuged within 10 minutes following withdrawal and plasma was stored at -80 °C until analysis at Attoquant Diagnostics (Vienna, Austria). To determine soluble (s)ACE2 enzyme activity, diluted plasma was spiked with Angiotensin (Ang)-II and incubated at 37 °C for 1 hour in the presence and absence of the specific ACE2 inhibitor MLN-4760, after which Ang-1-7 concentrations were quantified using liquid chromatography–mass spectrometry (LC-MS). The calculated difference between Ang-1-7 formation rate with/without the inhibitor results in ACE2-specific Ang-1-7-generating capacity and thus plasmatic sACE2 activity. sACE2 quantity was subsequently calculated using the linear calibration obtained from a standard curve of hrsACE2 in plasma obtained from healthy volunteers (5). Ang-II levels were compared to those in non-incubated control samples to assure that the substrate was present in excess during the incubation period, assuring a stable Ang 1-7 formation rate.

Furthermore, equilibrium analysis, reflecting overall plasmatic renin–angiotensin system (RAS) activity, was performed by adding stable-isotope-labeled internal standards for angiotensin metabolites (Ang-I, Ang-II, Ang-1-7, Ang-1-5) to plasma samples followed by incubation at 37 °C for 1 hour and quantification of angiotensin peptide levels by liquid chromatography-mass spectrometry. Data are presented as median [interquartile range] for continuous variables, and as counts and

percentages for categorical variables. Between-group differences were evaluated using Mann-Whitney-U and Fisher exact tests. A two-sided p-value <0.05 was considered significant. Data were analyzed using Graphpad Prism 8.3 (Graphpad Software, San Diego, USA).

Results

Baseline characteristics were comparable between COVID-19 patients and healthy controls: age 61.5 [56.3-68.0] vs. 58.0 [51.0-60.5] years, p=0.22; BMI 27.7 [25.2-30.9] vs. 25.6 [24.3-28.1] kg/m^2, p=0.20; sex (male) 7/10 (70%) vs. 4/5 (80%), p=0.59. Half of the patients previously used ACE-inhibitor/ARB therapy, 60% had a history of cardiovascular disease. All patients were critically ill and had respiratory failure, reflected by admission sequential organ failure assessment (SOFA) scores of 6 [5-8] and PaO_2/FiO_2 ratios of 180 [111-242], respectively.

As depicted in Figure 1, COVID-19 patients displayed markedly increased sACE2 levels compared with controls (13-fold increase, p<0.001). Moreover, the equilibrium analysis yielded increased formation of Ang-1-7 (12-fold increase, p<0.01, Figure 1), while Ang-II formation was reduced (3-fold decrease, p<0.01, Figure 1). No differences in Ang-I and Ang-1-5 formation were observed (Figure 1). Furthermore, no differences between patients previously on ACE-inhibitors/ARBs and non-users were present (data not shown).

Discussion

Our data reveal increased non-classical angiotensin pathway activity in the blood of critically ill COVID-19 patients, reflected by increased sACE2 levels as well as decreased Ang-II and enhanced Ang-1-7 formation in the equilibrium assay compared to healthy controls. Whether this enhanced circulating sACE2 activity accurately reflects responses in the lung remains to be determined, as systemic angiotensin responses do not necessarily reflect local (paracrine) responses (6). Since SARS-CoV-2 binds and cleaves membrane-bound ACE2 upon cell entry (2, 7), increased sACE2 activity might actually reflect reduced pulmonary enzyme activity, with cleaved sACE2 ending up in the circulation. Hence, our findings should be compared to measurements in the pulmonary compartment (e.g. bronchoalveolar fluid), which were unfortunately not available from our cohort.

sACE2 as well as Ang-1-7 administration were already found to protect from pulmonary injury in different murine ARDS models (8, 9). As the ACE2/Angiotensin-1-7 axis possesses potent anti-inflammatory properties (10), the increased sACE2 levels in our cohort might also represent a failing physiological response aimed at reducing inflammation-mediated pulmonary injury. To address this, serial assessment of sACE2 activity in COVID-19 patients with varying disease severities should be performed to determine the kinetics of enhanced sACE2 activity and if it could function as a biomarker for SARS-CoV-2 disease progression.

Whether increased sACE2 levels negatively affect blood pressure in critically ill patients is currently incompletely understood. In the context of septic shock, increased classical RAAS activity is a physiologic and potentially life-saving response aimed at maintaining organ perfusion (11). For instance, it is essential to maintain glomerular filtration, especially during periods of attenuated renal perfusion (12). As such, impaired classical RAAS activity induced by increased sACE2 levels could putatively induce hypotension in COVID-19 patients and might explain the high incidence of acute kidney injury observed in critically ill COVID-19 patients (2).

Recently, a case report described the successful treatment of a COVID-19 patient with human-recombinant-soluble (hrs)ACE2 therapy (13). A phase 2/3 study investigating hrsACE2 therapy is currently ongoing (ClinicalTrials.gov Identifier: NCT04335136). Based on our data showing marked variation of circulating ACE2 levels in critically ill COVID-19 patients, sACE2 measurements may be valuable to identify patients amenable to hrsACE2 therapy in a personalized medicine approach.

Along these lines, sACE2 measurements might serve as a population enrichment strategy in such trials.

A major limitation of the current study is the small sample size. Therefore, our results should be interpreted as hypothesis-generating. However, it represents the first study to assess both classical and non-classical blood angiotensin metabolism in critically ill COVID-19 patients and provides initial evidence of non-classical RAAS activation in these patients.

Acknowledgements
Not applicable

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics declaration
The study was carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent. All patients or legal representatives were informed about the study details and could decline to participate.

Competing interests
The authors declare no competing interests or conflicts of interest.

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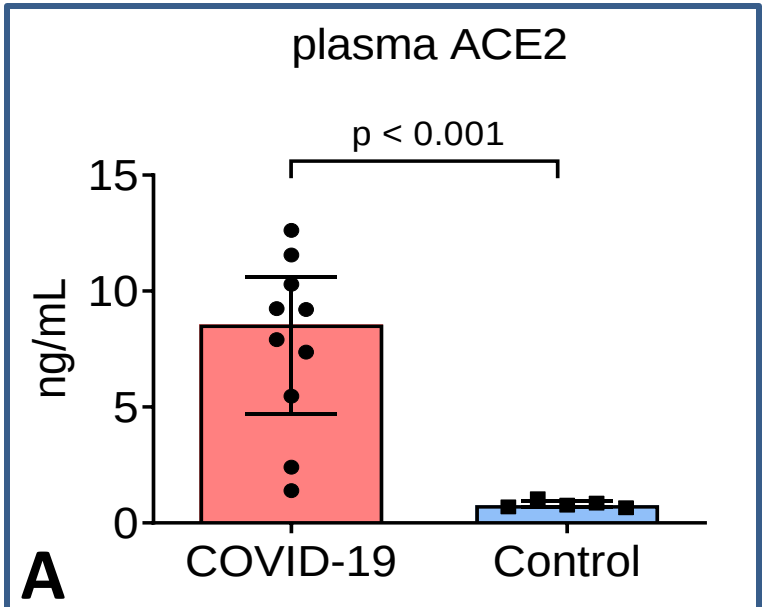
Figure 1. ***A****. Plasma sACE2 levels.* ***B.*** *Overview of all measured angiotensin enzymes/metabolites in critically-ill COVID-19 patients as well as age matched healthy controls.* ***C.*** *Alterations in classical/non-classical angiotensin pathways in critically ill COVID-19 patients. Median and interquartile levels are displayed, p-values were calculated using Mann Whitney-U tests. Arrows in panel C indicate significantly attenuated/increased levels of the respective metabolites in COVID-19 patients compared to healthy controls, while the equal sign represents a non-significant difference between groups. ACE2 = angiotensin converting enzyme 2, ACE/CHY = Ace converting enzyme/Chymase, Ang = Angiotensin.*



Measurement	COVID-19 patients	Healthy controls	p-value
Plasma ACE2 (ng/mL)	8.5 [4.7-10.6]	0.8 [0.7-1.0]	<0.001
Angiotensin-I (pmol/L)	93.9 [46.4-299.2]	42.5 [37.4-95.2]	0.165
Angiotensin-II (pmol/L)	52.0 [29.9-78.1]	137.4 [105.1-495.1]	0.008
Angiotensin-1-7 (pmol/L)	23.9 [5.4-39.7]	2.0 [2.0-3.3]	0.004
Angiotensin-1-5 (pmol/L)	9.0 [5.7-13.8]	*4.3 [2.6-13.9]*	0.458

B