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Early View

Research letter

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Increased circulating levels of angiotensin-(1-7) in severely ill COVID-19 patients

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The mono-carboxypeptidase Angiotensin-Converting Enzyme 2 (ACE2) is a major player in the Renin-Angiotensin System (RAS) as it converts the decapeptide Angiotensin I (Ang I) to Ang-(1-9), and Angiotensin II (Ang II) to Ang-(1-7) (Figure 1A) [1]. ACE2 is also a target for the new human coronavirus SARS-CoV-2, which is responsible for the dramatic ongoing COVID-19 pandemic [2]. It has been suggested that following SARS-CoV-2/ACE2 internalization, Ang II level increases [3] in parallel to a decrease of Ang-(1-7) level [4]. These changes would be expected both at tissue and circulatory levels. Considering that Ang-(1-7) has many beneficial effects, including anti-inflammatory, anti-thrombogenic and anti-fibrotic activities [1], it has been hypothesized that Ang-(1-7) administration would improve the clinical outcome of COVID-19 patients. Aiming to test this hypothesis, a Phase I/II clinical trials (NCT04633772) has been initiated with a planned Phase III clinical trial (NCT04332666).

This report aims at answering the key question whether the circulating levels of angiotensin peptides are indeed altered in COVID-19 patients using LC-MS/MS (Figure 1B). This is important since there are no data in the literature on the concentration of circulating angiotensin peptides using direct measurements in COVID-19 patients. Others reported that Ang II is higher in the plasma of COVID-19 patients using ELISA method to quantify Ang II [3]. However, this method has been recently criticized by Chappell et al. due to its poor specificity for the measurement of Ang II and Ang-(1-7) in human plasma [5]. Also, Kintscher et al. have recently reported the "plasma angiotensin peptide profiling" in COVID-19 patients [6] but, even though they used a mass spectrometry-based strategy, they collect the blood using neither protease inhibitors nor under denaturation conditions to "quench" the activity of plasma proteases. Actually, they even incubated the heparinized plasma samples at 37^{o} C to increase proteases activities and thus measure what they call "peptides' equilibrium concentrations" (named "equilibrium method" here). This method allows only the indirect measurement of RAS peptides as it does not account for the important role of enzymes on the endothelium, for example, in the production or hydrolysis of RAS peptides in the circulation. Here, we are reporting the direct measurements of the RAS peptides Ang I, Ang II, Ang-(1-7) and Ang-(1-5) in arterial blood samples from 19 patients with severe COVID-19 and 19 non-COVID-19 volunteers. The study protocol has been approved by the Ethics Committee of the Federal University of Minas Gerais, Belo Horizonte, Brazil (CAAE 34080720.0.1001.5149). Written consents were obtained from

the patients or their relatives for blood sampling. The COVID-19 patients were recruited in two hospitals (Mater Dei Hospital and Eduardo de Menezes Hospital, Belo Horizonte, Minas Gerais, Brazil). Arterial blood samples were obtained from patients admitted at the hospitals' Intensive Care Unit (ICU) upon admission and before starting any additional treatment (medication, oxygen therapy, etc.) in the ICU. The arterial samples from non-COVID-19 subjects were collected from healthy volunteers (n=6) or before cardiac catheterization (n=13). Importantly, only the arterial blood samples from patients with no diagnostic for cardiovascular diseases, *as per* the catheterization results, were included in the non-COVID-19 group. Three COVID-19 patients and three non-COVID-19 individuals were previously using ACE inhibitors (ACEi) (Figure 1B) and the treatment was not interrupted at any time.

A mass spectrometry-based approach was used to quantify the RAS peptides. Inactivation of plasma proteases and potential SARS-CoV-2 in the blood samples was achieved by mixing 2 mL of arterial blood sample with 8 mL of denaturation solution (4M guanidine thiocyanate in 0.5 % TFA). Samples (1,200 µL) were cleaned up using C18 solid-phase extraction (Sep-Pak, Waters), reconstituted in 60 µL 0.1% formic acid (concentration factor = 20-fold), and analyzed by LC-MS/MS (Xevo TQ-S, Waters) using the multiple reaction monitoring (MRM) mode (Figure 1C). The calibration curve was obtained using a stock solution containing synthetic peptides (Bachem, CA) of all RAS peptides used in this study. The applied calibration curve model ($y = ax + b$) proved accurate over the concentration range from 10 to 1000 pg/mL ($r = 0.997$). The limit of quantitation (LOQ), inter- and intra-variability of this method has been previously reported [7]. Data are shown as mean ± SEM and the parametric t test was used for the statistical analyses.

Arterial concentrations of the angiotensin peptides are shown in Figure 1D. In COVID-19 patients, the arterial concentration of Ang II (6.03 ± 1.18 vs. 10.7 ± 1.87 pg/mL; $P = 0.0381$) and Ang-(1-5) (3.43 ± 0.75 vs. 19.3 ± 5.80 pg/mL; $P = 0.0084$) were significantly lower than in the non-COVID-19 volunteers. Surprisingly, the blood levels of Ang-(1-7) were significantly higher in COVID-19 patients (14.0 ± 2.32 vs. 7.49 ± 1.42 pg/mL; $P = 0.0214$). No significant difference was observed for Ang I (31.2 ± 6.23 pg/mL vs. 40.8 ± 9.54 pg/mL; $P = 0.3959$). ACEi therapy did not significantly change the observed results, as shown by the calculated values excluding the data from individuals under ACEi treatment (Figure 1E): Ang I = 34.5 ± 7.07 vs. 45.2 ± 10.8

pg/mL; *P* = 0.4023; Ang II = 6.00 ± 1.33 vs. 11.2 ± 2.07; *P* = 0.0407; Ang-(1-7) = 12.9 ± 2.02 vs. 6.47 ± 1.03 pg/mL; *P* = 0.0080; Ang-(1-5) = 3.75 ± 0.86 vs. 17.5 ± 6.29 pg/mL; *P* = 0.0330.

Although we did not measure the tissular levels of RAS peptides, our findings contrast with the initial hypothesis that the interaction of SARS-CoV-2 with ACE2 would result in higher Ang II and lower Ang-(1-7) levels compared to non-COVID-19 subjects [4].Recent studies using the equilibrium method to measure the ACE2 activity [8, 9] are in line with the results presented here, as they reported higher Ang-(1-7) and lower Ang II plasma levels in severe COVID-19. For general clinical studies including non-severe COVID-19 patients, it seems that all circulating RAS peptides are reduced due to decreased activity of renin [10]. The role of ACE2 in the observed results is questionable as previous studies suggested that one of the main routes to produce Ang-(1-7) in the circulation is by ACE2-independent ways [11, 12]. The observed significant decrease of Ang II and increase of Ang-(1-7) arterial levels in severe COVID-19 patients (Figure 1D) is probably due to a direct dysregulation of RAS pathways in COVID-19 rather than a direct consequence of ACEi usage (Figure 1E). The Ang-(1-7)/Ang II ratio, which is an estimation of Ang II → Ang-(1-7) conversion, was 3-fold higher in COVID-19 patients (2.79 ± 0.682 vs. 0.878 ± 0.201; *P* = 0.0141), which may suggest an increased ACE2 or other Ang-(1-7)-forming activity in COVID-19. Increased soluble ACE2 in severe COVID-19 patients have been recently reported [9, 13, 14]. Reindl-Schwaighofer [9] reported that ACE2 level increased in the course of the disease reaching its maximum peak after ~10 days of hospitalization. Increased ACE2 correlated with increased Ang-(1-7) and decreased Ang II levels [9], suggesting its important role to control the circulating RAS peptides in severe COVID-19.

In contrast to a previous report [3], we observed a significative reduction of Ang II concentration in COVID-19 patients, which may add more data against the reliability of ELISA to measure Ang II in human plasma [5]. The Ang II/Ang I ratio was not significantly altered in the COVID-19 patients (0.205 ± 0.0322 vs. 0.292 ± 0.0517; *P* = 0.1518), which may suggest that ACE activity is not altered in COVID-19. Unfortunately, we were unable to perform direct measurements of enzymatic activities in this study due to the denaturation conditions that we used to collect the samples, but our estimation of increased ACE2 activity in severe COVID-19 blood samples is in line with previous reports [9, 14]. The remaining question to be answered,

though, is whether the observed increased Ang-(1-7) and decreased Ang II levels are a direct effect of SARS-CoV2 or a consequence of inflammation due to the infection that triggers Ang-(1-7) synthesis via Ang II metabolization.

Limitations of our study includes the small cohort included (n = 19 for each group) and the lack of direct activity measurements of the RAS-related enzymes. Nonetheless, this is the first report of the direct measurement of RAS peptides in the arterial circulation of severe COVID-19 patients. Although future studies are obviously necessary to better understand the effects of this disease on RAS pathways, our data provide new insights for the interpretation and planning of future therapies to modulate the RAS in the context of COVID-19.

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References

1. Santos RAS, Sampaio WO, Alzamora AC, Motta-Santos D, Alenina N, Bader M, Campagnole-Santos MJ. The ACE2/Angiotensin-(1-7)/MAS Axis of the Renin-Angiotensin System: Focus on Angiotensin-(1-7). *Physiological reviews* 2018: 98(1): 505-553.
2. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Muller MA, Drosten C, Pohlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020: 181(2): 271-280 e278.
3. Liu Y, Yang Y, Zhang C, Huang F, Wang F, Yuan J, Wang Z, Li J, Li J, Feng C, Zhang Z, Wang L, Peng L, Chen L, Qin Y, Zhao D, Tan S, Yin L, Xu J, Zhou C, Jiang C, Liu L. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. *Science China Life sciences* 2020: 63(3): 364-374.
4. Peiro C, Moncada S. Substituting Angiotensin-(1-7) to Prevent Lung Damage in SARS-CoV-2 Infection? *Circulation* 2020: 141(21): 1665-1666.
5. Chappell MC, Pirro NT, South AM, Gwathmey TM. Concerns on the Specificity of Commercial ELISAs for the Measurement of Angiotensin-(1-7) and Angiotensin II in Human Plasma. *Hypertension* 2021.
6. Kintscher U, Slagman A, Domenig O, Rohle R, Konietschke F, Poglitsch M, Mockel M. Plasma Angiotensin Peptide Profiling and ACE (Angiotensin-Converting Enzyme)-2 Activity in COVID-19 Patients Treated With Pharmacological Blockers of the Renin-Angiotensin System. *Hypertension* 2020: 76(5): e34-e36.
7. Paquette K, Fernandes RO, Xie LF, Cloutier A, Fallaha C, Girard-Bock C, Mian MOR, Lukaszewski MA, Masse B, El-Jalbout R, Lapeyraque AL, Santos RA, Luu TM, Nuyt AM. Kidney Size, Renal Function, Ang (Angiotensin) Peptides, and Blood Pressure in Young Adults Born Preterm. *Hypertension* 2018: 72(4): 918-928.
8. van Lier D, Kox M, Santos K, van der Hoeven H, Pillay J, Pickkers P. Increased blood Angiotensin Converting Enzyme 2 activity in critically ill COVID-19 patients. *ERJ Open Res* 2021.
9. Reindl-Schwaighofer R, Hodlmoser S, Eskandary F, Poglitsch M, Bonderman D, Strassl R, Aberle JH, Oberbauer R, Zoufaly A, Hecking M. Angiotensin-Converting Enzyme 2 (ACE2) Elevation in Severe COVID-19. *American journal of respiratory and critical care medicine* 2021.
10. Kutz A, Conen A, Gregoriano C, Haubitz S, Koch D, Domenig O, Bernasconi L, Mueller B, Schuetz P. Renin-angiotensin-aldosterone system peptide profiles in patients with COVID-19. *European journal of endocrinology* 2021: 184(4): 543-552.
11. Serfozo P, Wysocki J, Gulua G, Schulze A, Ye M, Liu P, Jin J, Bader M, Myohanen T, Garcia-Horsman JA, Batlle D. Ang II (Angiotensin II) Conversion to Angiotensin-(1-7) in the Circulation Is POP (Prolyloligopeptidase)-Dependent and ACE2 (Angiotensin-Converting Enzyme 2)-Independent. *Hypertension* 2020: 75(1): 173-182.
12. Santos RA, Brosnihan KB, Jacobsen DW, DiCorleto PE, Ferrario CM. Production of angiotensin-(1-7) by human vascular endothelium. *Hypertension* 1992: 19(2 Suppl): II56-61.
13. Burns K, Cheng M, Lee T, McGeer A, Sweet D, Tran K, Lee T, Murthy S, Boyd J, Singer J, Walley K, Patrick D, Lamontagne F, Marshall J, Haljan G, Fowler R, Winston B, Russell J. Sustained dysregulation of the plasma renin-angiotensin system in acute COVID-19. 2021.

14. Patel SK, Juno JA, Lee WS, Wragg KM, Hogarth PM, Kent SJ, Burrell LM. Plasma ACE2 activity is persistently elevated following SARS-CoV-2 infection: implications for COVID-19 pathogenesis and consequences. *The European respiratory journal* 2021.

Figure Caption

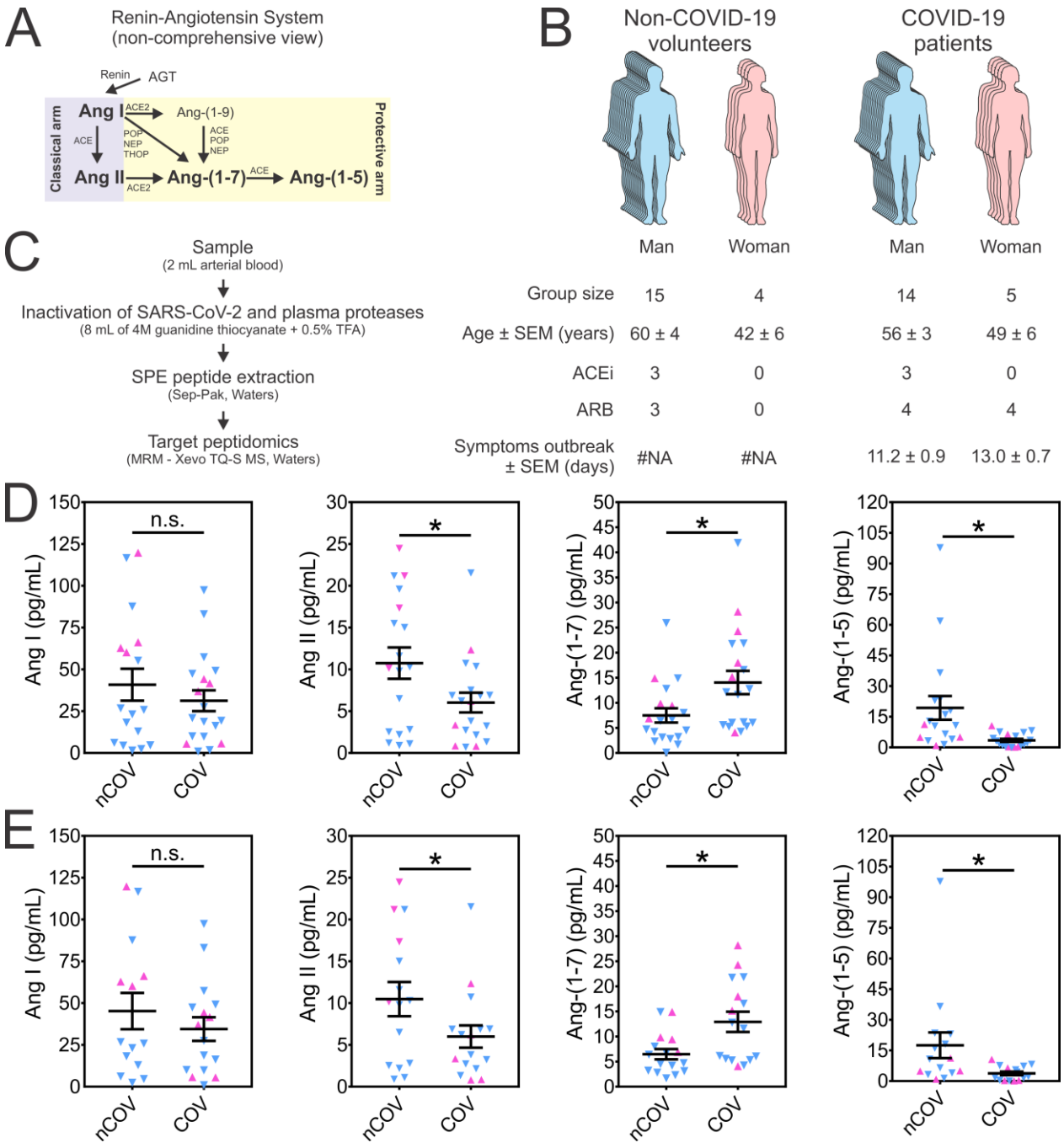

Figure 1. Circulating renin-angiotensin peptides in SARS-CoV-2 infection. (**A**) A non-comprehensive view of the formation of the peptides from the RAS. Peptides measured in this study are in bold. (**B**) Epidemiologic parameters of the subjects included in this study (COVID-

19 patients and non-COVID-19 volunteers). (**C**) A simplified schematic view of the methodology employed to quantify the selected peptides from the RAS. (**D**) Arterial blood concentration of the selected RAS peptides (Ang I, Ang II, Ang-(1-7) and Ang-(1-5)) from COVID-19 patients and non-COVID-19 volunteers. (**E**) Arterial blood concentration of the RAS peptides excluding individuals under ACEi treatment. Man is represented by (▼) and woman by (▲). Data are presented as mean ± SEM. Parametric t test was used for the statistical analyses. *$P < 0.05$. Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; ACEi, ACE inhibitor; AGT, angiotensinogen; ARB, Ang II receptor (AT1) antagonist; COV, COVID-19 patients; MRM, multiple reaction monitoring; nCOV, non-COVID-19 volunteers; NEP, neutral endopeptidase; POP, prolyl oligopeptidase; RAS, renin-angiotensin system; SPE, solid phase extraction; THOP, thimet oligopeptidase.