Circulating Plasma Concentrations of ACE2 in Primary Aldosteronism and Cardiovascular Outcomes

Running title: pACE2 vs. clinical outcomes in PA after Adrenalectomy

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Key words: pACE2, ADAM17, TMPRSS2, primary aldosteronism, adrenalectomy, MRA

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Abbreviations:

ACEi, ACE inhibitor;
ACE2, angiotensin-converting enzyme 2;
ADAM17, metalloprotease 17;
Af, atrial fibrillation;
Ang II, angiotensin II;
APA, aldosterone-producing adenoma;
ARR, aldosterone-renin ratio;
AT1R, angiotensin I receptor;
biPA, bilateral PA;
BP, blood pressure;
CABG, coronary artery bypass graft;
CKD, chronic kidney disease.
CHF, congestive heart failure;
CVD, cardiovascular diseases;
EH, essential hypertension;
GAM, generalized additive model;
GEO, Gene Expression Omnibus;
NCBI, National Center for Biotechnology Information;
PA, primary aldosteronism;
PAC, plasma aldosterone concentration;
PASO, Primary Aldosteronism Surgery Outcome;
PBMCs, peripheral blood mononuclear cells,
PCR, polymerase chain reaction;
PRA, plasma renin activity;
MACE, major cardiovascular events;
MRA, minorcorticoid receptor antagonist;
MI, myocardial infarction;
RAAS, renin- angiotensin -aldosterone system;
uPA, unilateral PA;

Context: The plasma concentrations of angiotensin-converting enzyme 2 ([pACE2) has been independently associated with cardiovascular diseases.

Objective: Higher [pACE2] concentrations could be found in patients with primary aldosteronism (PA) and might lead to increased cardiovascular events.

Methods: Using an inception observational cohort, we examined [pACE2] among 168 incident patients with PA. The expression of ACE2, serine protease 2 (*TMPRSS2*), and metalloprotease 17 (*ADAM17)* were assessed in peripheral blood mononuclear cells (PBMCs).

Results: Incident PA and EH patients had similarly elevated [pACE2] (47.04±22.06 vs. 46.73±21.06 ng/ml, p= 0.937). Age was negatively (β, -2.15, p= 0.033) and higher serum potassium level (β, 2.29, p= 0.024) was positively correlated with higher [pACE2] in PA patients. Clinical complete hypertension-remission after adrenalectomy (PASO criteria) was achieved in 36 (50%) of the 72 surgically-treated uPA patients. At follow-up, the [pACE2] decreased in surgically-treated patients who had (p< 0.001) or had no (p= 0.006) hypertension-remission, but the [pACE2] attenuation was not significant in uPA (p= 0.085) and biPA (p= 0.409) administered with minerocorticoid receptor antagonist (MRA). Persistently elevated [pACE2] (> 23ng/ml) after targeted treatments was related to all-cause mortality and cardiovascular events among PA patients (HR, 8.8, p=0.04); with a mean followed up of 3.29 years. *TMPRSS2* mRNA expression was higher in uPA (p= 0.018) and EH (p= 0.038) patients than that in normotensive controls; it was also decreased after adrenalectomy (p< 0.001).

Conclusions: PA and EH patients had elevated [pACE2] and higher expression of *TMPRSS2* mRNA compared to those of normotensive population. Persistently elevated [pACE2] (> 23ng/ml) after targeted treatments was associated the risk of mortality and incident

cardiovascular events.

Keywords: angiotensin-converting enzyme 2 (ACE2), aldosterone, hyperaldosteronism, primary aldosteronism, COVID-19

Introduction

Primary aldosteronism (PA) is the most common cause of secondary hypertension and is reportedly present in 5%–20% or more of hypertensive patients(1,2). PA occurs independent of the physiological autoregulation of renin- angiotensin- aldosterone system (RAAS) and is not suppressed by sodium loading. Despite similar severity of hypertension, PA patients have higher cardiovascular morbidities and mortality than those without it(2).

Angiotensin-converting enzyme 2 (ACE2) is present on endothelial cells and has been identified as an important RAAS counter-regulator, capable of mitigating deleterious actions mediated by angiotensin II (Ang II) and angiotensin I receptor (AT1R)(3). The plasma form of ACE2 (pACE2) is derived via proteolytic shedding of membrane-bound ACE2. However, elevated plasma ACE2 concentrations [pACE2] could play a pivotal detrimental role in the regulation of blood pressure (BP), diabetes, heart failure, coronary heart disease, and chronic kidney disease(4-6). Higher [pACE2] has been associated with an increased risk of greater disease severity(7), all-cause mortality, cardiovascular and even non-cardiovascular deaths(8). Elevated [pACE2] is the highest-ranked predictor of mortality compared with other established risk factors for cardiovascular diseases (CVD)(7,8), long-term cardiac death(9) and has been identified as a

circulating indicator of diabetes, biological ageing, coagulopathy, and mortality(7).

As a novel negative regulator of the RAAS and as the SARS-CoV-2 targeted receptor for the virus to infect humans, ACE2 provides a fundamental connection between viral infection, CVD and immunity(10). Additionally, plasma ACE2-SARS-CoV-2 fusion particles could also be threatening since they can interact with endothelial membrane-bound ACE2, resulting in endothelial damage(11). The imbalance of ACE2 was shed by desintegrin and metalloproteinase domain 17 (ADAM17), along with specific genetic factors that are mainly associated with type II transmembrane serine protease (TMPRSS2) expression(12). The interactions of ACE2, ADAM17 and TMPRSS2 in concert facilitate SARS-CoV-2 viral entry(12) setting the stage for the development of COVID-19 infection.

Despite overwhelming evidence that MRA or adrenalectomy treatment reduces morbidity and mortality in PA patients, the potential impact on the susceptibility for ACE2 expression has encouraged further investigations into the effect of respective targeted treatments, especially during the COVID-19 pandemic period. Given that the over-activity of aldosterone and dysregulation of the RAAS are implicated in the pathophysiology of PA(13) and the data on [pACE2] before and/or after targeted treatments with adrenalectomy or MRA is lacking, this study aimed to understand whether [pACE2] could be differentially expressed or regulated in PA patients and be associated with the outcomes by various treatments. We further identified the expressions of ACE2, ADAM17 and TMPRSS2 in patients' peripheral blood mononuclear cells (PBMCs) to gain insight into the hypertensive aldosterone- phenotype of PA patients.

Materials and Methods

Data sources and study population

Patients aged >18 years who had been diagnosed with PA were prospectively recruited from January 2017 to January 2020 and were monitored until January 2021. We matched essential hypertension (EH) patients as control group.

Screening, confirmation, and the subtype identification of incident patients with PA were performed in patients with hypertension according to the standard TAIPAI protocol and aldosteronism consensus in Taiwan(14). All original anti-hypertensive medications were discontinued for at least 21 days before PA screening and confirmatory tests. Doxazosin and/or diltiazem were administered to control markedly high BP during the work-up stage when required. The diagnosis of PA in hypertensive patients was based on the inappropriate hypersecretion of aldosterone and according to the fulfillment of the standard criteria(15). (methods detailed in the Supplementary Files, sfigure 1(16))

Confirmation tests

Fulfillment of the following three criteria confirmed a diagnosis of PA: (1) autonomous excess aldosterone production evidenced with an aldosterone-renin ratio (plasma aldosterone concentration (PAC)/plasma renin activity (PRA); ARR) > 35(ng/dL)/(ng/mL/h); (2) a TAIPAI score larger than 60%; or (3) post-saline loading PAC > 16 ng/dL, or PAC/PRA > 35 (ng/dL)/(ng/mL/h) shown in a post-captopril/losartan test(14).

Peripheral blood mononuclear cells (PBMC) isolation

Whole blood samples were collected from PA, EH patients and normotensive controls and subjected to PBMC isolation using the Ficoll density-gradient separation approach, as previously reported(17).

Ethical approval of the study protocol

The study complied with the Declaration of Helsinki and was approved by the National Taiwan University Hospital Research Ethics Committee (No. 200611031R, 201901114RIND). All participants received comprehensive written information and signed a consent form before their inclusion in the study.

Measurement of plasma ACE2 concentrations

The plasma concentrations of ACE2 ([pACE2]) were measured using a commercially available sandwich enzymatic immunoassay via following the manufacturer's recommendations (Wuhan Fine Biotech Co., Ltd., Wuhan, China; FineTest Cat# EH0027, RRID:AB_2920799, https://scicrunch.org/resolver/AB_2920799)(18,19). The range of the kit is 0.391–25ng/ml, and the sensitivity is 0.234ng/ml (Supplementary Methods).

Outcome measurements

Primary composite outcome were all-cause mortality and cardiovascular events included *de-novo* (incident) major cardiovascular events (MACE), atrial fibrillation (Af) and/or congestive heart failure (CHF) after the index date of PA confirmation. MACE was defined as the incidence of major cardiovascular events that include non-fatal myocardial infarction (MI), coronary artery bypass graft (CABG), nonfatal stroke, positive findings in coronary angiography(20,21). To corroborate long-term outcome events, we have further validated TAIPAI records with the

Taiwan National Health Insurance Research Database (referring to Supplement).

Secondary outcome was according to the Primary Aldosteronism Surgery Outcome (PASO) consensus on clinical and biochemical outcomes (stable 1) (16,22). Patients were evaluated monthly for the first 3 months postoperatively and every 3 months thereafter. PA patients treated with MRA were monitored every 3 months.

Determination of ACE2 / ADAM17/ TMPRSS2 expression

Total RNA was extracted from PBMCs using a column-based method with Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA, USA). RNA quality was assessed by the Nanodrop ((Thermo Fisher Scientific, Waltham, MA, USA) to ensure that the yield of RNA was sufficient for polymerase chain reaction (PCR) sequencing analysis. Reverse transcription was performed using 2 ug of total RNA, and real-time PCR of *ACE2/ADAM17/TMPRSS2* was performed with a Fast SYBR™ Green Master Mix (Thermo Fisher Scientific) by using a CFX96 Real-Time PCR Detection System and CFX Manager Software (Bio-Rad, Hercules, CA, USA) (sTable 2)(16). Relative transcript levels were obtained with normalization to GAPDH transcript levels.

Sample size calculation

The study was paired sample designed to have a type I error level of 0.01 and type II error level of 0.01. We showed the minimum required number of pairs was 154 and the power was 90%. (supplementary methods)

Statistical analysis

A two-tailed *p* value <0.05 was considered statistically significant. Cox regression models with time-varying covariates accounted for their influences on risk of outcome of interesting. Time-

varying covariates took the value 0 before the start of surgery treatment and could switch to 1 at the start of treatment. Date of censoring was defined as the earliest of the date of death or cardiovascular events of study subjects during follow-up, date of follow-up termination whichever comes the earliest. We also calculated E-values to assess how strong an unmeasured confounder would be necessary to disregard an observed treatment outcome relationship(23). Continuous variables are expressed as mean ± standard deviation or median (interquartile interval) as appropriate. A normal distribution was attained by appropriate transformations of skewed variables as PRA and ARR.

Statistical analyses were performed using Stata 14.2 MP (Stata Corporation, College Station, TX, USA) and R software, version 3.4.4 (Free Software Foundation, Inc., Boston, MA, USA).

Results

Baseline characteristics

We enrolled 168 consecutive patients of newly identified PA (56.0% women; mean 54.5 years), 40 patients of EH (52.5% women; mean 50.2 years), and 24 normotensive patients (54.2% women; mean 52 years) during the study period (Table 1). PA patients had higher BP, higher PAC, higher ARR, lower PRA and lower serum potassium concentration ([K]) than those of the EH patients. Before enrollment, more PA patients were administered with β-blocker, and fewer with ACEi than EH patients. EH patients had lower LogARR, lower systolic BP (SBP), higher [K], and higher PRA than either unilateral PA (uPA) or bilateral PA (biPA) (Figure 1). Furthermore, 38 uPA, 29 EH and 24 normotensive patients agreed to have their mRNA expressions in PBMCs evaluated (stable 3)(16). Patients of PA and EH had higher expression of ACE2 than

normotensive individuals.

Factors related to pACE2

uPA, biPA and EH patients had similar [pACE2] that were significantly higher than that of the normotensive controls (54.7± 20.8, 49.7± 21.7, 53.9± 16.6, vs. 30.7± 13.9 ng/ml, respectively; all $p< 0.05$; Figure 1). EH patients had similar K levels, but had higher PAC, SBP and lower PRA as well as ARR than normotensive controls (Figure 1).

sTable 4 (16)summarizes the approaches used to model the relationships between [pACE2] and one or more underlying clinical and biochemical parameters. Age was negatively (β, -2.15, p= 0.033), while potassium levels (β, 2.29, p= 0.024) were positively associated with [pACE2] in the multivariate linear regression model.

A correlation matrix showed limited biomarkers directly associated (Figure 1a), and the linear correlation network depicted very few baseline characteristics that were closely correlated with pACE2 (Figure 1b, sFigure 2)(16). A generalized additive model (GAM) plot showed that plasma potassium levels were positively yet non-linearly correlated to pACE2 among patients with PA ($p< 0.005$) (sFigure 3)(16).

Characteristics of PA patients before and after targeted treatments

In those who underwent adrenalectomy, regardless of their complete clinical success (hypertension-remission, n= 36) or not (n= 36), their [pACE2] and PAC were significantly attenuated, while their potassium level and PRA levels were increased in comparison to their pre-operative data (Table 2, Figure 2a). The [pACE2] after adrenalectomy, in both the hypertension-remission and hypertension-uncured groups, were significantly reduced and

similar to those of normotensive controls (34.6±23.7, 36.7±19.5, vs. 30.7±13.9 ng/ml, respectively, both P> 0.05). Intriguingly, in multivariate regression modules, pACE2 were neither related to clinical hypertension-remission (p= 0.891) or biochemical remission (p= 0.555) according to the PASO criteria.

There were 96 incident patients with PA (50 uPA; 46 biPA) treated with MRA for at least one year during the study period. After MRA treatment, the PRA and potassium levels were increased, while BPs were decreased. However, the [pACE2] did not significantly change in uPA (p= 0.085) or biPA (p= 0.409) patients after MRA treatment (Table 2) (Figure 2b).

Post treatment [pACE2] associated with all-cause mortality and incident cardiovascular events

After a follow-up of 3.29±0.57 years among PA patients, 5 (3.0%) expired, 14 (8.3%) had MACE, 5 (3.0%) had Af, while 7 (4.2%) had CHF. Since the median concentration of pACE2 in healthy volunteers is 23ng/mL (22, 23), we validated if [pACE2] > 23ng/ml is a risk predictor. In the Cox proportional hazard model, [pACE2] > 23ng/ml after targeted treatments was a risk factor associated with all-cause mortality and cardiovascular events. (HR, 8.8, p=0.004).

The relative risk for all-cause mortality and cardiovascular events was 4.2, while E-value for the point estimates was 7.9. This analysis indicated no substantial unmeasured confounding.

Subgroup analysis: factors affecting [pACE2]

To delineate the [pACE2] in PA versus EH patients, we further performed subgroup analysis. The forest plot showed that the use of anti-hypertensive medications, even RAAS inhibitors, the duration of hypertension, diabetic status, the statuses of hypokalemia, larger adenomas, chronic kidney disease etc. did not confound the [pACE2] (Figure 2c).

The expression of cellular *ACE2/ ADAM17/TMPRSS2* among PBMCs

We showed that the expression of cellular *ACE2* and *ADAM17* mRNA in PBMCs was not different among uPA, EH patients and normotensive controls. However, *TMPRSS2* expression was lower in the normotensive controls (0.66±0.42 mRNA folds change (FC)) than that in uPA (1.66 ±1.80 FC, p= 0.018) and EH (1.45±1.61FC, p= 0.038) patients. Of note, there was no difference in the expression of *ACE2* and *TMPRSS2* between PA and EH patients (p= 0.696). The chronological changes of *TMPRSS2* mRNA in PBMCs decreased after adrenalectomy (n=10, 2.41± 2.52 to 1.60 ±1.69 FC, p< 0.001), but even though the cellular TMPRSS2 mRNA expression at one year in uPA patients who was treated with MRA showed a trend of decrease, there was no statistically significant change (n=10, 1.91±1.90 to 1.57±1.75 FC, p= 0.054). (Figure 3)

To further explore the interplay of *ACE2/ADAM17/TMPRSS2* and RAAS, we used expression profile available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (From GEO Query DataSets for GSE71994). The expression of cellular*TMPRSS2* is positively correlated with that of cellular *ACE2* but negatively correlated with that of *ADAM17* in PBMCs. (supplementary methods, sFigure 4)(16)

Discussion

We found that PA patients, including both uPA and BiPA, had elevated [pACE2] similar to that of EH patients; their [pACE2] were higher than that of the normotensive controls. We also revealed that adrenalectomy attenuated [pACE2] in uPA patients, regardless of hypertension-remission or not; similar observation was not found in uPA or biPA patients who underwent MRA treatment. The [pACE2] were positively, yet non-linearly, correlated with the younger patients and serum potassium levels in PA patients. After targeted treatments, higher level of

[pACE2] was associated with a greater risk of long-term mortality and incident cardiovascular events. We further showed that there was higher *TMPRSS2* expression in the PBMCs of uPA and EH patients than that of the normotensive population, but their *ADAM17* and *ACE2* mRNA expression in PBMCs were all similar. Arguably, we speculate that elevated [pACE2] and cellular TMPRSS2 expression might be associated with increased risk and severity to SARS-CoV-2 infection in PA patients due to its disease mechanism involving the binding of its spike protein to ACE2(24,25). **(sFigure 5)(16)**

High [pACE2] in PA and EH patients and its clinical relevance

In a previous observational study, [pACE2] was positively associated with biomarkers reflecting myocardial injury and neurohormonal activation, cardiac injury, heat failure(26), stroke(27) and all-cause mortality. Importantly, [pACE2] are usually low in healthy population(28), and higher in patients with CVD,(29) which could correlate with the extent of tissue damages or CVD progression. In our patients who underwent targeted treatments, their post-treatment [pACE2] could predict their cardiovascular outcomes. High [pACE2] was correlated with makers of inflammation as well as endothelial dysfunction(30).

The association between SARS-CoV-2 and ACE2 points to the frequent involvement of hypertension during COVID-19 pathogenesis (31) and mechanisms that directly link to COVID-19 features in the lung, including inflammation, oxidative stress and fibrosis(32). A recent paper confirmed that small molecules halofuginone and homoharringtonine could block TMPRSS2 activity and lead to marked resistance to SARS-CoV-2 infection—a piece of direct evidence showing attenuating TMPRSS2 expression/activity maybe the key to prevent or treat the infection(33); our finding of diminished TMPRSS2 expression after adrenalectomy in uPA

patients could hypothetically achieve similar benefit.

Our study found that unilateral PA patients had attenuated [pACE2] and TMPRSS2 levels after adrenalectomy, regardless of their hypertension-remission. Thus, theoretically when these uPA patients have mitigated TMPRSS2 expression and lower [pACE2] in the circulation and tissue fluid after adrenalectomy, their risks of SARS-CoV-2 infection would also be decreased.

Factors related to [pACE2]

Our study showed that [pACE2] had a nonlinear association with younger PA patients and those PA patients with relatively higher serum [K] is noteworthy, as it may be due to the lower potassium concentration and younger population of PA patients in nature. ACE2 was reported to decrease the RAAS activity and thus related to attenuated potassium excretion from renal collecting tubules(34). Interestingly, hypokalemia appears to be a prominent biological marker of ACE2 down-regulation in patients with Covid-19 infection(35).

[pACE2] decreased in PA patients treated with adrenalectomy, but not with MRA

MRAs increases ACE2 mRNA expression and activity in patients with chronic heart failure as well as in a hypertensive rat model(36-38). In heart failure patients, the ACE2 activity and ACE mRNA are increased after short-term MRA treatment(36); however, the long-term expression of ACE2, including [pACE2] changes, after MRA treatment has been lacking. Decrease in pACE2 during a weight loss diet intervention is associated with amelioration in metabolic health, fat mass, and markers of angiotensin peptide. In this study, we showed unprecedentedly that [pACE2] did not significantly change after MRA treatment in patients with uPA and biPA. In recent studies, adrenalectomy in patients with uPA was found to decrease glucocorticoid

secretion, restore osteoporosis, attenuate adverse metabolic risks and improve the quality of life (39,40), attributed to decreased glucocorticoid levels in addition to mineralocorticoid excess(41). Thus, we propose that adrenalectomy should be the treatment of choice for feasible uPA patients, and every effort should be made for early diagnosis of PA, especially uPA, in order to prevent its associated long-term morbidities, and to benefit from the high possibility of hypertension-remission and biochemical advantages like the decrease of [pACE2] in uPA patients after ipsilateral adrenalectomy.

[pACE2] and ACE2 activity

A previous study measured ACE2 catalytic activity, by way of a quenched fluorescent substitute assay, showed that it could be related to CVD(36,42). Most importantly, augmented [pACE2] is associated with adverse cardiac risks(43) and outcomes(44). However, the pathophysiological mechanisms to explain the apparent discrepancy between the negative prognostic impact of [pACE2] versus the protective effects of member-bound ACE2 remains unexplored. It is likely that there could be complex interactions between cellular expressions, enzymatic shedding, and impaired pACE2 plasma clearance and therefore changes of truncated pACE2 concentrations(8).

Subgroup analysis

ARBs have been reported to alter ACE2 expression more consistently in several studies, both at the mRNA and protein levels(45). Yet, in heart failure patients, [pACE2] was not associated with ACE inhibitor (ACEi) or ARBs use(24). In line with this, our finding suggested that PA patients who were treated with ARBs, ACEi or MRA before the PA confirmation period did not interfere

with our conclusion in parallel subgroup analyses. [pACE2] has been demonstrated to decrease in men without chronic kidney disease (CKD) and that it is independently associated with other classical CV risk factors, such as advanced age and diabetes(46). Our forest plots showed that increased [pACE2] in PA patients was independent of their comorbidities of DM, CVD or CKD.

The expression of ACE2, ADAM17 and TMPRSS2 in PBMCs

*The decreased expression of ACE2 and TMPRSS2 in circulating PBMCs have implications for lower ris*k of SARS-CoV-2 infection and/or the severity of COVID-19(33,47). As pACE2 holds the binding site for SARS-CoV-2, it is possible that sequestration of SARS-CoV-2 by pACE2 may enable cell entry of the virus into tissues where member-bound ACE2 is poorly expressed. The attachment of the S protein of SARS-CoV-2 to ACE2 triggers ADAM17 activation, and leads to increasing membrane ACE2 down-modulation, and reducing surface ACE2 expression. The cytoplasmic tail cleavage of membranous ACE2 was achieved by synergistic action of TMPRSS2 and ADAM17(47). There were reports indicating increased expression of TMPRSS2 induced by hormones or the coexistent specific genetic variants(48,49), which may lead to exacerbation of membranous ACE2 cleavage—possibly enabling SARS-CoV-2 to enter the cells. This finding was supported by the positive correlation between *TMPRSS2* and *ACE2,* while negative correlation between *TMPRSS2* and *ADAM17* from the GEO expression profile in hypertension patients from our results. As TMPRSS2 is expressed outside of the lung and can therefore contribute to the extrapulmonary spread of viruses, our results raised a suspicion that PA patients could be more likely to suffer from SARS-CoV-2 infection because of their higher level of TMPRSS2 expression. This is further attested by the recent paper *t*hat small molecules halofuginone and homoharringtonine blocked TMPRSS2 activity and led to marked resistance to SARS-CoV-2

infection; such direct evidence of attenuating TMPRSS2 expression and lowering risks of infection could be achieved among the uPA patients who undergo ipsilateral adrenalectomy(33). Thus, we speculated that the enriched *TMPRSS2* in PBMCs is correlated with the p*ACE2* expression.

Limitation

This study has several limitations. First, while ACE2 is the main cellular port of entry for SARS-CoV-2, it is imperative to note that we have not directly measured the susceptibility for a SARS-CoV-2 infection by way of ACE2 *in vitro* or for a deleterious disease progression *in patients of PA*. Some risk factors, e.g. male gender, diabetes, were reported to be independent risks correlating to [pACE2](7), but it was not found in our analysis. This was partly due to specific characteristics of our patients with PA. Second, although we held all anti-hypertensive medications that could interfere with the RAAS during the screening and confirmatory stages, there are some physiological states that could also disconcert the [pACE2]. Third, [pACE2] may not necessarily be parallel to membrane-bound ACE2 expression because ACE2 shedding is mediated by disintegrin and ADAM-17 and may not be relevant to the total extent of tissue-bound ACE2 activity. The equilibrium between circulating pACE2 and membrane-bound ACE2 remains, to the best of our knowledge, incompletely understood(51). Assays using fluorogenic substrates demonstrate that pACE2 can hydrolyze angiotensin-I and angiotensin-II analogues. The truncated plasma form of ACE2 could further indicate the production of angiotensin-(1–9) and angiotensin-(1–7), by mass spectrometry(52). Fourth, we hypothesized that the increased expression of TMPRSS2 mRNA from PBMCs could lead to the susceptibility to SARS-CoV-2

infection. It warrants further exploration of TMPRSS2 as a potential target for viral spread and infectivity; even recent article has shown attenuating TMPRSS2 expression by small molecules lowered the risks of SARS-CoV-2 infection. Epidemiologic studies in large cohorts of COVID-19 cases, even with patients of PA and appropriate controls, are required to confirm this hypothesis. Fifth, short follow-up time and the low number of subjects with MACE makes the study susceptible to an alpha error. However, the E-values of our analysis are greater than known relative risk, unmeasured confounding cannot explain away the ACE2 level and composite outcomes. Importantly, the power analysis showed our enrolled number of PA patients could achieve a power of 90%. Sixth, we did not acknowledge the status of somatic mutation and pACE2 level. Seventh, limited number of EH as well as normotensive were enrolled in this study. Finally, the different comorbidities in the patient's cohorts may substantially confound our results. However, we used the paired t analysis comparing patients' biochemistry data at baseline with their post-treatment data. We also performed multiple univariate logistic and linear regression analysis of [pACE2] to determine potential confounders. Further studies are warranted to evaluate the direct relationship and mechanism of [pACE2] and the susceptibility or severity of COVID-19 disease in PA patients.

Perspectives

MAS is a high affinity functional receptor for angiotensin 1-7 (Ang 1-7) (53). The question of whether MAS, like ACE2, is also differentially up-regulated in PA patients, will be an interesting subject for future studies to address. In hypertensive patients with concomitant COVID-19 infection, ACE2 levels at presentation could not be used in prognosis and mortality of COVID-19

patients(54). Whether up-regulation of [pACE2] has important functional consequences in PA patients in regard to cardiovascular diseases and metabolic abnormalities remains to be determined. However, our emerging data raised the intriguing possibility that targeted therapeutic approaches to PA by way of modulating the arm of RAAS may be achieved through changes to [pACE2]. We demonstrated specific augmented [pACE2] via mononuclear TMPRSS2 in uPA and hypertensive patients. Given the detrimental role of pACE2 in SARS-CoV-2 entry and/or subsequent disease severity at the site of infection and distant cells, monitoring [pACE2] in SARS-CoV-2 comorbid patients could be crucial. Recently, viral entry through angiotensin II type 1 receptor and arginine vasopressin receptor 1B with ADAM17-mediated cleavage of ACE2 has recently been reported as a novel mechanism of SARS-CoV-2 infection(25). These pre-clinical findings suggest that patients with aldosterone-enhanced hypertension may have increased binding affinity to SARS-CoV-2 which might explain why hypertension is a risk factor for higher susceptibility to develop COVID-19. Our report showed that it could be very worthwhile to measure [pACE2] in PA patients, not only as the counter regulator of RAAS, a marker of targeted treatments, but also as an important therapeutic frontier related to cardiovascular events for PA patients.

Conclusions

Our findings suggest that pACE2 concentrations were significantly elevated in EH and various PA patients. The post-treatment persistently elevated pACE2 level > 23 ng/mL was associated with the risk of long-term mortality and incident cardiovascular events. Adrenalectomy in uPA patients, regardless of hypertension-remission, attenuates pACE2 levels; such changes are not

found in uPA or biPA patients who underwent MRA treatment. We further demonstrated the higher expression of *TMPSSR2* in EH and uPA patients compared to that of normotensive controls; such expression was attenuated after adrenalectomy in uPA patients.

Data Availability

Some or all data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Figure legends

Figure 1. (A) The correlation matrix among the levels of various biomarkers. The correlations between the blood pressure, body mass index (BMI) and levels of aldosterone profiles at index enrollment when holding drugs that interfere RAAS were examined. On the contents of the diagonal are the value (logit) of the correlation. Blue color depicted positive correlation; while red color depicted negative correlation.

(B) Correlation network visualized the correlations of pACE2 versus other biochemical and baseline characteristics. pACE-2 was not correlated with other clinical parameters and did not cluster with previously recognized factors by Spearman correlation. Each path represented a correlation between the two variables that it joined. An orange path represented a positive correlation, and a blue path corresponded to a negative correlation.
* The R function network_plot() was used to visualize and explore correlations (***r***). The width and transparency of the path represent the strength of the correlation (wider and less transparent indicated stronger correlation).

Abbreviations: ACE, angiotensin-converting enzyme; ARR, aldosterone to renin ratio; b, before confirmation test; BiPA, bilateral primary aldosteronism; BMI, body mass index; b, before confirmation test; Cre, creatinine; dBP, diastolic blood pressure ; EH, essential hypertension, K, potassium; PAC, plasma aldosterone concentration; p, post-confirmation test; PRA, plasma renin activity; sBP, systolic blood pressure; uPA, unilateral primary aldosteronism,

(C). The differences of baseline biochemistry data in patients with PA, essential hypertension, when compared with normotensive controls. Violin plots showed the difference of plasma ACE2 levels, plasma aldosterone concentration, plasma renin activity, aldosterone/ratio, serum potassium concentrations and systemic blood pressure.
Abbreviations: ACE, angiotensin-converting enzyme; ARR, aldosterone to renin ratio; BiPA, bilateral primary aldosteronism; EH, essential hypertension, K, potassium; PAC, plasma aldosterone concentration; N, normotensive population; PRA, plasma renin activity; uPA, unilateral primary aldosteronism.
¶ the analysis consisted independent t-tests with normotensive population.
§ Log transformation was applied for skewed distributions, such as ARR and PRA.
*, $p<0.05$

Figure 2

Violin plots showed the differences of pACE2 levels of
(A) in patients with essential hypertension (EH), uPA patients at index date, who underwent MRA therapy for 1 year, who had complete clinical remission after adrenalectomy, who were uncured at 1 year after adrenalectomy, and normotensive controls†.
(B) biPA patients with essential hypertension, biPA at index date, who underwent MRA therapy after 1 year, and normotensive controls†.
¶compared with uPA patients who received MRA for at least one year.
§ compared with uPA patients who were clinical remission after adrenalectomy for one year.
‡ compared with uncured uPA patients after adrenalectomy for one year.
++ compared with biPA patients received MRA for at least one year.
† using unpaired t test.
Abbreviations: pACE, plasma angiotensin-converting enzyme; APA, aldosterone producing adenoma, BiPA, bilateral PA, EH, essential hypertension, K, potassium; MRA, minorcorticoid receptor antagonist; PAC, plasma aldosterone concentration; PRA, plasma renin activity; uPA, unilateral PA.

(C). Forest plot depicts odds ratio (OR) and 95% confidence interval (CI) derived from multivariate logistic regression analyzing the risk of increased pACE2 expression compared with essential hypertension for multiple clinical variables¶.
Abbreviations: ACE, angiotensin-converting enzyme, ACEi, angiotensin-converting enzyme inhibitor, APA, aldosterone-producing adenoma; ARB, Angiotensin Receptor Blocker; HTN, hypertension; eGFR, estimated Glomerular filtration rate; OR, odds ratio.
¶ OR was adjusted with age, gender, body mass index, blood pressure, plasma aldosterone concentration, plasma renin activity.

Figure 3. Plots depict (A) Quantity expression of the mRNA folds between unilateral PA, essential hypertension and normotensive patients (B) the temporal fold change of the mRNA before, post operation and MRA treatment in unilateral PA by using a quantitative real-time polymerase chain reaction with gene-specific primers in the complementary DNA synthesis.
Abbreviations, ACE2, angiotensin-converting enzyme 2; ADAM17, Tumor Necrosis Factor-α Convertase; APA, aldosterone producing adenoma; EH, essential hypertension, N, normotensive controls; OP, operation; TMPRSS2, type II transmembrane serine protease.
*, $p<0.05$

Tables for Circulating Plasma Concentrations of ACE2/ *TMPRSS2/ ADAM17* in Primary Aldosteronism and Cardiovascular Outcomes

by Wu et al.

Table 1. Baseline characteristics of patients with essential hypertension and primary aldosteronism.

Characteristics	***EH***	**PA**	***Normotension***	***p(EH vs PA)***	***p(PA vs Nor)***
Case numbers, n (%)	40	168	24		
Age (yr)	50.15±13.49	54.48±11.05	52.0±11.4	0.122	0.313
Female, n (%)	21 (52.5%)	94 (56.0%)	13 (54.2%)	0.665	0.845
Body mass index (kg/m^2)	24.72 [22.25-27.64]	24.82 [22.06-27.56]	23.5 [20.9- 25.7]	0.88	<0.001
Smoker, n (%)	4 (10.0%)	11 (6.5%)	2 (9.1%)	0.48	0.635
Duration of hypertension (yr) (log)	0.70 [0 - 1]	0.78 [0.3 - 1.18]	NA	0.031	NA
Systolic blood pressure (mmHg)	143.12±20.60	152.51±20.59	117.1±12.4	0.01	<0.001
Diastolic blood pressure (mmHg)	86.53±15.01	91.36±13.34	71.0±10.4	0.046	<0.001
Diabetes mellitus, n (%)	5 (12.50%)	33 (19.76%)	NA	0.287	NA
Creatinine (mg/dL)	0.8 [0.75 - 0.9]	0.8 [0.7 - 1]	0.7 [0.6 - 0.9]	0.595	0.011
Serum potassium (mEq/L)	4.07±0.35	3.73±0.62	4.2±0.4	<0.001	<0.001
Plasma ACE2 level (ng/ml)	54.7± 20.8	53.9± 16.6	30.7± 13.9	0.937	0.007
Before confirmation†					
α-Blocker user	8 (20.0%)	34 (20.2%)	NA	0.96	NA
β-Blocker user	8 (20.0%)	63 (37.5%)	NA	0.04	NA
ARB user	19 (47.5%)	97 (57.7%)	NA	0.226	NA
ACEi user	10 (25.0%)	8 (4.8%)	NA	<0.001	NA
Plasma aldosterone concentration (ng/dL)	34.75 [20.44 - 47.67]	43.95 [30.32 - 60.44]	13.5 [11.8 - 16.4]	0.036	<0.001
Plasma renin activity (ng/mL/hr)	2.2 [0.2 - 6.3]	0.2 [0.1 - 0.6]	1.12 [0.58 - 1.34]	<0.001	<0.001
Log ARR§	1.47±0.84	2.32±0.69	1.71±0.43	<0.001	<0.001

Data are presented as the mean [standard deviation] for normally distributed data and median [interquartile range] for non-normally distributed data.

Abbreviations: ACE, angiotensin-converting enzyme, ACEi, angiotensin-converting enzyme inhibitor, APA, aldosterone-producing adenoma; ARB, Angiotensin Receptor Blocker; ARR, aldosterone to renin ratio; EH, essential hypertension; PA, primary hyperaldosteronism; yr, year.

† All anti-hypertensive medications that will interfere the RAAS were discontinued before PA confirmation tests.

§Log transformation was applied for skewed distributions, such as ARR.

Table 2. Baseline clinical and biochemical characteristics of PA patients after targeted treatment¶.

Disease Type	***uPA***						***uPA***			***biPA***		
Biochemistry and clinical data†	**Pre-OP**	***Post-OP Clinical remission***	***p***	**Pre-OP**	***Post-OP Uncured HTN***	***p***	***Pre-MRA***	***Post-MRA***	***p***	***Pre-MRA***	***Post-MRA***	***p***
Case number, n	36			36			50			46		
Serum potassium (mmol/L)	3.8±0.7	4.4±0.3	<0.001	3.8±0.6	4.2±0.4	<0.001	3.62±0.51	4.22±0.45	<0.001	3.85±0.51	4.26±0.47	<0.001
Plasma aldosterone level (ng/dL)	55.7±43.4	31.1±19.7	0.007	44.9±23.7	28.0±22.2	0.012	56.6±32.6	63.4±37.7	0.293	47.9±23.9	51.6±36.8	0.496
Plasma renin activity (ng/mL/hr)	0.21±0.19	3.37±2.41	<0.001	0.79±0.42	3.44±0.72	0.001	0.55±1.01	1.72±1.97	<0.001	0.57±0.74	2.36±2.17	<0.001
Systolic blood pressure (mmHg)	139.8±16.8	121.7±11.7	<0.001	164.1±19.5	142.2±19.2	<0.001	153.8±223.6	146.0±23.0	0.025	151.6±15.9	144.0±18.1	0.009
Diastolic blood pressure (mmHg)	84.1±11.8	77.1±8.2	<0.001	98.0±13.4	87.0±14.0	<0.001	90.2±15.8	86.9±13.6	0.231	92.2± 10.9	87.7±11.4	0.007
Plasma ACE2 (ng/ml)	56.8±24.6	31.2±14.4	<0.001	52.5±15.9	36.7±19.5	0.006	45.7±22.8	39.9±27.7	0.085	49.7± 21.7	45.4±29.9	0.409

Abbreviations: ACE, angiotensin-converting enzyme, OP, operation, MRA, minorcorticoid receptor antagonist.

¶the analysis consisted of paired t-tests.

† Data after holding the medications that will interfere the renin-angiotensin-aldosterone system

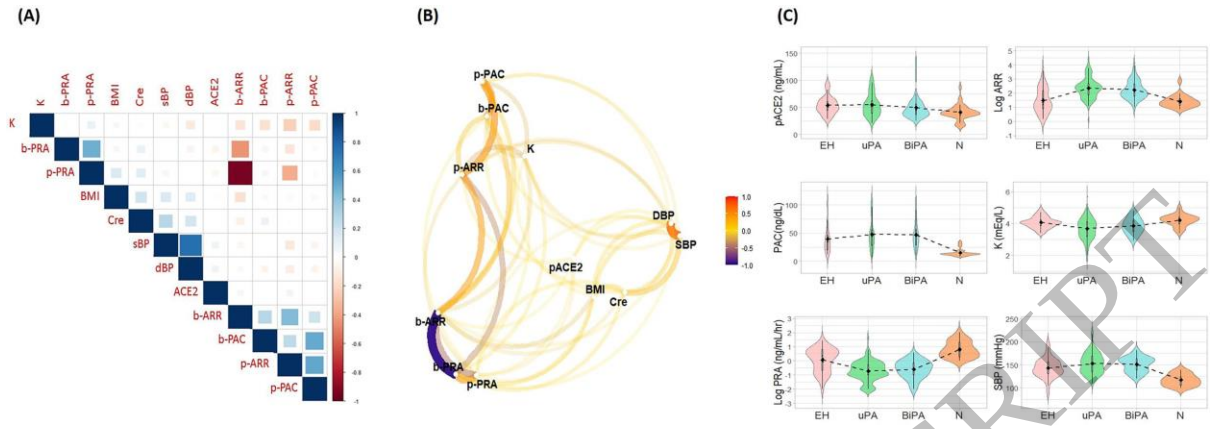

Figure 1
159x56 mm (x DPI

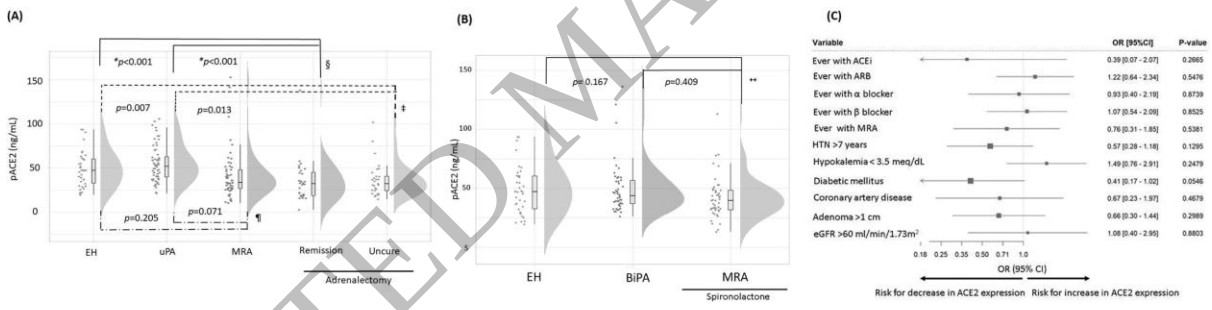

Figure 2
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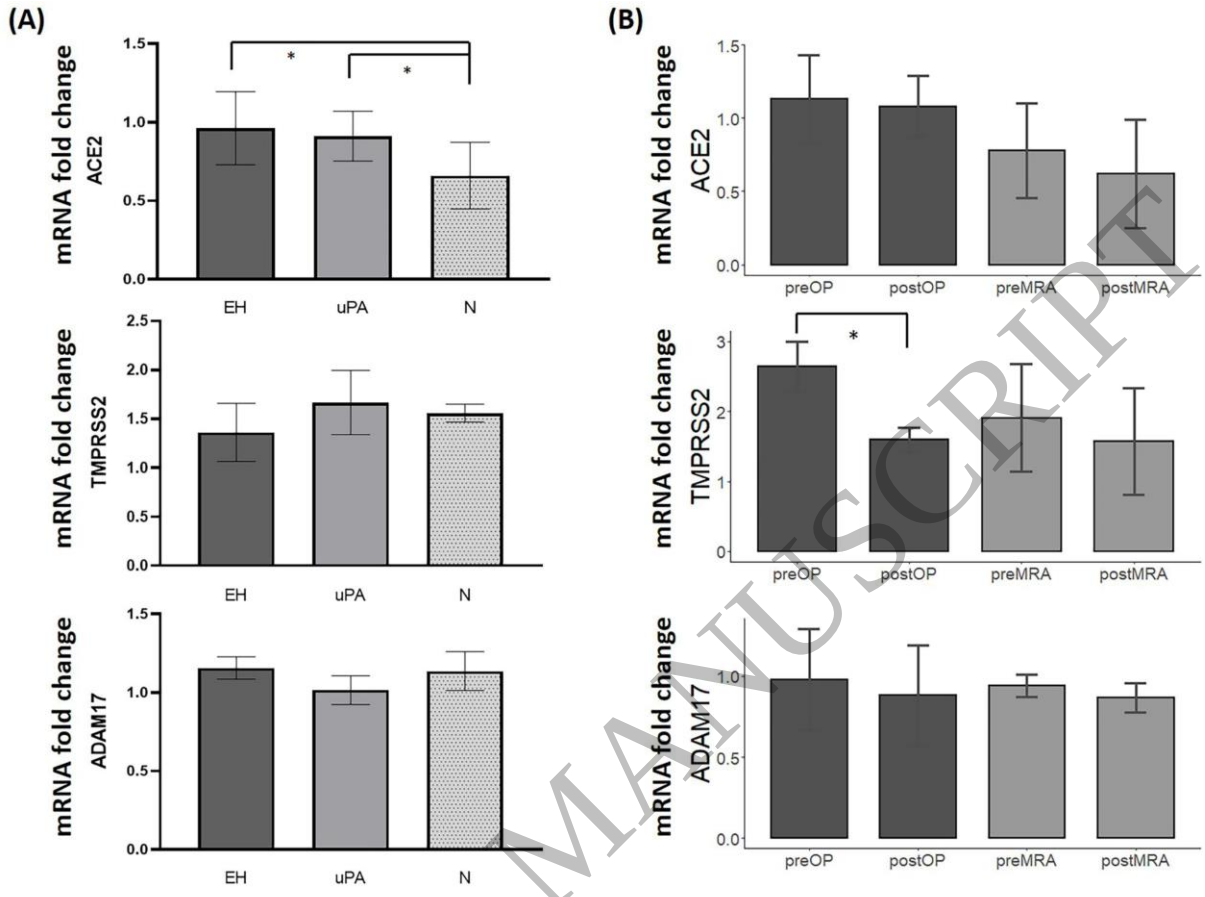

Figure 3
159x117 mm (x DPI)