The Role of Plasma ACE2 and Angiotensin Peptides in Heart Failure and Coronavirus Disease

by

Kaiming Wang

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Medicine

University of Alberta

Abstract

Background

The discovery of angiotensin-converting enzyme 2 (ACE2) introduced an alternative protective arm of the renin-angiotensin system (RAS), the ACE2/Angiotensin 1-7 (Ang 1-7)/Mas receptor axis, to counterbalance the more renowned pathogenic angiotensin-converting enzyme (ACE)/Angiotensin II (Ang II)/AT_1 receptor axis that predominates in disease states due to RAS hyperactivation. As a crucial endogenous regulator of the RAS, ACE2 possesses a catalytically active ectodomain exposed to the circulation that hydrolyzes various vasoactive peptides including, Ang II and angiotensin I (Ang I), generating Ang 1-7 and angiotensin 1-9 (Ang 1-9), respectively. Notably, ACE2 has garnered international attention as the cellular receptor for severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2, becoming one of the most researched and well-known human proteins. A soluble form of ACE2 is shed from the membrane through proteolytic cleavage by a disintegrin and metalloprotease 17 (ADAM17), resulting in diminished ACE2-mediated tissue protection under pathological conditions. We propose that the dysregulation of plasma angiotensin peptides and ACE2 is a molecular signature of cardiovascular and inflammatory diseases and can further serve as prognostic markers for adverse clinical outcomes in heart failure (Chapter 2) and coronavirus disease-2019 (Chapter 3).

Methods and Results

In Chapter 2, we prospectively enrolled 110 patients with heart failure from outpatient clinics and the emergency department to perform comprehensive profiling of circulating and equilibrium levels of plasma angiotensin peptides using liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques. We found that an elevation in the ratio between Ang 1-7 to Ang II, effector peptides of the protective and harmful axis of the RAS, respectively, served as an

independent and incremental predictor of beneficial outcomes, better survival rate, and reduced hospitalization duration. Although multiple peptidases participate in the proteolytic processing of Ang 1-7 and Ang II, ACE2 is a direct regulator of the Ang 1-7/Ang II ratio, and its dysregulation contributes to RAS imbalance resulting in adverse clinical outcomes.

In Chapter 3, we prospectively enrolled 242 consecutive patients admitted to hospital wards designated for COVID-19 or intensive care units to measure plasma soluble ACE2 and other established disease markers using enzyme-linked immunosorbent assay and angiotensin peptides through LC-MS/MS at admission and repeated sampling on day seven. We found that an upward trajectory in soluble ACE2 was independently associated with an increased risk of mortality and incidence of acute myocardial injury and circulatory shock. In contrast, patients who survived the COVID-19 hospitalization were characterized by a substantial reduction in soluble ACE2. Additionally, we found a prominent elevation in plasma Ang I and Ang 1-7 levels in patients with COVID-19 accompanied by downregulated plasma ACE activity at hospital admission, likely due to the extensive pulmonary vascular endothelial injuries during SARS-CoV-2 infections.

Conclusions

Heart failure and COVID-19 shares mutual disease pathophysiology in forms of dysregulation in ACE2 and the RAS. Importantly, plasma angiotensin peptide and soluble ACE2 levels can be effectively monitored through disease progression to facilitate a personalized biomarker-guided approach for risk stratification and the implementation of medical therapies against these conditions. Moreover, our data suggest a potential role of ADAM17 inhibition and the development of novel strategies to enhance the beneficial ACE2/Ang 1-7/Mas axis to improve patient outcomes in cardiovascular and inflammatory diseases.

Preface

This thesis is an original work by Kaiming Wang. All of the research shown in this thesis that are published in peer reviewed journals are provided below.

The second chapter is published in Circulation Heart Failure as "Wang K, Basu R, Poglitsch M, Bakal JA, Oudit GY. **Elevated angiotensin 1-7/angiotensin II ratio predicts favorable outcomes in patients with heart failure**. *Circ Heart Fail.* 2020; 13(7):e006939". Ethics approval for this study was received from the University of Alberta Health Research Ethics Board, project name "Peptide biomarkers of cardiovascular diseases", No. Pro00026480, January 16th, 2012. I was responsible for collection of research data, performing experiments and statistical analyses, drafting the manuscript, and responding to reviewers.

The third chapter is published in Hypertension as "Wang K, Gheblawi M, Nikhanj A, Munan M, MacIntyre E, O'Neil C, Poglitsch M, Colombo D, Del Nonno F, Kassiri Z, Sligl W, Oudit GY. **Dysregulation of ACE (Angiotensin-Converting Enzyme)-2 and renin-angiotensin peptides in SARS-CoV-2 mediated mortality and end-organ injuries**. *Hypertension.* 2022; 79(2):365-378." Ethics approval for this study was received from the University of Alberta Health Research Ethics Board, project name "CoCollab" No. Pro00100207, July 6th, 2020, and "The renin-angiotensin system and COVID-19: Defining risk and mechanisms of injury", No. Pro00100319, May 6th, 2020. I was responsible for obtaining informed consent from patients, coordinating biospecimen collections, analysis of research data, performing experiments and statistical analyses, drafting the manuscript, and responding to reviewers.

Acknowledgements

I was very fortunate for the wonderful opportunity to complete my graduate studies in an incredibly supportive training environment. For that, I would like to first express my deepest gratitude to my supervisor, Dr. Gavin Y. Oudit, who nurtured my budding interest in research and medicine through his outstanding mentorship and guidance both within and outside the lab. He provided me with ample opportunities to explore my interests while always taking the time to review my progress and address my concerns. His unparalleled passion and dedication to the field are truly inspirational and always drove me to be the best version of myself. I will be forever grateful for this rewarding experience and the once-in-a-lifetime opportunity.

I want to express my sincere appreciation for my committee members, Dr. Finlay McAlister and Dr. Scot Simpson for their invaluable feedback, support, and continuous encouragement during my studies. Thank you to Dr. Paul Jurasz for kindly agreeing to be my external examiner.

I would also like to thank Dr. Bruce Ritchie for supporting my career development and allowing me to work alongside the amazing team at the Canadian BioSample Repository, along with Dr. Wendy Silgl for making COVID-19 biobanking a reality. I am also grateful to Dr. Jeffery Bakal and Erik Youngson for their input and assistance with biostatistics.

A heartfelt gratitude goes to all the present and former members of the Oudit lab for the collegial environment, friendships, and always being there to brighten my day during difficult times. We were truly in this together!

Finally, I would like to express my gratitude to my parents for being my biggest fans and always having faith in my abilities no matter what the outcomes were. I am also extremely thankful for my friends; without them, I would certainly not be the person I am today.

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

1.1. Background

Heart failure is a complex medical syndrome accompanied by repeated hospitalization and high mortality rates. At least 600,000 Canadians are currently suffering from heart failure, with 50,000 new diagnoses each year, causing an enormous burden on patients and our healthcare system.[1] More recently, the COVID-19 pandemic has taken center stage leading to over 500 million individuals infected with SARS-CoV-2 and 6 million death worldwide. SARS-CoV-2 infections damages multiple organs beyond the respiratory system, including the cardiovascular system.[2-7] SARS-CoV-2 enters human cells by binding to the transmembrane enzyme, ACE2, internalizing into the cells with a higher binding affinity for ACE2 compared to the initial SARS-CoV.[8-11] However, ACE2 is also a crucial enzyme required for optimal cardiovascular function; it converts Ang II, a pathogenic peptide, into Ang 1-7, a peptide with cardiovascular protective effects.[12] Hence, loss of ACE2 causes accumulation of Ang II and diminished Ang 1-7, an imbalance in the RAS leading to cardiovascular pathologies, such as heart failure.[12-16] However, the dynamic state of plasma ACE2 and angiotensin peptides under these disease conditions and their relationship to clinical outcomes in patients is not well understood.

1.2. Discovery of ACE2

Following the initial and seminal discovery of renin in 1898 by Tigerstedt and Bergman, the RAS now encompasses a complex network of enzymes, peptides, and receptors.[10,16-23] While many metallopeptidases cluster in small inter-related gene families (e.g., the neprilysin family), unusually, no human homolog of the vasoactive zinc-peptidase angiotensin converting enzyme

(ACE) had been identified at the turn of the century. Almost simultaneously, in 2000, two independent approaches searching for such ACE homologs revealed the existence of a close relative of the *ACE* gene designated *ACEH*[20] or *ACE2*[21]. *ACEH* was cloned from a human lymphoma cDNA library and the identical *ACE2* from a human HF ventricular cDNA library, the latter emphasizing a potential role for ACE2 in CV pathologies. Expression of the *ACE2* gene was initially established in the heart, kidney and, testis, but subsequent studies have shown a much broader distribution, including the upper airways, lungs, gut, and liver (**Figure 1.1.A**). Sequence comparison of ACE and ACE2 strongly suggested that ACE2, like ACE, was an integral transmembrane protein (and ectoenzyme) with a transmembrane anchor close to the C-terminus (type I membrane protein). A close evolutionary relationship existed between the *ACE* and *ACE2*, genes and it was presumed that the two proteins would have similar substrate specificities and involvement in the RAS.

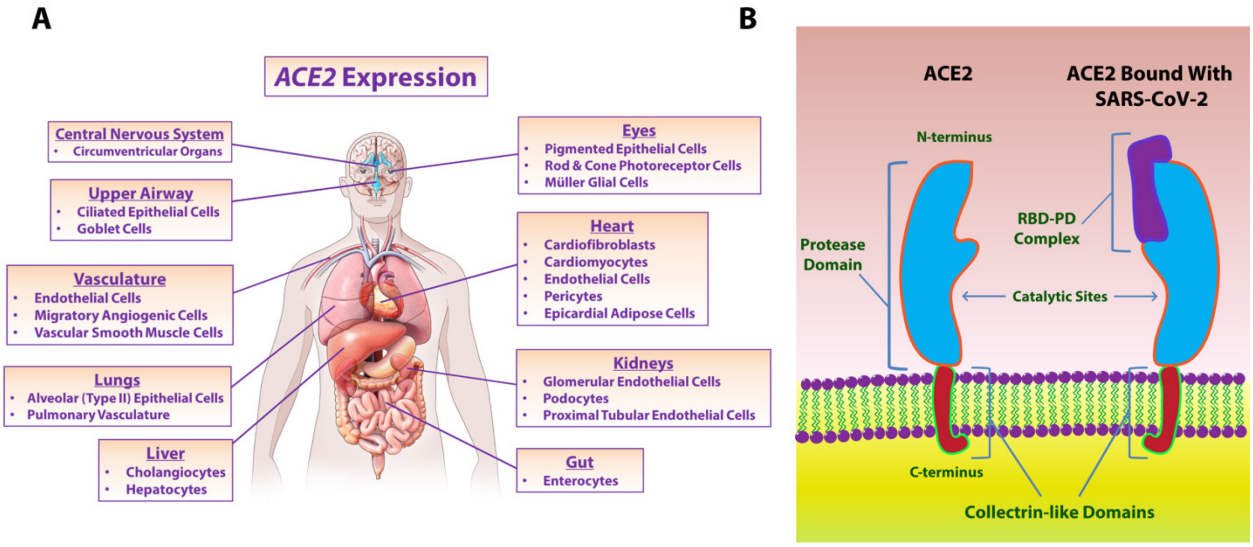


Figure 1.1. ACE2 expression throughout the body and schematic of ACE2 primary domains. (**A**) ACE2 is expressed in the vascular system (endothelial cells, migratory angiogenic cells, and vascular smooth muscle cells), heart (cardiofibroblasts, cardiomyocytes, endothelial cells, pericytes, and epicardial adipose cells) and kidneys (glomerular endothelial cells, podocytes and

proximal tubule epithelial cells). ACE2 is also expressed and functions in the local RAS of the liver (cholangiocytes and hepatocytes), retina (pigmented epithelial cells, rod and cone photoreceptor cells and Müller glial cells), enterocytes of the intestines, circumventricular organs of the central nervous system, upper airway (goblet and ciliated epithelial cells), and alveolar (Type II) epithelial cells of the lungs and pulmonary vasculature. (**B**) ACE2 has an extracellular facing N-terminal domain and a C-terminal transmembrane domain with a cytosolic tail. The N-terminal portion of the protein contains the claw-like protease domain (PD), while the C-terminal domain is referred to as the Collectrin-like domain (CLD). The receptor-binding domain (RBD) of SARS-CoV-2 binds with the PD of ACE2, forming the RBD-PD complex distinct from the ACE2 catalytic site.

As it turned out, important differences occur, particularly in the active site regions of the enzymes, such that the two enzymes counterbalance rather than reinforce each other's actions. Many subsequent studies over the next twenty years have revealed their inter-relationship, respective roles in the RAS, and multiple physiological and pathological actions from vasoactive peptide metabolism, importantly including not only Ang II but also apelin, to intestinal amino acid transport affecting innate immunity, to lung function and brain amyloid metabolism (converting Aβ43 to Aβ42, a substrate for ACE).[7,12,24] Another unexpected twist in ACE2 biology was its identification in 2003 as the cell-surface receptor for the then newly identified SARS-CoV that led to more than 8000 cases of SARS and almost 800 deaths,[25] and as the receptor for SARS-CoV-2 that is currently devastating many countries worldwide.[9,26]

1.3. *ACE2* Gene and Biochemistry

Unlike the *ACE* gene, which is located on human chromosome 17, the 40kb *ACE2* gene is located on chromosome Xp22 and contains 18 exons, most of which resemble exons in the *ACE* gene. Whereas somatic ACE contains two active sites, ACE2 possesses only a single catalytic domain. Both ACE and ACE2 act as zinc metallopeptidases but of differing substrate specificities defining

their distinct and counterbalancing roles in the RAS. Whereas ACE cleaves C-terminal dipeptide residues from susceptible substrates (a peptidyl dipeptidase), ACE2 acts as a simple carboxypeptidase able to hydrolyze Ang I, forming Ang 1–9 and Ang II to Ang 1–7 (**Figure 1.1.B**). ACE2 does not cleave bradykinin, further distinguishing its specificity from that of ACE while it is also insensitive to conventional ACE inhibitors.[20,24] The C-terminal domain of ACE2 which has no similarity with ACE, is a homolog of a renal protein, collectrin, which regulates the trafficking of amino acid transporters to the cell surface, endowing ACE2 with multiple and distinctive physiological functions. It is the multiplicity of physiological roles that ACE2 plays that has allowed it to be hijacked by SARS-CoV-2 as a receptor, resulting in the COVID-19 pandemic.[10,19] Structural studies have revealed the structures of both the SARS-CoV and much more recently, the SARS-CoV-2 in complex with ACE2 (**Figure 1.1.B**).[27,28] In the case of SARS-CoV-2, the major spike glycoprotein (S1) binds to the N-terminal region of ACE2. The knowledge of the biology and physiology of ACE2 accumulated over the last 20 years since its discovery should provide a major stimulus to understanding some of the key steps in SARS-CoV-2 infection and its ultimate prevention.

1.4. Regulation of ACE2

Given the canonical role of ACE2 as a negative regulator of the RAS and receptor for SARS-coronaviruses, the cellular control of ACE2 is an active area of investigation. As an X-linked gene capable of escaping X chromosome inactivation, the dimorphic expression of *ACE2* is implicated in sex-based differences in health and diseases.[29,30] Interestingly, the anticipated female bias in *ACE2* expression appears to be negated by the effects of transcriptional control, such as that of sex

hormones.[30] Precisely, delineating the complex physiological regulation of ACE2 requires assessment at the transcriptional, post-transcriptional, and post-translational levels.

Transcriptional control encompasses many facets with agonist activation of the androgen receptor (AR) capable of initiating nuclear translocation and binding to enhancer elements distal to the *ACE2* gene to upregulate gene expression.[31] Therefore, male mice had increased ACE2 activity compared to females in the heart and kidney, which was reversed when female animals were ovariectomized and restored upon exogenous treatment with 17β-estradiol.[32,33] Aside from the sex hormones, transcriptional control is also mediated by the apelin pathway, for example. Although the mechanism is not elucidated entirely, apelin upregulates *ACE2* expression presumably through downstream signaling of the apelin receptor, initiating nuclear translocation of GATA transcription factors.[34,35] In accordance, exogenous administration of apelin restores *ACE2* expression in the failing hearts of *APLN* knockout animals subjected to transthoracic aortic constriction.[34]

Epigenetic control of the ACE2 locus is mediated by DNA methylation and histone modifications. Aberrant DNA hypermethylation, presumably promoting transcriptional repression, at CpG4 and CpG5 within the promoter of *ACE2* has been described in essential hypertension.[36,37] Histone post-translational modification, such as lysine-specific histone demethylase 5B (*KDM5B)* that demethylates lysine 4 of histone 3 (H3K4), was positively associated with *ACE2* expression in the lungs.[38] In contrast, enhancer of zeste homolog 2 (EZH2) catalyzes H3K27 trimethylation (H3K27me3) to suppress *ACE2* expression in human embryonic stem cells.[39] Finally, miRNAs represent a nidus of post-transcriptional control through translational repression of *ACE2*. Based on a predicted miRNA binding domains on the 3' untranslated region (UTR), *in silico* modeling identified miR-125a-5p, miR-200c, and miR-200b

and miR-429 as putative regulators of *ACE2*.[40] miR-421 reduces ACE2 protein levels and activity in cell lines, and miRNA-143 is inversely correlated to ACE2 activity in a study following aerobic exercise training in humans.[41,42]

The ACE2 protein is controlled post-translationally by targeted degradation and proteolytic shedding. In pulmonary arterial hypertension (PAH), murine double minute 2 (MDM2), an E3 ubiquitin ligase, targets ACE2 for proteasomal degradation by ubiquitination.[43] Interestingly, AMP-activated protein kinase (AMPK) phosphorylates the intracellular domain of ACE2 and thereby stabilizes its membrane expression.[43,44] ADAM17 (A Disintegrin and Metalloproteinase-17) mediates ectodomain shedding of membrane-bound ACE2, detectable as plasma soluble ACE2. Similarly, ADAM17 activation is central to SARS-CoV-2 pathogenesis and upregulated by Ang II through its actions on the AT_1R creating a deleterious positive feedback loop, which we will parallel in latter sections.[45] Thus, multiple transcriptional, post-transcriptional, and post-translational regulators control ACE2 expression in different cell types and in a sex-specific and plausibly age-dependent manner. Moreover, ACE2 expression changes, as predicted from its central role in cardiovascular and fluid homeostasis, in multiple diseases from heart failure, hypertension, diabetes, or obesity.[46-49]

1.5. Physiological Roles of ACE2

A. Negative Regulator of the RAS

Discovery of ACE2 resulted in a paradigm-changing concept in all aspects of the RAS. ACE2 is a monocarboxypeptidase that converts Ang I into a nonapeptide, Ang 1–9, and Ang II into a heptapeptide, Ang 1–7 (**Figure 1.2.A**). This distinct enzymatic pathway for degradation of Ang I and Ang II negatively regulates RAS activation and mitigates the deleterious actions mediated by Ang II and AT_1R.[12] This is of particular significance in pathological conditions where the RAS is

overstimulated. Ang 1–7 is a biologically active peptide whose vast array of effects are opposite to those attributed to Ang II.[50-56] Furthermore, ACE2 can antagonize ACE independent formation of Ang II, such as from mast cell chymase.[57,58] In 2003, an endogenous orphan receptor, Mas (MasR), was identified as the Ang 1–7 receptor. A779, a MasR antagonist, blocks the majority of Ang 1–7 effects.[52,59-62] Ang 1–9 has also shown beneficial biological effects via the AT_2R that result in cardioprotection.[63-66] Thus, the ACE2/Ang 1–7/MasR axis has emerged as a physiological counter regulator of the activated RAS.[51,67-71] The cardioprotective effects of ACE2 taken together can be attributed to i) degradation of Ang I to Ang 1–9, whereby limiting action of ACE on its substrate, ii) reducing Ang II detrimental effects through degradation of the peptide, and iii) formation of Ang 1–7 which exercises cardioprotective effects. Formation of Ang 1–7 is an important mechanism of ACE2 mediated protection, as antagonism of Ang 1–7 using A779 prevented beneficial effects of rhACE2 in murine model of systolic dysfunction.[72] Diminished ACE2 activity results in activation of the Ang II/AT1R axis, contributing to the increased progression of CVD. Elevated ACE2 level and activity result in the formation of Ang 1–9 and Ang 1–7, leading to protection against CVD. (**Figure 1.2.A**).

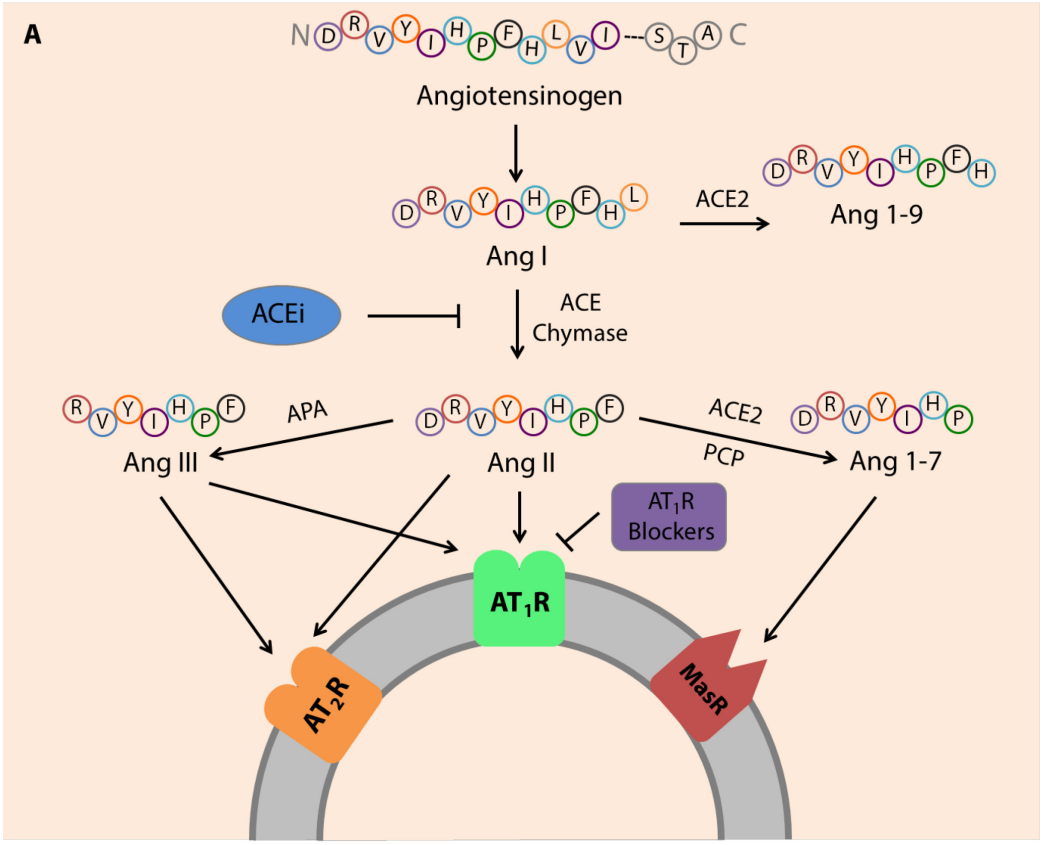
B. Interaction with Apelin Peptides

The apelin family of peptides act through the apelin receptors mediating protection against CVD.[73,74] The X-linked *APLN* gene encodes a 77 amino acids pre-pro-apelin that is subsequently cleaved by endopeptidases to various bioactive peptides from 13 to 36 amino acids in length. CVD, including HF and hypertension, is characterized by an apelin deficient state in both human myocardium and plasma.[75-77] Apelin KO mice exhibit increased infarct size and systolic dysfunction following coronary ligation and reduced myocardial contractility concomitant with

increased susceptibility to HF in pressure-overload models.[78,79] Reduced myocardial *Ace2* mRNA and ACE2 protein levels in apelin KO mice, which were rescued by infusion of apelin-13, suggest a crucial regulatory role of apelin in *Ace2* gene expression.[34] Apelin signaling through the apelin receptors specifically increased *Ace2* promoter activity leading to an increase in *Ace2* mRNA and protein.[34,80,81] These effects are consistent with the ability of the pyr-apelin-13 peptide to negatively regulate Ang II-mediated superoxide production, myocardial hypertrophy, dysfunction, and fibrosis[81] and analogs of apelin-17 preventing abdominal aortic rupture in low-density lipoprotein receptor KO models induced by Ang II infusion.[82] However, ACE2 through its monocarboxypeptidase activity cleaves and inactivates bioactive apelin peptides apelin-13 and apelin-36 through a negative feedback mechanism in the heart and vasculature (**Figure 1.2.B**).[24,83] Due to the short half-life of endogenous apelin peptides in the plasma, synthetic apelin peptide analogs resistant to ACE2 degradation and retaining their binding capability to endogenous apelin receptors elicit protection in the CV system are being explored as potential new therapies.[73,82]

C. Chaperone for B^0AT1 (SLC6A19) Amino Acid Transporter

B^0AT1 is highly expressed in the intestines and kidneys with function in the absorption of neutral amino acids.[84] The ACE2-B(0)AT1 complex is assembled as a dimer of heterodimers, with the collectrin-like domain of ACE2 mediating homo-dimerization.[10] ACE2 has a RAS-independent function, regulating intestinal amino acid homeostasis, expression of antimicrobial peptides, and the gut microbiome.[85] ACE2 is necessary for the expression of the Hartnup transporter in the intestine, and the differential functional association of mutant B(0)AT1 transporters with ACE2 in the intestine regulates the phenotypic heterogeneity of human Hartnup disorder.[84]


A
Angiotensinogen
Ang I
ACE2
Ang 1-9
ACEi
ACE
Chymase
APA
Ang III
Ang II
ACE2
PCP
Ang 1-7
AT1R
Blockers
AT1R
AT2R
MasR

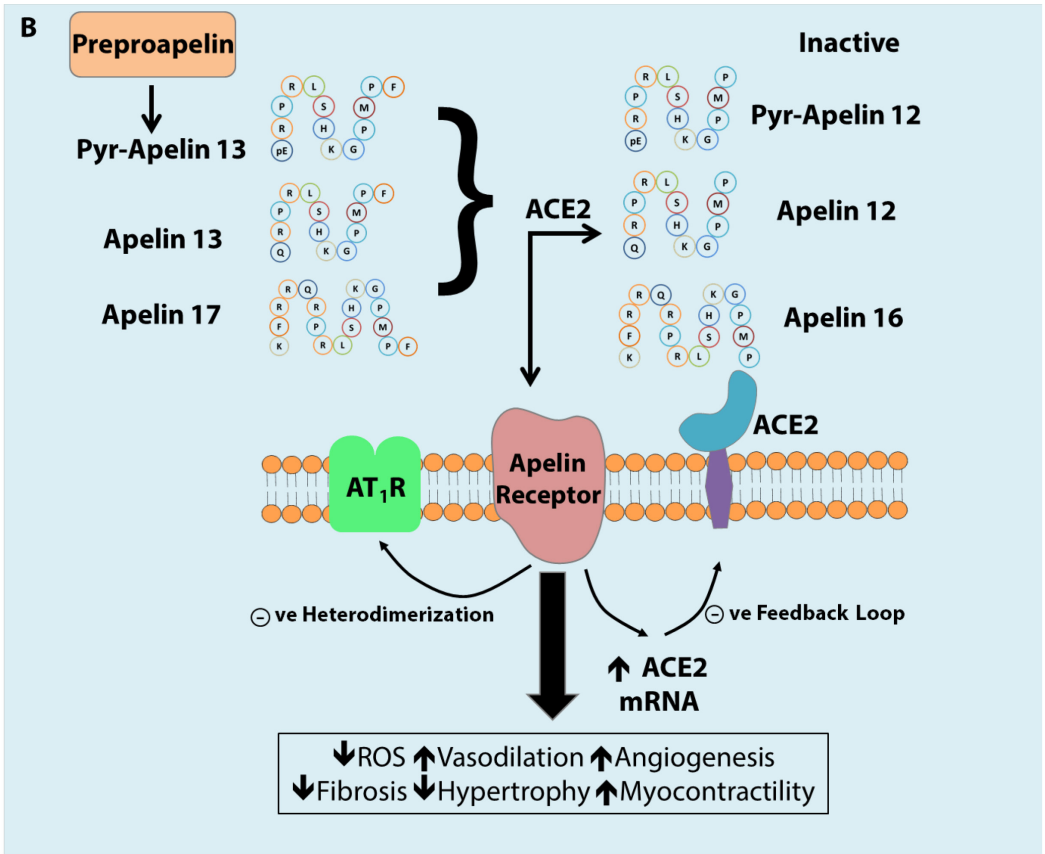

B
Preproapelin
Pyr-Apelin 13
Apelin 13
Apelin 17
Inactive
Pyr-Apelin 12
Apelin 12
Apelin 16
ACE2
ACE2
AT1R
Apelin
Receptor
ve Heterodimerization
ve Feedback Loop
ACE2
mRNA
ROS Vasodilation Angiogenesis
Fibrosis Hypertrophy Myocontractility

Figure 1.2. ACE2 role in the RAS peptide cascade and its interaction with the apelinergic peptide system. (**A**) Angiotensinogen is processed by renin into angiotensin I (Ang I) which is further cleaved by angiotensin-converting enzyme (ACE) or mast cell chymase into angiotensin II (Ang II). Ang II can go on to affect the cardiovascular system predominantly through the angiotensin type 1 receptor (AT_1R) or via the angiotensin type 2 receptor (AT_2R). Alternatively, Ang II can be degraded by the carboxypeptidase angiotensin-converting enzyme 2 (ACE2) or the prolyl carboxypeptidase (PCP) into angiotensin 1–7 (Ang 1–7). Ang 1–7 mediates protective effects throughout tissues which host the Mas receptor (MasR). Ang 1–7 can be further formed through the activity of ACE2 on Ang I forming Ang 1-9 which is then cleaved by either ACE or neprilysin (NEP). (**B**) Stimulation of the apelin receptor by apelin peptides leads to cardiovascular protective effects while disrupting Ang II signaling by sequestration of the AT_1R through receptor heterodimerization. Apelin is inactivated by ACE2 cleavage of its C-terminal phenylalanine while stimulation of the apelin receptor promotes ACE2 mRNA transcription presenting apelin's role as a positive regulator of ACE2.

1.6. ACE2 as the Receptor for SARS-CoV-2

In 2003, ACE2 was recognized as a candidate cell-surface receptor for the then newly identified SARS-CoV that eventually led, as far as is known, to around 8000 cases of confirmed infections and almost 800 deaths.[25] Since multiple other receptors were proposed, the role of ACE2 as the critical in vivo SARS-CoV receptor was definitively shown in genetic mouse experiments. Subsequently, ACE2 was rapidly identified as the receptor for SARS-CoV-2 that devastated countries worldwide during the COVID-19 pandemic.[9] Once again, mutant mouse experiments, as well as knock-out studies in human organoids have confirmed that ACE2 is the essential entry receptor for SARS-CoV-2, not excluding that other candidate receptors might modulate SARS-CoV-2 entry into the cells.[86] Thus, all carefully performed genetic experiments came to the same conclusion: ACE2 is the essential receptor for SARS-CoV2 infections *in vitro* and *in vivo*.

SARS-CoV and SARS-CoV-2 belong to the betacoronavrius genera originating in bats.[87] Recombination in the bat coronavirus genome permits spillover to animals, with palm civets identified as a likely intermediate host before human adaptation for SARS-CoV.[88,89] The *S* gene that encodes the spike protein is the most frequent site for recombination to enhance receptor binding affinities for ACE2, suggesting the evolution of the receptor-binding domain (RBD) as a key event for cross-species transmission.[90,91] By contrast, ACE2 orthologs are highly conserved across vertebrate species and can even be found in insects and certain bacterial species, reflective of its cardinal physiological activities.[92,93] Consequently, this results in broad host susceptibility, thereby driving zoonotic transmission and viral evolution of SARS-CoV-2. Both *in silico* and *in vivo* analyses have confirmed the susceptibility of many domestic animals, livestock, and wildlife, including cats, dogs, ferrets, hamsters, sheep, and deer to wildtype SARS-CoV-2 but not in rodents or pigs, which carries significant implications for agriculture, animal conservation, and scientific research.[92-96]

Multiple betacoronaviruses have been identified, sharing an ancestral origin in ACE2 binding and substantial evolutionary plasticity carrying the potential to spill over into human and other mammalian species in the future.[97] As a natural reservoir for SARS coronaviruses, bat species demonstrate extensive *ACE2* polymorphisms at an accelerated evolutionary pace compared to other mammalian lineages due to the ongoing selection pressures between the viral S-protein and ACE2 during long periods of coexistence. In contrast, *ACE2* variations are present but exceedingly rare in humans at the population level.[93,98] Although structural and functional evaluations suggest specific human *ACE2* variants may enhance or disrupt S-protein binding.[99] Furthermore, single mutations in the human *ACE2* receptor-binding interface can profoundly diminish its catalytic activity, thus explaining the scarcity of human *ACE2* polymorphisms that prevents an evolutionary

escape from SARS-CoV-2 infection.[100,101] Importantly, SNPs in *ACE2* that apparently alter its expression are associated with the clinical severity of COVID-19, providing that ACE2 expression levels are associated with disease pathogenesis.[102,103] Nevertheless, ACE2 expression is modified by sex, age, and a multitude of physiological and pathophysiological conditions, representing a possible explanation of how pre-existing co-morbidities could affect susceptibility and severity of COVID-19, and possibly, though there are not enough case numbers, SARS.

The diverse viral tropism and extra-pulmonary manifestations of SARS-CoV-2 have prompted interest in the tissue expression and regulation of ACE2. Specific to the infective process, the overall *ACE2* expression is relatively low in the respiratory tract and shows a progressive reduction from the nasal epithelium towards the lower respiratory tract, which correlates with patterns of viral infectivity.[104] The mRNA expression of *ACE2* is high in the nasal goblet and ciliated cells but remains detectable in the basal, ciliated, club, and alveolar cells of the lower airway.[47] Notably, while the lower respiratory tract and pneumocytes served as primary replication sites for SARS-CoV, SARS-CoV-2 can efficiently replicate in tissues of the upper respiratory tract and retains replication capabilities in the lower airway, which contributes to a more efficient transmission and infection dynamics compared to SARS-CoV.[105,106] Additionally, entry of SARS-CoV-2 into host cells requires the presence of ACE2 co-expressed with accessory proteases for spike protein priming and activation.[8] SARS-CoV-2 has evolved to utilize many host proteases, including cathepsin L, cathepsin B, furin, and TMPRSS2 (transmembrane protease serine 2) for S-protein priming and potentiating cellular infection.[46,107] Of note, the omicron variant appears to be largely independent of TMPRSS2 cleavage, which allows a different endosomal dominated viral entry making therapeutic inhibition of this protease largely ineffective against this variant.[108] Besides the respiratory epithelium, immunohistochemistry of animals and humans, as

well as genes expression studies, including a meta-analysis of single-cell RNA-sequencing datasets from The Human Cell Atlas revealed that *ACE2*$^{+}$ cells are highly present in enterocytes of the intestine, proximal tubules of the kidney, cardiomyocytes and associated fibroblasts and vascular smooth muscle cells, defined endothelial cells and pericytes, including cells of the choroid plexus constitution part of the blood-brain barrier, as well as lymphocytes and macrophages.[109]

At the intersection between SARS-CoV-2 infections and consequent organ injury lies the ACE2 protein. Although in organ damage, multiple pathways are ultimately involved, such as immune activation, autoimmunity, and changes in the coagulation, which explains why nearly all clinical trials have failed in treating severe COVID-19 and making it paramount to advance treatments that target multiple pathways. Despite being the critical receptor of SARS coronaviruses, a plethora of studies have shown that ACE2 protects multiple tissues from damage in response to a deregulated local RAS and possibly other deregulated peptide systems such as apelin, bradykinin, or neuroactive peptides. In particular, ACE2 confers protection in the pulmonary system, and downregulation of ACE2 contributes to increased severity and pathogenesis of acute and chronic lung injury.[110,111] Importantly, treatment of multiple cell types, organoids, or even mice with recombinant spike and even RBD protein of SARS-CoV and SARS-CoV-2 led to ACE2 downregulation from the cell surface, concomitant with worsened pulmonary function and exacerbation of lung pathology.[111-113] However, ACE2 downregulation is also observed independent of direct viral interaction in pathogenic avian influenza A H5N1 and H7N9 virus infections or non-infectious acid injury, leading to greater disease severity and elevation in plasma AngII levels.[114,115] In comparison, less pathogenic human influenza viruses and coronaviruses causing limited non-severe upper-respiratory-tract infections, including H1N1 and HCoV-NL63, apparently do not alter ACE2 expression.[113,114,116]

ACE2 exists in two forms – a full-length form that is membrane-bound and a shorter soluble form that is shed into body fluids and circulates typically in the blood in very small amounts.[117] Both forms contain the same sequence used by the RBD of the SARS-CoV-2 S-protein, but soluble ACE2 lacks the transmembrane domain necessary for anchoring to the cell membrane.[21,118] Pathological induction of the ADAM17 cascade during SARS-CoV-2 infection through direct viral mechanisms or the ensuing inflammatory response triggers aberrant proteolytic cleavage of ACE2 into the circulation, thereby reducing its tissue expression and blocking its local counter-regulatory effects on the RAS (**Figure 1.3.**).[116,117,119] Additionally, activation of the AngII pathway through AT_1R and TNFα signaling through the TNF receptor (TNFR) upregulates ADAM17 activity, leading to a detrimental positive feedback loop in infected tissues.[45,120] Furthermore, stimulation of p38 MAPK by inflammation and cellular stress directly promotes ADAM17 mediated ACE2 ectodomain shedding.[121] Thus, inflammation, cellular stress, or AngII induction appear to trigger ADAM17, thereby increasing soluble ACE2 in the plasma. Persistent elevation in soluble ACE2, reflective of putative sustained pathological ADAM17 activation, was associated with increased severity, mortality, and incidence of acute myocardial injury.[122,123] Collectively, these findings provide a molecular bridge between inflammation, cellular stress, and ACE2 downregulation from the cell membrane via shedding and internalization observed during SARS-CoV-2 infection as a contributor to multi-organ pathologies and elevated risk of mortality.

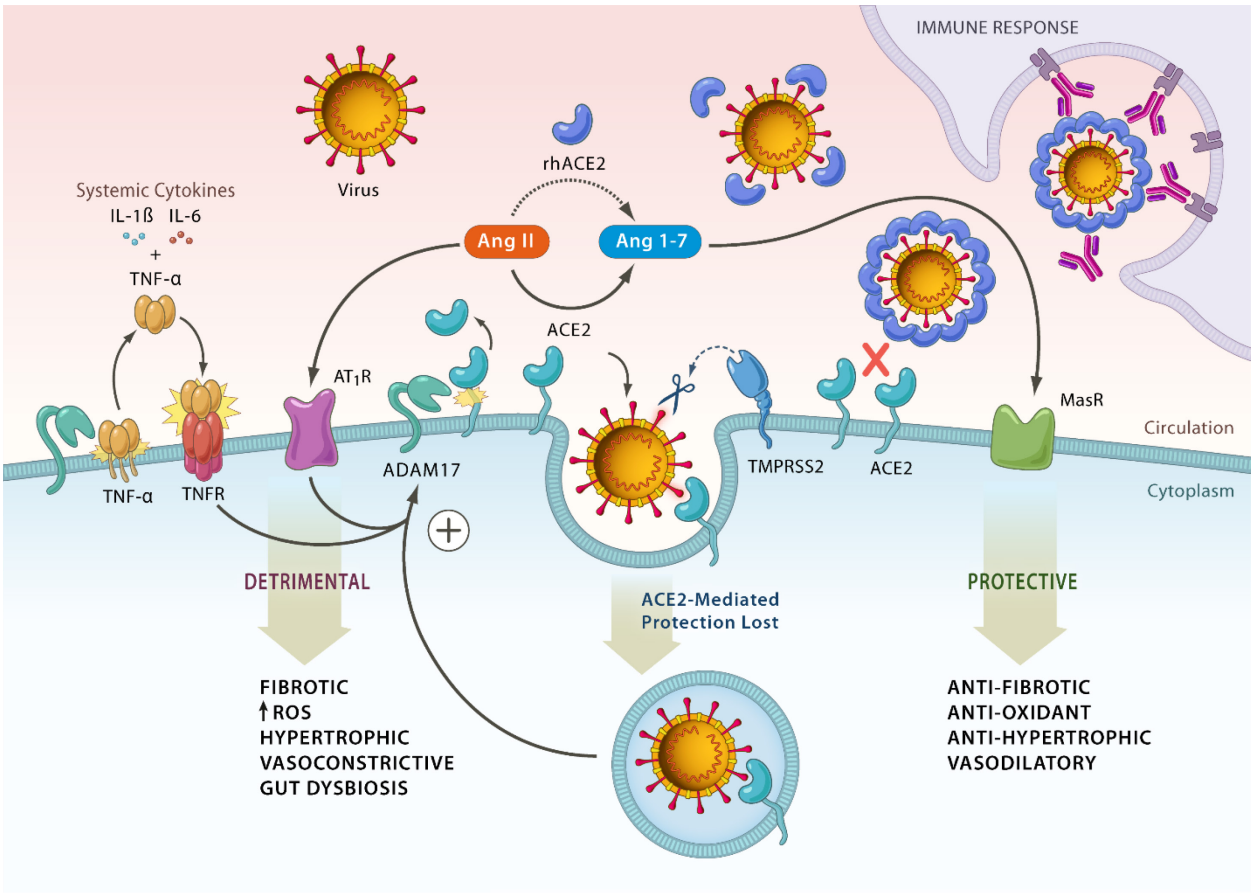


Figure 1.3. Role of ACE2 in the pathogenesis of COVID-19 and the inflammatory response. ACE2-mediated cardiovascular protection is lost following endocytosis of the enzyme along with SARS-CoV-2 viral particles. Ang II levels elevate with increased activity of angiotensin 1 receptors (AT_1R) at the cost of ACE2/Ang 1–7 driven pathways leading to adverse fibrosis, hypertrophy, increased reactive oxygen species (ROS), vasoconstriction, and gut dysbiosis. ADAM17 mediated proteolytic cleavage of ACE2 is upregulated by endocytosed SARS-CoV-2 spike proteins. Activation of the AT_1R by elevated Ang II levels also further increases ADAM17 activity. ADAM17 correspondingly also cleaves its primary substrate releasing soluble TNF-α into the extracellular region where it has auto- and paracrine functionality. TNF-α activation of its Tumor Necrosis Factor Receptor (TNFR) represents a third pathway elevating ADAM17 activity. TNF-α along with systemic cytokines released due to SARS-CoV-2 infection and in conjunction with comorbidities such as diabetes and hypertension can lead to a cytokine storm.

1.7. ACE2 in Cardiovascular and Lung Disease

A. ACE2 and Heart Disease

Cardiovascular disease is the leading cause of death worldwide and a major public health concern. Heart disease is characterized by the activation of several signaling pathways associated with pathological hypertrophy and maladaptive ventricular remodeling. In the heart, ACE2 is localized to cardiomyocytes, cardiac fibroblasts, epicardial adipose tissue, and the coronary vascular endothelium[45,124,125]; Ang 1–7/MasR is also present on cardiomyocytes, cardiac fibroblasts, and endothelial and vascular smooth muscle cells.[59,126-128] Genetic *Ace2* deletion resulted in exacerbation of Ang II-mediated cardiorenal fibrosis and oxidative stress in the heart and kidney of hypertensive mice while administration of recombinant human ACE2 (rhACE2) remarkably rescued the Ang II-induced hypertension, pathological hypertrophy, oxidant injury and cardiac dysfunction.[13,14]

Various ACE2 polymorphisms are linked to CVD.[129] Post-MI remodeling, and coronary artery disease are common causes of HF.[12,130] Notably, MI increases ACE2 mRNA expression in human patients, mice and rat models[15,131], whereas genetic ACE2 deletion results in worsening of MI-induced cardiac dysfunction, infarct size, matrix metalloproteinase (MMP)2/MMP9 activation and extracellular matrix disruption.[15,131] Loss of ACE2 leads to increased neutrophilic infiltration in the infarct and peri-infarct regions, resulting in upregulation of inflammatory cytokines, interferon-γ, interleukin (IL)-6, and the chemokine, monocyte chemoattractant protein-1 (MCP-1), as well as increased phosphorylation of ERK1/2 and JNK1/2 signaling pathways, changes that were blocked with an ARB ultimately resulting in improvement in myocardial function.[15] In contrast, overexpression of ACE2 and the action of Ang 1–7 ameliorates MI-induced cardiac

remodeling.[132,133] Importantly, heterozygote loss of ACE2, as seen in explanted human hearts from patients with dilated cardiomyopathy, was sufficient to increase susceptibility to heart disease.[134]

HF with preserved ejection fraction (HFpEF) is a proinflammatory state closely linked to obesity-related cardiac and microvascular dysfunction for which there are no approved therapies.[125,135,136] Epicardial adipose tissue (EAT) is a primary source of inflammatory cytokines that could have detrimental effects on the heart.[135] Loss of ACE2 increases macrophage polarization to proinflammatory M1-phenotype (alternatively activated, $CD11c^{+}$) in EAT from patients with HFpEF, with decreases in polarization to anti-inflammatory, M2-phenotype macrophages and worsening of HFpEF in response to diet-induced obesity.[135] Importantly, Ang 1–7, decreased macrophage polarization in EAT and preserved the cardiac function of obese *Ace2* KO mice.[125,137] Ang 1–7 has potent anti-inflammatory effects in adipose tissue of obese type 2 diabetic mice and protects against diabetic cardiomyopathy and nephropathy.[137-139] The ACE2/Ang 1–7 axis also promotes browning of adipose tissue leading to improved metabolic effects and weight loss, which can confer further benefits to the CV system.[140,141]

B. ACE2 and Vascular Disease

Blockade of the deleterious arm of the RAS has been the mainstay of the therapeutic management of hypertensive individuals. An increase in ACE2 and the vasoprotective axis of the RAS by angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs) clearly reinforces this view. Furthermore, increased ACE2 expression protects against hypertension, while ACE2 deficiency exacerbates hypertension. Renal *Ace2* expression is inversely related to blood pressure in experimental models of hypertension.[16] In the spontaneously hypertensive rat (SHR) and stroke-prone SHR, renal *Ace2* mRNA levels are reduced compared to normotensive Wistar-Kyoto rats.[16] These studies support the essential role of ACE2 in maintaining

healthy blood pressure. Lentiviral overexpression of ACE2 results in increased expression of anti-hypertensive components of the RAS and attenuates elevated blood pressure.[142,143] Pretreatment with rhACE2 prevented hypertension induced by Ang II and decreased plasma Ang II while increasing plasma Ang 1–7 levels.[144] ACE2 and ADAM17 were selectively knocked down from all neurons (AC-N), which revealed a reduction of inhibitory inputs to AC-N presympathetic neurons relevant for blood pressure regulation. Mice with ACE2 selectively knocked down from Sim1 neurons in mice exhibited a blunted blood pressure elevation and preserved ACE2 activity during the development of salt-sensitive hypertension.[145] The metalloproteinase ADAM17 is responsible for mediating ACE2 shedding from the cell membrane-bound domain, which can be promoted by Ang II, and release of ACE2 as a soluble form in plasma[45,117,145] impairing brain ACE2 compensatory activity and thus contributing to the development of neurogenic hypertension.[146] Genetic *Ace2* deficiency is associated with the upregulation of putative mediators of atherogenesis and enhances responsiveness to proinflammatory stimuli suggestive of a key role of ACE2 in suppressing vascular inflammation and atherosclerotic disease.[147] In addition, ACE2 inhibition blocks neuropeptide catestatin-mediated protective effects in the development of atherosclerosis in *ApoE*$^{-/-}$ mice fed a high-fat diet.[148]

C. ACE2 and Diabetic Cardiovascular Complications

The counter-regulatory role of the ACE2/Ang 1–7/MasR axis of the RAS has been well-characterized in the progression of diabetic complications, including CV and kidney disease.[12,149,150] Support for the importance of ACE2 in diabetes comes from its impact on diabetic complications wherein diabetes-induced vascular dysfunction is strongly associated with a shift in the RAS Axis towards the profibrotic, proinflammatory arm of RAS with a reduction in the protective arm (**Figure 1.4.**). Loss of the protective effects of the RAS is related to the regulation

of tissue and circulating levels of Ang II and their sequelae in the context of diabetes.[151,152] Alterations within the RAS are considered pivotal for the development of both diabetic micro and macrovascular complications.[12,153]

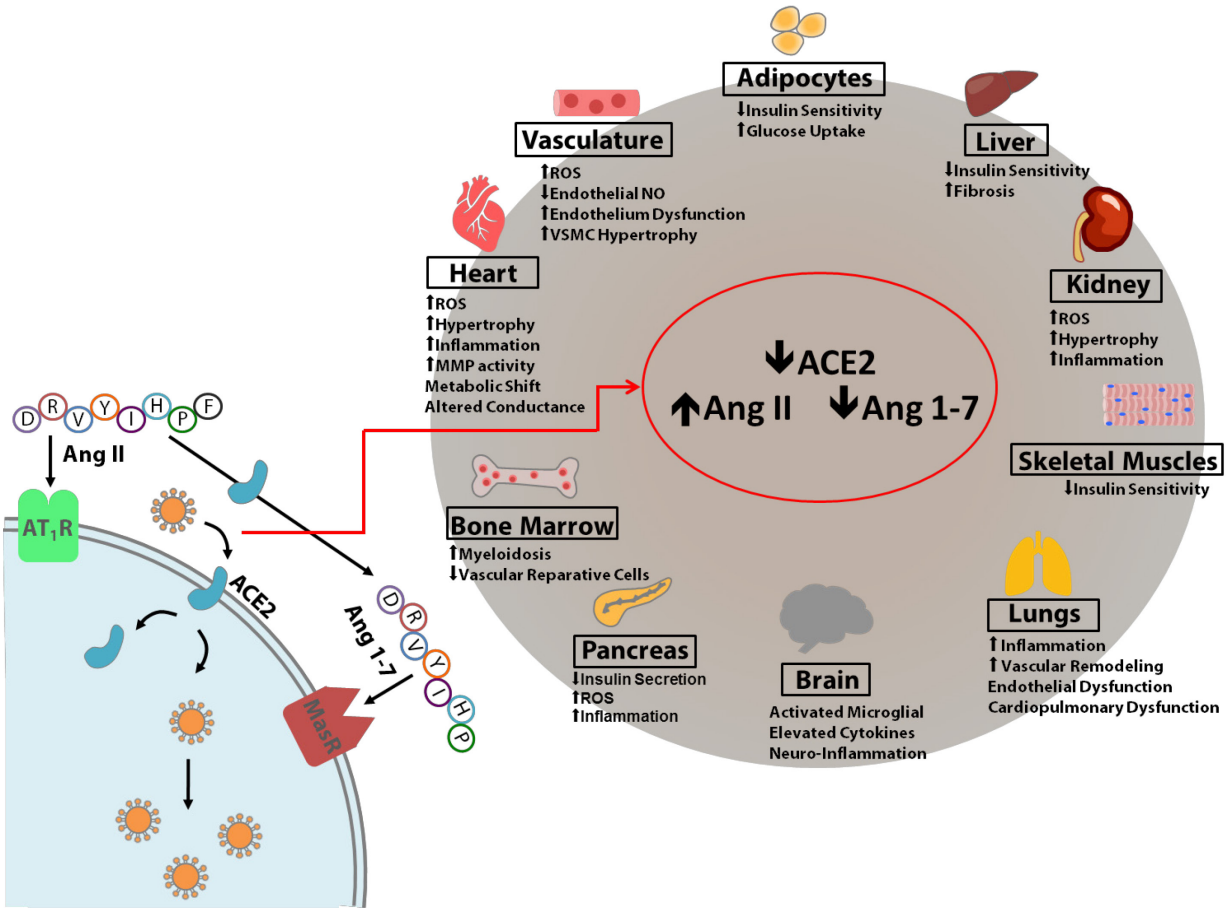


Figure 1.4. Loss of ACE2 exacerbates diabetic cardiovascular complications via a multitude of disease mechanisms. The loss of ACE2 action in diabetic states elevates Ang II and lowers Ang 1–7 levels in tissues and systemically. Increased Ang II/AT_1R signaling drives multiple pathologies in various end-organs elevating reactive oxygen species (ROS) and promoting fibrosis, hypertrophy, and inflammation aggravated by the loss of the protective effects of Ang 1–7. Ang II stimulation also systemically alters metabolic profiles and modulates insulin sensitivity in affected tissues.

The blockade of the proinflammatory and profibrotic arms of the RAS provides significant renoprotection in both experimental models of diabetes and in patients. While the loss of ACE2 worsens diabetic kidney injury[154], rhACE2 is therapeutic in an animal model of diabetic nephropathy[149] and experimental Alport syndrome.[155] ACEi in T1D and angiotensin receptor blockade with losartan and irbesartan in T2D retard the progression of nephropathy.[156] In diabetic renal tubules, ACE2 gene expression is decreased by ~50%, which would reduce Ang 1–7 formation and allow Ang II accumulation hence directly increasing the expression of TGF-β and connective tissue growth factor (CTGF), leading to tubulointerstitial fibrosis.[157] RAS blockade retards renal damage and ACE inhibitor therapy, as mentioned above, resulting in a compensatory increase in ACE2, leading to renoprotection.[13] Therefore support for the loss of ACE2 contributing to vascular complications in diabetes comes from strong clinical and experimental evidence.[12]

Retinopathy, the most common complication of diabetes and one of the leading causes of blindness in working-age adults, is linked to activation of oxidative stress, profibrotic, and proinflammatory arm of the RAS which can be effectively curtailed by the ACE2/Ang 1–7 axis in experimental models.[158,159] Increased secretion of proinflammatory cytokines by bone marrow mesenchymal stem cells (MSCs) skews hematopoiesis towards the generation of an increased number of myelo-monocytic cells.[160] Target tissues of diabetic complications secrete CCL2 in response to high glucose-induced stress[161] facilitating the homing of $CCR2^{+}$cells to these regions and promoting the development of vascular complications.[162-167] In addition to an increase in myeloidosis, diabetics with complications have reduced bone marrow-derived vascular reparative cells and circulating angiogenic cells ($CD34^{+}$cells).[168] Levels of *Ace2* mRNA were also a significant predictor of the presence of microvascular disease.[168] Diabetic individuals that remained free of retinopathy despite >40 years of poor glycemic control had higher mRNA levels

for genes of the vasoprotective axis (ACE2/Mas) compared to age, sex, and glycemia-matched diabetics with retinopathy.[169] In dysfunctional $CD34^+$ cells from diabetic individuals, activation of the protective arm of RAS, by exposing the cells to Ang1–7 corrected their dysfunction by restoring bioavailable NO and reducing ROS. Ang1–7 gene modification of $CD34^+$ cells restored the in vivo vasoreparative function of these cells in a mouse retinal ischemia-reperfusion injury model.[169] Moreover, intraocular administration of AAV-ACE2 or Ang1–7 reduced diabetes-induced retinal vascular leakage and inflammation, thus preventing retinopathy.[159]

D. ACE2 and Lung Disease

Lung epithelial cells express high levels of ACE2, which positively correlates with airway epithelial differentiation.[110,170,171] Involvement of ACE2 in acute respiratory distress syndrome (ARDS), which is triggered by multiple diseases including SARS-CoV and SARS-CoV-2, has been established in multiple animal models.[111,172] *Ace2* KO mice exhibit severe pathology of ARDS.[110,171] Additional *Ace* deficiency, or treatment with AR1R blockers of *Ace2* KO mice rescues them from ARDS implicating the benefit of ACE2 and the critical balance of the protective vs. proinflammatory and fibrotic axes of the RAS.[111] These findings are consistent with evidence of a beneficial effect of rhACE2 on pulmonary blood flow and oxygenation in a pig model of LPS induced ARDS.[173] Age-related loss of ACE2 in the lungs correlates with the increased mortality and worsened phenotype in elderly patients with COVID-19.[170]

ACE2 has been implicated in acute lung injury (ALI) by inducing an imbalance in the RAS. Evidence includes that in ALI (i) a decrease in pulmonary ACE2 and an increase in Ang II levels occurs; (ii) supplementation with ACE2 or inhibition of Ang II improves outcomes; and (iii) a lack or decrease of pulsmonary ACE2 aggravates viral-induced ALI. ACE2 is also involved in pulmonary hypertension (PH) and fibrosis.[171] Increasing ACE2 activity using rhACE2 reduced

bleomycin-induced inflammation and fibrosis, resulting in improved lung function and exercise capacity[171], and the ACE2 activator, DIZE, protects animals from PH and fibrosis.[174] Moreover, oral feeding of a bioencapsulated form of ACE2 protects and arrests the progression of PH.[175] Validation of this protective effect comes from a small human study that showed that PH is characterized by reduced ACE2 activity and supplementation of these individuals with rhACE2 improved pulmonary hemodynamics and reduced oxidative and inflammatory markers.[176] Collectively, these studies unequivocally establish the conceptual framework that ACE2 is a central player in normal pulmonary function, and its imbalance leads to pulmonary diseases.

1.8. Soluble ACE2 as a Disease Biomarker

Studies found that male sex, advanced age, smoking, higher blood pressure and body mass index lead to elevated soluble ACE2 concentrations and also correspond to risk for severe COVID-19.[177-179] In a study including 10,753 participants, higher soluble ACE2 concentration was independently associated with a greater risk of all-cause mortality and incidences of myocardial infarction, diabetes, and heart failure beyond traditional cardiac risk factors.[178] Moreover, in prospective studies of patients with heart failure, atrial fibrillation, coronary artery disease, and aortic stenosis, elevated soluble ACE2 activity reflected impaired functional status and was associated with adverse clinical outcomes.[58,180-182] Together, these observations suggest that elevated soluble ACE2 signifies pathology reflecting the loss of the protective arm of the RAS in local tissues and membrane-bound ACE2. However, whether soluble ACE2 retains regulation over the systemic RAS and exhibits functions beyond serving a disease biomarker remains to be determined.

1.9. Research Hypothesis and Objectives

Although the ACE2/Ang 1-7/Mas axis has been demonstrated in various preclinical heart failure models to elicit vasodilatory, anti-fibrotic, anti-hypertrophic, antioxidant, and anti-inflammatory effects, the dynamic state of this organ protective pathway in the clinical setting remains unclear. In accordance, a significant barrier to translating our findings from preclinical models into efficacious therapies for heart failure patients is our current lack of RAS biomarkers to enable a personalized precision medicine-based initiation of novel therapies such as recombinant human ACE2 (rhACE2), Ang 1-7 analogs, Mas activators aimed at enhancing the protective function of this pathway. Therefore, identifying plasma RAS-based biomarkers from patients with heart failure is warranted to help with risk-stratification and prognostication. Furthermore, we were affected by the COVID-19 pandemic during my studies. Despite posing significant challenges to ongoing projects, this also presented a once-in-a-lifetime opportunity to contribute to the global cause of identifying pathways underlying SARS-CoV-2 pathophysiology through biomarker research. Since SARS-CoV-2 targets ACE2 as its cellular receptor, and previous studies have demonstrated the downregulation of membrane ACE2 expression with the initial SARS-CoV infection, we hypothesize that, like heart failure, elevated levels of plasma soluble ACE2 can serve as a novel biomarker for prognostication of adverse clinical outcomes and organ injuries in COVID-19. Conversely, an elevated Ang 1-7/Ang II ratio as a signature of enhanced or preserved ACE2 functionality may lead to more favorable patient outcomes in heart failure. The specific objectives of this thesis are as below:

1. To establish clinical significance and prognostic value of plasma circulating and equilibrium levels of angiotensin peptides in heart failure

2. To determine the prognostic value of plasma soluble ACE2 and other substrates of ADAM17 activity (sTNFRI and sTNFRII) at baseline and repeated sampling for COVID-19-related mortality and multi-organ injuries.
3. To investigate the perturbations in the systemic angiotensin peptide profile during SARS-CoV-2 infection.

Chapter 2. Elevated Angiotensin 1-7/Angiotensin II Ratio Predicts Favorable Outcomes in Patients with Heart Failure

Authors:

Kaiming Wang[a,b], Ratnadeep Basu[a,b], Marko Poglitsch[c], Jeffrey A. Bakal[d], Gavin Y. Oudit[a,b]

Affiliations:

[a]Division of Cardiology, Department of Medicine, University of Alberta, Edmonton, Canada

[b]Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Canada

[c]Attoquant Diagnostics, Vienna, Austria

[d]Alberta Strategy for Patient Oriented Research (SPOR) Unit, University of Alberta, Edmonton, Canada

This work has been published as: Wang K, Basu R, Poglitsch M, Bakal JA, Oudit GY. **Elevated angiotensin 1-7/angiotensin II ratio predicts favorable outcomes in patients with heart failure**. *Circ Heart Fail.* 2020; 13(7):e006939

2.1. Abstract

Background: ACE2 and Ang 1-7 are endogenous negative regulators of the renin-angiotensin system (RAS) exerting cardioprotective effects in models of heart failure (HF). Recombinant human ACE2 markedly increased plasma Ang 1-7 and lowered Ang II levels in phase II clinical trials. We hypothesize that the dynamic state of this RAS protective arm could influence long-term outcomes in patients with HF.

Methods: 110 HF patients were prospectively enrolled from our outpatient clinic and the emergency department. Comprehensive circulating and equilibrium levels of plasma angiotensin peptide profiles were assessed using novel liquid chromatography-mass spectrometry/mass spectroscopy techniques. Plasma aldosterone, BNP, active renin concentration and clinical profiles were captured at baseline. During a median follow-up of 5.1 years (IQR: 4.7 to 5.7 years), composite clinical outcomes were assessed using all-cause in-patient hospitalizations and mortality.

Results: Circulating and equilibrium angiotensin peptide levels strongly correlated in our patient cohort. Adjusting for covariates, elevated equilibrium (HR: 0.38; 95% CI: 0.18 to 0.81; p=0.012) and circulating (HR: 0.38; 95% CI: 0.18 to 0.80; p=0.011) Ang 1-7/Ang II ratios were associated with improved survival. Lower hospitalization duration was also associated with elevated equilibrium (p<0.001) and circulating (p=0.023) Ang 1-7/Ang II ratios. Importantly, individual Ang 1-7 and Ang II peptide levels failed to predict all-cause mortality or hospitalization duration in our patient cohort.

Conclusions: We extensively profiled plasma angiotensin peptides in patients with HF and identified elevated Ang 1-7/Ang II ratio, as an independent and incremental predictor of beneficial outcomes, higher survival rate, and decreased hospitalization duration. These findings provide

important clinical evidence supporting strategies aiming to promote the beneficial Ang 1-7/Mas axis concurrent with RAS blockade therapies inhibiting the detrimental Ang II/AT_1 receptor axis.

Brief Title: Prognostic value of Ang 1-7/Ang II ratios in HF

Subject Terms: ACE/Angiotensin Receptors/Renin Angiotensin System, Heart Failure, Mortality/Survival

2.2. Novelty and Clinical Implications

WHAT IS NEW?

- Beneficial arm of the renin-angiotensin system (RAS) mediated through the Ang 1-7/Mas receptor axis is well-established in experimental models of heart failure (HF) and our study provides crucial clinical evidence supporting the importance of this pathway in patients with HF.
- Dynamic state of the RAS reflected through equilibrium and circulating plasma Ang 1-7/Ang II ratio serves as an independent and incremental predictor of mortality, but not the individual angiotensin peptide levels in patients with HF.
- Elevated plasma Ang 1-7/Ang II ratio is associated with lowered hospitalization duration per year

WHAT ARE THE CLINICAL IMPLICATIONS?

- Angiotensin II as the "bad peptide" of the RAS mediates HF progression, while angiotensin 1-7 as the "good peptide" offers protection in HF.
- Plasma Ang 1-7/Ang II ratio is an important prognostic tool in risk stratification across the broad spectrum of HF.
- Novel HF therapeutics should aim to enhance the Ang 1-7/Mas receptor axis as a complement to current RAS blockade which are aimed primarily at blocking Ang II effects.

2.3. Introduction

Pathological activation of the renin-angiotensin system (RAS) resulting in chronic elevation of angiotensin II (Ang II) and aldosterone levels mediates the development and progression of heart failure (HF).[183-185] Pharmacological blockade of the detrimental RAS pathways yielded several key classes of medical therapy for HF with reduced ejection fraction (HFrEF) including angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB) and angiotensin receptor/neprilysin inhibitors (ARNI).[186,187]

However, the prospect of enhancing protective neurohumoral pathways is frequently overlooked during the development of novel HF therapeutics.[188] Additionally, management of HF with preserved ejection fraction (HFpEF) and acutely decompensated HF patients remains clinically challenging. Within the angiotensin peptide family, angiotensin 1-7 (Ang 1-7) acting via the G protein-coupled receptor, Mas, exerts potent antihypertrophic, antifibrotic, antioxidant and vasoprotective actions to counter-act the detrimental effects of Ang II signalling with promising clinical significance in the settings of both HFrEF and HFpEF.[12] Being a well-studied mediator of the beneficial RAS axis, angiotensin converting enzyme 2 (ACE2) is responsible for the endogenous conversion of Ang I and Ang II into angiotensin peptides Ang 1-9 and Ang 1-7, respectively.[12,16,189] Our ability to harness the power of ACE2 to convert systemic and tissue Ang II into Ang 1-7 has been validated in preclinical models, explanted human hearts, healthy subjects and recently in clinical trials using recombinant human ACE2 (rhACE2).[14,68,124,176,189-191]

To highlight the importance of targeting the Ang 1-7/Mas axis as novel HF therapies, clinical significance and prognostic value of this endogenous RAS protective arm needs to be established in HF. Hence, we generated RAS fingerprints of plasma angiotensin peptides to

examine the prognostic abilities of individual peptides and the Ang 1-7/Ang II ratio in the RAS protective arm across a broad spectrum of HF patients.

2.4. Methods

Study Population

Our prospective study followed 110 HF patients visiting the University of Alberta Hospital emergency department (n=46) and the Mazankowski Alberta Heart Institute outpatient clinics (n=64) between October 2012 and April 2015. All enrolled patients had documented diagnosis of HF representing an entire spectrum of NYHA functional class I to class IV, with patients having either reduced or preserved ejection fraction. HF patients using any investigational drugs within the last 4 weeks were excluded from the present study. Patient demographics and clinical characteristics at baseline were collected through review of their electronic medical records (EMR). Our study was in accordance with the ethical principles of the Declaration of Helsinki, and the University of Alberta Health Research Ethics Board guidelines (Pro00026480). Written and informed consent was obtained from all participants.

Plasma Collection and Laboratory Measurements

Neurohormonal assessments have been described by our previous study.[189] Briefly, venous blood was drawn into pre-labelled vials containing either angiotensin-stabilizing protease inhibitor cocktail (RAS-Enzyme-Inhibitor-Cocktail, Attoquant Diagnostics, Vienna), lithium heparin or ethylenediaminetetraacetic acid (EDTA) and stored at -80°C for subsequent analysis of circulating and equilibrium angiotensin peptides, plasma aldosterone, B-type natriuretic peptide (BNP), and active renin concentration. Plasma angiotensin peptide levels were measured through liquid chromatography-mass spectrometry/mass spectroscopy analysis (Acquity UPLC® C18, Waters,

Massachusetts). Details on assay sensitivities and recovery rates of individual angiotensin peptides were reported previously.[189] A chemiluminescence based assay (IDS-iSYS #IS-3300) was performed to measure plasma aldosterone levels from lithium heparin containing samples. Plasma renin concentration was measured using a quantitative sandwich enzyme-linked immunoradiometric assay (Cisbio Bioassays, model 18) with EDTA containing samples. A similar quantitative sandwich enzyme-linked two site chemiluminescent immunoenzymatic assay (Triage BNP #98200) was performed to measure plasma BNP.

Outcome Assessment

Clinical outcomes including inpatient hospitalizations and all-cause mortality events were obtained from linked healthcare administrative databases available through the Alberta Health Services. The primary outcome was long-term all-cause mortality with a median follow-up of 5.1 years (IQR: 4.7 to 5.7 years) since enrollment date. Patients were followed until death or appropriately censored at the end of the study follow-up duration. All-cause mortality was obtained through the use of Alberta provincial registry database and verified by follow-up review of patient's electronic medical records. Secondary outcomes are duration of all-cause inpatient hospitalizations obtained through the Discharge Abstract Database that provides detailed acute inpatient care and hospitalization records anywhere within the province of Alberta, including admission and discharge dates, dispositions and up to 25 diagnoses and 20 interventions.

Statistical Analysis

Descriptive statistics were presented as medians with interquartile range (IQR) for continuous data and absolute numbers with percentages for categorical data. Pair-wise comparisons between groups were carried out using the nonparametric Mann-Whitney *U* test or Kruskal-Wallis test

when appropriate for scalar values and the Fisher exact test for nominal values. The correlations between equilibrium and circulating levels of plasma angiotensin peptides were assessed using Spearman's correlation coefficient with natural logarithmically transformed peptide levels.

We stratified our HF population into above and below median cohorts based on either individual plasma Ang 1-7 and Ang II levels or the Ang 1-7/Ang II ratio. The primary endpoint (all-cause mortality) among the below and above median patient cohorts was compared using the Kaplan-Meier curve and the log-rank test. Univariate Cox regression analysis provided unadjusted hazard ratios (HR) for each individual predictors of all-cause mortality in our patient cohort, including traditional cardiovascular risk factors. Statistically significant predictors were subsequently assessed using an all possible subset variable selection to generate the most optimized multivariate model based on the Akaike Information Criterion (AIC).[192] 65535 possible models were examined, both the equilibrium and circulating ratios appeared in all top 5 variable models respectively. The best model that included key prognostic parameters in patients with HF including age, BNP and NYHA functional class was subsequently incorporated into the multivariate Cox regression analysis to adjust for relevant covariates and evaluation of independent predictors of all-cause mortality.

The incremental prognostic value of plasma Ang 1-7/Ang II ratio was assessed using Harrell's C-statistics and the category free net reclassification improvement (NRI) analysis with clinical variables from the adjusted model. Secondary endpoints as assessed through all-cause hospitalization events between the above and below median cohorts were compared using a box and whisker plot and the Mann-Whitney *U* test for average yearly hospitalization duration during follow-up. Statistical significance was considered based on two tailed $p<0.05$. All statistical

analyses were performed using SPSS version 26 software (IBM Corporation, Armonk, New York) and R 3.6.1 (Vienna, Austria).

2.5. Results

Baseline characteristics

Patients were prospectively enrolled in the study from the emergency department or outpatient cardiology clinics. RAS fingerprints of plasma angiotensin peptides were generated in 110 patients, median age was 71 years (IQR: 58-80 years), with 72 male patients (65.5%). Overall, 51 patients (46.4%) were clinically classified as NYHA functional class I or II, 42 patients (38.2%) as NYHA functional class III, and 17 patients (15.4%) as NYHA functional class IV (**Table 2.1.**). Atrial fibrillation and hypertension were major comorbidities in the overall cohort with 58 (52.7%) and 60 (54.5%) patients, respectively. RAS blockade therapies with ACE inhibitors (69.2%) or ARBs (15.4%) were common, while patients taking ARNI were excluded from the study. A total of 40 patients (36.4%) reached the primary endpoint during a median follow-up of 5.1 years (IQR: 4.7 to 5.7 years). In general, the survivor group with 70 patients (63.6%) was characterized by having a younger age (65 years [IQR: 55-73 years], $p<0.001$), higher BMI (31.6 kg/m^2 [26.7-34.8 kg/m^2], $p=0.034$), hemoglobin (137 g/L [IQR: 122-150 g/L], $p=0.008$), estimated glomerular filtration rate (GFR, 60 ml/min/m^2 [IQR:43-67 ml/min/m^2], $p=0.005$) and lower BNP levels (225 pg/ml [IQR: 80-478 pg/ml], $p=0.005$) . Additionally, survivors had lower systolic blood pressure (118 mmHg [IQR: 104-132 mmHg], $p=0.004$), less usage of loop diuretics (n=45 [64.3%], $p=0.027$), less symptom burden as reflected by greater number of patients in the NYHA class I or II (n=42 [60.0%], $p<0.001$) with lower prevalence of atrial fibrillation (n=30 [42.9%], $p=0.009$),

chronic kidney disease (CKD) (n=21 [30.0%], p=0.003), hypertension (n=31 [44.3%], p=0.005) and chronic obstructive pulmonary disease (COPD) (n=16 [22.9%], p=0.006).

Equilibrium and circulating angiotensin peptide levels

A newly developed liquid chromatography-mass spectrometry/mass spectroscopy technique provided an unique opportunity to profile angiotensin peptide levels in HF patient plasma samples.[189] Circulating levels represent a snapshot of the dynamic state of angiotensin peptides in patient's circulation and were measured from vials containing enzyme inhibitors completely blocking angiotensin peptide metabolism. In comparison, equilibrium angiotensin peptide levels were measured from lithium-heparin plasma after resting for 30 minutes under room condition, thus allowing sufficient time for equilibration and enzyme activation. Median equilibrium peptide levels were higher than circulating levels across the analyzed angiotensin peptides (**Figure 2.1.**). Although Ang I (50.0 to 261.2 pg/ml) and Ang II (4.9 to 37.2 pg/ml) increased markedly during *ex vivo* equilibration of plasma samples compared to their circulating state, the increase in Ang 1-7 (2.6 to 7.6 pg/ml) and Ang 1-9 levels (1.6 to 3.0 pg/ml) from circulating to equilibrium state was much less pronounced. The suppressed plasma Ang II relative to Ang I levels in both equilibrium and circulating analysis reflects the prevalence of ACE inhibitor use in our study cohort. Correlation analysis revealed equilibrium and circulating angiotensin peptide levels correlated strongest for Ang I (r^2=0.90, p<0.001), which remained strong and significant with Ang II (r^2=0.75, p<0.001), Ang 1-7 (r^2=0.66, p<0.001) and Ang 1-9 (r^2= 0.48, p<0.001) (**Supplemental Figure 2.1.**).

Angiotensin peptides and survival

HF patients were stratified into above and below median cohorts based on equilibrium and circulating levels of Ang II and Ang 1-7 or the associated Ang 1-7/Ang II ratio. Kaplan-Meier analysis of all-cause mortality for HF patients with equilibrium Ang 1-7/Ang II ratios above the median showed higher survival rates over the follow-up period than those below the median (76.4% vs. 50.9%; p=0.004); similar results were observed for the circulating Ang 1-7/Ang II ratios (72.7% vs. 54.5%; p=0.041) (**Figure 2.2.**). Importantly, Ang 1-7 and Ang II peptide levels alone failed to display significant differences in survival rates between the above and below median cohorts for both circulating and equilibrium levels during the follow-up period (**Supplemental Figures 2.2. and 2.3.**).

Unadjusted hazards showed above median equilibrium (HR: 0.39; 95% CI: 0.20 to 0.76; p=0.005) and circulating (HR: 0.52; 95% CI: 0.27 to 0.99; p=0.045) Ang 1-7/Ang II ratios strongly predicted lower all-cause mortality in our HF patient cohort. Plasma BNP, hemoglobin levels, NYHA functional class, age, body mass index, estimated GFR, systolic blood pressure, use of ACE inhibitor, beta blocker and loop diuretics, in addition to the presence of atrial fibrillation, CKD, hypertension, and COPD were additional significant predictors of all-cause mortality; while HF etiology, gender, plasma aldosterone, active renin levels and LVEF were not significant (**Table 2.2. and 2.3.; Supplemental Tables 2.1. and 2.2.**). In the adjusted multivariate analysis incorporating significant covariates from the unadjusted model, which produced the best possible subset incorporating age, BNP and NYHA functional class, above median equilibrium Ang 1-7/Ang II ratios remained as an independent predictor of lower all-cause mortality (HR: 0.38; 95% CI: 0.18 to 0.81; p=0.012) (**Table 2.2.**). Similar prognostic importance was found with circulating Ang 1-7/Ang II ratio through the multivariate analysis (HR: 0.38; 95% CI: 0.18 to 0.80; p=0.011)

(**Table 2.3.**). Atrial fibrillation was another independent predictor of all-cause mortality alongside both equilibrium and circulating ratios in the adjusted model. Once again, the individual plasma angiotensin peptide levels for Ang 1-7 and Ang II failed to predict all-cause mortality for both equilibrium and circulating levels in our patient cohorts (**Supplemental Table 3.3.**).

For estimation of the incremental prognostic value of the plasma Ang 1-7/Ang II ratio in predicting all-cause mortality, we compared the adjusted multivariate models, which included all predictors of all-cause mortality from the univariate analysis with and without the diminished Ang 1-7/Ang II ratio (below median cohort). In nested models, the Harrell's C-statistics increased from 0.78 to 0.81 (p=0.039) for all-cause mortality when accounting for equilibrium Ang 1-7/Ang II ratios and increased from 0.78 to 0.79 (p=0.040) for circulating ratios. Net reclassification analysis showed considerable improvement in risk prediction for all-cause mortality at 5 years provided by both the equilibrium (+45.0% [95% CI: 7.3% to 82.7%]) and circulating Ang 1-7/Ang II ratios (+24.3% [95% CI: 0.4% to 59.6%]) respectively.

Angiotensin peptides and hospitalization duration

The above median cohort of both equilibrium and circulating Ang 1-7/Ang II ratios was associated with shorter hospitalization duration on average per year of the 5-year follow-up period (**Figure 2.3.**). Median yearly hospitalization duration was much higher in the below median cohort at 8.1 days (IQR: 1.9-23.0 days) compared to 1.9 days (IQR: 1.1-2.8 days) for the above median cohort based on equilibrium Ang 1-7/Ang II ratios (p<0.001). Similarly, with circulating Ang 1-7/Ang II ratios, below median cohort had a median yearly hospitalization duration of 7.8 days (IQR:0.7-22.7 days), compared to 1.8 days (IQR: 0.1-9.4 days) of the above median cohort (p=0.023). The individual peptide levels mirrored our findings from the primary endpoint assessment and failed to display associations with hospitalization durations (**Supplemental Figures 2.4. and 2.5.**).

2.6. Discussion

A comprehensive assessment of plasma angiotensin peptides in the RAS demonstrates that the dynamic state of the endogenous RAS protective arm as reflected through the plasma Ang 1-7/Ang II ratio serves as a predictor of all-cause mortality and hospitalization duration in HF patients during long-term follow-up. In the multivariate Cox regression analysis, elevated plasma Ang 1-7/Ang II ratio provided independent prognostic value beyond adjusted covariates including age, plasma BNP, NYHA functional class and other conventional cardiovascular risk factors, with significant improvements in risk prediction based on c-statistics and the reclassification analysis indicative of the incremental prognostic power of the ratios. These findings highlight the translational potential of exploring the protective Ang 1-7/Mas axis as both a novel therapeutic target and prognostic marker for HF patients.

The major effector peptide of the beneficial RAS pathway is Ang 1-7, the concentration of which is affected by a variety of molecular enzymes participating in angiotensin metabolism. Molecular changes within its formation and degradation pathways through RAS enzyme expression or pharmacologic treatments directly modulate plasma Ang 1-7 levels. An extensively characterized enzyme involved in Ang 1-7 formation is ACE2. ACE2 is a monocarboxypeptidase and homologue of ACE that serves as an endogenous counter-regulator of the RAS, having dual functions behind its protective effects, (i) degrading Ang I and Ang II to limit activation of the adverse ACE/Ang II/AT_1R axis, and (ii) generating Ang 1-7 to stimulate the beneficial ACE2/Ang 1-7/Mas axis.[12,189] Preclinical evidence suggest an important role of the ACE2/Ang 1-7/Mas axis to ameliorate adverse left ventricular remodelling, myocardial fibrosis and dysfunctions.[13,14] This protective axis further mediates additional benefits in settings of prevalent HF risk factors and

comorbidities including coronary artery disease, diabetes, hypertension and obesity, yet it remains severely underutilized in clinical translations.[15,125,138,193,194]

Elevated human plasma ACE2 activity is associated with increase in HF severity, myocardial dysfunction independent of HF etiology, while also serving as an independent prognostic marker for adverse clinical outcomes in chronic HF patients.[195,196] Furthermore, elevated levels of plasma ACE2 protein was positively associated with incident HF through proteomic biomarker analysis.[197] Indeed, the proteolytic cleavage and release of tissue ACE2 by Ang II induced TACE/ADAM-17 protease activity into the circulation represents a positive feedback loop leading to loss of ACE2 mediated counter-regulatory protection within the tissue RAS.[45,134] Clinical trials with intravenous infusion of rhACE2 in patients with pulmonary arterial hypertension and acute lung injury lead to prompt increase in Ang 1-7/Ang II ratio reflecting ACE2 therapeutic actions.[176,191] In experimental murine models, increased *Ace2* tissue mRNA expression and activity is associated with an increase in plasma Ang 1-7, while changes in plasma Ang II levels depends on the mode of RAS inhibition.[198,199] Moreover, during SARS-CoV-1 and SARS-CoV-2 infections, ACE2 as the viral cellular receptor is downregulated through endocytosis alongside viral particles and increased TACE/ADAM-17 mediated proteolytic cleavage from the membrane, representing loss of ACE2 from the tissue RAS with a corresponding increase in plasma Ang II levels observed in COVID-19 patients which linearly correlated with the SARS-CoV-2 viral load.[7,116,200,201]

Importantly, increases in the Ang 1-7/Ang II ratio are not exclusively mediated by ACE2. Beside other enzymes involved in Ang 1-7 formation, including neutral endopeptidases (NEP), prolyl endopeptidases (PEP) and prolyl carboxypeptidases (PCP), pharmacologic inhibition of ACE interferers with angiotensin metabolism at multiple stages.[202,203] Firstly, ACE inhibitors

block formation of Ang II from Ang I, thereby inhibiting the Ang II/AT_1R axis and lead to an increase in Ang I levels. Moreover, ACE inhibitors block the conversion of Ang 1-7 to Ang 1-5, which is mainly driven by the N-domain of ACE.[24,202] Together with an increased Ang 1-7 formation rate via NEP, which is driven by increased availability of Ang I as a substrate, Ang 1-7 levels significantly increase in the presence of ACE inhibitors, resulting in an elevated of Ang 1-7/Ang II ratio and upregulation of the Ang 1-7/Mas axis. In comparison, ARNI (sacubitril/valsartan) serves as a dual inhibitor of both NEP and AT_1R resulting in circulating and equilibrium angiotensin profiles resembling ARB therapy.[204,205] NEP inhibition further prevents generation of the beneficial Ang 1-7, in combination with the elevated Ang II levels from AT_1R blockade depresses the Ang 1-7/Ang II ratio in the absence of an effective ACE2 mediated counter-regulation of the RAS peptides.

The findings of individual angiotensin peptide levels failed to display prognostic significance provides valuable insights into the RAS protective arm, as it showed that pathophysiological RAS activation is especially detrimental if the increase in Ang II is not counter-balanced by an associated increase in the beneficial Ang 1-7. Which has great clinical implications in the settings of incomplete RAS blockade due to alternative tissue production of Ang II through ACE independent pathways such as chymase-mediated Ang II production.[189,206]

The prognostic abilities of the plasma Ang 1-7/Ang II ratio generated using equilibrium peptides appear greater in comparison to circulating peptides in the univariate analysis. As such, the equilibrium angiotensin peptide levels may account for the functional reserve of the RAS in HF patients available under pathophysiological stress, and thus provides additional information beyond the circulating angiotensin peptide profiles. Moreover, a special protease inhibitor cocktail is required to determine circulating angiotensin peptide levels, as such sampling is more pragmatic

for the equilibrium analysis, because it is compatible with standard serum or lithium-heparin sampling, underlining its potential utility for comprehensive biochemical evaluation of the RAS in clinical settings. Interestingly, equilibrium and circulating angiotensin peptide levels displayed strong correlations supporting the interconnected nature for both assays.[189,205]

In addition to providing accurate prognostic information and the ability to inform clinical decision-making, biomarkers may further serve an essential role in guiding the development of novel HF therapeutics and improving HF clinical trial efficacy.[189,207] The remarkable success from targeted inhibition of NEP using ARNI for treating HFrEF patients is inseparable from the initial discovery of the tremendous prognostic ability held by the natriuretic peptides, notably, BNP and NT-proBNP in HF patients.[208,209] Currently, blockade of the detrimental Ang II/AT_1R axis with ACE inhibitors, ARBs and ARNI represents class I recommendation for the treatment of HFrEF patients.[187] In our patient cohort, plasma Ang 1-7/Ang II ratio remained as an independent and incremental predictor of all-cause mortality after adjusting for relevant clinical covariates, which solidifies the significance of the beneficial Ang 1-7/Mas axis in survival of patients with HF. Moreover, Ang 1-7 treatment improved endothelial dysfunction in obese humans,[210] and is currently in clinical trial for management of cognitive impairment in HF patients (NCT03159988).[211] As such, the important prognostic value of the plasma Ang 1-7/Ang II ratio from our findings support the concurrent effort to examine plasma Ang 1-7 together with Ang II levels for HF prognosis and risk stratification, while introducing novel therapeutic interventions such as rhACE2, Ang 1-7 supplementation and Mas agonists to promote the Ang 1-7/Mas axis together with present RAS blockade therapies in clinical management of HF and the associated comorbidities.[51,212-214]

HF is a constantly evolving disease spanning across a broad spectrum of phenotypes which cannot be fully represented by conventional categorizations such as those based on LVEF, rather the dynamic changes in functional and structural components are better reflected through the use of appropriate biomarkers to guide personalized therapies.[215] An additional strength of the present study is the inclusive nature of our HF cohort, by incorporating acute decompensated HF and patients with reduced or preserved ejection fraction, we successfully demonstrated clinical utility of the plasma Ang 1-7/Ang II ratio in HF patients regardless of their etiology or clinical severity. We recognize the current analysis is limited by the sample size of our patient pool, thus hindering our ability to perform further subgroup analysis. This is especially relevant for patients using ACE inhibitors versus ARB that have differential implications on the Ang 1-7/Ang II ratio based on their divergent approach for achieving RAS blockade. In all 17 patients treated with ARBs, Ang II was the major angiotensin metabolite detected in equilibrium analysis, with markedly lower levels of Ang I detected. In our total cohort, this picture shifted with Ang I levels being 7-fold higher than Ang II levels, indicating efficient ACE inhibition in the majority of our patients. As expected, patients treated with ACE inhibitors tended to accumulate above the median for Ang 1-7/Ang II ratio, suggesting that ACE inhibition contributes to activation of the beneficial RAS axis in HF. Future studies should examine the relative activities and contributions of endogenous peptidases including ACE, ACE2, PEP, PCP and NEP in angiotensin peptide metabolism and their effects on the equilibrium Ang 1-7/Ang II ratio.

2.7. Conclusion

Our study utilized the novel mass spectrometry technique in examining the prognostic abilities of the RAS endogenous protective arm and observed elevated Ang 1-7/Ang II ratio serves as an independent predictor of all-cause mortality after accounting for conventional cardiovascular risk

factors, and was associated with shorter hospitalization durations per year during a 5 year follow-up period. In comparison, the observation that individual plasma Ang 1-7 and Ang II levels alone failed to display prognostic value provides exciting opportunities to apply precision medicine through the concurrent assessment of both peptides for prognosis and risk stratification in HF patients. As equilibrium angiotensin peptide levels can be measured in standard lithium-heparin plasma or serum without the need of the special protease inhibitor cocktail required for circulating measurements, while holding similar prognostic power as circulating Ang 1-7/Ang II ratios in the multivariate analysis, equilibrium Ang 1-7/Ang II ratio may be preferentially assessed in routine clinical practices.

Figures and Tables

Figures

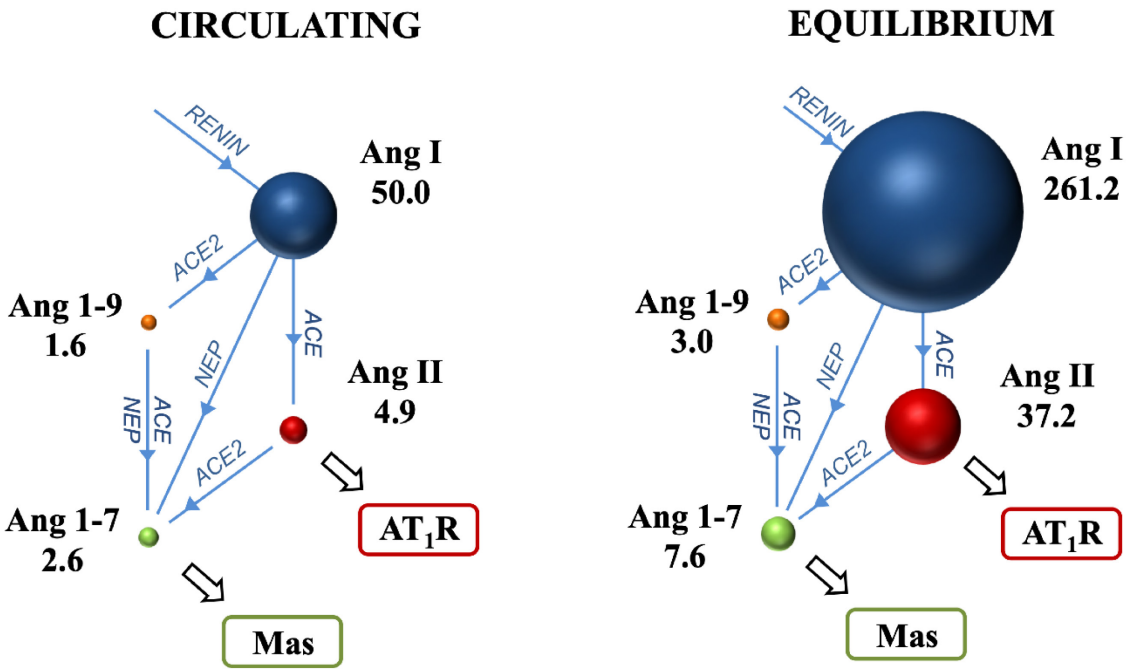


Figure 2.1. Global representation of median equilibrium and circulating angiotensin peptide levels from our heart failure cohort. Circulating levels reflect the snapshot of the dynamic angiotensin profiles within the circulation collected in presence of protease inhibitors completely blocking angiotensin peptide metabolism (n=110). Equilibrium levels reflect the resultant angiotensin peptide levels from *ex vivo* equilibration. Median circulating and equilibrium levels of indicated angiotensin peptides are given in pg/ml and are reflected by the size of spheres. Blue arrows connecting the spheres indicate enzymes catalyzing the conversion between connected peptides as indicated.

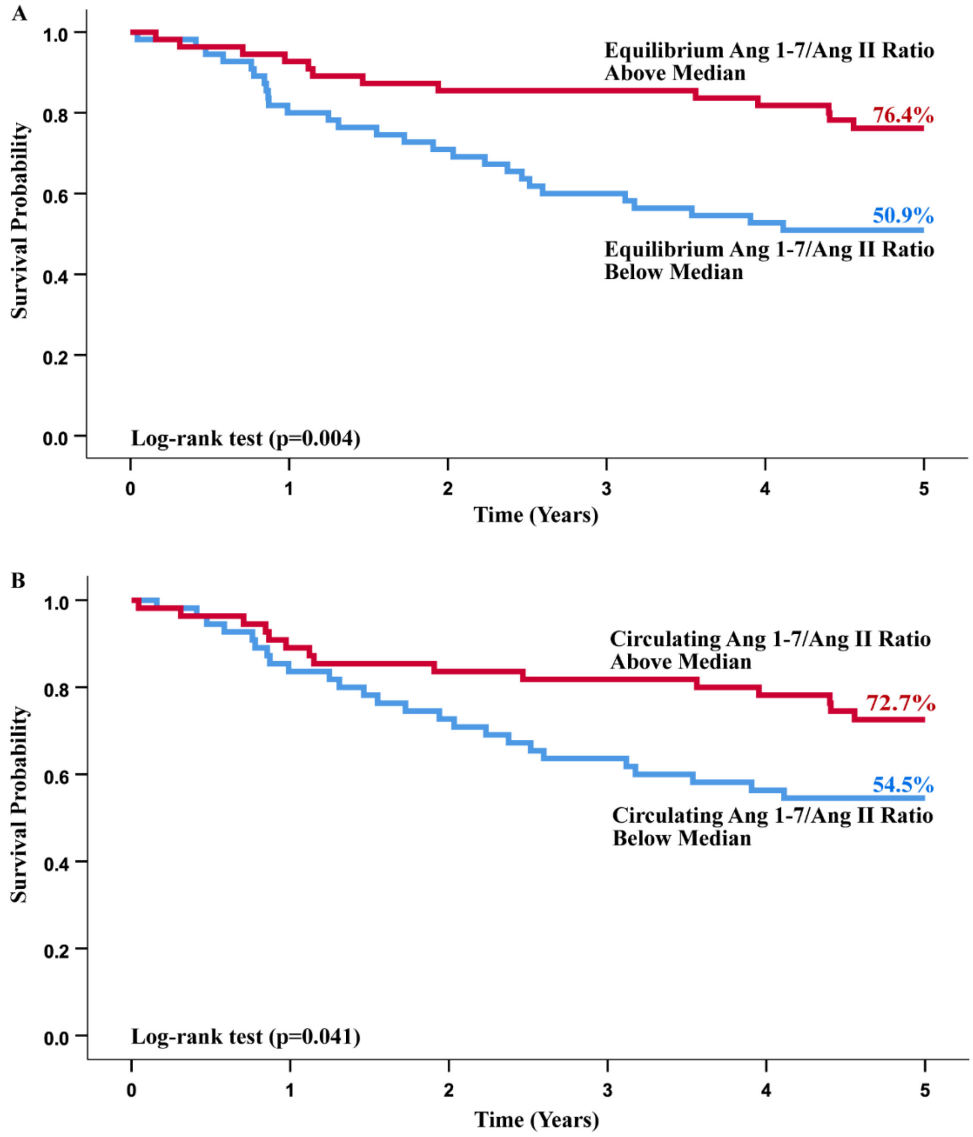


Figure 2.2. Kaplan-Meier analysis of primary outcomes (all-cause mortality) with Ang 1-7/Ang II ratios. (A) Heart failure (HF) patients stratified based on below and above median cohorts of Ang 1-7/Ang II ratios based on equilibrium peptide levels. Above median cohort had a 5-year survival rate of 76.4%, compared to below median cohort with a survival rate of 50.9% (p=0.004) (n=110). (B) HF patients stratified based on below and above median cohorts of Ang 1-7/Ang II ratios based on circulating peptide levels. Above median cohort had a 5-year survival rate of 72.7%, compared to below median cohort with a survival rate of 54.5% (p=0.041).

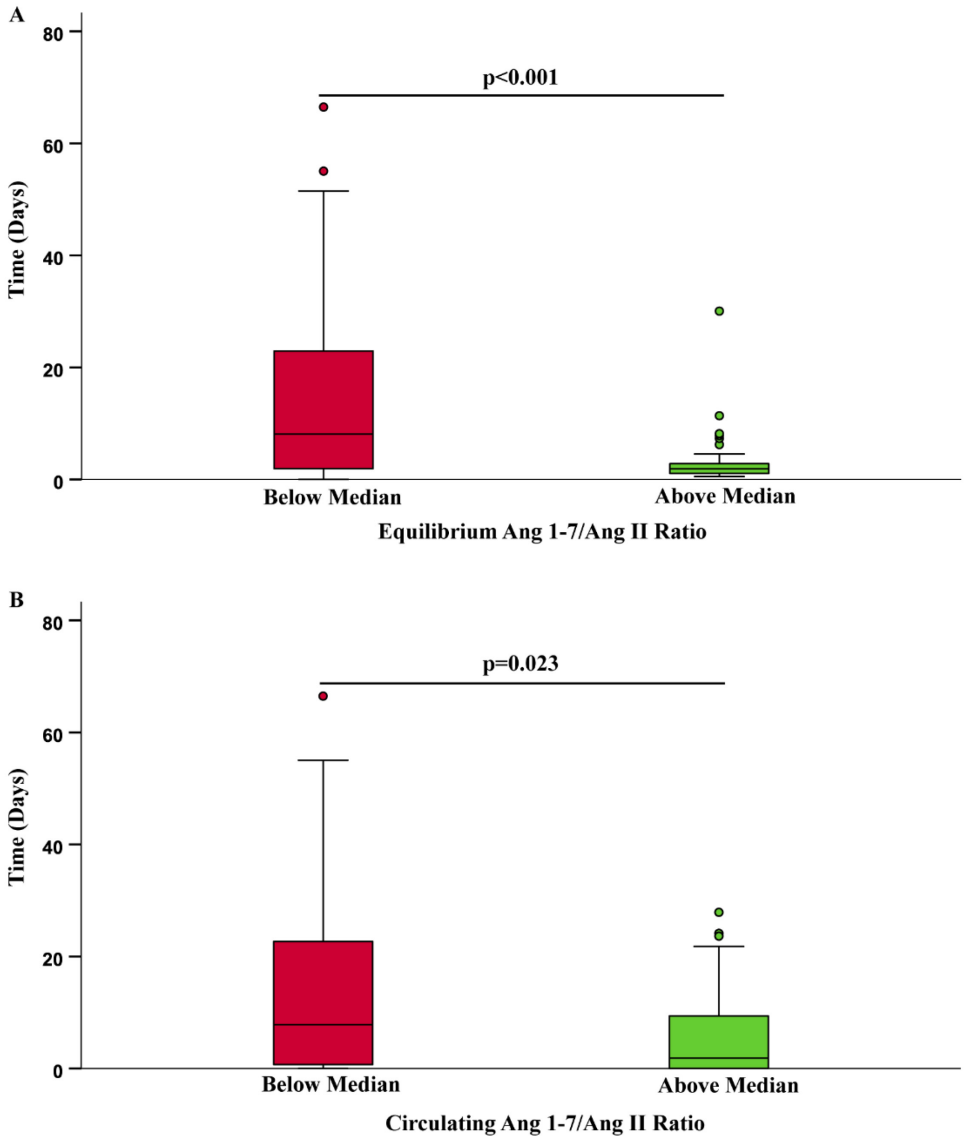


Figure 2.3. Side by side comparison of secondary outcomes (yearly hospitalization duration) with Ang 1-7/Ang II ratios. (A) Heart failure (HF) patients stratified based on below and above median cohorts of Ang 1-7/Ang II ratios based on equilibrium peptide levels (n=110). Above median cohort was associated with shorter hospitalization duration per year over the 5-year follow-up ($p<0.001$). (B) HF patients stratified based on below and above median cohorts of Ang 1-7/Ang II ratios based on circulating peptide levels. Above median cohort was associated with shorter hospitalization duration per year over the 5-year follow-up ($p=0.023$).

Tables

Table 2.1. Patient baseline clinical characteristics (n=110)

	Entire Cohort (n=110)	All-cause Mortality (n=40)	Survivors (n=70)	p Value
Demographics				
Age (years)	71 (58-80)	78 (72-83)	65 (55-73)	<0.001
BMI (kg/m^2)	30.3 (25.0-34.2)	27.1 (24.0-31.7)	31.6 (26.7-34.8)	0.034
Male	72 (65.5)	25 (62.5)	47 (67.1)	0.68
Laboratory Tests				
Equilibrium Ang 1-7 (pg/ml)	7.6 (2.6-47.9)	4.9 (2.6-42.7)	12.7 (2.7-47.9)	0.40
Equilibrium Ang II (pg/ml)	37.2 (7.5-126.9)	55.4 (4.8-254.6)	35.4 (9.5-93.1)	0.30
Circulating Ang 1-7 (pg/ml)	2.6 (2.0-8.7)	2.4 (1.9-4.4)	3.5 (2.1-11.6)	0.09
Circulating Ang II (pg/ml)	4.9 (2.0-14.5)	6.5 (2.0-19.3)	4.4 (2.0-11.0)	0.29
Equilibrium Ang 1-7/Ang II	0.5 (<0.1-1.9)	0.2 (<0.1-1.4)	0.8 (0.1-1.9)	0.028
Circulating Ang 1-7/Ang II	1.0 (0.3-1.8)	0.6 (0.2-1.4)	1.1 (0.4-2.0)	0.010
Hemoglobin (g/L)	131 (117-145)	124 (112-135)	137 (122-150)	0.008
eGFR (ml/min/m^2)	53 (35-67)	43 (34-57)	60 (43-67)	0.005
Aldosterone (pmol/L)	359 (190-559)	359 (213-665)	356 (160-528)	0.73
BNP (pg/ml)	280 (94-653)	456 (198-1135)	225 (80-478)	0.005
Active renin (pg/ml)	40 (10-149)	30 (8-73)	58 (11-156)	0.17
Echocardiography				
LVEF (%)	40 (28-55)	41 (30-58)	39 (27-53)	0.30
Medications				
ACEI %MRDD	75 (69.2) 100 (50-100)	22 (55.0) 50 (25-94)	53 (75.7) 100 (75-100)	0.033 0.005
ARB %MRDD	17 (15.4) 50 (25-50)	8 (20.0) 50 (25-50)	9 (12.9) 50 (25-75)	0.41 0.42
Beta Blocker %MRDD	99 (90.0) 50 (25-100)	32 (80.0) 31 (25-100)	67 (95.7) 63 (28-100)	0.017 0.023
Loop Diuretics %MRDD	79 (71.8) 10 (7-13)	34 (85.0) 12 (7-13)	45 (64.3) 7 (7-13)	0.027 0.48
MRA %MRDD	43 (39.1) 50 (50-50)	11 (27.5) 50 (50-75)	32 (45.7) 50 (50-50)	0.07 0.28

Heart Failure Etiology				
Ischemic	51 (46.4)	18 (45.0)	33 (47.1)	0.85
Non-Ischemic	59 (53.6)	22 (55.0)	37 (52.9)	0.85
Functional Class				
NYHA Class I/II	51 (46.4)	9 (22.5)	42 (60.0)	<0.001
NYHA Class III	42 (38.2)	20 (50.0)	22 (31.4)	0.067
NYHA Class IV	17 (15.4)	11 (27.5)	6 (8.6)	0.013
Comorbidities				
AF	58 (52.7)	28 (70.0)	30 (42.9)	0.009
DM	37 (33.6)	15 (37.5)	22 (31.4)	0.54
CKD	45 (40.9)	24 (60.0)	21 (30.0)	0.003
HTN	60 (54.5)	29 (72.5)	31 (44.3)	0.005
COPD	36 (32.7)	20 (50.0)	16 (22.9)	0.006
Vitals				
Heart Rate	70 (60-85)	77 (63-92)	66 (60-80)	0.016
Systolic BP	121 (107-136)	128 (114-142)	118 (104-132)	0.004
Diastolic BP	72 (65-80)	75 (69-83)	70 (63-80)	0.15

BMI=body mass index; AF=atrial fibrillation; DM=diabetes mellitus; CKD=chronic kidney disease; HTN=hypertension; COPD=chronic obstructive pulmonary disease; ACEI=ACE inhibitor; ARB=angiotensin receptor blocker; MRA=mineralocorticoid receptor antagonist; MRDD= maximum required daily dose, eGFR=estimated glomerular filtration rate based on the MDRD equation, BP = blood pressure. Doses as % of MRDD shown in parentheses.

Table 2.2. Cox Regression Analysis for All-Cause Mortality of Plasma Ang 1-7/Ang II Ratio using Equilibrium Peptide Levels (n=110)

	Univariate Analysis		Multivariate Analysis	
Clinical Variables	**HR (95% CI)**	**p-Value**	**HR (95% CI)**	**p-Value**
Ang 1-7/Ang II Ratio (Above Median)	0.39 (0.20-0.76)	0.005	0.38 (0.18-0.81)	0.012
Age (per 5 years)	1.33 (1.15-1.54)	<0.001	1.09 (0.92-1.29)	0.32
BMI (per 5 kg/m^2)	0.72 (0.54-0.95)	0.022	0.84 (0.59-1.19)	0.32
Gender (Male)	0.83 (0.44-1.58)	0.57		
Hemoglobin (per 10 g/L)	0.85 (0.74-0.97)	0.016		
eGFR (per 10 ml/min/m^2)	0.79 (0.67-0.93)	0.005		
Ln (Aldosterone)	1.10 (0.72-1.67)	0.67		
Ln (BNP)	1.55 (1.19-2.02)	0.001	1.19 (0.84-1.69)	0.32
Ln (Active Renin)	0.88 (0.72-1.06)	0.17		
LVEF (per 5 %)	1.06 (0.95-1.17)	0.31		
ACEI	0.48 (0.26-0.90)	0.023		
ARB	1.44 (0.66-3.13)	0.36		
Beta Blocker	0.34 (0.16-0.75)	0.007		
Loop Diuretics	2.61 (1.09-6.21)	0.031		
MRA	0.52 (0.26-1.04)	0.07		
HF Etiology (Ischemic)	0.89 (0.48-1.66)	0.71		
NYHA Functional Class	2.29 (1.52-3.45)	<0.001	1.52 (0.87-2.65)	0.14
AF	2.57 (1.30-5.05)	0.006	2.31 (1.06-5.02)	0.035
DM	1.16 (0.61-2.19)	0.66		

CKD	2.75 (1.46-5.19)	0.002		
HTN	2.61 (1.30-5.24)	0.007		
COPD	2.40 (1.29-4.46)	0.006	1.83 (0.80-4.20)	0.15
Heart Rate (per 10 beats/minute)	1.15 (1.01-1.30)	0.030		
Systolic BP (per 10 mmHg)	1.24 (1.09-1.42)	0.002	1.10 (0.90-1.33)	0.36
Diastolic BP (per 10 mmHg)	1.17 (0.92-1.50)	0.21		

CI = confidence interval; HR = hazard ratio. Additional abbreviations are defined in Table 2.1.

Table 2.3. Cox Regression Analysis for All-Cause Mortality of Plasma Ang 1-7/Ang II Ratio using Circulating Peptide Levels (n=110)

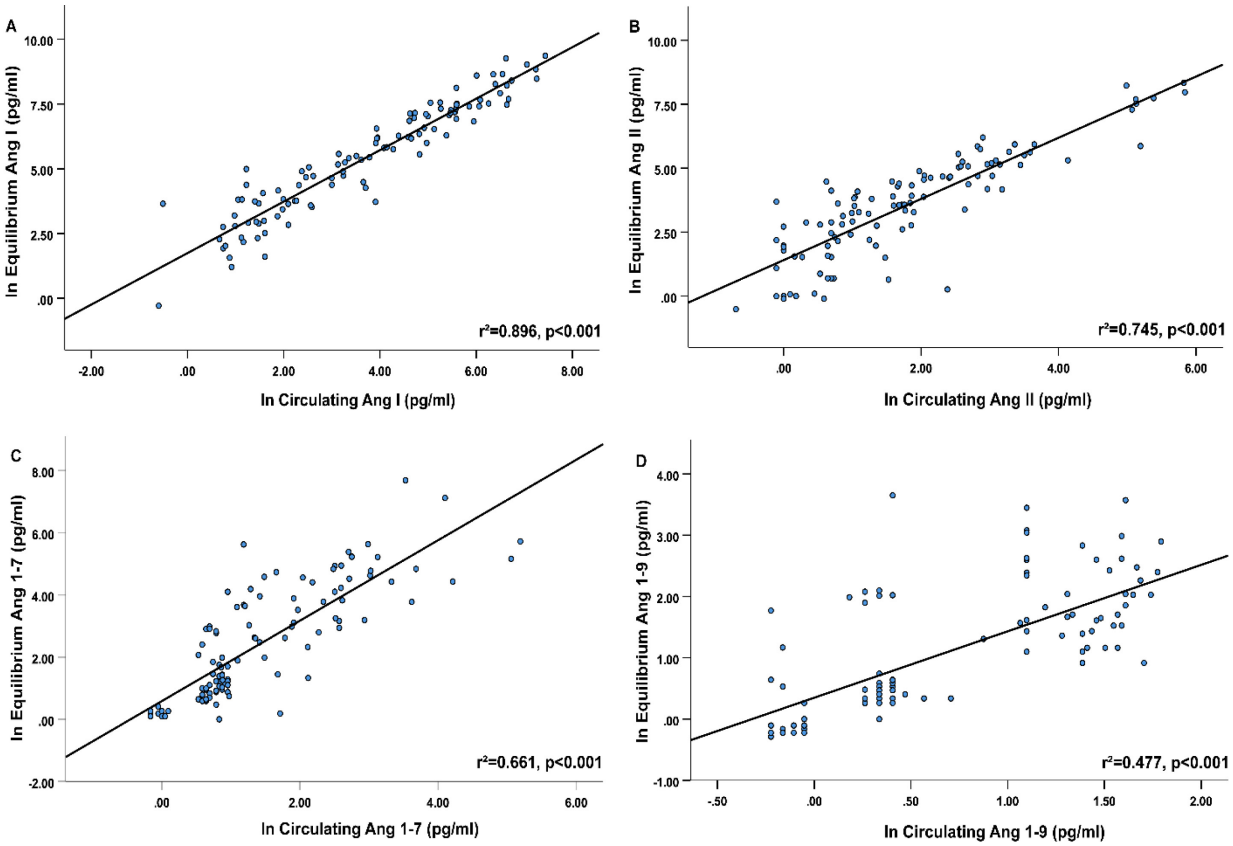
	Univariate Analysis		Multivariate Analysis	
Clinical Variables	**HR (95% CI)**	**p-Value**	**HR (95% CI)**	**p-Value**
Ang 1-7/Ang II Ratio (Above Median)	0.52 (0.27-0.99)	0.045	0.38 (0.18-0.80)	0.011
Age (per 5 years)	1.33 (1.15-1.54)	<0.001	1.09 (0.92-1.28)	0.33
BMI (per 5 kg/m^2)	0.72 (0.54-0.95)	0.022	0.80 (0.57-1.14)	0.22
Gender (Male)	0.83 (0.44-1.58)	0.57		
Hemoglobin (per 10 g/L)	0.85 (0.74-0.97)	0.016		
eGFR (per 10 ml/min/m^2)	0.79 (0.67-0.93)	0.005		
Ln (Aldosterone)	1.10 (0.72-1.67)	0.67		
Ln (BNP)	1.55 (1.19-2.02)	0.001	1.19 (0.85-1.65)	0.31
Ln (Active Renin)	0.88 (0.72-1.06)	0.17		
LVEF (per 5 %)	1.06 (0.95-1.17)	0.31		
ACEI	0.48 (0.26-0.90)	0.023		
ARB	1.44 (0.66-3.13)	0.36		
Beta Blocker	0.34 (0.16-0.75)	0.007		
Loop Diuretics	2.61 (1.09-6.21)	0.031		
MRA	0.52 (0.26-1.04)	0.07		
HF Etiology (Ischemic)	0.89 (0.48-1.66)	0.71		
NYHA Functional Class	2.29 (1.52-3.45)	<0.001	1.45 (0.83-2.51)	0.19
AF	2.57 (1.30-5.05)	0.006	2.42 (1.11-5.27)	0.026
DM	1.16 (0.61-2.19)	0.66		
CKD	2.75 (1.46-5.19)	0.002		

HTN	2.61 (1.30-5.24)	0.007		
COPD	2.40 (1.29-4.46)	0.006	1.69 (0.74-3.90)	0.22
Heart Rate (per 10 beats/minute)	1.15 (1.01-1.30)	0.030		
Systolic BP (per 10 mmHg)	1.24 (1.09-1.42)	0.002	1.01 (0.99-1.03)	0.14
Diastolic BP (per 10 mmHg)	1.17 (0.92-1.50)	0.21		

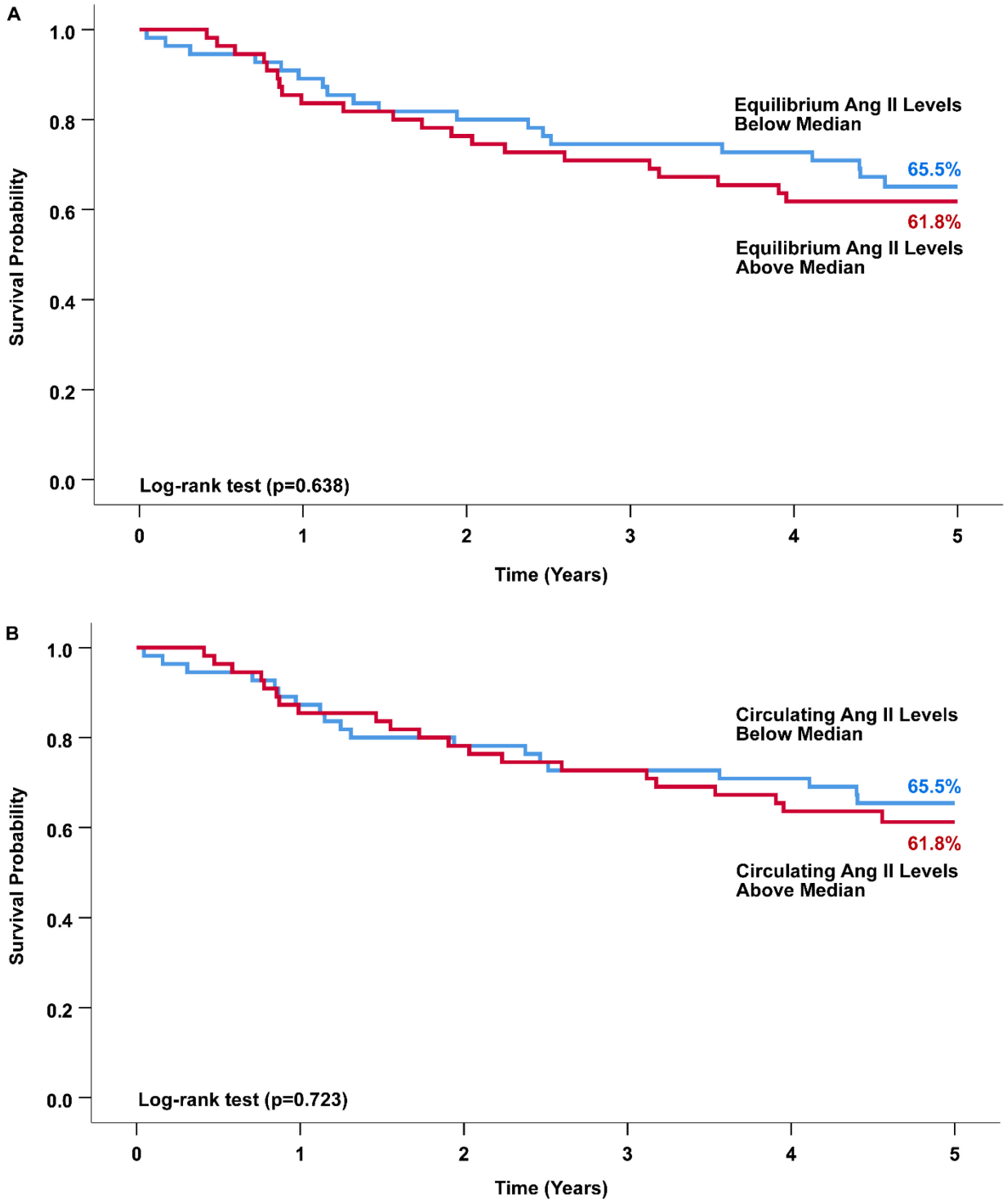
CI = confidence interval; HR = hazard ratio. Additional abbreviations are defined in Table 2.1.

Supplementary Material

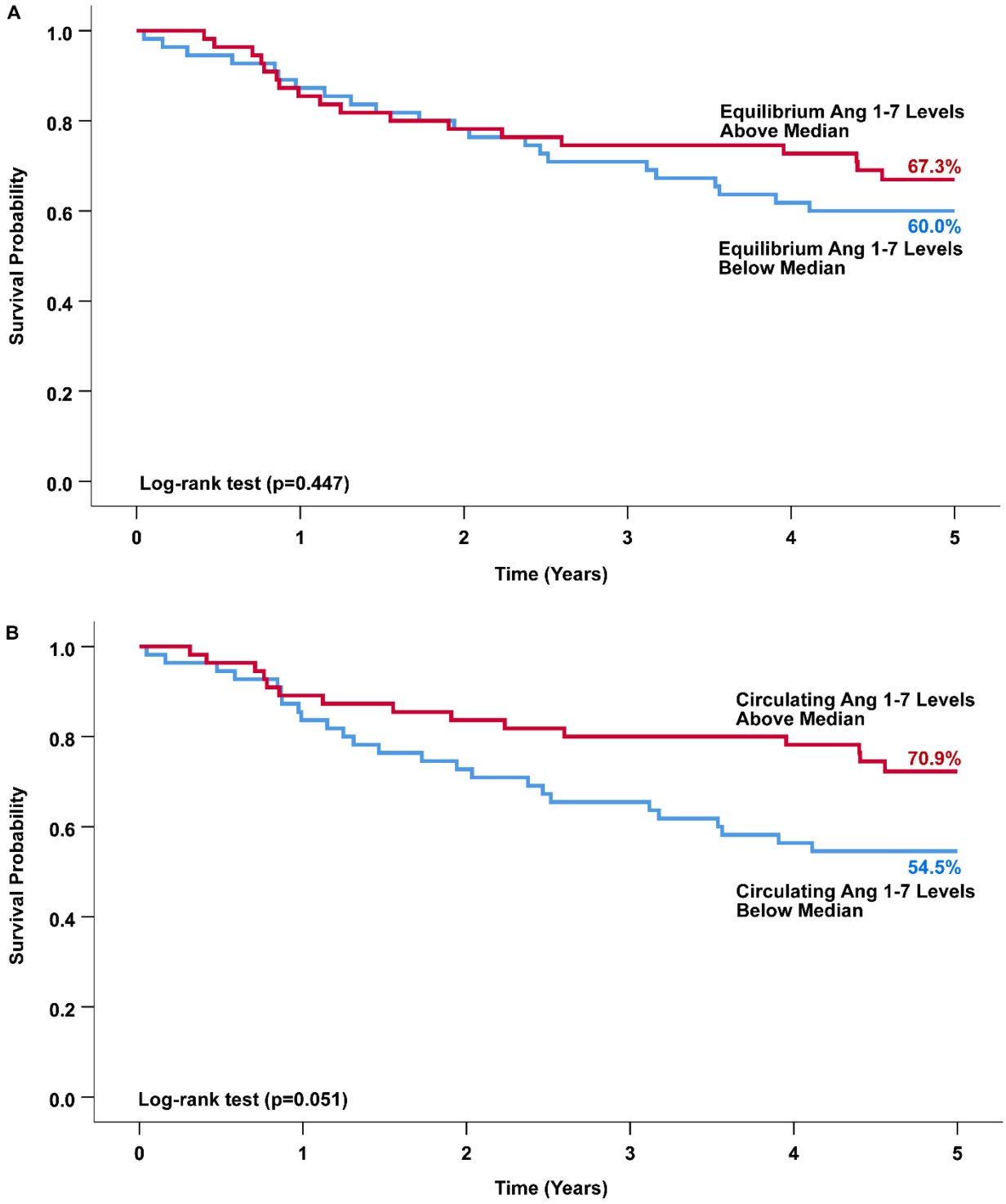
Supplemental Figures



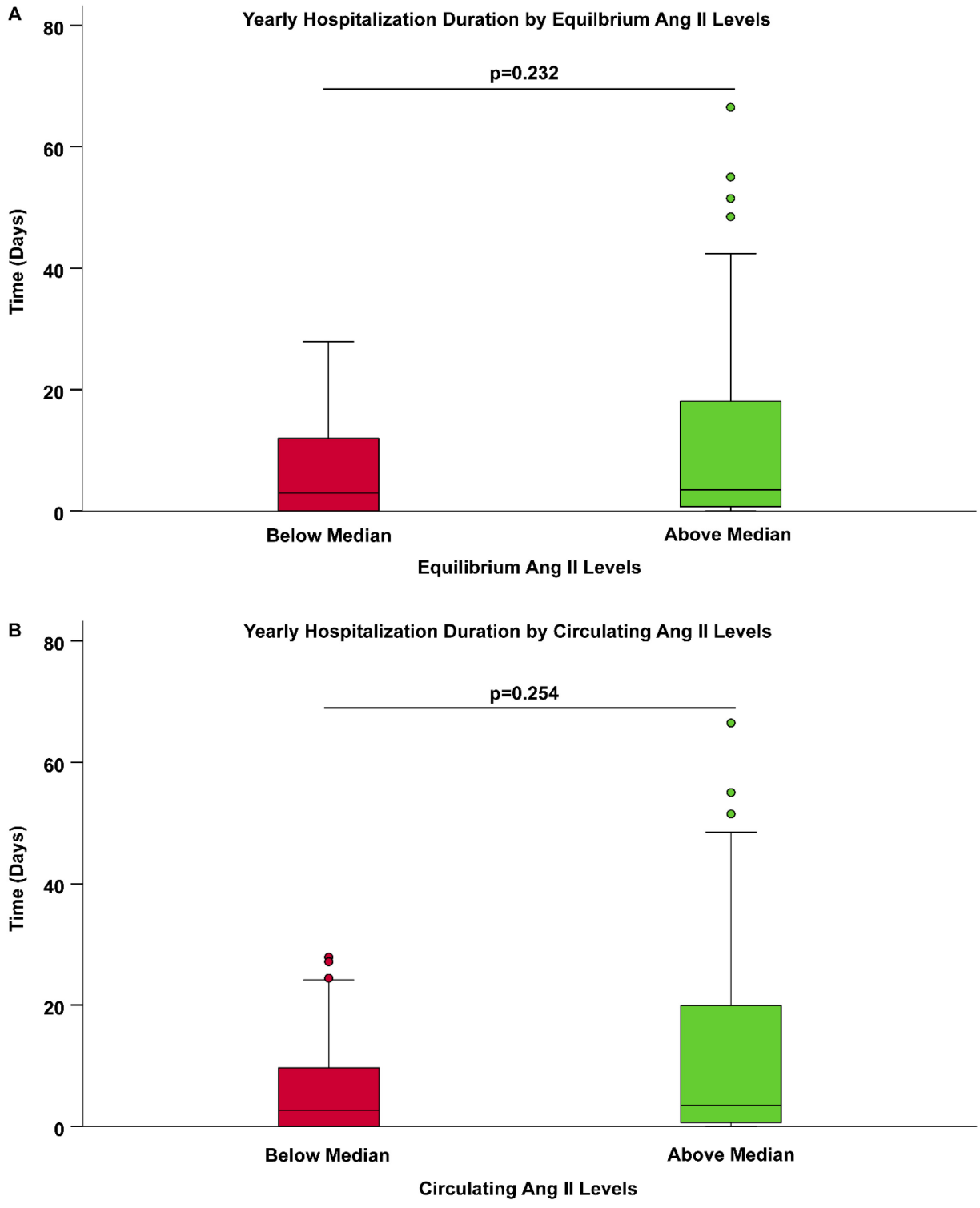
Supplemental Figure 2.1. Scatterplot of correlation between circulating and equilibrium angiotensin peptide levels for (A) Ang I (B) Ang II (C) Ang 1-7 (D) Ang 1-9.



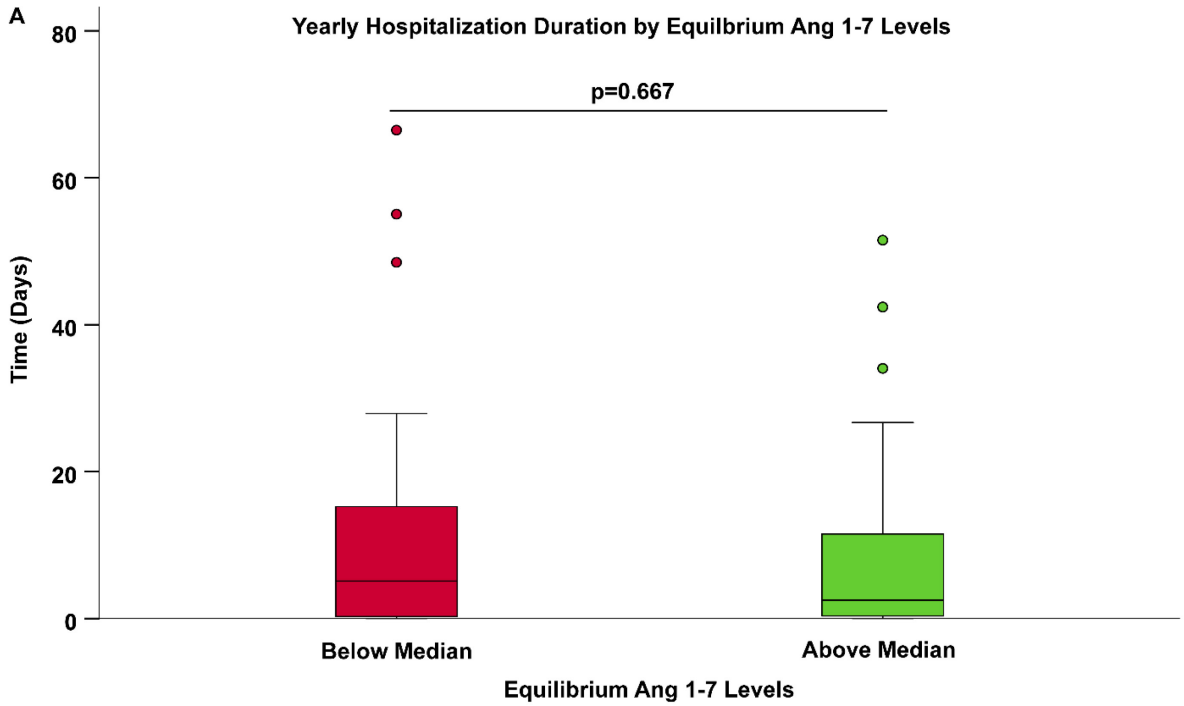
Supplemental Figure 2.2. Kaplan-Meier analysis of primary outcomes with plasma Ang II levels. When HF patients were stratified based on below and above median cohorts, both equilibrium (A) and Circulating (B) levels failed to display a statically significant difference in survival probability.

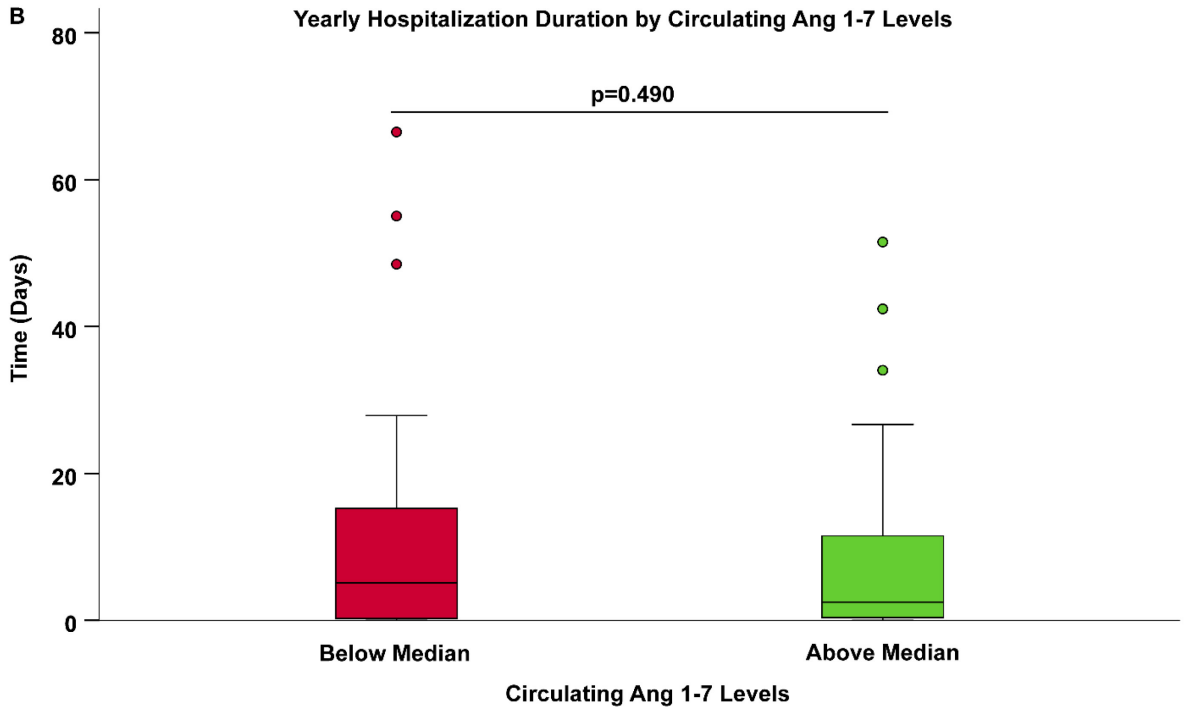


Supplemental Figure 2.3. Kaplan-Meier analysis of primary outcomes with plasma Ang 1-7 levels. HF patients were stratified based on below and above median cohorts, both equilibrium (A) and Circulating (B) levels failed to display a statically significant difference in survival probability.



Supplemental Figure 2.4. Comparison of secondary outcomes (yearly hospitalization duration) with plasma Ang II levels. HF patients were stratified based on below and above median cohorts, both equilibrium (A) and Circulating (B) levels failed to display a statically significant difference in hospitalization duration per year over the 5-year follow-up.





Supplemental Figure 2.5. Comparison of secondary outcomes (yearly hospitalization duration) with plasma Ang 1-7 levels. HF patients were stratified based on below and above median cohorts, both equilibrium (A) and Circulating (B) levels failed to display a statically significant difference in hospitalization duration per year over the 5-year follow-up.

Supplemental Tables

Supplemental Table 2.1. Patient Characteristics for Patient Cohorts Stratified by Median Equilibrium Ang 1-7/Ang II ratios (n=110)

	Below Median (n=55)	Above Median (n=55)	p-value
Demographics			
Age (years)	73 (62-79)	66 (57-80)	0.25
BMI (kg/m^2)	27.8 (24.0-33.2)	31.0 (27.8-35.0)	0.08
Gender (Male)	34 (61.8)	38 (69.1)	0.55
Labs			
Hemoglobin (g/L)	126 (112-141)	137 (122-150)	0.016
eGFR (ml/min)	44 (31-68)	60 (46-67)	0.08
Sodium (mmol/L)	138 (137-140)	139 (138-141)	0.031
Potassium (mmol/L)	4.0 (3.7-4.3)	4.3 (4.1-4.7)	0.001
Plasma biomarkers			
Aldosterone (pmol/L)	361 (166-634)	346 (198-527)	0.54
BNP (pg/ml)	369 (133-909)	215 (85-485)	0.05
Active renin (pg/ml)	30 (10-124)	59 (8-167)	0.33
Echocardiography			
LVEF (%)	43 (33-55)	38 (26-55)	0.040
Medications, % of MRDD			
ACE Inhibitor	21 (38.2)	54 (98.2)	<0.001
% MRDD	50 (25-100)	100 (75-100)	0.033
ARB	17 (30.9)	0 (0)	<0.001
% MRDD	50 (25-50)	-	-
Beta Blocker	49 (89.1)	50 (90.9)	1.00
% MRDD	50 (25-100)	75 (41-100)	0.59
Loop Diuretics	46 (83.6)	33 (60)	0.010
% MRDD	13 (7-13)	7 (7-13)	0.81

MRA % MRDD	17 (30.9) 50 (50-100)	26 (47.3) 50 (50-50)	0.12 0.11
Heart Failure Etiology			
Ischemic	30 (54.5)	21 (38.2)	0.13
Non-Ischemic	25 (45.5)	34 (61.8)	0.13
Functional Class			
NYHA Class I/II	18 (32.7)	33 (60.0)	0.007
NYHA Class III	28 (50.9)	14 (25.5)	0.010
NYHA Class IV	9 (16.4)	8 (14.5)	1.00
Comorbidities			
Atrial Fibrillation	30 (54.5)	28 (50.9)	0.85
DM	20 (36.4)	17 (30.9)	0.69
CKD	30 (54.5)	15 (27.3)	0.006
HTN	34 (61.8)	26 (47.3)	0.18
COPD	16 (29.1)	20 (36.4)	0.54

BMI=body mass index; AF=atrial fibrillation; DM=diabetes mellitus; CKD=chronic kidney disease; HTN=hypertension; COPD=chronic obstructive pulmonary disease; ACEI=ACE inhibitor; ARB=angiotensin receptor blocker; MRA=Mineralocorticoid Receptor Antagonist; MRDD= Maximum required daily dose, eGFR=estimated glomerular filtration rate based on the MDRD equation. Doses as % of MRDD shown in parentheses. Kruskal-Wallis test was used for comparing statistical viability of continuous parameters between study cohorts, and Chi-squared test was used for categorical data. P-value less than 0.05 was considered statistically significant.

Supplemental Table 2.2. Patient Characteristics for Patient Cohorts Stratified by Median Circulating Ang 1-7/Ang II ratios (n=110)

	Below Median (n=55)	Above Median (n=55)	p-value
Demographics			
Age (years)	72 (61-79)	70 (57-81)	0.69
BMI (kg/m^2)	28.6 (24.2-33.8)	30.9 (26.9-34.2)	0.36
Gender (Male)	33 (60.0)	39 (70.9)	0.32
Labs			
Hemoglobin (g/L)	130 (116-143)	131 (119-148)	0.64
eGFR (ml/min)	49 (34-60)	60 (43-67)	0.09
Sodium (mmol/L)	138 (137-141)	139 (138-141)	0.09
Potassium (mmol/L)	4.1 (3.8-4.3)	4.3 (3.9-4.7)	0.034
Plasma biomarkers			
Aldosterone (pmol/L)	390 (276-656)	286 (156-515)	0.09
BNP (pg/ml)	388 (79-850)	225 (112-485)	0.23
Active renin (pg/ml)	41 (13-124)	39 (8-167)	0.91
Echocardiography			
LVEF (%)	41 (33-55)	39 (25-55)	0.40
Medications, % of MRDD			
ACE Inhibitor	27 (49.1)	48 (87.3)	<0.001
% MRDD	50 (25-100)	100 (75-100)	0.26
ARB	15 (27.3)	2 (3.6)	0.001
% MRDD	50 (25-50)	119 (84-153)	-
Beta Blocker	49 (89.1)	50 (90.9)	1.00
% MRDD	50 (25-100)	63 (25-100)	0.94
Loop Diuretics	46 (83.6)	33 (60)	0.010
% MRDD	10 (7-13)	7 (7-13)	0.66
MRA	17 (30.9)	26 (47.3)	0.12
% MRDD	50 (50-50)	50 (50-50)	0.72

Heart Failure Etiology			
Ischemic	27 (49.1)	24 (43.6)	0.70
Non-Ischemic	28 (50.9)	31 (56.4)	0.70
Functional Class			
NYHA Class I/II	20 (36.4)	31 (56.4)	0.06
NYHA Class III	26 (47.3)	16 (29.1)	0.08
NYHA Class IV	9 (16.4)	8 (14.5)	1.00
Comorbidities			
Atrial Fibrillation	29 (52.7)	29 (52.7)	1.00
DM	24 (43.6)	13 (23.6)	0.043
CKD	30 (54.5)	15 (27.3)	0.006
HTN	31 (56.4)	29 (52.7)	0.85
COPD	16 (29.1)	20 (36.4)	0.54

BMI=body mass index; AF=atrial fibrillation; DM=diabetes mellitus; CKD=chronic kidney disease; HTN=hypertension; COPD=chronic obstructive pulmonary disease; ACEI=ACE inhibitor; ARB=angiotensin receptor blocker; MRA=Mineralocorticoid Receptor Antagonist; MRDD= Maximum required daily dose, eGFR=estimated glomerular filtration rate based on the MDRD equation. Doses as % of MRDD shown in parentheses. Kruskal-Wallis test was used for comparing statistical viability of continuous parameters between study cohorts, and Chi-squared test was used for categorical data. P-value less than 0.05 was considered statistically significant.

Supplemental Table 2.3. Univariate Cox Regression Analysis for All-Cause Mortality with Individual Peptide Levels (n=110)

	Univariate Analysis	
Clinical Variables	**HR (95% CI)**	**p-Value**
Equilibrium Ang-(1-7)	0.79 (0.42-1.47)	0.45
Circulating Ang-(1-7)	0.53 (0.28-1.00)	0.01
Equilibrium Ang II	1.16 (0.62-2.16)	0.64
Circulating Ang II	1.12 (0.60-2.08)	0.72

CI = confidence interval; HR = hazard ratio. Additional abbreviations are defined in Supplemental Table 2.1.

Chapter 3. Dysregulation of ACE (Angiotensin-converting enzyme)-2 and renin-angiotensin peptides in SARS-CoV-2 mediated mortality and end-organ injuries

Authors:

Kaiming Wang[1,2], Mahmoud Gheblawi[2,3], Anish Nikhanj[1,2], Matt Munan[4], Erika MacIntyre[4,5], Conar O'Neil[6], Marko Poglitsch[7], Daniele Colombo[8], Franca Del Nonno[8], Zamaneh Kassiri[3], Wendy Sligl[4,6], and Gavin Y. Oudit[1,2,3*]

Affiliations:

[1]Division of Cardiology, Department of Medicine, University of Alberta, Edmonton, AB, Canada; [2]Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, AB, Canada; [3]Department of Physiology; [4]Department of Critical Care Medicine; [5]Division of Respirology, Department of Medicine; [6]Division of Infectious Diseases, Department of Medicine, University of Alberta, Edmonton, AB, Canada; [7]Attoquant Diagnostics, Vienna, Austria; [8]Pathology Unit, National Institute for Infectious Diseases "Lazzaro Spallanzani", IRCCS, Rome, Italy;

This work has been published as: Wang K, Gheblawi M, Nikhanj A, Munan M, MacIntyre E, O'Neil C, Poglitsch M, Colombo D, Del Nonno F, Kassiri Z, Sligl W, Oudit GY. **Dysregulation of ACE (Angiotensin-Converting Enzyme)-2 and renin-angiotensin peptides in SARS-CoV-2 mediated mortality and end-organ injuries**. *Hypertension.* 2022; 79(2):365-378.

3.1. Abstract

Angiotensin-converting enzyme 2 (ACE2) as the target for SARS-CoV-2 also negatively regulates the renin-angiotensin system (RAS). Pathological activation of a disintegrin and metalloproteinase-17 (ADAM17) may potentiate inflammation and diminish ACE2 mediated tissue protection through proteolytic shedding, contributing to SARS-CoV-2 pathogenesis. We aim to examine plasma soluble ACE2 (sACE2) and angiotensin profiles in relation to outcomes by enrolling consecutive patients admitted for COVID-19 with baseline blood collection at admission and repeated sampling at seven days. The primary outcome was 90-day mortality, and secondary outcomes were the incidence of end-organ injuries. Overall, 242 patients were included, the median age was 63 (52-74) years, 155 (64.0%) were males, and 57 (23.6%) patients reached the primary endpoint. Baseline sACE2 was elevated in COVID-19 but was not associated with disease severity or mortality. In contrast, an upward trajectory of sACE2 at repeat sampling was independently associated with an elevated risk of mortality and incidence of acute myocardial injury and circulatory shock. Similarly, an increase in soluble tumor necrosis factor receptor levels was also associated with adverse outcomes. Plasma Ang I, Ang 1-7 levels, and the Ang 1-7/Ang II ratio were elevated during SARS-CoV-2 infection related to downregulation of ACE activity at baseline. Moreover, patients having an upward trajectory of sACE2 were characterized by an imbalance in the Ang 1-7/Ang II ratio. The observed dysregulation of ACE2 and angiotensin peptides with disease progression suggest a potential role of ADAM17 inhibition and enhancing the beneficial Ang 1-7/Mas axis to improve outcomes against SARS-CoV-2 infection.

Brief Title: ACE2 and angiotensin peptide profiles in COVID-19

Subject Terms: SARS-CoV-2, COVID-19, ACE2, Renin-angiotensin system, Mortality, Angiotensin peptide, ADAM17

3.2. Novelty and Relevance

WHAT IS NEW?

- An increase in soluble angiotensin-converting enzyme 2 (sACE2) at repeat sampling seven days from admission is linked to an imbalance between angiotensin peptides of the beneficial Ang 1-7/Mas axis and detrimental Ang II/AT_1R axis.
- Elevated plasma Ang I and Ang 1-7 levels in patients with COVID-19 is reflective of diminished pulmonary ACE activity during SARS-CoV-2 infections.

WHAT IS RELEVANT?

- Proteolytic release of soluble ACE2 (sACE2) by ADAM17 into the circulation diminishes ACE2 mediated protection against the tissue RAS, contributing to multiorgan injuries and severe extrapulmonary manifestations of SARS-CoV-2 mediated pathology.
- The dynamic state of sACE2 may provide important prognostic insight for both COVID-19 and cardiovascular diseases based on shared disease pathophysiology.

3.3. Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative virus behind the coronavirus disease 2019 (COVID-19) pandemic, targets angiotensin-converting enzyme 2 (ACE2) as its cellular receptor.[9] We proposed that ACE2 serves as a double-edged sword during SARS-CoV-2 infections by facilitating viral entry while simultaneously mediating crucial protective effects through generating angiotensin 1-7 (Ang 1-7) from angiotensin II (Ang II) to counter-regulate the renin-angiotensin system (RAS).[46,216] A disintegrin and metalloproteinase 17 (ADAM17) is a ubiquitously expressed metalloproteinase that mediates ectodomain shedding of a wide array of surface proteins, including ACE2, thereby releasing it into the circulation.[117] Accumulation of Ang II, inflammatory cytokines, and SARS coronavirus infections upregulates ADAM17 activity,[45,116,120] creating a deleterious positive feedback loop resulting in diminished ACE2 mediated organ protection.[46,111,217] Moreover, ADAM17 plays a crucial role in regulating immune responses through the proteolytic release of membrane-bound tumor necrosis factor-alpha (TNFα) and its receptors (TNFRI and TNFRII).[218,219]

Cardiovascular complications are prevalent in patients with COVID-19. In a pooled analysis of 11,685 hospitalized patients, acute myocardial injury (AMI) was present in 20% of all cases and is associated with greater risks of mortality.[220] SARS-CoV-2 infection is associated with ventricular dysfunction, arrhythmias, acute coronary syndromes, accompanied by elevated cardiac troponin and N-terminal pro-brain natriuretic peptide levels.[6,221] Furthermore, cardiac infections by SARS-CoV-2 occurred in 62% of autopsy cases from patients succumbing to COVID-19,[222] which aligns with our previous report of myocardial infiltration by SARS-CoV during the SARS epidemic.[168] However, the implications of ACE2 and RAS dysregulation in the pathogenesis of SARS-CoV-2 and its effects on clinical outcomes remain unclear.

In this study, we investigated the prognostic value of sACE2 at baseline and during repeat sampling, alongside sTNFRI and sTNFRII levels as surrogate markers of ADAM17 activity for COVID-19 related mortality and end-organ injuries. Additionally, we provided evidence for an imbalance in systemic angiotensin peptide profiles related to impairment in angiotensin-converting enzyme (ACE) and ACE2 functionality by generating RAS fingerprints in prospectively recruited patients hospitalized with COVID-19.

3.4. Methods

Study Participants

The COVID-19 Surveillance Collaboration (CoCollab) Study prospectively enrolled 242 consecutive patients newly admitted to hospital wards designated for COVID-19 (n=137) and intensive care units (ICU, n=105) between September 30th, 2020 and April 30th, 2021 (**Supplemental Figure 3.1.**). Our centers included the University of Alberta Hospital and Misericordia Community Hospital in Edmonton, Canada encompassing a catchment of approximately 2 million adults. All enrolled patients were ≥18 years of age with a laboratory-confirmed COVID-19 diagnosis based on a positive SARS-CoV-2 real-time polymerase chain reaction assay from nasopharyngeal swabs or lower respiratory samples. Patients under any investigational therapy or who had received vaccinations for SARS-CoV-2 were excluded from the present study. Comparisons were made with previously obtained age and sex-matched healthy controls (n=38). Our study was conducted in accordance with the ethical principles for the Declaration of Helsinki with approval from the University of Alberta Health Research Ethics Board (Pro00100319 and Pro00100207). Written informed consent was obtained from all

participants enrolled from hospital wards. A waiver of consent was granted for participants from ICUs, followed by a regained capacity consent signed whenever possible.

Plasma Collection and Laboratory Measurements

Venous blood sampling was done in the morning by trained phlebotomists and transported to the Canadian Biosample Repository located at the University of Alberta within one hour for immediate processing. Samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) or lithium heparin and centrifuged at 1500G for 10 minutes at room temperature, and plasma was subsequently aliquoted for storage at -80 °C. Baseline sampling was performed immediately after hospital admission, while repeat sampling occurred at seven days, either in the hospital or from their location of residence by the study team if patients were discharged. Plasma samples were available from all 242 participants at baseline and 187 participants at follow-up. ACE2, TNFα, interleukin-6 (IL-6), neprilysin, TNFRI, and TNFRII levels were assessed in EDTA plasma using commercially available human ELISA kits from R&D Systems, Minneapolis, MN, USA (DY933-05, HSTA00E, HS600C, DY1182, DRT100, DRT200, respectively) according to manufacturer's instructions. Samples above the dynamic range of the R&D Systems ACE2 assay were diluted by a factor of 10, and the limit of detection for ACE2 was 300 pg/mL. High sensitivity troponin I (hs-TnI) levels were measured directly by the Alberta Precision Laboratories using the Beckman Access hs-TnI immunoassay with a detection limit of 3 ng/L.

Angiotensin peptides and angiotensin-converting enzyme activity measurement

Equilibrium concentrations of the peptides Ang I, Ang II, Ang 1-9, Ang 1-7, and Ang 1-5, as well as the level of the steroid aldosterone were measured in lithium-heparin plasma by liquid chromatography with tandem mass spectrometry analysis (LC-MS/MS, Attoquant Diagnostics,

Vienna). Samples were spiked with stable isotope labelled (peptides) or deuterated (aldosterone) internal standards after equilibration, and analytes were extracted using C18-based solid-phase extraction. Extracted samples were analyzed using mass spectrometry analysis using a reversed analytical column (Acquity UPLC C18, Waters) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters Xevo TQ/S, Milford, MA) in multiple reaction monitoring mode. Internal standards were used to correct for analyte recovery across the sample preparation procedure in each individual sample. Analytic concentrations were calculated from integrated chromatograms considering the corresponding response factors determined in appropriate calibration curves in plasma matrix, when integrated signals exceeded a signal-to-noise ratio of 10. The lower limits of quantification were 3 pmol/L (Ang I), 2 pmol/L (Ang II), 2 pmol/L (Ang 1-9), 2 pmol/L (Ang 1-7), 2 pmol/L (Ang 1-5), and 13.9 pmol/L (aldosterone), respectively. In addition, a previously validated surrogate measure of plasma renin was derived from the total concentration of all five angiotensin metabolites, Ang I, Ang II, Ang 1-9, Ang 1-7, Ang 1-5.[223]

Circulating angiotensin-converting enzyme (ACE) activity were determined using mass spectrometry by first spiking Ang I to lithium-heparin plasma samples diluted in an ACE activity assay buffer (phosphate-buffered saline (pH 7.4) with specific inhibitors to stabilize Ang I (substrate) and Ang II (product), followed by incubation at 37 ℃ for 1 hour in the presence or absence of the ACE inhibitor, Lisinopril. The incubation is stopped by acidification and Ang II concentration was determined by LC-MS/MS and calculated the ACE dependent Ang II formation.

Outcome Assessment

Detailed clinical characteristics, including demographics, laboratory measurements, vital signs, presenting symptoms, comorbidities, medications, and outcomes were collected through review of electronic medical records. The primary outcome of interest was all-cause mortality at 90 days.

Secondary outcomes of interest included new onset of acute respiratory distress syndrome (ARDS), acute kidney injury (AKI), AMI, and circulatory shock following initial hospitalization. ARDS was defined based on the Berlin Definition,[224] AKI was defined based on the KDIGO clinical practice guidelines,[225] and AMI was defined as hs-TnI levels above the upper reference limit of the assay (>20ng/L). Patients were followed until death or appropriately censored at the end of study follow-up.

Statistical Analysis

Descriptive statistics were presented as n (%) for categorical data, compared using the χ^2 test, and medians with interquartile range (IQR) for continuous data, compared using the Mann-Whitney *U* or Kruskal-Wallis test with pairwise comparisons when appropriate. Correlations between biomarkers were assessed using the Spearman correlation coefficient. Logistic regression models were used to determine the association of baseline and changes in sACE2 levels during repeat sampling with clinical characteristics. Biomarkers were classified as a dichotomous variable: above or below median for baseline and increased or decreased for divergent trajectories during repeat sampling. Statistically significant variables through univariable logistic regression analyses were subsequently examined in the adjusted model, which included age, sex, body mass index (BMI), history of diabetes, hypertension, chronic kidney disease (CKD), cardiovascular disease (CVD), and the use of angiotensin-converting enzyme inhibitors (ACEI) or Ang II receptor blockers (ARB). CVD included a history of myocardial infarction, coronary artery disease, heart failure (HF), and arrhythmias. Adjusted variables were selected apriori based on their clinical significance and potential confounding on ACE2 expression.

The association between sACE2, sTNFRI, and sTNFRII with the primary outcome was assessed using Cox proportional hazards regression and Kaplan-Meier survival analyses.

Univariable analyses were conducted to generate the crude hazard ratios (HR) followed by multivariable adjustments in Model 1 for age, sex, BMI, history of diabetes, hypertension, CKD, CVD, and the use of ACEI or ARB. Model 2 was subsequently adjusted for all variables in Model 1, along with hs-TnI, TNFα, IL-6, platelets, neutrophils, C-reactive protein, and lymphocytes as markers of cardiac injury, inflammation, and viremia. The changes in disease markers during repeat sampling were excluded from Model 2 due to strong multicollinearity with their corresponding baseline values. Secondary outcomes were assessed using multivariable logistic regression to evaluate associations between the incidence of end-organ injuries with measured biomarkers through the same progressive models as survival analyses.

The impact of ACE2 dysregulation on angiotensin peptides was examined using a nearest-neighbor matching strategy to generate 1:1 propensity-score matched cohorts of patients having either increased or decreased sACE2 during repeat sampling without replacement. Forty-three matched pairs were identified from 55 patients having increased sACE2 and 132 patients with decreased sACE2 at follow-up. Standardized differences were used to assess for a suitable balance between propensity-matched cohorts. In addition, covariates described in Model 1 of the survival analyses were adjusted as potential confounders on ACE2 expression. Statistical significance was considered based on 2-tailed $p<0.05$. All statistical analyses were performed using SPSS version 26 (IBM Corporation, Armonk, New York) and R 3.6.2 (Vienna, Austria).

3.5. Results

Patient Characteristics

A total of 242 consecutive patients were prospectively enrolled in our study from September 2020 to April 2021. Of which, 136 remained non-ventilated survivors, while 106 patients either required

mechanical ventilation and/or have died (**Table 3.1.**). The median age was 63 years (IQR, 52-74 years), with 155 males (64.0%), and time from COVID-19 diagnosis to baseline collection of 7 days (IQR, 3-10 days). The most common comorbidities were hypertension (134 patients, 55.4%), diabetes (111 patients, 45.9%), and CVD (55 patients, 22.7%). Management for COVID-19 during hospital stay included the use of dexamethasone (211 patients, 87.2%), antibiotics (200 patients, 82.6%), and supplemental oxygen (201 patients, 83.1%). Patients with greater disease severity reflected by the need for mechanical ventilation and/or death were more likely males with an abnormal chest x-ray and having a higher prevalence of CVD. Laboratory abnormalities associated with greater disease severity included lower glomerular filtration rate, hemoglobin, platelet, and lymphocyte counts, along with higher white blood cell counts, neutrophil counts, C-reactive protein, and lactate dehydrogenase levels (**Table 3.1.**). Importantly, the use of ACEI and ARB were not different between the cohorts.

Temporal Profile of sACE2, sTNFRI, and sTNFRII

Baseline sACE2 was elevated in COVID-19 compared to age and sex-matched healthy controls (2578 [720-15529] versus 1649 [300-7157] pg/mL, p=0.004, **Figure 3.1.A**). However, patients in the mechanical ventilation/death cohort had comparable sACE2 levels as non-ventilated survivors (2393 [763-14294] versus 3013 [703-16253] pg/mL, p=0.64). Presence of CVD (aOR, 2.50 [95% CI, 1.10-5.68], p=0.03) and reduced white blood cell counts (aOR, 0.76 [95% CI, 0.59-0.98], p=0.04) were independently associated with patients having above-median sACE2 levels in the multivariable logistic regression model (**Supplemental Tables 3.1. and 3.2.**). Other cardiac and inflammatory biomarkers measured, including hs-TnI, sTNFRI, sTNFRII, IL-6, and TNFα

showed a notable stepwise increase from healthy controls to non-ventilated survivors and subsequently those in the mechanical ventilation/death cohort (**Figure 3.1.B-F**).

At repeat sampling, the temporal profile of sACE2, sTNFRI, and sTNFRII showed a substantial increase in biomarkers related to ADAM17 activity in deceased patients (**Figure 3.2.A-C**). In total, 55 patients experienced an increase in sACE2 during follow-up (**Supplemental Table 3.3.**), which was independently associated with a reduction in platelet counts (aOR, 0.96 [95% CI, 0.92-0.99], p=0.02) in the multivariable model (**Supplemental Table 3.4.**). sACE2 decreased by a median of 1149 pg/mL (IQR, 80-5230 pg/mL) during follow-up in survivors, but increased by 745 pg/mL (IQR, 108-7731 pg/mL) in deceased patients (p<0.001, **Figure 3.2.A**). Similarly, sTNFRI and sTNFRII levels rose in deceased patients by 558 pg/mL (IQR, 142-2841 pg/mL; p<0.001) and 1076 pg/mL (IQR, 98-2200 pg/mL; p<0.001), respectively (**Figure 3.2.B-C**). Importantly, changes in sTNFRI were positively correlated with changes in sACE2 (r^2=0.20, p=0.006) and sTNFRII (r^2=0.61, p<0.001), tracking closely with the trajectories of sACE2 (**Supplemental Figure 3.2.**). Moreover, sACE2, sTNFRI, and sTNFRII levels remained elevated in the mechanical ventilation/death cohort, reflective of pathological ADAM17 activation and ectodomain shedding (**Supplemental Figure 3.3.**). These results demonstrate that severe COVID-19 results in persistently elevated plasma sACE2 levels, which correlates with markers of ADAM17 activation.

Plasma levels of sACE2, sTNFRI and sTNFRII and Outcomes

Over a median duration of 90 (IQR, 88-90) days, 57 deaths (23.6%) occurred in the overall cohort at baseline, and 34 deaths (18.2%) occurred in patients available for repeat sampling. An increase in sACE2 at repeat sampling was associated with >7-fold greater hazard for mortality in the

unadjusted model (HR, 7.47 [95% CI, 3.56-15.66]), with a 43.6% mortality rate in these patients (**Figure 3.2.D and Table 3.2.**). Compared with patients having a reduction in biomarker levels, an increase in sTNFRI and sTNFRII was also associated with a higher risk of mortality (**Figure 3.2.E-F**). After adjustment for established biomarkers (Model 2) in addition to relevant clinical characteristics in Model 1, upward trajectories of sACE2 (aHR, 3.94 [95% CI, 1.51-10.30]), sTNFRI (aHR, 8.70 [95% CI, 3.01-25.13]) and sTNFRII (aHR, 7.92 [95% CI, 2.50-25.12]) remained significant predictors of 90-day mortality (**Table 3.2.**). In the analysis of secondary outcomes, an increase in sACE2 was further associated with the incidence of AMI (aHR, 3.91 [95% CI, 1.39-10.97]), and circulatory shock (aHR, 6.11 [95% CI, 1.26-19.57]) following multivariable adjustments (**Table 3.2.**).

Association between baseline biomarkers and outcomes were analyzed according to the below and above median cohorts (**Supplemental Figure 3.4.**). Baseline sACE2 levels were not associated with the primary outcome of interest (**Table 3.2.**). In comparison, above median sTNFRI and sTNFRII levels were associated with more than double the risk of mortality in unadjusted models, but the association was attenuated and no longer significant with the incorporation of clinical characteristics and disease biomarkers in Model 2 (**Table 3.2.**). Elevated sTNFRI and sTNFRII levels were also associated with the incidence of ARDS, AKI, and circulatory shock following adjustment with clinical characteristics in Model 1. However, only circulatory shock remained independently associated with sTNFRI and sTNFRII levels in Model 2. Moreover, baseline sTNFRI and sTNFRII levels showed a positive correlation with the length of hospital stay while the biomarkers examined did not correlate with the P/F ratio, consistent with the lack of association with ARDS in fully adjusted models (**Supplemental Table 3.5.**). Collectively, our results demonstrate that in patients with severe COVID-19, the early stage of

aberrant ACE2 shedding reflects the infectious stage of the illness, while persistent elevation in sACE2 results in progressive end-organ injury due to loss of tissue ACE2 and correlates with mortality.

Renin-angiotensin System Dysregulation During SARS-CoV-2 Infection

Equilibrium levels of angiotensin peptides were profiled in 38 healthy controls, 45 non-ventilated survivors, and 43 patients requiring mechanical ventilation and/or had died due to COVID-19 (**Figure 3.3.A** and **Supplemental Table 3.6.**). Median levels of Ang I, Ang 1-7, and the Ang 1-7/Ang II ratio were elevated in patients with COVID-19 but did not differ based on disease severity (**Figure 3.3.B, 3D-E**). In comparison, plasma Ang II levels were lower in non-ventilated survivors than healthy controls (47.7 [IQR, 16.9-106.9] versus 101.8 [IQR, 68.4-140.4] pmol/L, p=0.004, **Figure 3.3.C**). Moreover, plasma aldosterone levels were also suppressed in patients with COVID-19 (**Supplemental Figure 3.5.A**). We found that the observed dysregulation in baseline RAS profile is related to decreased plasma ACE activity during SARS-CoV-2 infection (**Figure 3.3.F**). In contrast, plasma neprilysin levels, an alternative enzyme capable of elevating Ang 1-7/Ang II ratio through the direct conversion of Ang I to Ang 1-7 were similar across all cohorts (**Supplemental Figure 3.6.**). Additionally, patients with COVID-19 had comparable plasma renin as healthy controls irrespective of disease severity (**Supplemental Figure 3.5.B**).

To determine the effects of aberrant ACE2 shedding on systemic angiotensin peptides, we characterized baseline and follow-up peptide profiles between 43 propensity score-matched pairs of patients having either increased or decreased trajectory of sACE2 at repeat sampling (**Supplemental Table 3.7.**). There were no differences in angiotensin peptide levels or RAS enzyme activity between the two cohorts at baseline (**Figure 3.4. and Supplemental Figure 3.7.**).

Overall, changes in angiotensin peptide levels were dynamic during the follow-up period. Plasma Ang II levels decreased by a median of 4.2%, while Ang 1-7, Ang 1-7/Ang II ratio, and Ang I increased by 63.9%, 52.4%, and 62.6%, respectively, during repeat sampling (**Figure 3.4.**). Notably, despite having comparable changes in individual Ang II and Ang 1-7 levels, patients with increased sACE2 had suppressed Ang 1-7/Ang II ratio compared to those with decreased sACE2 during follow-up (**Figure 3.4.E**). These results demonstrate that the derangement in baseline angiotensin profile is driven by reduced ACE activity, likely reflects the initial dominant pulmonary involvement in patients with COVID-19. In contrast, during disease progression with greater loss of tissue ACE2 and a corresponding rise in sACE2, Ang 1-7/Ang II ratio was reduced, indicative of diminished tissue ACE2 mediated protection.

3.6. Discussion

Elucidating potential therapeutic targets to protect from organ injury and reduce mortality remains an important focus of current COVID-19 research. In this prospective cohort of patients hospitalized with COVID-19, elevated sACE2 at baseline served as a disease signature but was not associated with severity or mortality. Instead, we found sACE2 levels decreased drastically in survivors but increased in patients who died due to COVID-19. An increase in sACE2 at repeat sampling was independently associated with 90-day mortality after adjustment for clinical characteristics and established markers of cardiac injury, inflammation, and viremia. Multivariable analysis of secondary outcomes revealed a further association of AMI and circulatory shock with increased sACE2. Moreover, a similar rise in sTNFRI and sTNFRII was also associated with adverse clinical outcomes. Together these findings suggest a crucial role of pathological ADAM17 activation and ACE2 shedding in SARS-CoV-2 pathogenesis, contributing to organ injuries and mortality (**Figure 3.5.**).

An important distinction between SARS coronaviruses and another human coronavirus, HCoV-NL63, which also hijacks ACE2 as the receptor but causes only mild respiratory symptoms is the induction of ADAM17.[116,226] Our data shows augmented release of sACE2 by ADAM17 begins early on during SARS-CoV-2 infection based on elevated sACE2 levels at admission in patients with COVID-19. In addition, sustained rise in sACE2 was associated with an elevated risk of mortality and incidence of AMI. Whereas survivors demonstrated a substantial reduction in sACE2 levels, suggesting competent regulation over aberrant ACE2 shedding and inflammation. Furthermore, upregulation of ADAM17 is linked to CVD progression by releasing various cytokines, growth factors, and adhesion molecules, leading to fibrosis, inflammation, and adverse cardiac remodeling.[227,228] Inhibiting ADAM17 activity using pharmacological inhibitors or through knockdown by siRNA successfully suppressed cellular infections by SARS-CoV-2 *in vitro*.[226] As such, ADAM17 inhibition early on in SARS-CoV-2 infection possesses the therapeutic potential to attenuate sACE2 release and prevent further loss of ACE2 mediated cardiovascular protection.

Changes in cellular expression of the examined biomarkers may also contribute towards the observed elevations at baseline and differential trajectories during follow-up. As the immune response is cardinal to SARS-CoV-2 pathogenesis, the expression and release of TNFα, along with its receptors, must be tightly regulated to protect against the pathological sequelae of excessive inflammation and tissue damage. Evidently, IFNγ is the dominant cytokine produced by $CD4^{+}$T cells during SARS-CoV-2 infection and a central mediator of the host antiviral response,[229] which has been shown to upregulate the synthesis and expression of TNF receptors.[230,231] In the fully adjusted model, increased sTNFRI and sTNFRII retained prognostic significance beyond established markers of inflammation and viremia, possibly attributed to its reflection of both the

shedding and concomitant cellular responses to various cytokines during infections. In comparison, ACE2 expression patterns demonstrated tissue specificity in response to SARS-CoV-2 infections. In the pulmonary endothelial and alveolar epithelial cells, ACE2 was upregulated in COVID-19 and influenza-related ARDS, which may represent an increase in susceptibility through viral-induced pathways or a compensatory mechanism for tissue protection.[232,233] However, prominent downregulation of ACE2 expression from SARS-CoV-2 infection was observed in the vascular endothelium accompanied by immune infiltration and myocardial fibrosis in autopsy heart tissues of patients succumbing to COVID-19.[234,235] Loss of ACE2 impairs vascular tone, increased monocyte-endothelial adhesion, macrophage activation, vascular permeability and oxidative stress, which exacerbates endothelial dysfunctions caused by SARS-CoV-2.[150,236] Severe presentations of COVID-19 include coagulopathy, thrombosis, and endotheliitis in the microvasculature,[237-239] where ACE2 exerts potent anti-thrombotic, anti-inflammatory, and antioxidant effects through degradation of Ang II and concurrent activation of the beneficial Ang 1-7/Mas axis.[46] Our findings of persistent sACE2 elevation as an independent predictor of AMI and circulatory shock supports the histological findings related to a reduction in ACE2 mediated myocardial and vascular protection as a driver of adverse outcomes beyond ARDS and initial pulmonary injuries.

Our study also revealed the associations between elevated baseline sACE2 levels with a history of CVD and the presence of leukopenia, while changes in sACE2 at repeat sampling were associated with reduced platelet counts in the multivariable regression models. Earlier studies reported thrombocytopenia was associated with a worse prognosis in COVID-19.[240,241] Although platelets are hyperactivated to secrete proinflammatory cytokines and promote hypercoagulation in this setting,[242] the putative link to ACE2 downregulation has yet to be established. In the PURE

epidemiology study of 10,753 participants, increased sACE2 was positively associated with mortality, incidents of HF, myocardial infarction, stroke, and diabetes beyond traditional cardiovascular risk factors.[178] Studies on smaller cohorts also highlighted the independent prognostic value of sACE2 in coronary artery disease, atrial fibrillation, and HF.[179,180,182,196] Therefore, sACE2 has emerged as a novel biomarker in both CVD and COVID-19. Prospective and serial measurements of sACE2 can serve as a tool for risk stratification of patients to enable a biomarker-guided approach for the development of personalized therapies against SARS-CoV-2.

Whether upregulation of the RAS is implicated in SARS-CoV-2 pathogenesis has been debated ever since the emergence of the COVID-19 pandemic. Our comprehensive profiling of angiotensin peptides revealed a lack of overt RAS activation in COVID-19 regardless of disease severity. However, an imbalance in angiotensin peptides was observed, characterized by an elevation in plasma Ang I, Ang 1-7 levels, and the Ang 1-7/Ang II ratio. Furthermore, reduction in Ang II levels was seen in non-ventilated survivors, concomitant with suppressed aldosterone levels, suggesting an absence of pathological Ang II/Aldosterone crosstalk in SARS-CoV-2 infection. In agreement with recent studies, we found a reduction in ACE activity as a dominant driver of systemic RAS imbalance in COVID-19 at admission, as other RAS enzymes examined thus far, including prolyloligopeptidase, renin, and neprilysin, were not substantially altered.[232,243] Indeed, the angiotensin peptide profiles in COVID-19 closely resembled HF patients receiving chronic treatment with ACE inhibition.[58] ACE catalytically cleaves Ang I into Ang II and Ang 1-7 into Ang 1-5. Therefore, impaired ACE activity leads to a corresponding increase in Ang I and Ang 1-7 levels through reduced degradation.[202] Since pulmonary ACE represents the primary site of systemic Ang II formation,[244,245] downregulation of ACE activity in the lungs is exceedingly plausible in settings of extensive pulmonary vascular endothelial injury induced by SARS-CoV-2

infections.[233,246] Accordingly, based on the impaired ACE activity and an absence of systemic Ang II/aldosterone stimulation in COVID-19, benefits from *de novo* initiation of ACEI and ARB remains questionable, and awaiting results from ongoing clinical trials (NCT04335786 and NCT04366050).[247]

The prominent role of tissue RAS in influencing systemic angiotensin peptide profiles is evident in the imbalance of Ang 1-7/Ang II ratio in patients with increased sACE2 at repeat sampling. Ang 1-7 is a potent regulator of endothelial function through activating nitric oxide synthase and can directly counter-regulate Ang II/AT_1R signalling to protect against SARS-CoV-2 mediated endothelial inflammation, fibrosis, and apoptosis.[59,238] Moreover, the underlying importance behind balancing the detrimental Ang II/AT_1R axis and the beneficial Ang 1-7/Mas axis is demonstrated through the independent prognostic value of the Ang 1-7/Ang II ratio in patients with HF, as individual levels of Ang 1-7 and Ang II lacked clinical significance.[248] ACE2 predominantly functions in the tissues since the physiological activity of sACE2 is extremely low and may be masked by endogenous inhibitors in the human plasma.[249,250] Accordingly, elevation in sACE2 naturally reflects increased shedding of tissue ACE2 and diminished protection against the tissue RAS,[111,217] which increases susceptibility to myocardial infarction, HF, hypertension, and Ang II-mediated microvascular complications, inflammation, fibrosis, cardiac dysfunction, and oxidative stress.[14,16]

Limitations

Our study prospectively enrolled patients from designated COVID-19 wards and ICUs, resulting in a relatively severe cohort of hospitalized patients. Therefore, our findings need to be extended to asymptomatic individuals and those not requiring hospitalization. Furthermore, although repeat collections occurred in a relatively short timeframe from admission, 16 patients

still died before serial sampling could occur, which results in survival bias in the follow-up cohort. Regarding the quantification of angiotensin peptides and enzymes, utilization of selective inhibitor cocktails blocking further angiotensin metabolism could have facilitated a more direct assessment of circulating RAS peptides and enzymatic activities, providing additional information beyond the present equilibrium measurements. Additionally, biomarkers and angiotensin peptide profiles in hospitalized patients may be influenced by COVID-19 treatment regiment, such as dexamethasone and antibiotics, multiple comorbidities, blood pressure, or fluid volume status, which is not fully captured by age and sex-matched healthy controls. The multivariable regression models did not account for the trajectories in clinical disease markers or validated disease progression indexes such as the National Early Warning Score 2, which may lead to overestimation in the effects of temporal changes in the examined biomarkers. As ADAM17 is a membrane-bound metalloproteinase, a direct assessment of its cellular activity was not possible in the present clinical setting as it would require the procurement of myocardial specimens. Demonstration of increased ADAM17 activity in the myocardial tissue and experimental animal models would further validate the approach of ADAM17 inhibition as a novel therapeutic strategy in limiting the adverse outcomes of SARS-CoV-2 infections.

3.7. Conclusion

In patients hospitalized with COVID-19, we demonstrate an association between the temporal profile of sACE2 following admission with mortality beyond established markers of cardiac injury, inflammation, and viremia. Furthermore, we found a dysregulation in angiotensin peptide profiles linked to downregulated ACE and ACE2 activity in patients with COVID-19. Findings from this study provide a rationale for the potential utility of ADAM17 inhibitors, genetic overexpression of ACE2, ACE2 activators, recombinant human ACE2, Ang 1-7 analog, and Mas-receptor agonists

aimed at preventing ACE2 shedding, restoring ACE2 expression and enhancing the Ang 1-7/Mas axis functionality to improve outcomes against SARS-CoV-2 infections.

Figures and Tables

Figures

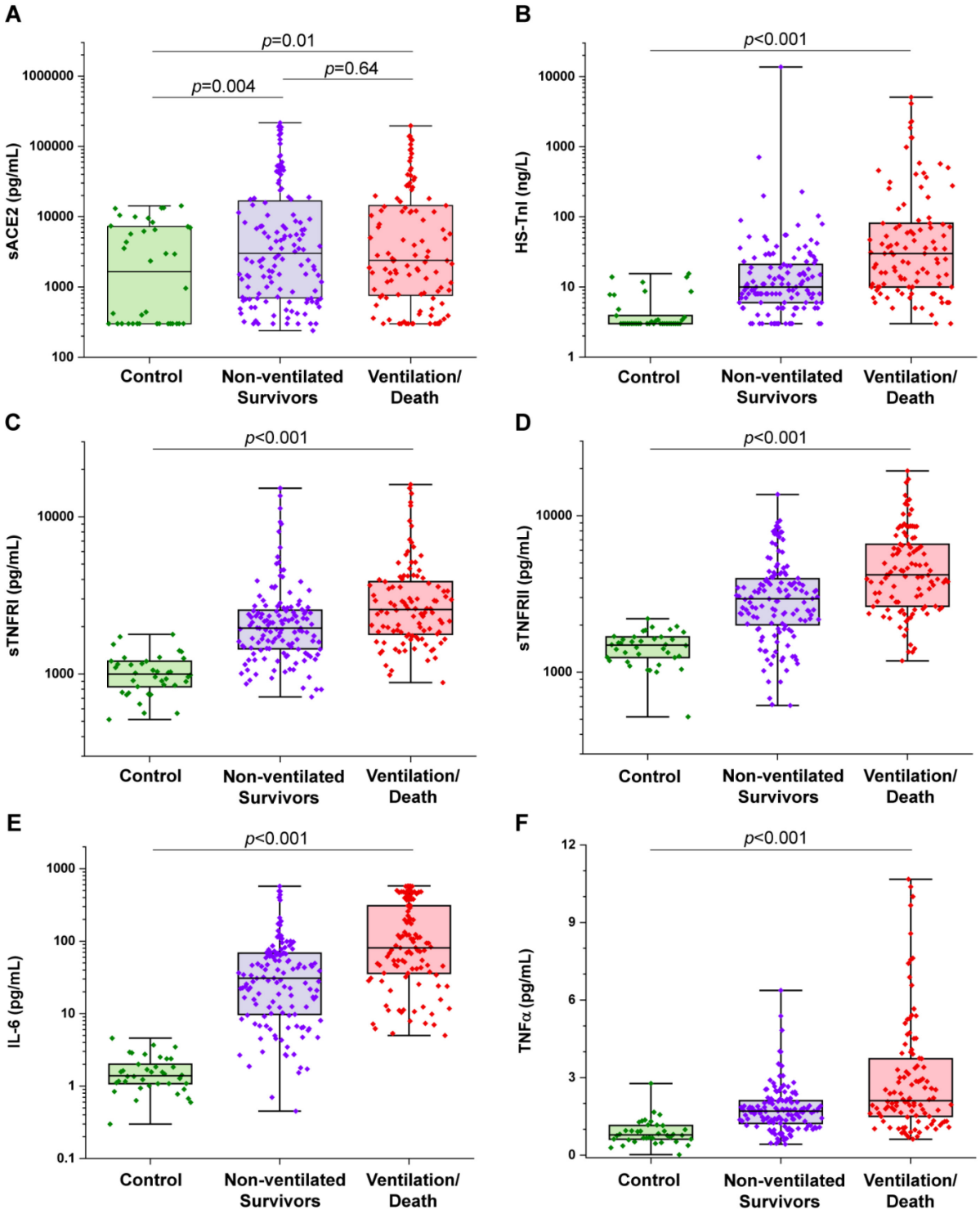


Figure 3.1. Concentration of plasma biomarkers at baseline in patients with COVD-19 (n=242) compared with age and sex-matched healthy controls (n=38). Box and whisker plots of plasma soluble angiotensin-converting enzyme 2 (sACE2, A), high sensitivity cardiac troponin I (Hs-TnI, B), soluble tumor necrosis factor receptor I (sTNFRI, C) and II (sTNFRII, D), interleukin-6 (IL-6, E) and tumor necrosis factor alpha (TNFα, F) concentration stratified by disease severity (n=136 for non-ventilated survivors and n=106 for mechanical ventilation and/or death) in comparison to age and sex-matched healthy controls. $p<0.001$ across the cohorts and in pair-wise comparisons.

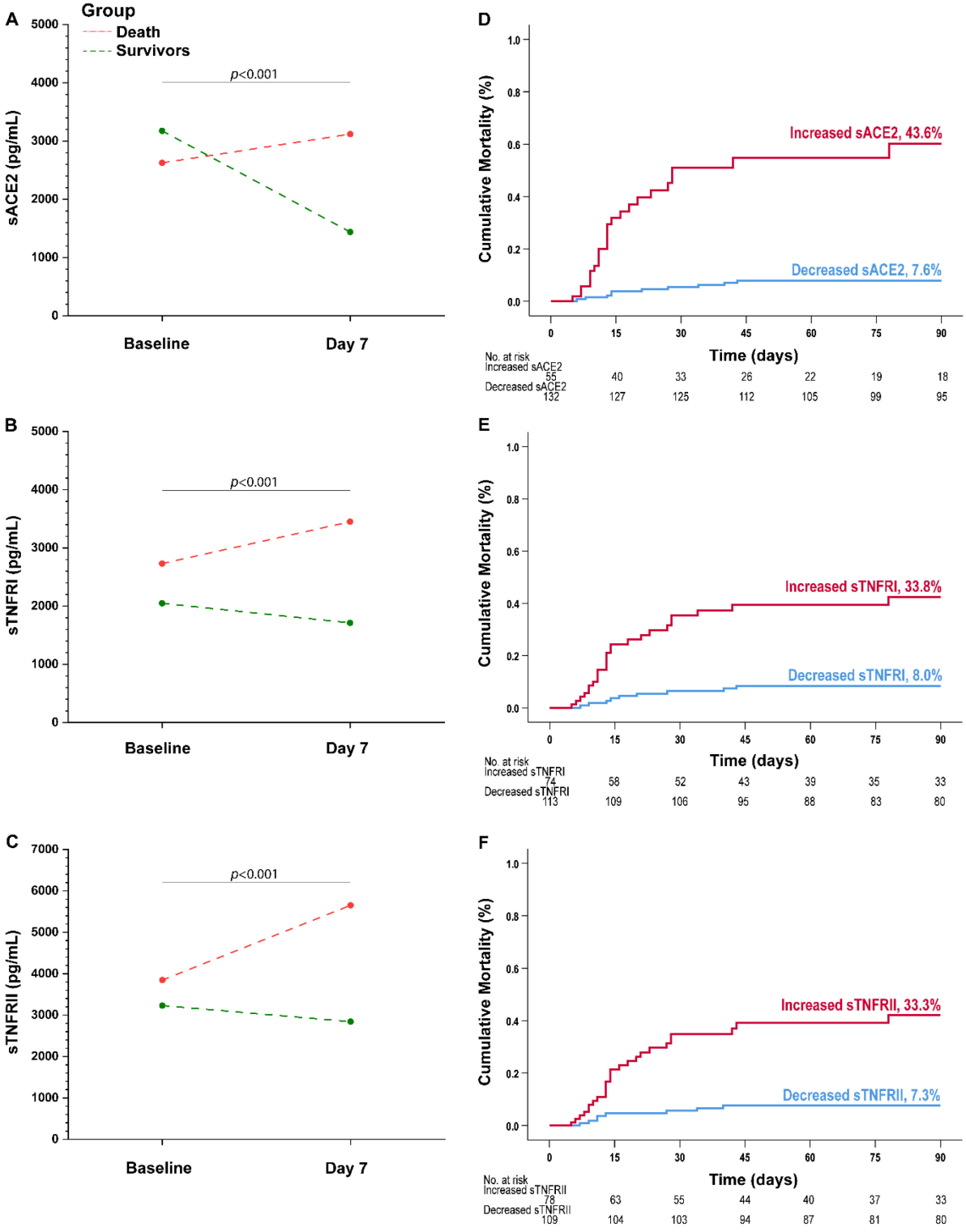

A
Group
Death
Survivors
p<0.001
sACE2 (pg/mL)
Baseline
Day 7
B
p<0.001
sTNFRI (pg/mL)
Baseline
Day 7
C
p<0.001
sTNFRII (pg/mL)
Baseline
Day 7
D
Cumulative Mortality (%)
Increased sACE2, 43.6%
Decreased sACE2, 7.6%
Time (days)
No. at risk
Increased sACE2 55 40 33 26 22 19 18
Decreased sACE2 132 127 125 112 105 99 95
E
Cumulative Mortality (%)
Increased sTNFRI, 33.8%
Decreased sTNFRI, 8.0%
Time (days)
No. at risk
Increased sTNFRI 74 58 52 43 39 35 33
Decreased sTNFRI 113 109 106 95 88 83 80
F
Cumulative Mortality (%)
Increased sTNFRII, 33.3%
Decreased sTNFRII, 7.3%
Time (days)
No. at risk
Increased sTNFRII 78 63 55 44 40 37 33
Decreased sTNFRII 109 104 103 94 87 81 80

Figure 3.2. Temporal changes in soluble ACE2, TNFRI and TNFRII between baseline and repeat sampling in association with the primary outcome. Soluble angiotensin-converting enzyme 2 (sACE2, A), soluble tumor necrosis factor receptor I (sTNFRI, B) and soluble tumor necrosis factor receptor II (sTNFRII, C) increased significantly during follow-up in deceased patients compared to survivors, $p<0.001$. Association of divergent trajectories in sACE2 (D), sTNFRI (E) and sTNFRII (F) at repeat sampling with 90-day mortality related to the role of ectodomain shedding by ADAM17 in COVID-19 pathophysiology. Log-rank $p<0.001$.

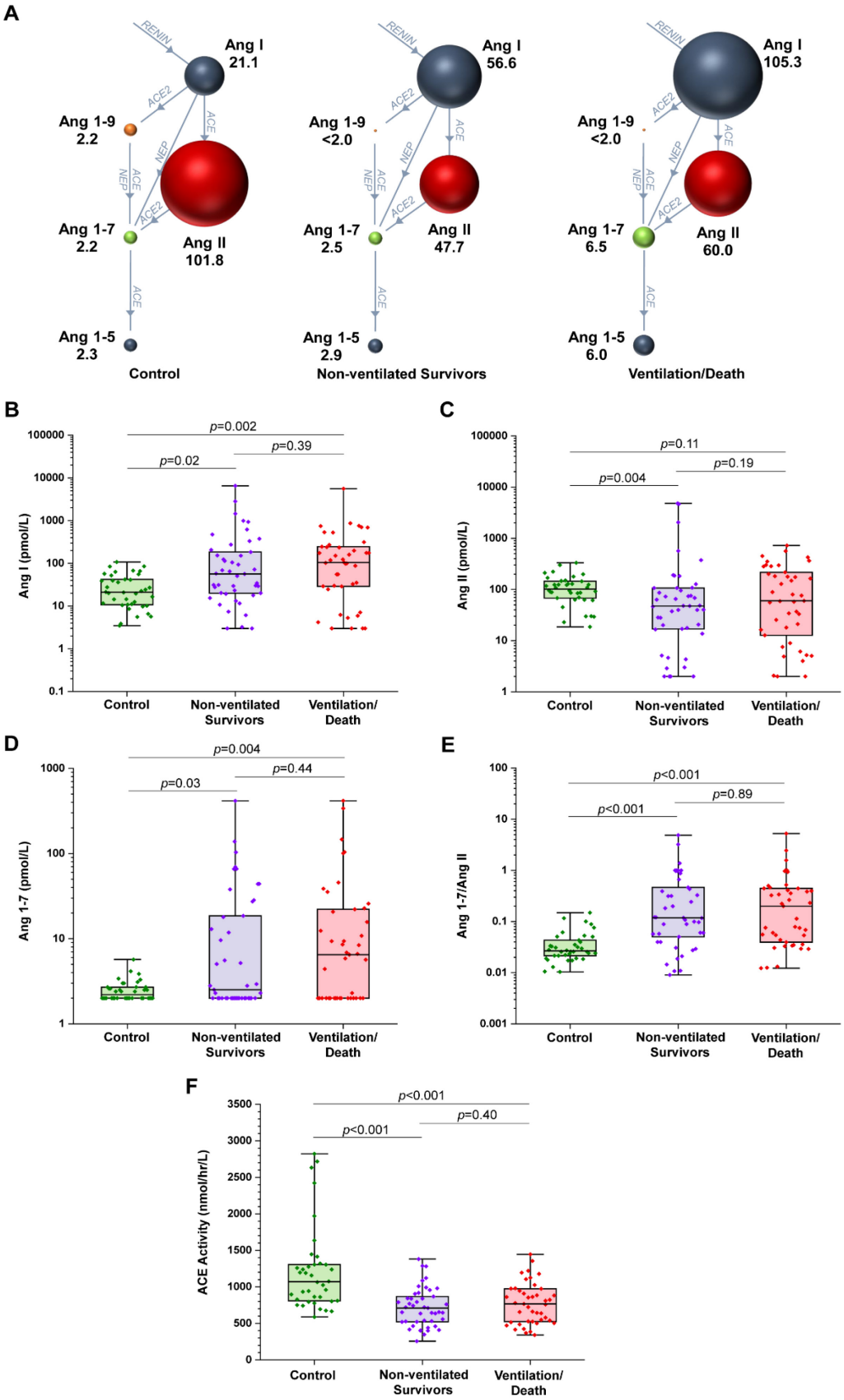

A
RENIN
Ang I
21.1
ACE2
ACE
Ang 1-9
2.2
NEP
ACE
ACE2
Ang 1-7
2.2
Ang II
101.8
ACE
Ang 1-5
2.3
Control
Ang I
56.6
Ang 1-9
<2.0
Ang 1-7
2.5
Ang II
47.7
Ang 1-5
2.9
Non-ventilated Survivors
Ang I
105.3
Ang 1-9
<2.0
Ang 1-7
6.5
Ang II
60.0
Ang 1-5
6.0
Ventilation/Death
B
Ang I (pmol/L)
p=0.002
p=0.39
p=0.02
C
Ang II (pmol/L)
p=0.11
p=0.19
p=0.004
D
Ang 1-7 (pmol/L)
p=0.004
p=0.44
p=0.03
E
Ang 1-7/Ang II
p<0.001
p=0.89
p<0.001
F
ACE Activity (nmol/hr/L)
p<0.001
p=0.40
p<0.001
Control
Non-ventilated Survivors
Ventilation/ Death

Figure 3.3. Profile of angiotensin peptides and angiotensin-converting enzyme activity at baseline in patients with COVD-19 compared with age and sex-matched healthy controls. A. Global representation of median angiotensin peptide levels in 38 healthy controls, 45 non-ventilated survivors, and 43 patients requiring mechanical ventilation and/or had died due to COVID-19 at baseline. Box and whisker plots for plasma levels of (B) Ang I, (C)Ang II, (D) Ang 1-7, (E) Ang 1-7/Ang II Ratio, and (F) Angiotensin-converting enzyme activity.

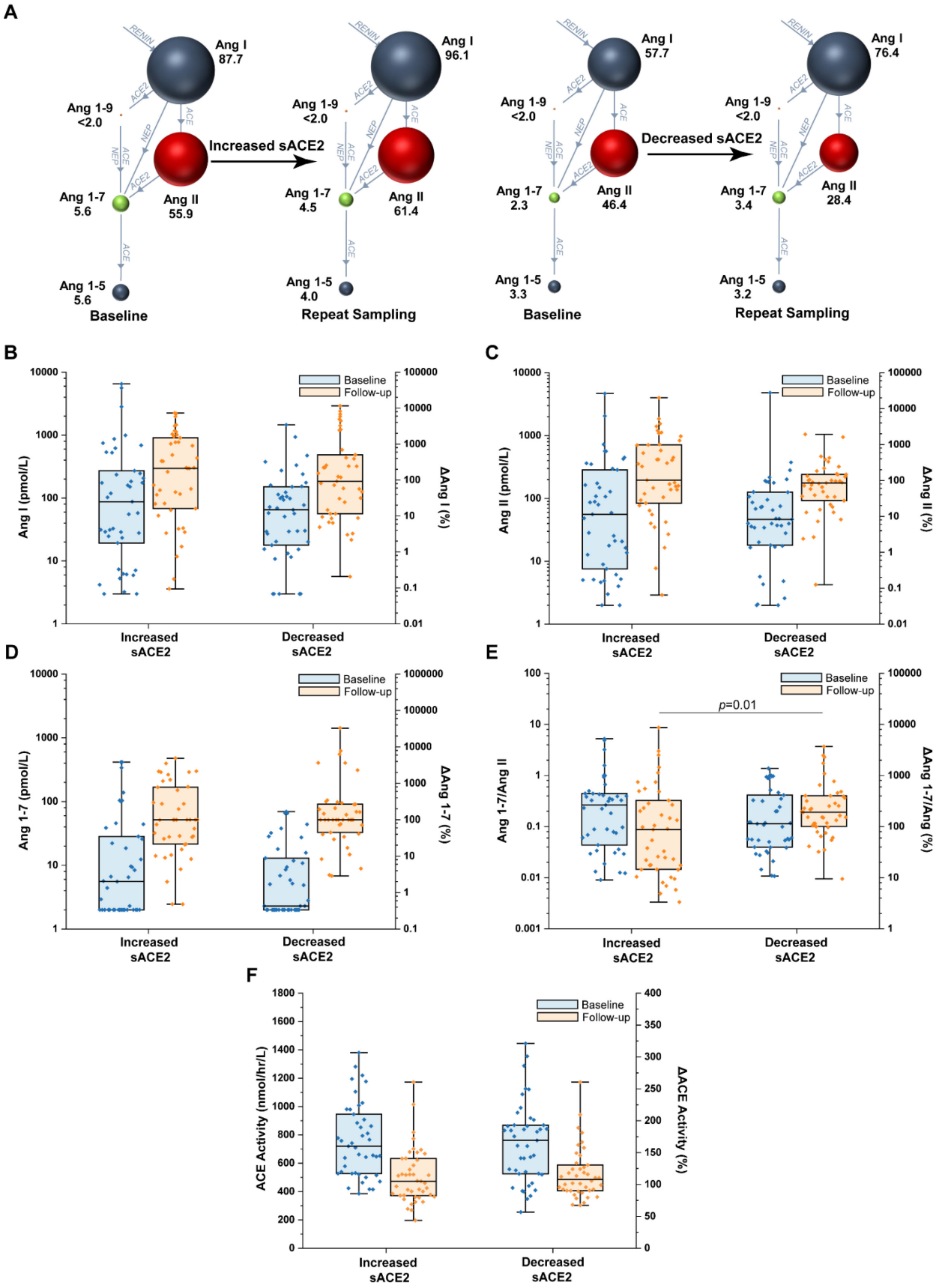

A
Ang I 87.7
Ang 1-9 <2.0
Ang 1-7 5.6
Ang II 55.9
Ang 1-5 5.6
Baseline
Increased sACE2
Ang I 96.1
Ang 1-9 <2.0
Ang 1-7 4.5
Ang II 61.4
Ang 1-5 4.0
Repeat Sampling
Ang I 57.7
Ang 1-9 <2.0
Ang 1-7 2.3
Ang II 46.4
Ang 1-5 3.3
Baseline
Decreased sACE2
Ang I 76.4
Ang 1-9 <2.0
Ang 1-7 3.4
Ang II 28.4
Ang 1-5 3.2
Repeat Sampling
RENIN
ACE2
ACE
NEP
B
Ang I (pmol/L)
ΔAng I (%)
C
Ang II (pmol/L)
ΔAng II (%)
D
Ang 1-7 (pmol/L)
ΔAng 1-7 (%)
E
Ang 1-7/Ang II
ΔAng 1-7/Ang (%)
p=0.01
F
ACE Activity (nmol/hr/L)
ΔACE Activity (%)
Baseline
Follow-up
Increased sACE2
Decreased sACE2

Figure 3.4. Profile of angiotensin peptides and angiotensin-converting enzyme activity in propensity score-matched cohorts of patients having either increased or decreased soluble ACE2 levels at baseline and during repeat sampling. A. Global representation of median angiotensin peptide levels in 43 propensity score-matched pairs based on soluble angiotensin-converting enzyme 2 (sACE2) trajectories at baseline and during follow-up. Box and whisker plots showing baseline (blue) and percentage changes (yellow) in plasma levels of (B) Ang I, (C) Ang II, (D) Ang 1-7, (E) Ang 1-7/Ang II Ratio, and (F) Angiotensin-converting enzyme activity.

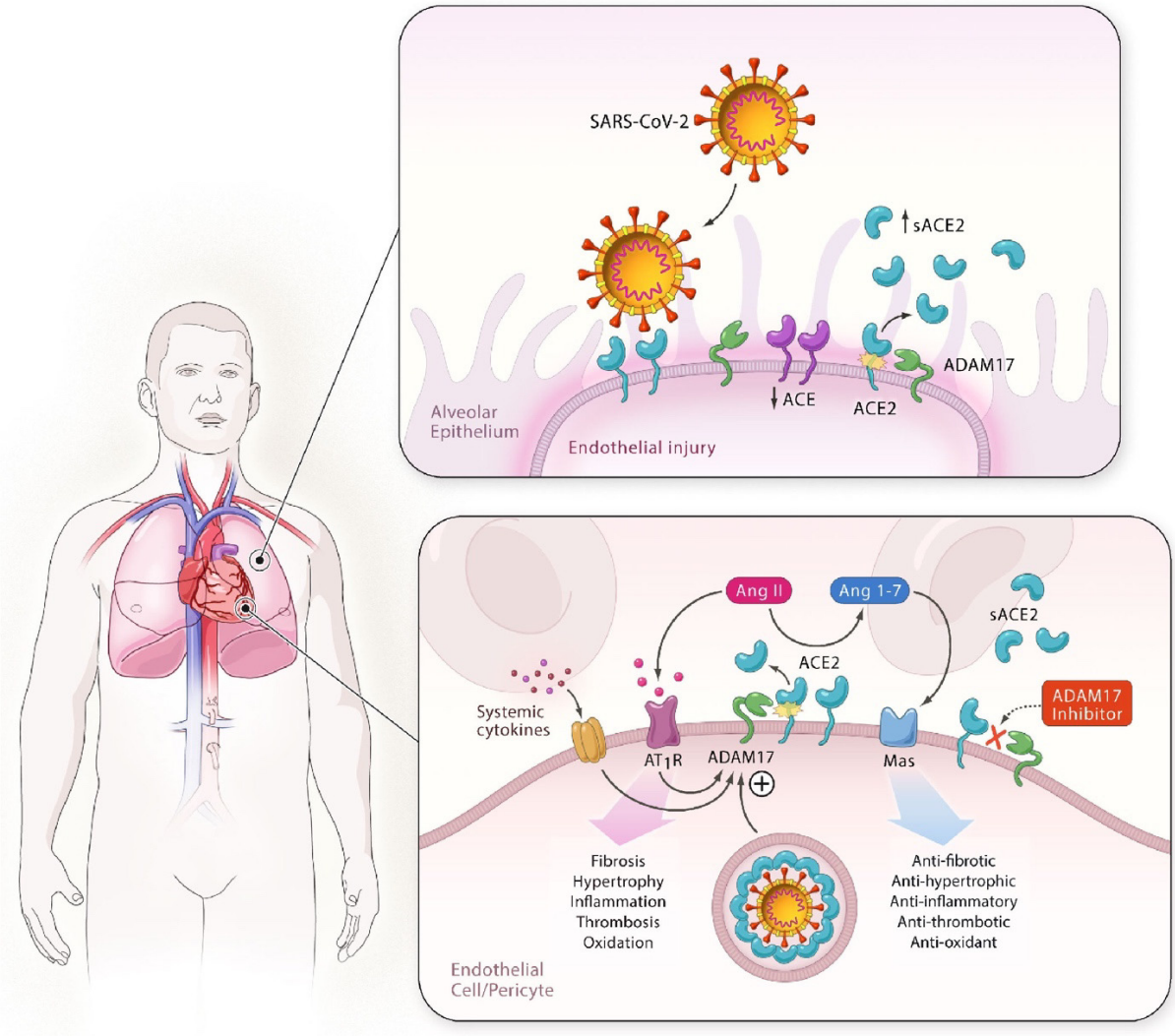


Figure 3.5. The potential role of ADAM17 dysregulation for aberrant ACE2 shedding in COVID-19 mediated pathophysiology. Elevation in soluble ACE2 (sACE2) through ADAM17 mediated proteolytic cleavage and reduction in ACE activity through pulmonary endothelial injury serves as signatures of the initial SARS-CoV-2 infection. However, persistent pathological upregulation of ADAM17 from systemic cytokines, Ang II and SARS-CoV-2 infections leads to further increase in soluble ACE2 (sACE2) levels reflective of reduced tissue ACE2 expression, which is associated with elevated risk of myocardial injury and mortality. ADAM17 inhibitor may have therapeutic potential in this setting through attenuating sACE2 release and preventing further loss of ACE2 mediated cardiovascular protection.

Tables

Table 3.1. Patient baseline clinical characteristics by disease severity (n=242)

Characteristic	Entire Cohort (n=242)	Non-ventilated Survivors (n=136)	Mechanical Ventilation and/or Death (n=106)	p Value
Demographics				
Age (years)	63 (52-74)	62 (53-75)	63 (50-73)	0.39
Male sex	155 (64.0)	79 (58.1)	76 (71.7)	0.03
BMI (kg/m^2)	28.9 (25.7-35.0)	28.6 (24.7-35.3)	29.1 (26.0-34.0)	0.38
Days from diagnosis	7 (3-10)	8 (3-11)	7 (3-10)	0.13
Current or previous smoker	105 (43.4)	54 (39.7)	51 (48.1)	0.28
Symptoms				
Fever	95 (39.3)	49 (36.0)	46 (43.4)	0.29
Myalgia	56 (23.1)	37 (27.2)	19 (17.9)	0.09
Cough	150 (62.0)	80 (58.8)	70 (66.0)	0.29
Dyspnea	167 (69.0)	89 (65.4)	78 (73.6)	0.21
Diarrhea/Nausea	87 (36.0)	47 (34.6)	40 (37.7)	0.69
Abnormal CXR	185 (76.4)	96 (70.6)	89 (84.0)	0.02
Vitals				
HR (beats/min)	76 (65-87)	76 (65-85)	77 (64-93)	0.55
SBP (mmHg)	123 (111-139)	125 (115-138)	122 (107-140)	0.20
Temperature (°C)	36.5 (36.2-36.9)	36.4 (36.1-36.9)	36.6 (36.3-37.1)	0.02
RR (breaths/min)	20 (18-24)	20 (18-21)	22 (18-28)	0.002
Laboratory tests				
BNP (ng/L)	78 (42-219)	63 (40-161)	103 (51-298)	0.05
Hemoglobin (g/L)	124 (113-137)	129 (115-141)	120 (107-131)	0.001
RBC (10^{12}/L)	4.3 (3.8-4.7)	4.4 (3.9-4.8)	4.1 (3.6-4.6)	<0.001
Platelet (10^9/L)	243 (181-310)	257 (194-339)	228 (159-293)	0.004
WBC (10^9/L)	9.8 (6.9-12.3)	9.0 (6.7-11.4)	10.9 (7.6-14.0)	0.002
Neutrophil (10^9/L)	7.5 (5.5-10.5)	7.1 (5.0-9.2)	9.1 (5.8-11.9)	0.001
Lymphocytes (10^9/L)	0.9 (0.5-1.4)	1.0 (0.6-1.4)	0.7 (0.5-1.0)	0.002
CRP (mg/L)	71 (30-143)	60 (28-120)	78 (49-174)	0.008
LDH (U/L)	328 (254-446)	296 (230-353)	391 (291-555)	<0.001
GFR ($mL/min/1.73m^2$)	75 (51-98)	79 (58-99)	66 (35-97)	0.04
ALT (U/L)	38 (24-73)	37 (24-64)	41 (26-79)	0.25
AST (U/L)	45 (30-67)	42 (28-61)	50 (34-72)	0.04
ALP (U/L)	73 (54-105)	67 (52-95)	78 (56-108)	0.09
D-dimer (mg/L)	1.3 (0.8-2.8)	1.2 (0.8-2.3)	1.3 (0.9-3.7)	0.16
Medical History				

Diabetes	111 (45.9)	60 (44.1)	51 (48.1)	0.60
Hypertension	134 (55.4)	69 (50.7)	65 (61.3)	0.12
COPD	42 (17.4)	26 (19.1)	16 (15.1)	0.50
CKD	43 (17.8)	20 (14.7)	23 (21.7)	0.18
CVD	55 (22.7)	23 (16.9)	32 (30.2)	0.02
Cancer	37 (15.3)	21 (15.4)	16 (15.1)	0.99
Anxiety/Depression	48 (19.8)	27 (19.9)	21 (19.8)	0.99
Medications				
ACEIs or ARBs	98 (40.5)	51 (37.5)	47 (44.3)	0.29
Beta-blockers	47 (19.4)	20 (14.7)	27 (25.5)	0.05
Statins	98 (40.5)	57 (41.9)	41 (38.7)	0.69
Management				
Dexamethasone	211 (87.2)	116 (85.3)	95 (89.6)	0.34
Antibiotic	200 (82.6)	103 (75.7)	97 (91.5)	0.001
Supplemental O_2	201 (83.1)	108 (79.4)	93 (87.7)	0.12

Abbreviations: BMI, body mass index; CXR, chest X-ray; HR, heart rate; SBP, systolic blood pressure; RR, respiratory rate; BNP, B-type natriuretic peptide; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; GFR, glomerular filtration rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CVD, cardiovascular disease (includes previous history of myocardial infarction, coronary artery disease, heart failure, atrial and ventricular arrhythmia); ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

Table 3.2. Crude and multivariable adjusted odds ratio for incidences of primary and secondary outcomes according to soluble ACE2, TNFRI and TNFRII at baseline (n=242) and trajectories during repeat sampling (n=187)

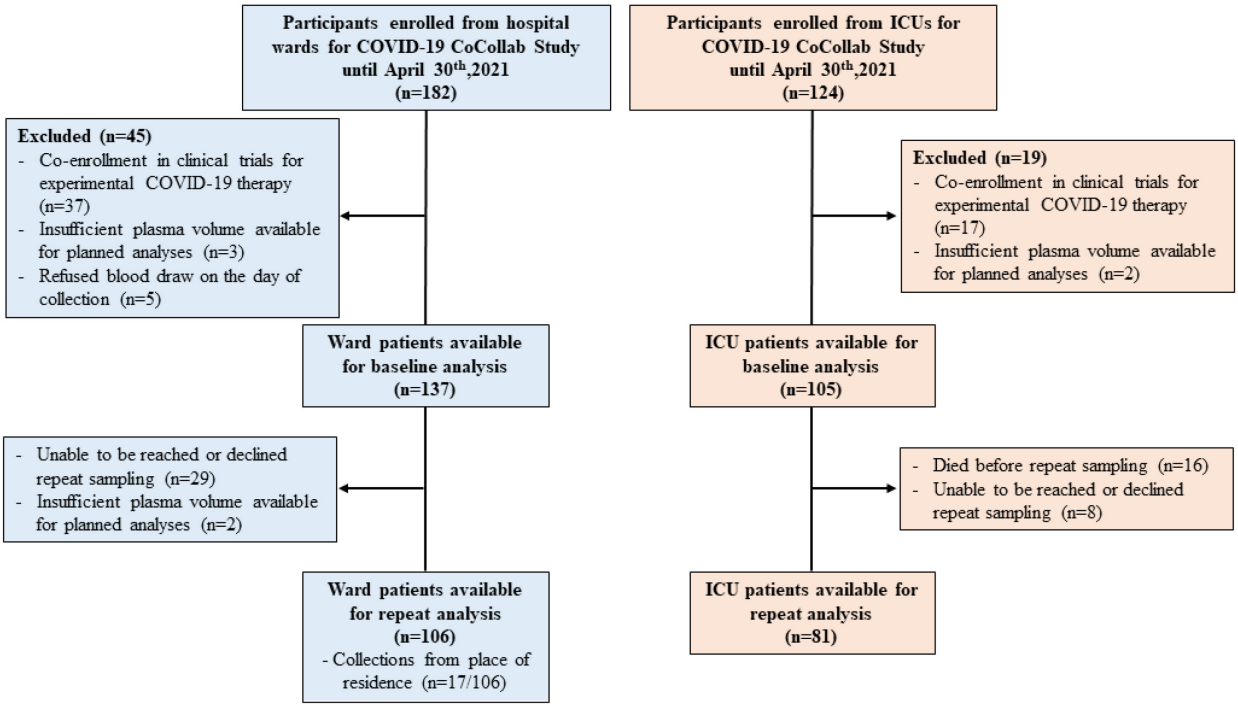
Primary Outcome	Crude HR (95% CI)	Model 1 aHR (95% CI)	Model 2 aHR (95% CI)
sACE2	0.81 (0.48-1.36)	0.73 (0.41-1.30)	1.65 (0.79-3.45)
ΔsACE2	7.47 (3.56-15.66)*	5.74 (2.62-12.58)*	3.94 (1.51-10.30)*
sTNFRI	2.58 (1.46-4.56)*	2.09 (1.02-4.26)*	1.22 (0.53-2.81)
ΔsTNFRI	5.02 (2.34-10.76)*	6.04 (2.49-14.66)*	8.70 (3.01-25.13)*
sTNFRII	2.68 (1.52-4.73)*	2.35 (1.17-4.69)*	1.38 (0.61-3.13)
ΔsTNFRII	5.32 (2.40-11.76)*	7.32 (2.90-18.48)*	7.92 (2.50-25.12)*
Secondary Outcomes	**Crude OR (95% CI)**	**Model 1 aOR (95% CI)**	**Model 2 aOR (95% CI)**
Acute Respiratory Distress Syndrome			
sACE2	0.69 (0.41-1.16)	0.48 (0.26-0.88)*	0.74 (0.35-1.57)
ΔsACE2	2.49 (1.32-4.67)*	2.72 (1.29-5.74)*	1.62 (0.64-4.11)
sTNFRI	1.41 (0.84-2.36)	2.27 (1.11-4.62)*	1.05 (0.42-2.63)
ΔsTNFRI	1.68 (0.92-3.06)	1.44 (0.72-2.89)	1.66 (0.67-4.11)
sTNFRII	2.41 (1.42-4.09)*	3.43 (1.69-6.96)*	2.05 (0.82-5.17)
ΔsTNFRII	1.50 (0.82-2.72)	1.27 (0.64-2.55)	1.34 (0.54-3.29)
Acute Kidney Injury			
sACE2	0.82 (0.48-1.40)	0.63 (0.33-1.19)	0.66 (0.30-1.45)
ΔsACE2	1.30 (0.69-2.44)	1.28 (0.60-2.75)	1.06 (0.40-2.79)
sTNFRI	5.22 (2.94-9.29)*	3.44 (1.65-7.15)*	4.66 (1.80-12.10)*
ΔsTNFRI	1.82 (0.99-3.33)	1.62 (0.76-3.44)	1.26 (0.47-3.41)
sTNFRII	3.17 (1.81-5.56)*	1.74 (0.87-3.46)	2.11 (0.85-5.22)
ΔsTNFRII	1.02 (0.56-1.86)	0.94 (0.44-2.00)	0.61 (0.23-1.65)
Acute Myocardial Injury			

sACE2	1.07 (0.63-1.82)	0.77 (0.41-1.46)	0.72 (0.33-1.58)
ΔsACE2	2.49 (1.31-4.75)*	2.61 (1.21-5.60)*	3.91 (1.39-10.97)*
sTNFRI	2.09 (1.22-3.57)*	1.43 (0.69-2.98)	0.86 (0.33-2.23)
ΔsTNFRI	1.48 (0.79-2.75)	1.67 (0.78-3.55)	3.11 (1.12-8.65)*
sTNFRII	2.79 (1.60-4.85)*	2.23 (1.10-4.50)*	2.41 (0.96-6.07)
ΔsTNFRII	1.33 (0.72-2.47)	1.41 (0.66-3.01)	1.80 (0.65-4.99)
Circulatory Shock			
sACE2	0.96 (0.44-2.08)	0.99 (0.43-2.25)	1.18 (0.37-3.77)
ΔsACE2	4.53 (1.78-11.51)*	5.02 (1.80-14.01)*	6.11 (1.26-19.57)*
sTNFRI	2.58 (1.17-5.70)*	3.61 (1.37-9.55)*	6.24 (1.45-26.87)*
ΔsTNFRI	1.62 (0.66-3.95)	1.49 (0.57-3.91)	1.56 (0.38-6.40)
sTNFRII	3.75 (1.54-9.12)*	4.24 (1.51-11.85)*	7.25 (1.50-37.10)*
ΔsTNFRII	2.28 (0.92-5.64)	2.33 (0.88-6.19)	3.65 (0.91-13.73)

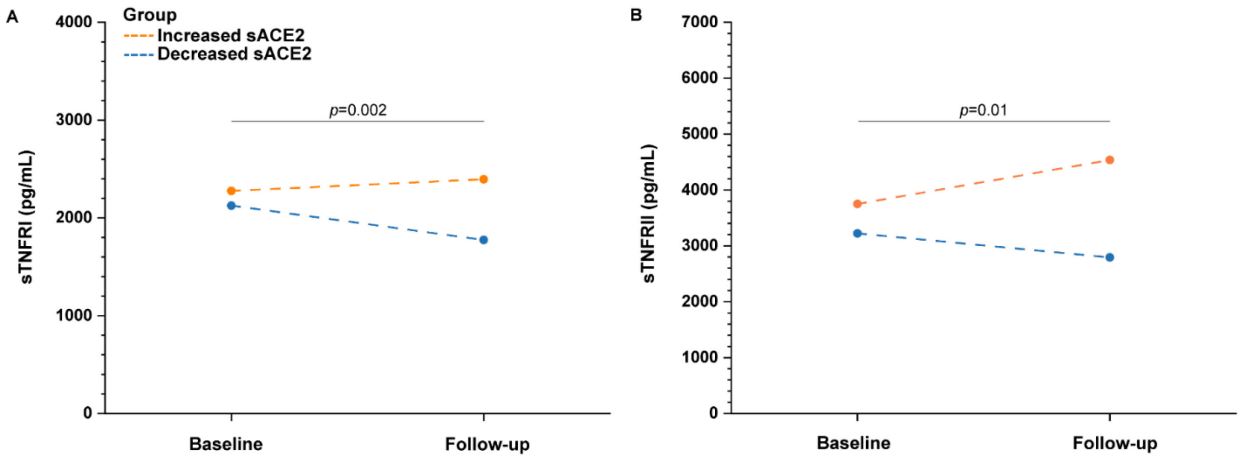
Abbreviations: CI, confidence interval; HR, hazard ratio; aHR, adjusted hazard ratio; OR, odds ratio; aOR; adjusted odds ratio; sACE2, soluble angiotensin-converting enzyme 2; sTNFRI, soluble tumor necrosis factor-alpha receptor I; sTNFRII, soluble tumor necrosis factor-alpha II. Biomarkers at baseline were classified according to above or below median cohorts, while changes in biomarkers at repeat sampling were classified based on increasing or decreasing trajectories. Median values for baseline sACE2, sTNFRI, and sTNFRII are 2.6 ng/mL, 2169 pg/mL, and 3396 pg/mL, respectively. sACE2, sTNFRI, and sTNFRII increased in 55, 74, and 78 patients, respectively at repeat sampling. Model 1 was adjusted for age, sex, body mass index, diabetes, hypertension, chronic kidney disease, cardiovascular disease and the use of either angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker. Model 2 was adjusted for all variables in Model 1 with the addition of high sensitivity cardiac troponin I, interleukin 6, tumor necrosis factor-alpha, C-reactive protein, platelet, neutrophil, and lymphocyte counts. *$p<0.05$

Supplementary Material

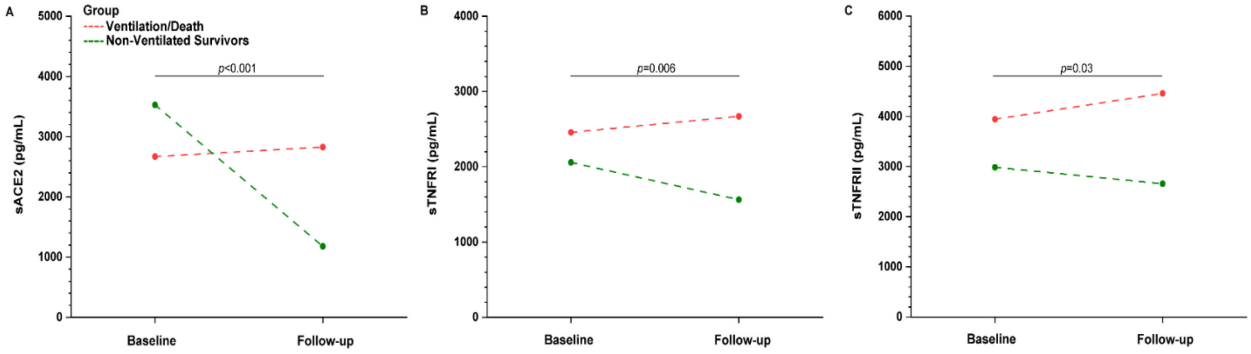
Supplemental Figures



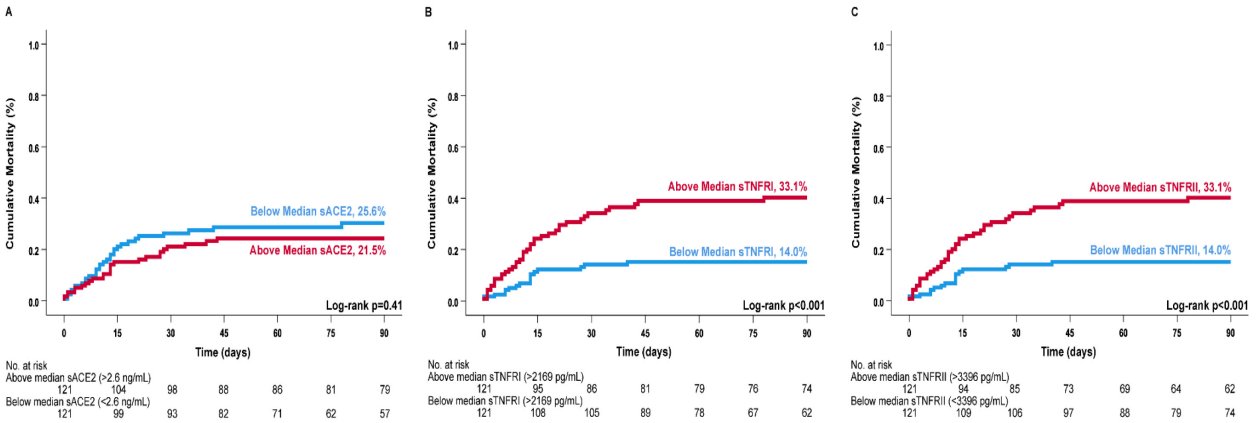
Supplemental Figure 3.1. Flow diagram of patients included for the COVID-19 CoCollab study.



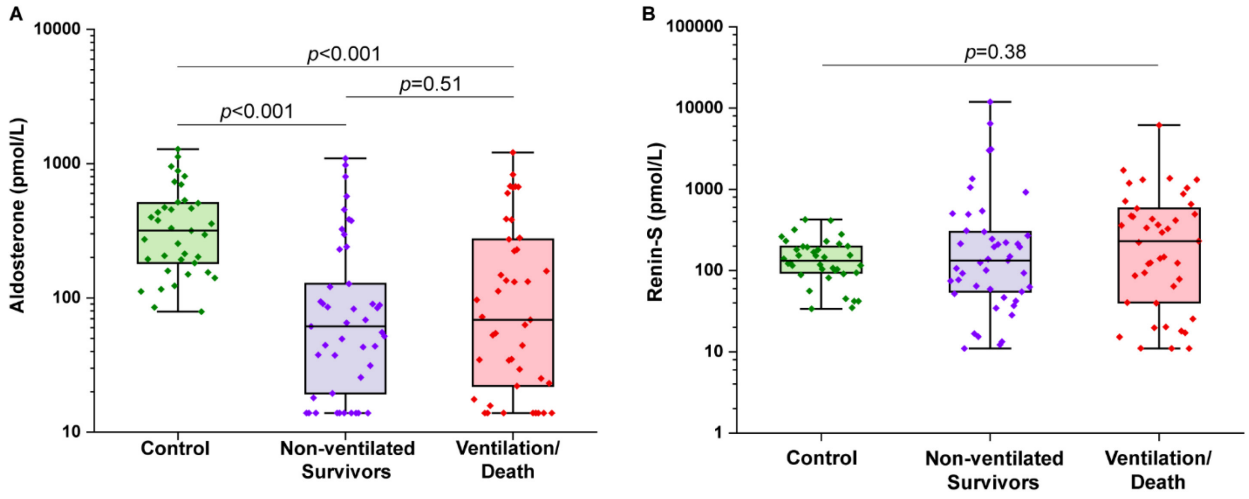
Supplemental Figure 3.2. Temporal profile of soluble TNFRI and TNFRII according to the trajectory of change in soluble ACE2.



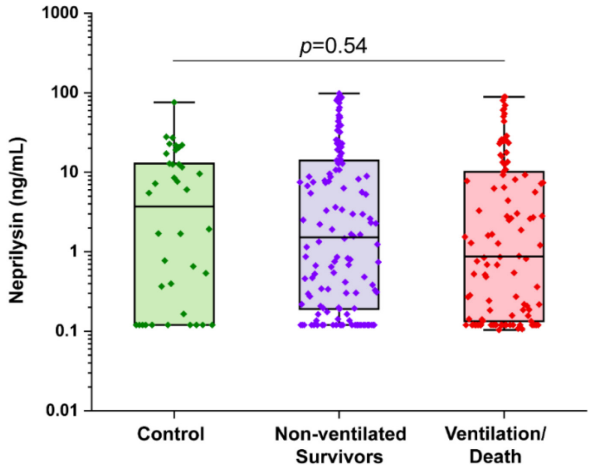
Supplemental Figure 3.3. Persistent elevation in soluble ACE2, TNFRI, and TNFRII in patients with severe COVID-19.



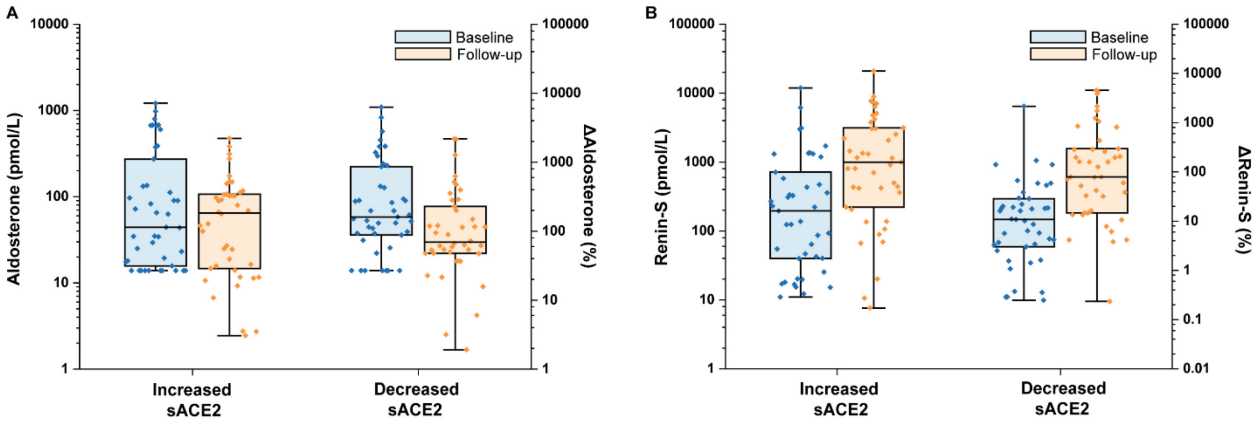
Supplemental Figure 3.4. Kaplan-Meier plots of all-cause mortality according to above and below median soluble ACE2, TNFRI, and TNFRII levels at baseline.



Supplemental Figure 3.5. Profile of aldosterone and plasma renin at baseline in patients with COVD-19 compared with age and sex-matched healthy controls. Box and whisker plot for concentration of aldosterone and plasma renin surrogate in 38 age and sex-matched healthy controls, 45 non-ventilated survivors and 43 patients requiring mechanical ventilation and/or have died due to COVID-19 at baseline.



Supplemental Figure 3.6. Profile of neprilysin at baseline in patients with COVD-19 compared with age and sex-matched healthy controls. Box and whisker plot for plasma neprilysin levels in 38 age and sex-matched healthy controls, 136 patients in non-ventilated survivors and 106 patients requiring mechanical ventilation and/or have died due to COVID-19 at baseline.



Supplemental Figure 3.7. Profile of aldosterone and plasma renin in propensity score-matched cohorts of patients having either increased or decreased soluble ACE2 levels. Box and whisker plot for concentration of aldosterone and plasma renin surrogate in in 43 propensity score-matched pairs based on soluble angiotensin-converting enzyme 2 (sACE2) trajectories at baseline and during follow-up.

Supplemental Tables

Supplemental Table 3.1. Patient clinical characteristics by baseline soluble ACE2 (n=242)

Characteristic	Below Median sACE2 (<2.6 ng/mL, n=121)	Above Median sACE2 (>2.6 ng/mL, n=121)	p Value
Demographics			
Age (years)	62 (51-74)	64 (53-73)	0.41
Male sex	75 (62.0)	80 (66.1)	0.59
BMI (kg/m^2)	28.1 (25.1-33.7)	29.9 (26.6-35.2)	0.08
Days from diagnosis	7 (3-13)	7 (3-10)	0.60
Current or previous smoker	53 (43.8)	52 (43.0)	0.79
Vitals			
HR (beats/min)	76 (65-89)	77 (64-87)	0.75
SBP (mmHg)	122 (111-136)	125 (112-140)	0.15
Temperature (°C)	36.6 (36.2-36.9)	36.5 (36.1-36.9)	0.23
RR (breaths/min)	20 (18-24)	20 (18-24)	0.75
Laboratory tests			
BNP (ng/L)	70 (41-161)	109 (45-275)	0.15
Hemoglobin (g/L)	123 (112-139)	127 (114-137)	0.93
RBC (10^{12}/L)	4.3 (3.8-4.8)	4.2 (3.8-4.6)	0.39
Platelet (10^9/L)	236 (175-307)	246 (183-321)	0.31
WBC (10^9/L)	10.3 (8.0-12.7)	8.8 (6.1-12.0)	0.01
Neutrophil (10^9/L)	8.4 (5.9-10.9)	7.1 (4.8-10.1)	0.02
Lymphocytes (10^9/L)	0.8 (0.5-1.3)	0.9 (0.5-1.4)	0.75
CRP (mg/L)	73 (32-153)	70 (29-122)	0.34
LDH (U/L)	311 (259-371)	353 (246-469)	0.50
GFR ($mL/min/1.73m^2$)	75 (51-100)	75 (51-97)	0.97
ALT (U/L)	42 (26-64)	36 (24-80)	0.62
AST (U/L)	45 (30-64)	46 (29-70)	0.91
ALP (U/L)	78 (56-106)	69 (50-98)	0.25
D-dimer (mg/L)	1.2 (0.8-3.1)	1.3 (0.9-2.5)	0.89
Medical History			
Diabetes	50 (41.3)	61 (50.4)	0.20
Hypertension	64 (52.9)	70 (57.9)	0.52
COPD	18 (14.9)	24 (19.8)	0.40
CKD	17 (14.0)	26 (21.5)	0.18
CVD	20 (16.5)	35 (28.9)	0.03
Cancer	19 (15.7)	18 (14.9)	0.86
Anxiety/Depression	26 (21.5)	22 (18.2)	0.63
Medications			
ACEIs or ARBs	49 (40.5)	49 (40.5)	0.99

Beta-blockers	21 (17.4)	26 (21.5)	0.52
Statins	50 (41.3)	48 (39.7)	0.90
Management			
Dexamethasone	105 (86.8)	106 (87.6)	0.85
Antibiotic	101 (83.5)	99 (81.8)	0.87
Supplemental O_2	102 (84.3)	99 (81.8)	0.73

Abbreviations: sACE2, soluble angiotensin-converting enzyme 2; BMI, body mass index; CXR, chest X-ray; HR, heart rate; SBP, systolic blood pressure; RR, respiratory rate; BNP, B-type natriuretic peptide; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; GFR, glomerular filtration rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CVD, cardiovascular disease (includes previous history of myocardial infarction, coronary artery disease, heart failure, atrial and ventricular arrhythmia); ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

Supplemental Table 3.2. Association of clinical characteristics with above median baseline soluble ACE2 (n=242)

	Univariable Analysis		Multivariable Analysis	
Characteristic	**OR (95% CI)**	**P Value**	**aOR (95% CI)**	**P Value**
Age (per 10 Yrs)	1.03 (0.87-1.22)	0.73	0.94 (0.76-1.16)	0.56
Male Sex	1.14 (0.68-1.93)	0.62	1.02 (0.53-1.95)	0.96
BMI (per 5 kg/m^2)	1.19 (0.98-1.46)	0.08	1.25 (0.99-1.57)	0.06
Diabetes	1.40 (0.84-2.33)	0.19	1.22 (0.63-2.38)	0.55
Hypertension	1.26 (0.76-2.09)	0.37	1.24 (0.53-2.91)	0.62
CKD	1.64 (0.84-3.21)	0.15	0.89 (0.37-2.16)	0.79
CVD	2.23 (1.19-4.16)	0.01	2.50 (1.10-5.68)	0.03
ACEIs or ARBs	1.04 (0.62-1.74)	0.88	0.72 (0.31-1.63)	0.43
WBC (per 10^9/L)	0.93 (0.88-0.98)	0.01	0.76 (0.59-0.98)	0.04
Neutrophil (per 10^9/L)	0.93 (0.88-0.99)	0.02	1.21 (0.92-1.58)	0.17

Abbreviations: OR, odds ratio; CI, confidence interval, BMI, body mass index; CKD, chronic kidney disease; CVD, cardiovascular disease (includes previous history of myocardial infarction, coronary artery disease, heart failure, atrial and ventricular arrhythmia); ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; WBC, white blood cells.

Supplemental Table 3.3. Patient clinical characteristics by the trajectory of change in soluble ACE2 (n=187)

Characteristic	Increased sACE2 (n=55)	Decreased sACE2 (n=132)	p Value
Demographics			
Age (years)	66 (58-77)	61 (51-72)	0.08
Male sex	39 (70.9)	82 (62.1)	0.31
BMI (kg/m^2)	27.8 (25.5-33.5)	29.3 (25.7-35.3)	0.51
Days from diagnosis	6 (2-10)	7 (3-10)	0.50
Current or previous smoker	31 (56.4)	54 (40.9)	0.13
Vitals			
HR (beats/min)	77 (64-86)	75 (64-86)	0.68
SBP (mmHg)	122 (108-140)	124 (111-139)	0.78
Temperature (°C)	36.7 (36.2-37.0)	36.5 (36.2-36.9)	0.37
RR (breaths/min)	20 (18-28)	20 (18-24)	0.05
Laboratory tests			
BNP (ng/L)	74 (49-207)	78 (40-197)	0.76
Hemoglobin (g/L)	124 (114-138)	125 (113-137)	0.67
RBC (10^{12}/L)	4.3 (3.8-4.8)	4.2 (3.8-4.6)	0.54
Platelet (10^9/L)	203 (139-248)	247 (193-329)	<0.001
WBC (10^9/L)	9.8 (6.4-11.6)	9.1 (7.2-12.1)	0.74
Neutrophil (10^9/L)	7.4 (5.7-10.1)	7.2 (5.0-9.9)	0.57
Lymphocytes (10^9/L)	0.7 (0.5-1.0)	0.9 (0.5-1.4)	0.04
CRP (mg/L)	75 (40-172)	63 (29-104)	0.14
LDH (U/L)	366 (256-492)	307 (258-395)	0.21
GFR ($mL/min/1.73m^2$)	66 (38-85)	84 (56-101)	0.003
ALT (U/L)	51 (31-86)	36 (22-67)	0.008
AST (U/L)	54 (40-74)	44 (26-67)	0.05
ALP (U/L)	80 (56-115)	69 (52-96)	0.38
D-dimer (mg/L)	1.3 (0.9-3.1)	1.4 (0.9-2.5)	0.66
Medical History			
Diabetes	24 (43.6)	63 (47.7)	0.63
Hypertension	31 (56.4)	63 (47.7)	0.34
COPD	7 (12.7)	22 (16.7)	0.66
CKD	11 (20.0)	20 (15.2)	0.52
CVD	15 (27.3)	29 (22.0)	0.45
Cancer	10 (18.2)	19 (14.4)	0.51
Anxiety/Depression	8 (14.5)	31 (23.5)	0.24
Medications			
ACEIs or ARBs	24 (43.6)	48 (36.4)	0.41

Beta-blockers	11 (20.0)	23 (17.4)	0.68
Statins	24 (43.6)	50 (37.9)	0.51
Management			
Dexamethasone	46 (83.6)	116 (87.9)	0.48
Antibiotic	47 (85.5)	109 (82.6)	0.83
Supplemental O_2	43 (78.2)	111 (84.1)	0.40

Abbreviations: sACE2, soluble angiotensin-converting enzyme 2; BMI, body mass index; CXR, chest X-ray; HR, heart rate; SBP, systolic blood pressure; RR, respiratory rate; BNP, B-type natriuretic peptide; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; GFR, glomerular filtration rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CVD, cardiovascular disease (includes previous history of myocardial infarction, coronary artery disease, heart failure, atrial and ventricular arrhythmia); ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

Supplemental Table 3.4. Association of clinical characteristics with increased soluble ACE2 (n=187)

	Univariable Analysis		Multivariable Analysis	
Characteristic	**OR (95% CI)**	**P Value**	**aOR (95% CI)**	**P Value**
Age (per 10 Yrs)	1.19 (0.96-1.47)	0.12	1.05 (0.81-1.38)	0.70
Male Sex	1.49 (0.75-2.93)	0.25	1.18 (0.52-2.70)	0.69
BMI (per 5 kg/m^2)	0.91 (0.72-1.17)	0.47	1.03 (0.78-1.37)	0.82
Diabetes	0.85 (0.45-1.60)	0.61	0.58 (0.25-1.33)	0.20
Hypertension	1.42 (0.75-2.66)	0.28	1.61 (0.57-4.60)	0.37
CKD	1.40 (0.62-3.16)	0.42	0.82 (0.25-2.69)	0.74
CVD	1.33 (0.65-2.74)	0.44	1.05 (0.41-2.67)	0.92
ACEIs or ARBs	1.36 (0.71-2.57)	0.35	0.99 (0.36-2.71)	0.99
GFR (per 10 $mL/min/1.73m^2$)	0.87 (0.79-0.96)	0.006	0.89 (0.77-1.03)	0.11
Platelet (per $10^8/L$)	0.95 (0.92-0.98)	0.001	0.96 (0.92-0.99)	0.02

Abbreviations: OR, odds ratio; CI, confidence interval; BMI, body mass index; CKD, chronic kidney disease; CVD, cardiovascular disease (includes previous history of myocardial infarction, coronary artery disease, heart failure, atrial and ventricular arrhythmia); ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; GFR, glomerular filtration rate.

Supplemental Table 3.5. Correlation analysis between baseline and changes in soluble ACE2, TNFRI, and TNFRII during repeat sampling with length of hospital stay and P/F ratio

	sTNFRI (pg/mL)	sTNFRII (pg/mL)	sACE2 (pg/mL)	ΔsTNFRI (pg/mL)	ΔsTNFRII (pg/mL)	ΔsACE2 (pg/mL)
Length of Hospital Stay	r^2=0.33; p<0.001	r^2=0.38; p<0.001	r^2=-0.02; p=0.81	r^2=-0.03; p=0.68	r^2=-0.04; p=0.61	r^2=-0.01; p=0.88
P/F Ratio	r^2=-0.14; p=0.11	r^2=-0.17; p=0.06	r^2=-0.15; p=0.08	r^2=0.03; p=0.77	r^2=-0.14; p=0.17	r^2=-0.16; p=0.11

Abbreviations: sACE2, soluble angiotensin-converting enzyme 2; sTNFRI, soluble tumor necrosis factor-alpha receptor I; sTNFRII, soluble tumor necrosis factor-alpha II. Spearman's correlation coefficients are reported for absolute values of each examined biomarker with the length of hospital stay (n=190) and P/F ratio (n=133). Patients who died during the same period of hospitalization were excluded from the analysis for length of hospital say.

Supplemental Table 3.6. Patient clinical characteristics according to disease status for renin-angiotensin peptide profiles

Characteristic	Healthy Control (n=38)	Non-ventilated Survivors (n=45)	Mechanical Ventilation and/or Death (n=43)	p Value
Demographics				
Age (years)	62 (53-72)	67 (59-79)	64 (50-71)	0.19
Male sex	24 (63.2)	28 (62.2)	30 (69.8)	0.37
BMI (kg/m^2)		28.6 (24.7-31.7)	28.5 (26.0-33.2)	0.48
Days from diagnosis		6 (3-9)	4 (2-7)	0.07
Current or previous smoker		23 (51.1)	22 (51.2)	0.99
Vitals				
HR (beats/min)		77 (64-83)	84 (61-104)	0.18
SBP (mmHg)		122 (113-139)	122 (111-140)	0.79
Temperature (°C)		36.4 (36.1-36.8)	36.6 (36.4-37.4)	0.07
RR (breaths/min)		20 (18-20)	21 (18-24)	0.09
Laboratory tests				
BNP (ng/L)		72 (42-164)	114 (50-357)	0.19
Hemoglobin (g/L)		130 (117-139)	118 (107-132)	0.04
RBC ($10^{12}/L$)		4.3 (3.9-4.8)	4.0 (3.5-4.6)	0.05
Platelet ($10^9/L$)		261 (201-305)	233 (145-308)	0.12
WBC ($10^9/L$)		8.5 (6.4-10.8)	11.0 (7.5-13.2)	0.02
Neutrophil ($10^9/L$)		7.1 (4.5-8.9)	8.2 (5.7-10.9)	0.05
Lymphocytes ($10^9/L$)		0.9 (0.6-1.3)	0.7 (0.5-1.0)	0.13
CRP (mg/L)		54 (28-137)	70 (39-138)	0.61
LDH (U/L)		296 (222-347)	424 (258-576)	0.01
GFR ($mL/min/1.73m^2$)		76 (62-95)	72 (30-98)	0.20
ALT (U/L)		37 (24-64)	38 (23-80)	0.61

AST (U/L)		42 (27-63)	63 (36-81)	0.10
ALP (U/L)		65 (51-90)	98 (73-123)	0.008
D-dimer (mg/L)		1.6 (0.9-2.8)	1.4 (1.0-2.8)	0.85
Medical History				
Diabetes		16 (35.6)	24 (55.8)	0.09
Hypertension		17 (37.8)	25 (58.1)	0.09
COPD		10 (22.2)	6 (14.0)	0.41
CKD		6 (13.3)	10 (23.3)	0.28
CVD		11 (24.4)	17 (39.5)	0.17
Cancer		6 (13.3)	5 (11.6)	0.99
Anxiety/Depression		9 (20.0)	8 (18.6)	0.99
Medications				
ACEIs or ARBs		8 (17.8)	9 (20.9)	0.79
Beta-blockers		6 (13.3)	10 (23.3)	0.28
Statins		12 (26.7)	16 (37.2)	0.36
Management				
Dexamethasone		36 (80.0)	37 (86.0)	0.57
Antibiotic		30 (67.7)	40 (93.0)	0.003
Supplemental O_2		35 (77.8)	36 (83.7)	0.59

Abbreviations: BMI, body mass index; CXR, chest X-ray; HR, heart rate; SBP, systolic blood pressure; RR, respiratory rate; BNP, B-type natriuretic peptide; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; GFR, glomerular filtration rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CVD, cardiovascular disease (includes previous history of myocardial infarction, coronary artery disease, heart failure, atrial and ventricular arrhythmia); ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

Supplemental Table 3.7. Comparison of clinical characteristics between patients having increased and decreased trajectories in soluble ACE2 in propensity score-matched samples

Characteristic	Increased sACE2 (n=43)	Decreased sACE2 (n=43)	P Value	Standardized Differences
Age (years)	70 (61-77)	69 (60-77)	0.80	0.02
Male sex	30 (69.8)	31 (72.1)	0.81	0.05
BMI (kg/m^2)	27.8 (25.5-33.0)	27.8 (25.3-32.3)	0.71	0.05
Diabetes	25 (58.1)	26 (60.5)	0.83	0.05
Hypertension	26 (60.5)	27 (62.8)	0.83	0.05
CKD	7 (16.3)	8 (18.6)	0.78	0.06
CVD	18 (41.9)	19 (44.2)	0.83	0.05
ACEIs or ARBs	23 (53.5)	21 (48.8)	0.67	0.09

Abbreviations: sACE2, soluble angiotensin-converting enzyme 2; BMI, body mass index; CKD, chronic kidney disease; CVD, cardiovascular disease (includes previous history of myocardial infarction, coronary artery disease, heart failure, atrial and ventricular arrhythmia); ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

Chapter 4. Summary and Future Perspective

4.1. Overview of Research

Comprehensive examinations of plasma angiotensin peptide profile, sometimes referred to as RAS fingerprinting, can be reliably conducted through state-of-the-art liquid chromatography tandem mass spectrometry methods. In this thesis, we measured plasma angiotensin peptide levels concurrent with related RAS and disease biomarkers in patients with heart failure and COVID-19 (Chapter 2 and Chapter 3, respectively). Importantly, we determined the association between novel RAS-biomarkers identified through our studies with adverse clinical outcomes, including mortality, hospitalization, and end-organ injuries to facilitate risk-stratification and delivery of personalized management for individuals suffering from these conditions.

4.2. Clinical Implications of Novel Findings

The RAS plays a fundamental role in regulating systemic blood pressure and fluid volume homeostasis.[251] While Ang II is traditionally considered the effector peptide of the RAS based on its ability to stimulate salt and water reabsorption, promote vasoconstriction, cell migration, hyperplasia, inflammation, oxidative stress, and tissue remodeling through the AT_1R, other angiotensin peptides also exert numerous physiological effects. Notably, Ang 1-7 elicits the majority of its cardioprotective actions through the Mas receptors and has been shown to directly antagonize many of the detrimental effects mediated by Ang II signaling.[12,252] Systemic infusion of Ang 1-7 in rats attenuated the progression of heart failure and preserved cardiac function following myocardial infarction.[253] Further, Ang 1-7 infusion protected against the development of diastolic dysfunction in diabetic db/db mice,[138] and Ang II-mediated adverse cardiac remodeling in rats.[254] In patients, Ang 1-7 infusion alleviated obesity-related vascular dysfunction through

improving endothelium-dependent vasodilatory response to insulin.[210] However, given the limitations imposed by rapid peptidase processing of Ang 1-7, cyclic analogs, formulation with inclusion compounds, and bioencapsulation have been explored to increase its bioavailability.[175,255,256] Alternatively, multiple Mas activators were also developed, such as AVE0991, CGEN-856, CGEN-861, and CGEN-856S, capable of eliciting potent vasodilatory, anti-inflammatory, and anti-hypertrophic cardioprotective effects.[255,257]

Although initially believed to be biologically inactive, Ang 1-9, Ang 1-5, and Ang 3-8 are other components of the alternative RAS, having demonstrated counter-regulatory effects in balancing pathophysiological RAS activation through various *in vitro* and *in vivo* experiments.[65,258,259] For example, Ang 1-9 attenuated cardiac hypertrophy and fibrosis via AT_2R, Ang 1-5 is protective against ischemic-perfusion injury through the Mas receptor, and Ang 3-8 exerted vaso-protective effects via signaling through the AT_4R. Therefore, the protective axis of the RAS and its related angiotensin peptides plays a central role in preventing and halting the development of various cardiovascular diseases, making them attractive therapeutic targets.

As an endogenous regulator of the RAS to maintain the balance between harmful and protective RAS axes, loss-of-function experiments using ACE2 knockout mice and ACE2 inhibitors have shown increased susceptibility to myocardial infarction, hypertension and Ang II-mediated myocardial hypertrophy, microvascular complications, inflammation, fibrosis, diastolic and systolic dysfunction, and oxidative stress.[12,14] Importantly, partial loss of ACE2, as seen in explanted human hearts from patients with heart failure and dilated cardiomyopathy, is sufficient to enhance the susceptibility to heart disease.[12] Conversely, gain-of-function experiments with recombinant ACE2 have shown protective roles in various models of cardiovascular disease, including hypertension, diabetes, and heart failure.[12,14] The safety and tolerability of recombinant

human ACE2 (rhACE2) have already been demonstrated in healthy volunteers,[190] and in two pilot trials in patients with acute respiratory disease syndrome and pulmonary arterial hypertension, respectively.[176,191] Administration of rhACE2 led to prompt systemic conversion of Ang II to Ang 1-7, associated with a reduction in biomarkers of inflammation and oxidative stress.[176,191] Moreover, the therapeutic value of rhACE2 was evaluated in explanted human hearts with dilated cardiomyopathy and in plasma from patients with acute and chronic heart failure, where treatment resulted in changes in angiotensin peptide metabolism favoring Ang 1-7 and Ang 1-9 while reducing Ang II levels.[58] Following myocardial infarction, rhACE2 prevents cardiomyocyte apoptosis secondary to hypoxia and inhibits the proliferation of myofibroblasts, thus limiting fibrosis and improving hemodynamic parameters, including left ventricular ejection fraction.[260] Furthermore, genetic ablation of *Ace2* exacerbated cardiac fibrosis and oxidative stress in hypertensive mice, which was rescued by administration of rhACE2.[13,14]

Based on current heart failure guidelines, blockade of pathophysiological pathways including the RAS, aldosterone, and sympathetic nervous system using ACE inhibitors, angiotensin receptor blockers (ARB), angiotensin receptor-neprilysin inhibitor (ARNI), mineralocorticoid receptor antagonist (MRA), beta-blockers, and more recently, sodium-glucose co-transport 2 (SGLT2) inhibitors represent the cornerstone of modern-day therapeutics for the management of heart failure with reduced ejection.[188,261,262] Nevertheless, the possibility of enhancing protective neurohumoral pathways using the aforementioned approaches such as Ang 1-7 analogs, Mas activators, and rhACE2 as synergistic therapies to current heart failure treatments is an exciting prospect to explore. However, the clinical significance of these pathways, in this case, the ACE2/Ang 1-7/Mas axis, must be first established through prospectively conducted biomarker studies to guide their clinical implementation.

Accordingly, in Chapter 2, we prospectively enrolled 110 patients with heart failure presenting to the University of Alberta Hospital emergency department (n=46) and Mazankowski Alberta Heart Institute outpatient clinics (n=64) for angiotensin peptide profiling. The participants spanned a wide spectrum of heart failure severity from New York Heart Association (NYHA) functional class I to class IV and encompassed individuals with reduced or preserved ejection fraction. For angiotensin peptide analysis, blood was drawn into pre-labeled collection tubes with either lithium heparin for the equilibrium profile or a protease inhibitor cocktail containing broad-spectrum inhibitors against metalloproteases, aspartic proteases, cysteine proteases, serine proteases, and specific inhibitors against renin and aminopeptidases A and N, completely blocking angiotensin peptide metabolism to derive the circulating profile. Based on the Spearman correlation coefficient, we found that the equilibrium levels were strongly related to their circulating counterparts across all four peptides examined, Ang I, Ang II, Ang 1-7, and Ang 1-9. Indeed, we saw a near-identical prognostic value based on above and below median Ang 1-7/Ang II ratios for all-cause mortality using either equilibrium (aHR, 0.38 [95% CI, 0.18-0.81], p=0.01) or circulating (aHR, 0.38 [95% CI, 0.18-0.80], p=0.01) peptide levels after adjusting for conventional cardiovascular risk factors. Notably, incorporation of either equilibrium (+45.0% [95% CI, 7.3%-82.7%]) or circulating (+24.3% [95% CI, 0.4%-59.6%]) Ang 1-7/Ang II ratio provided substantial improvement in risk prediction of the nested multivariable model for all-cause mortality at five years according to our net reclassification analysis. These findings also translated to a lowered yearly hospitalization duration associated with an above-median Ang 1-7/Ang II ratio for both equilibrium and circulating peptide profiles. Therefore, a crucial implication for future research and clinical translation is the ability to gather accurate and comprehensive information regarding the dynamic state of the RAS based on standard lithium-heparin sampling commonly

used in clinical practice, which is much more pragmatic than the special protease inhibitor cocktail required for circulating analyses. Another important finding from our study is that the individual levels of Ang II or Ang 1-7 failed to display any prognostic significance while their ratios did. This finding suggests that patients could still have favorable outcomes if the activated disease-mediating RAS pathway is adequately counterbalanced by a corresponding upregulation of the RAS protective arm, which validates our approach to enhance the ACE2/Ang 1-7/Mas axis concurrent with the existing blockade of the detrimental ACE/Ang II/AT_1R axis. Additionally, it also highlights the potential utility of ACE2/Ang 1-7/Mas enhancers such as Ang 1-7 analog, Mas activators, and rhACE2 in patients with incomplete RAS blockade due to alternative tissue Ang II production through ACE independent mechanisms, such as mast cell chymase, which has been shown to be upregulated in explanted hearts from patients with chronic heart failure.[58]

In Chapter 3, we examined the state of soluble ACE2 and angiotensin peptides through COVID-19 disease progression by prospectively enrolling patients from COVID-19 wards (n=137) and intensive care units (n=105) having a positive SARS-CoV-2 real-time polymerase chain reaction assay with blood collection performed at admission and day seven follow-up. SARS-CoV-2 infection activates a strong inflammatory response and, in severe cases, can trigger cytokine release syndrome resulting in myocarditis-like and vasculitis phenotypes.[263-268] Cardiovascular diseases characterized by microvascular dysfunction, myocarditis, myocardial damage, and heart failure are common in patients with COVID-19.[233,241,269-274] Among the activated proinflammatory pathways is the induction of ADAM17, which is a multifunctional membrane metalloprotease.[116,275] The pathogenic role of ADAM17 dysregulation has already been demonstrated in vascular diseases[228,276] and cardiomyopathies.[277,278] ADAM17 mediates the proteolytic cleavage of membrane-bound ACE2 into the circulation,[45,279] which is enhanced by

Ang II and SARS-CoV-2 cell entry, creating a deteriorating positive feedback loop in the infected organs.[116,217] Importantly, another human coronavirus, HCoV-NL63, which also uses ACE2 as a receptor, but causes only common cold symptoms, fails to activate ADAM17.[113,116] In comparison, SARS coronaviruses both induced ADAM17 proteolytic activity and upregulated *ADAM17* mRNA expression, resulting in ACE2 downregulation associated with the loss of protection against tissue RAS.[113,116,280] In our study, we found an elevation in plasma soluble ACE2 at admission served as a signature of COVID-19 but was not associated with mortality or end-organ injuries. Furthermore, there was a stepwise increase in sTNFRI and sTNFRII, both are substrates of ADAM17, alongside IL-6, TNFα, and hsTnI levels reflective of the hyperinflammatory condition and myocardial injuries during SARS-CoV-2 infection. The temporal nature of our study design allowed for the crucial finding that an upward trajectory in soluble ACE2 levels between sampling was independently associated with increased risk of mortality and incidence of acute myocardial injury beyond established clinical risk factors and disease biomarkers of inflammation (IL-6 and TNFα) and myocardial injury (hsTnI). Moreover, patients who survived the COVID-19 hospitalization had a drastic reduction in soluble ACE2 levels during repeat sampling, concurrent with decreased sTNFRI and sTNFRII levels. Therefore, competent regulation over ADAM17-mediated shedding of ACE2 and other inflammatory mediators led to favorable clinical outcomes in COVID-19. Conversely, sustained pathological ADAM17 activation potentiates the dysregulated inflammatory response and leads to loss of ACE2-mediated organ protection leading to impaired vascular tone, increased vascular permeability, oxidative stress, and endothelial dysfunction, which are drivers of adverse clinical outcomes in SARS-CoV-2 infection.

Our comprehensive profiling of angiotensin peptides in COVID-19 using LC-MS/MS techniques revealed an increase in plasma Ang I and Ang 1-7 levels and a surprising reduction in

Ang II levels, which goes against some previous reports from the early days of the pandemic.[200,281] However, these studies measured Ang II levels using commercial antibody-based assays, which are generally not isoform-specific and lack the sensitivity required to distinguish differences from single amino acid changes.[282] Our finding of downregulated Ang II levels is exceeding plausible based on the extensive pulmonary vascular endothelial injuries observed in patients during acute COVID-19 and the predominant effects of the pulmonary circulation in systemic Ang II generation.[123,244] Therefore, in severe COVID-19, RAS inhibition may potentiate worsening hypotension and renal injury in the setting of circulatory shock, given the already suppressed Ang II levels. However, beyond the acute infectious phase, pathological loss of ACE2 contributes to disease progression, which is reflected through the reduction in Ang 1-7/Ang II ratio from our propensity-matched cohorts at repeat sampling, through ADAM17-mediated ectodomain shedding into the circulation, perpetuated by an Ang II-mediated positive feedback mechanism that is suppressed with AT_1R blockers.[44,45,123] Whereas ACE inhibition disrupts the generation of Ang II from Ang I and reduces the proteolytic degradation of Ang 1-7 into Ang 1-5 to compensate for ACE2 dysregulation with disease progression, thus supporting their potential utility to alleviate the risk of incident cardiovascular diseases in the long-term sequelae of COVID-19.[58,248,283] Overall, dysregulation of ACE2 and RAS peptides plays a vital role in COVID-19 disease progression and is associated with adverse outcomes. As such, novel therapeutics inhibiting ADAM17 pathways, restoring tissue ACE2 expression, and enhancing the Ang 1-7/Mas axis could protect against organ injuries and improve outcomes for patients with COVID-19.

4.3. Future Direction

The devastating toll of SARS-CoV-2 infections extends beyond the infectious phase and represents an emerging public health crisis. Notably, recovering patients six months post-infection showed a stepwise increase in the risk of mortality and development of new pulmonary, nervous system, metabolic, gastrointestinal, and cardiovascular diseases based on acute COVID-19 severity.[284] Several mechanisms have been proposed for long COVID pathogenesis, including vascular dysfunction, chronic immune activation, autoantibodies, and sustained downregulation of tissue ACE2.[285] Indeed, advancing age, female sex, elevated body mass index, and pre-existing conditions such as hypertension and diabetes are established risk factors for developing adverse clinical sequelae of COVID-19 in recovering patients, potentially related to the differential expression and regulation of ACE2 under these conditions.[286,287] Moreover, the development of cardiovascular complications is a major source of concern in long-COVID, where ACE2 dysregulation during the acute phase was associated with increased mortality and acute myocardial injury.[123,283] In particular, the population burdens of heart failure are drastically increased in patients recovering from COVID-19, a finding that extends to infected patients who were not hospitalized during the acute infectious phase. Therefore, it is prudent to consider enhancing organ protective factors, such as stimulating the ACE2/Ang 1-7/Mas axis, as a sustainable strategy to improve the health outcomes of existing patients with heart failure or those recovering from COVID-19 that are suffering from severe cardiovascular sequelae.

Bibliography

1. Tran DT, Ohinmaa A, Thanh NX, Howlett JG, Ezekowitz JA, McAlister FA, Kaul P. The current and future financial burden of hospital admissions for heart failure in Canada: a cost analysis. *CMAJ Open*. 2016;4:E365-E370. doi: 10.9778/cmajo.20150130
2. Botly LCP, Martin-Rhee M, Kasiban A, Swartz RH, Mulvagh SL, Lindsay MP, Goia C, Smith EE, Hill MD, Field TS, et al. COVID-19 Pandemic: Global Impact and Potential Implications for Cardiovascular Disease in Canada. *CJC Open*. 2020;2:265-272. doi: 10.1016/j.cjco.2020.06.003
3. Calvillo-Arguelles O, Ross HJ. Cardiac considerations in patients with COVID-19. *CMAJ*. 2020;192:E630. doi: 10.1503/cmaj.200562
4. Shi S, Qin M, Shen B, Cai Y, Liu T, Yang F, Gong W, Liu X, Liang J, Zhao Q, et al. Association of Cardiac Injury With Mortality in Hospitalized Patients With COVID-19 in Wuhan, China. *JAMA Cardiol*. 2020;5:802-810. doi: 10.1001/jamacardio.2020.0950
5. Liu PP, Blet A, Smyth D, Li H. The Science Underlying COVID-19: Implications for the Cardiovascular System. *Circulation*. 2020;142:68-78. doi: 10.1161/CIRCULATIONAHA.120.047549
6. Guo T, Fan Y, Chen M, Wu X, Zhang L, He T, Wang H, Wan J, Wang X, Lu Z. Cardiovascular Implications of Fatal Outcomes of Patients With Coronavirus Disease 2019 (COVID-19). *JAMA Cardiol*. 2020;5:811-818. doi: 10.1001/jamacardio.2020.1017
7. Wang K, Gheblawi M, Oudit GY. Angiotensin Converting Enzyme 2: A Double-Edged Sword. *Circulation*. 2020. doi: 10.1161/CIRCULATIONAHA.120.047049

8. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell*. 2020;181:271-280 e278. doi: 10.1016/j.cell.2020.02.052

9. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. 2020;579:270-273. doi: 10.1038/s41586-020-2012-7

10. Yan R, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science*. 2020;367:1444-1448. doi: 10.1126/science.abb2762

11. Shang J, Ye G, Shi K, Wan Y, Luo C, Aihara H, Geng Q, Auerbach A, Li F. Structural basis of receptor recognition by SARS-CoV-2. *Nature*. 2020;581:221-224. doi: 10.1038/s41586-020-2179-y

12. Patel VB, Zhong JC, Grant MB, Oudit GY. Role of the ACE2/Angiotensin 1-7 Axis of the Renin-Angiotensin System in Heart Failure. *Circulation research*. 2016;118:1313-1326. doi: 10.1161/CIRCRESAHA.116.307708

13. Zhong J, Guo D, Chen CB, Wang W, Schuster M, Loibner H, Penninger JM, Scholey JW, Kassiri Z, Oudit GY. Prevention of angiotensin II-mediated renal oxidative stress, inflammation, and fibrosis by angiotensin-converting enzyme 2. *Hypertension*. 2011;57:314-322. doi: 10.1161/HYPERTENSIONAHA.110.164244

14. Zhong J, Basu R, Guo D, Chow FL, Byrns S, Schuster M, Loibner H, Wang XH, Penninger JM, Kassiri Z, et al. Angiotensin-converting enzyme 2 suppresses pathological

hypertrophy, myocardial fibrosis, and cardiac dysfunction. *Circulation*. 2010;122:717-728, 718 p following 728. doi: 10.1161/CIRCULATIONAHA.110.955369

15. Kassiri Z, Zhong J, Guo D, Basu R, Wang X, Liu PP, Scholey JW, Penninger JM, Oudit GY. Loss of angiotensin-converting enzyme 2 accelerates maladaptive left ventricular remodeling in response to myocardial infarction. *Circ Heart Fail*. 2009;2:446-455. doi: 10.1161/CIRCHEARTFAILURE.108.840124

16. Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, et al. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature*. 2002;417:822-828. doi: 10.1038/nature00786

17. Bernstein KE, Xiao HD, Frenzel K, Li P, Shen XZ, Adams JW, Fuchs S. Six truisms concerning ACE and the renin-angiotensin system educed from the genetic analysis of mice. *Circulation research*. 2005;96:1135-1144. doi: 10.1161/01.RES.0000169536.73576.66

18. Page IH, Helmer OM. Angiotonin-Activator, Renin- and Angiotonin-Inhibitor, and the Mechanism of Angiotonin Tachyphylaxis in Normal, Hypertensive, and Nephrectomized Animals. *J Exp Med*. 1940;71:495-519. doi: 10.1084/jem.71.4.495

19. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell*. 2020. doi: 10.1016/j.cell.2020.02.058

20. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-

insensitive carboxypeptidase. *J Biol Chem*. 2000;275:33238-33243. doi: 10.1074/jbc.M002615200

21. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res*. 2000;87:E1-9. doi: 10.1161/01.res.87.5.e1
22. Skeggs LT, Jr., Kahn JR, Shumway NP. The preparation and function of the hypertensin-converting enzyme. *J Exp Med*. 1956;103:295-299. doi: 10.1084/jem.103.3.295
23. Goldblatt H, Lynch J, Hanzal RF, Summerville WW. Studies on Experimental Hypertension : I. The Production of Persistent Elevation of Systolic Blood Pressure by Means of Renal Ischemia. *J Exp Med*. 1934;59:347-379. doi: 10.1084/jem.59.3.347
24. Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *The Biochemical journal*. 2004;383:45-51. doi: 10.1042/BJ20040634
25. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*. 2003;426:450-454. doi: 10.1038/nature02145
26. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020. doi: 10.1016/S0140-6736(20)30566-3

27. Li F, Li W, Farzan M, Harrison SC. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science*. 2005;309:1864-1868. doi: 10.1126/science.1116480

28. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, Graham BS, McLellan JS. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science*. 2020;367:1260-1263. doi: 10.1126/science.abb2507

29. Tukiainen T, Villani AC, Yen A, Rivas MA, Marshall JL, Satija R, Aguirre M, Gauthier L, Fleharty M, Kirby A, et al. Landscape of X chromosome inactivation across human tissues. *Nature*. 2017;550:244-248. doi: 10.1038/nature24265

30. Viveiros A, Rasmuson J, Vu J, Mulvagh SL, Yip CYY, Norris CM, Oudit GY. Sex differences in COVID-19: candidate pathways, genetics of ACE2, and sex hormones. *Am J Physiol Heart Circ Physiol*. 2021;320:H296-H304. doi: 10.1152/ajpheart.00755.2020

31. Qiao Y, Wang XM, Mannan R, Pitchiaya S, Zhang Y, Wotring JW, Xiao L, Robinson DR, Wu YM, Tien JC, et al. Targeting transcriptional regulation of SARS-CoV-2 entry factors ACE2 and TMPRSS2. *Proc Natl Acad Sci U S A*. 2020. doi: 10.1073/pnas.2021450118

32. Dalpiaz PL, Lamas AZ, Caliman IF, Ribeiro RF, Jr., Abreu GR, Moyses MR, Andrade TU, Gouvea SA, Alves MF, Carmona AK, et al. Sex Hormones Promote Opposite Effects on ACE and ACE2 Activity, Hypertrophy and Cardiac Contractility in Spontaneously Hypertensive Rats. *PLoS One*. 2015;10:e0127515. doi: 10.1371/journal.pone.0127515

33. Liu J, Ji H, Zheng W, Wu X, Zhu JJ, Arnold AP, Sandberg K. Sex differences in renal angiotensin converting enzyme 2 (ACE2) activity are 17beta-oestradiol-dependent and sex chromosome-independent. *Biol Sex Differ*. 2010;1:6. doi: 10.1186/2042-6410-1-6

34. Sato T, Suzuki T, Watanabe H, Kadowaki A, Fukamizu A, Liu PP, Kimura A, Ito H, Penninger JM, Imai Y, et al. Apelin is a positive regulator of ACE2 in failing hearts. *J Clin Invest*. 2013;123:5203-5211. doi: 10.1172/JCI69608

35. Chatterjee P, Gheblawi M, Wang K, Vu J, Kondaiah P, Oudit GY. Interaction between the apelinergic system and ACE2 in the cardiovascular system: therapeutic implications. *Clin Sci (Lond)*. 2020;134:2319-2336. doi: 10.1042/CS20200479

36. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet*. 2009;10:295-304. doi: 10.1038/nrg2540

37. Fan R, Mao SQ, Gu TL, Zhong FD, Gong ML, Hao LM, Yin FY, Dong CZ, Zhang LN. Preliminary analysis of the association between methylation of the ACE2 promoter and essential hypertension. *Mol Med Rep*. 2017;15:3905-3911. doi: 10.3892/mmr.2017.6460

38. Pinto BGG, Oliveira AER, Singh Y, Jimenez L, Goncalves ANA, Ogava RLT, Creighton R, Schatzmann Peron JP, Nakaya HI. ACE2 Expression Is Increased in the Lungs of Patients With Comorbidities Associated With Severe COVID-19. *J Infect Dis*. 2020;222:556-563. doi: 10.1093/infdis/jiaa332

39. Li Y, Li H, Zhou L. EZH2-mediated H3K27me3 inhibits ACE2 expression. *Biochemical and biophysical research communications*. 2020;526:947-952. doi: 10.1016/j.bbrc.2020.04.010

40. Nersisyan S, Shkurnikov M, Turchinovich A, Knyazev E, Tonevitsky A. Integrative analysis of miRNA and mRNA sequencing data reveals potential regulatory mechanisms

of ACE2 and TMPRSS2. *PLoS One*. 2020;15:e0235987. doi: 10.1371/journal.pone.0235987

41. Lambert DW, Lambert LA, Clarke NE, Hooper NM, Porter KE, Turner AJ. Angiotensin-converting enzyme 2 is subject to post-transcriptional regulation by miR-421. *Clin Sci (Lond)*. 2014;127:243-249. doi: 10.1042/CS20130420

42. Fernandes T, Hashimoto NY, Magalhaes FC, Fernandes FB, Casarini DE, Carmona AK, Krieger JE, Phillips MI, Oliveira EM. Aerobic exercise training-induced left ventricular hypertrophy involves regulatory MicroRNAs, decreased angiotensin-converting enzyme-angiotensin ii, and synergistic regulation of angiotensin-converting enzyme 2-angiotensin (1-7). *Hypertension*. 2011;58:182-189. doi: 10.1161/HYPERTENSIONAHA.110.168252

43. Shen H, Zhang J, Wang C, Jain PP, Xiong M, Shi X, Lei Y, Chen S, Yin Q, Thistlethwaite PA, et al. MDM2-Mediated Ubiquitination of Angiotensin-Converting Enzyme 2 Contributes to the Development of Pulmonary Arterial Hypertension. *Circulation*. 2020;142:1190-1204. doi: 10.1161/CIRCULATIONAHA.120.048191

44. Deshotels MR, Xia H, Sriramula S, Lazartigues E, Filipeanu CM. Angiotensin II mediates angiotensin converting enzyme type 2 internalization and degradation through an angiotensin II type I receptor-dependent mechanism. *Hypertension*. 2014;64:1368-1375. doi: 10.1161/HYPERTENSIONAHA.114.03743

45. Patel VB, Clarke N, Wang Z, Fan D, Parajuli N, Basu R, Putko B, Kassiri Z, Turner AJ, Oudit GY. Angiotensin II induced proteolytic cleavage of myocardial ACE2 is mediated by TACE/ADAM-17: a positive feedback mechanism in the RAS. *J Mol Cell Cardiol*. 2014;66:167-176. doi: 10.1016/j.yjmcc.2013.11.017

46. Gheblawi M, Wang K, Viveiros A, Nguyen Q, Zhong JC, Turner AJ, Raizada MK, Grant MB, Oudit GY. Angiotensin-Converting Enzyme 2: SARS-CoV-2 Receptor and Regulator of the Renin-Angiotensin System: Celebrating the 20th Anniversary of the Discovery of ACE2. *Circ Res*. 2020;126:1456-1474. doi: 10.1161/CIRCRESAHA.120.317015

47. Sungnak W, Huang N, Becavin C, Berg M, Queen R, Litvinukova M, Talavera-Lopez C, Maatz H, Reichart D, Sampaziotis F, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nat Med*. 2020;26:681-687. doi: 10.1038/s41591-020-0868-6

48. Reich HN, Oudit GY, Penninger JM, Scholey JW, Herzenberg AM. Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease. *Kidney Int*. 2008;74:1610-1616. doi: 10.1038/ki.2008.497

49. Chen L, Li X, Chen M, Feng Y, Xiong C. The ACE2 expression in human heart indicates new potential mechanism of heart injury among patients infected with SARS-CoV-2. *Cardiovasc Res*. 2020;116:1097-1100. doi: 10.1093/cvr/cvaa078

50. Mercure C, Yogi A, Callera GE, Aranha AB, Bader M, Ferreira AJ, Santos RA, Walther T, Touyz RM, Reudelhuber TL. Angiotensin(1-7) blunts hypertensive cardiac remodeling by a direct effect on the heart. *Circ Res*. 2008;103:1319-1326. doi: 10.1161/CIRCRESAHA.108.184911

51. Oudit GY, Penninger JM. Recombinant human angiotensin-converting enzyme 2 as a new renin-angiotensin system peptidase for heart failure therapy. *Curr Heart Fail Rep*. 2011;8:176-183. doi: 10.1007/s11897-011-0063-7

52. Alenina N, Xu P, Rentzsch B, Patkin EL, Bader M. Genetically altered animal models for Mas and angiotensin-(1-7). *Exp Physiol*. 2008;93:528-537. doi: 10.1113/expphysiol.2007.040345

53. Bader M. ACE2, angiotensin-(1–7), and Mas: the other side of the coin. *Pflügers Archiv - European Journal of Physiology*. 2012;465:79-85. doi: 10.1007/s00424-012-1120-0

54. Santos RAS, Oudit GY, Verano-Braga T, Canta G, Steckelings UM, Bader M. The renin-angiotensin system: going beyond the classical paradigms. *Am J Physiol Heart Circ Physiol*. 2019;316:H958-H970. doi: 10.1152/ajpheart.00723.2018

55. Chappell MC. Emerging evidence for a functional angiotensin-converting enzyme 2-angiotensin-(1-7)-MAS receptor axis: more than regulation of blood pressure? *Hypertension*. 2007;50:596-599. doi: 10.1161/HYPERTENSIONAHA.106.076216

56. Iyer SN, Ferrario CM, Chappell MC. Angiotensin-(1-7) contributes to the antihypertensive effects of blockade of the renin-angiotensin system. *Hypertension*. 1998;31:356-361. doi: 10.1161/01.hyp.31.1.356

57. Dell'Italia LJ, Collawn JF, Ferrario CM. Multifunctional Role of Chymase in Acute and Chronic Tissue Injury and Remodeling. *Circulation research*. 2018;122:319-336. doi: 10.1161/CIRCRESAHA.117.310978

58. Basu R, Poglitsch M, Yogasundaram H, Thomas J, Rowe BH, Oudit GY. Roles of Angiotensin Peptides and Recombinant Human ACE2 in Heart Failure. *J Am Coll Cardiol*. 2017;69:805-819. doi: 10.1016/j.jacc.2016.11.064

59. Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM. Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide

synthase activation via Akt-dependent pathways. *Hypertension*. 2007;49:185-192. doi: 10.1161/01.HYP.0000251865.35728.2f

60. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, et al. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A*. 2003;100:8258-8263. doi: 10.1073/pnas.1432869100

61. Tallant EA, Ferrario CM, Gallagher PE. Angiotensin-(1-7) inhibits growth of cardiac myocytes through activation of the mas receptor. *American journal of physiology Heart and circulatory physiology*. 2005;289:H1560-1566. doi: 10.1152/ajpheart.00941.2004

62. Zhang Y, Li B, Wang B, Zhang J, Wu J, Morgan T. Alteration of cardiac ACE2/Mas expression and cardiac remodelling in rats with aortic constriction. *Chin J Physiol*. 2014;57:335-342. doi: 10.4077/CJP.2014.BAD268

63. Flores-Munoz M, Godinho BM, Almalik A, Nicklin SA. Adenoviral delivery of angiotensin-(1-7) or angiotensin-(1-9) inhibits cardiomyocyte hypertrophy via the mas or angiotensin type 2 receptor. *PloS one*. 2012;7:e45564. doi: 10.1371/journal.pone.0045564

64. Flores-Munoz M, Work LM, Douglas K, Denby L, Dominiczak AF, Graham D, Nicklin SA. Angiotensin-(1-9) attenuates cardiac fibrosis in the stroke-prone spontaneously hypertensive rat via the angiotensin type 2 receptor. *Hypertension*. 2012;59:300-307. doi: 10.1161/HYPERTENSIONAHA.111.177485

65. Ocaranza MP, Lavandero S, Jalil JE, Moya J, Pinto M, Novoa U, Apablaza F, Gonzalez L, Hernandez C, Varas M, et al. Angiotensin-(1-9) regulates cardiac hypertrophy in vivo and in vitro. *J Hypertens*. 2010;28:1054-1064. doi: 10.1097/hjh.0b013e328335d291

66. Ocaranza MP, Rivera P, Novoa U, Pinto M, Gonzalez L, Chiong M, Lavandero S, Jalil JE. Rho kinase inhibition activates the homologous angiotensin-converting enzyme-angiotensin-(1-9) axis in experimental hypertension. *J Hypertens*. 2011;29:706-715. doi: 10.1097/HJH.0b013e3283440665

67. Oudit GY, Crackower MA, Backx PH, Penninger JM. The role of ACE2 in cardiovascular physiology. *Trends Cardiovasc Med*. 2003;13:93-101.

68. Lo J, Patel VB, Wang Z, Levasseur J, Kaufman S, Penninger JM, Oudit GY. Angiotensin-converting enzyme 2 antagonizes angiotensin II-induced pressor response and NADPH oxidase activation in Wistar-Kyoto rats and spontaneously hypertensive rats. *Exp Physiol*. 2013;98:109-122. doi: 10.1113/expphysiol.2012.067165

69. Oudit GY, Kassiri Z, Patel MP, Chappell M, Butany J, Backx PH, Tsushima RG, Scholey JW, Khokha R, Penninger JM. Angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in ACE2 null mice. *Cardiovascular research*. 2007;75:29-39. doi: 10.1016/j.cardiores.2007.04.007

70. Patel VB, Bodiga S, Fan D, Das SK, Wang Z, Wang W, Basu R, Zhong J, Kassiri Z, Oudit GY. Cardioprotective effects mediated by angiotensin II type 1 receptor blockade and enhancing angiotensin 1-7 in experimental heart failure in angiotensin-converting enzyme 2-null mice. *Hypertension*. 2012;59:1195-1203. doi: 10.1161/HYPERTENSIONAHA.112.191650

71. Danilczyk U, Penninger JM. Angiotensin-converting enzyme II in the heart and the kidney. *Circ Res*. 2006;98:463-471. doi: 10.1161/01.RES.0000205761.22353.5f

72. Patel VB, Takawale A, Ramprasath T, Das SK, Basu R, Grant MB, Hall DA, Kassiri Z, Oudit GY. Antagonism of angiotensin 1-7 prevents the therapeutic effects of recombinant human ACE2. *J Mol Med (Berl)*. 2015;93:1003-1013. doi: 10.1007/s00109-015-1285-z

73. Zhong JC, Zhang ZZ, Wang W, McKinnie SMK, Vederas JC, Oudit GY. Targeting the apelin pathway as a novel therapeutic approach for cardiovascular diseases. *Biochim Biophys Acta Mol Basis Dis*. 2017;1863:1942-1950. doi: 10.1016/j.bbadis.2016.11.007

74. Pitkin SL, Maguire JJ, Bonner TI, Davenport AP. International Union of Basic and Clinical Pharmacology. LXXIV. Apelin receptor nomenclature, distribution, pharmacology, and function. *Pharmacological reviews*. 2010;62:331-342. doi: 10.1124/pr.110.002949

75. Chong KS, Gardner RS, Morton JJ, Ashley EA, McDonagh TA. Plasma concentrations of the novel peptide apelin are decreased in patients with chronic heart failure. *Eur J Heart Fail*. 2006;8:355-360. doi: 10.1016/j.ejheart.2005.10.007

76. Chen MM, Ashley EA, Deng DX, Tsalenko A, Deng A, Tabibiazar R, Ben-Dor A, Fenster B, Yang E, King JY, et al. Novel role for the potent endogenous inotrope apelin in human cardiac dysfunction. *Circulation*. 2003;108:1432-1439. doi: 10.1161/01.CIR.0000091235.94914.75

77. Przewlocka-Kosmala M, Kotwica T, Mysiak A, Kosmala W. Reduced circulating apelin in essential hypertension and its association with cardiac dysfunction. *J Hypertens*. 2011;29:971-979. doi: 10.1097/HJH.0b013e328344da76

78. Kuba K, Zhang L, Imai Y, Arab S, Chen M, Maekawa Y, Leschnik M, Leibbrandt A, Markovic M, Schwaighofer J, et al. Impaired heart contractility in Apelin gene-deficient

mice associated with aging and pressure overload. *Circ Res*. 2007;101:e32-42. doi: 10.1161/CIRCRESAHA.107.158659

79. Wang W, McKinnie SM, Patel VB, Haddad G, Wang Z, Zhabyeyev P, Das SK, Basu R, McLean B, Kandalam V, et al. Loss of Apelin exacerbates myocardial infarction adverse remodeling and ischemia-reperfusion injury: therapeutic potential of synthetic Apelin analogues. *J Am Heart Assoc*. 2013;2:e000249. doi: 10.1161/JAHA.113.000249

80. Siddiquee K, Hampton J, McAnally D, May L, Smith L. The apelin receptor inhibits the angiotensin II type 1 receptor via allosteric trans-inhibition. *Br J Pharmacol.* 2013;168:1104-1117. doi: 10.1111/j.1476-5381.2012.02192.x

81. Zhang ZZ, Wang W, Jin HY, Chen X, Cheng YW, Xu YL, Song B, Penninger JM, Oudit GY, Zhong JC. Apelin Is a Negative Regulator of Angiotensin II-Mediated Adverse Myocardial Remodeling and Dysfunction. *Hypertension*. 2017;70:1165-1175. doi: 10.1161/HYPERTENSIONAHA.117.10156

82. Wang W, Shen M, Fischer C, Basu R, Hazra S, Couvineau P, Paul M, Wang F, Toth S, Mix DS, et al. Apelin protects against abdominal aortic aneurysm and the therapeutic role of neutral endopeptidase resistant apelin analogs. *Proc Natl Acad Sci U S A*. 2019;116:13006-13015. doi: 10.1073/pnas.1900152116

83. Wang W, McKinnie SM, Farhan M, Paul M, McDonald T, McLean B, Llorens-Cortes C, Hazra S, Murray AG, Vederas JC, et al. Angiotensin-Converting Enzyme 2 Metabolizes and Partially Inactivates Pyr-Apelin-13 and Apelin-17: Physiological Effects in the Cardiovascular System. *Hypertension*. 2016;68:365-377. doi: 10.1161/HYPERTENSIONAHA.115.06892

84. Camargo SM, Singer D, Makrides V, Huggel K, Pos KM, Wagner CA, Kuba K, Danilczyk U, Skovby F, Kleta R, et al. Tissue-specific amino acid transporter partners ACE2 and collectrin differentially interact with hartnup mutations. *Gastroenterology*. 2009;136:872-882. doi: 10.1053/j.gastro.2008.10.055

85. Hashimoto T, Perlot T, Rehman A, Trichereau J, Ishiguro H, Paolino M, Sigl V, Hanada T, Hanada R, Lipinski S, et al. ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation. *Nature*. 2012;487:477-481. doi: 10.1038/nature11228

86. Monteil V, Kwon H, Prado P, Hagelkruys A, Wimmer RA, Stahl M, Leopoldi A, Garreta E, Hurtado Del Pozo C, Prosper F, et al. Inhibition of SARS-CoV-2 Infections in Engineered Human Tissues Using Clinical-Grade Soluble Human ACE2. *Cell*. 2020;181:905-913 e907. doi: 10.1016/j.cell.2020.04.004

87. Hu B, Zeng LP, Yang XL, Ge XY, Zhang W, Li B, Xie JZ, Shen XR, Zhang YZ, Wang N, et al. Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. *PLoS Pathog*. 2017;13:e1006698. doi: 10.1371/journal.ppat.1006698

88. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, Luo SW, Li PH, Zhang LJ, Guan YJ, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science*. 2003;302:276-278. doi: 10.1126/science.1087139

89. Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, Mazet JK, Hu B, Zhang W, Peng C, et al. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature*. 2013;503:535-538. doi: 10.1038/nature12711

90. Li W, Zhang C, Sui J, Kuhn JH, Moore MJ, Luo S, Wong SK, Huang IC, Xu K, Vasilieva N, et al. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. *EMBO J*. 2005;24:1634-1643. doi: 10.1038/sj.emboj.7600640

91. Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol*. 2019;17:181-192. doi: 10.1038/s41579-018-0118-9

92. Liu Y, Hu G, Wang Y, Ren W, Zhao X, Ji F, Zhu Y, Feng F, Gong M, Ju X, et al. Functional and genetic analysis of viral receptor ACE2 orthologs reveals a broad potential host range of SARS-CoV-2. *Proc Natl Acad Sci U S A*. 2021;118. doi: 10.1073/pnas.2025373118

93. Damas J, Hughes GM, Keough KC, Painter CA, Persky NS, Corbo M, Hiller M, Koepfli KP, Pfenning AR, Zhao H, et al. Broad host range of SARS-CoV-2 predicted by comparative and structural analysis of ACE2 in vertebrates. *Proc Natl Acad Sci U S A*. 2020;117:22311-22322. doi: 10.1073/pnas.2010146117

94. Sit THC, Brackman CJ, Ip SM, Tam KWS, Law PYT, To EMW, Yu VYT, Sims LD, Tsang DNC, Chu DKW, et al. Infection of dogs with SARS-CoV-2. *Nature*. 2020;586:776-778. doi: 10.1038/s41586-020-2334-5

95. Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, Liu R, He X, Shuai L, Sun Z, et al. Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science*. 2020;368:1016-1020. doi: 10.1126/science.abb7015

96. Hale VL, Dennis PM, McBride DS, Nolting JM, Madden C, Huey D, Ehrlich M, Grieser J, Winston J, Lombardi D, et al. SARS-CoV-2 infection in free-ranging white-tailed deer. *Nature*. 2021. doi: 10.1038/s41586-021-04353-x

97. Starr TN, Zepeda SK, Walls AC, Greaney AJ, Alkhovsky S, Veesler D, Bloom JD. ACE2 binding is an ancestral and evolvable trait of sarbecoviruses. *Nature*. 2022;603:913-918. doi: 10.1038/s41586-022-04464-z

98. Guo H, Hu BJ, Yang XL, Zeng LP, Li B, Ouyang S, Shi ZL. Evolutionary Arms Race between Virus and Host Drives Genetic Diversity in Bat Severe Acute Respiratory Syndrome-Related Coronavirus Spike Genes. *J Virol*. 2020;94. doi: 10.1128/JVI.00902-20

99. Suryamohan K, Diwanji D, Stawiski EW, Gupta R, Miersch S, Liu J, Chen C, Jiang YP, Fellouse FA, Sathirapongsasuti JF, et al. Human ACE2 receptor polymorphisms and altered susceptibility to SARS-CoV-2. *Commun Biol*. 2021;4:475. doi: 10.1038/s42003-021-02030-3

100. Chan KK, Dorosky D, Sharma P, Abbasi SA, Dye JM, Kranz DM, Herbert AS, Procko E. Engineering human ACE2 to optimize binding to the spike protein of SARS coronavirus 2. *Science*. 2020;369:1261-1265. doi: 10.1126/science.abc0870

101. Castiglione GM, Zhou L, Xu Z, Neiman Z, Hung CF, Duh EJ. Evolutionary pathways to SARS-CoV-2 resistance are opened and closed by epistasis acting on ACE2. *PLoS Biol*. 2021;19:e3001510. doi: 10.1371/journal.pbio.3001510

102. Sabater Molina M, Nicolas Rocamora E, Bendicho AI, Vazquez EG, Zorio E, Rodriguez FD, Gil Ortuno C, Rodriguez AI, Sanchez-Lopez AJ, Jara Rubio R, et al. Polymorphisms in ACE, ACE2, AGTR1 genes and severity of COVID-19 disease. *PLoS One*. 2022;17:e0263140. doi: 10.1371/journal.pone.0263140

103. Mohlendick B, Schonfelder K, Breuckmann K, Elsner C, Babel N, Balfanz P, Dahl E, Dreher M, Fistera D, Herbstreit F, et al. ACE2 polymorphism and susceptibility for

SARS-CoV-2 infection and severity of COVID-19. *Pharmacogenet Genomics*. 2021;31:165-171. doi: 10.1097/FPC.0000000000000436

104. Hou YJ, Okuda K, Edwards CE, Martinez DR, Asakura T, Dinnon KH, 3rd, Kato T, Lee RE, Yount BL, Mascenik TM, et al. SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract. *Cell*. 2020;182:429-446 e414. doi: 10.1016/j.cell.2020.05.042

105. Wolfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Muller MA, Niemeyer D, Jones TC, Vollmar P, Rothe C, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature*. 2020;581:465-469. doi: 10.1038/s41586-020-2196-x

106. Leung GM, Hedley AJ, Ho LM, Chau P, Wong IO, Thach TQ, Ghani AC, Donnelly CA, Fraser C, Riley S, et al. The epidemiology of severe acute respiratory syndrome in the 2003 Hong Kong epidemic: an analysis of all 1755 patients. *Ann Intern Med*. 2004;141:662-673. doi: 10.7326/0003-4819-141-9-200411020-00006

107. Millet JK, Whittaker GR. Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis. *Virus Res*. 2015;202:120-134. doi: 10.1016/j.virusres.2014.11.021

108. Meng B, Abdullahi A, Ferreira I, Goonawardane N, Saito A, Kimura I, Yamasoba D, Gerber PP, Fatihi S, Rathore S, et al. Altered TMPRSS2 usage by SARS-CoV-2 Omicron impacts infectivity and fusogenicity. *Nature*. 2022;603:706-714. doi: 10.1038/s41586-022-04474-x

109. Muus C, Luecken MD, Eraslan G, Sikkema L, Waghray A, Heimberg G, Kobayashi Y, Vaishnav ED, Subramanian A, Smillie C, et al. Single-cell meta-analysis of SARS-CoV-

2 entry genes across tissues and demographics. *Nat Med*. 2021;27:546-559. doi: 10.1038/s41591-020-01227-z

110. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, Yang P, Sarao R, Wada T, Leong-Poi H, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature*. 2005;436:112-116. doi: 10.1038/nature03712

111. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, Huan Y, Yang P, Zhang Y, Deng W, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med*. 2005;11:875-879. doi: 10.1038/nm1267

112. Lei Y, Zhang J, Schiavon CR, He M, Chen L, Shen H, Zhang Y, Yin Q, Cho Y, Andrade L, et al. SARS-CoV-2 Spike Protein Impairs Endothelial Function via Downregulation of ACE 2. *Circ Res*. 2021;128:1323-1326. doi: 10.1161/CIRCRESAHA.121.318902

113. Glowacka I, Bertram S, Herzog P, Pfefferle S, Steffen I, Muench MO, Simmons G, Hofmann H, Kuri T, Weber F, et al. Differential downregulation of ACE2 by the spike proteins of severe acute respiratory syndrome coronavirus and human coronavirus NL63. *J Virol*. 2010;84:1198-1205. doi: 10.1128/JVI.01248-09

114. Zou Z, Yan Y, Shu Y, Gao R, Sun Y, Li X, Ju X, Liang Z, Liu Q, Zhao Y, et al. Angiotensin-converting enzyme 2 protects from lethal avian influenza A H5N1 infections. *Nat Commun*. 2014;5:3594. doi: 10.1038/ncomms4594

115. Yang P, Gu H, Zhao Z, Wang W, Cao B, Lai C, Yang X, Zhang L, Duan Y, Zhang S, et al. Angiotensin-converting enzyme 2 (ACE2) mediates influenza H7N9 virus-induced acute lung injury. *Sci Rep*. 2014;4:7027. doi: 10.1038/srep07027

116. Haga S, Yamamoto N, Nakai-Murakami C, Osawa Y, Tokunaga K, Sata T, Yamamoto N, Sasazuki T, Ishizaka Y. Modulation of TNF-alpha-converting enzyme by the spike

protein of SARS-CoV and ACE2 induces TNF-alpha production and facilitates viral entry. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105:7809-7814. doi: 10.1073/pnas.0711241105

117. Lambert DW, Yarski M, Warner FJ, Thornhill P, Parkin ET, Smith AI, Hooper NM, Turner AJ. Tumor necrosis factor-alpha convertase (ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2). *J Biol Chem*. 2005;280:30113-30119. doi: 10.1074/jbc.M505111200

118. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme: cloning and functional expression as a captopril-insensitive carboxypeptidase. *Journal of Biological Chemistry*. 2000;275:33238-33243.

119. Yeung ML, Teng JLL, Jia L, Zhang C, Huang C, Cai JP, Zhou R, Chan KH, Zhao H, Zhu L, et al. Soluble ACE2-mediated cell entry of SARS-CoV-2 via interaction with proteins related to the renin-angiotensin system. *Cell*. 2021;184:2212-2228 e2212. doi: 10.1016/j.cell.2021.02.053

120. Adrain C, Zettl M, Christova Y, Taylor N, Freeman M. Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. *Science*. 2012;335:225-228. doi: 10.1126/science.1214400

121. Xu P, Derynck R. Direct activation of TACE-mediated ectodomain shedding by p38 MAP kinase regulates EGF receptor-dependent cell proliferation. *Mol Cell*. 2010;37:551-566. doi: 10.1016/j.molcel.2010.01.034

122. Reindl-Schwaighofer R, Hodlmoser S, Eskandary F, Poglitsch M, Bonderman D, Strassl R, Aberle JH, Oberbauer R, Zoufaly A, Hecking M. ACE2 Elevation in Severe COVID-

19. *Am J Respir Crit Care Med*. 2021;203:1191-1196. doi: 10.1164/rccm.202101-0142LE

123. Wang K, Gheblawi M, Nikhanj A, Munan M, MacIntyre E, O'Neil C, Poglitsch M, Colombo D, Del Nonno F, Kassiri Z, et al. Dysregulation of ACE (Angiotensin-Converting Enzyme)-2 and Renin-Angiotensin Peptides in SARS-CoV-2 Mediated Mortality and End-Organ Injuries. *Hypertension*. 2022;79:365-378. doi: 10.1161/HYPERTENSIONAHA.121.18295

124. Patel VB, Zhong JC, Fan D, Basu R, Morton JS, Parajuli N, McMurtry MS, Davidge ST, Kassiri Z, Oudit GY. Angiotensin-converting enzyme 2 is a critical determinant of angiotensin II-induced loss of vascular smooth muscle cells and adverse vascular remodeling. *Hypertension*. 2014;64:157-164. doi: 10.1161/HYPERTENSIONAHA.114.03388

125. Patel VB, Mori J, McLean BA, Basu R, Das SK, Ramprasath T, Parajuli N, Penninger JM, Grant MB, Lopaschuk GD, et al. ACE2 Deficiency Worsens Epicardial Adipose Tissue Inflammation and Cardiac Dysfunction in Response to Diet-Induced Obesity. *Diabetes*. 2016;65:85-95. doi: 10.2337/db15-0399

126. Santos RA, Castro CH, Gava E, Pinheiro SV, Almeida AP, Paula RD, Cruz JS, Ramos AS, Rosa KT, Irigoyen MC, et al. Impairment of in vitro and in vivo heart function in angiotensin-(1-7) receptor MAS knockout mice. *Hypertension*. 2006;47:996-1002. doi: 10.1161/01.HYP.0000215289.51180.5c

127. Sampaio WO, Henrique de Castro C, Santos RA, Schiffrin EL, Touyz RM. Angiotensin-(1-7) counterregulates angiotensin II signaling in human endothelial cells. *Hypertension*. 2007;50:1093-1098. doi: 10.1161/HYPERTENSIONAHA.106.084848

128. Iwata M, Cowling RT, Gurantz D, Moore C, Zhang S, Yuan JX, Greenberg BH. Angiotensin-(1-7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects. *American journal of physiology Heart and circulatory physiology*. 2005;289:H2356-2363. doi: 10.1152/ajpheart.00317.2005

129. Patel SK, Velkoska E, Freeman M, Wai B, Lancefield TF, Burrell LM. From gene to protein-experimental and clinical studies of ACE2 in blood pressure control and arterial hypertension. *Front Physiol*. 2014;5:227. doi: 10.3389/fphys.2014.00227

130. Gheorghiade M, Sopko G, De Luca L, Velazquez EJ, Parker JD, Binkley PF, Sadowski Z, Golba KS, Prior DL, Rouleau JL, et al. Navigating the crossroads of coronary artery disease and heart failure. *Circulation*. 2006;114:1202-1213. doi: 10.1161/CIRCULATIONAHA.106.623199

131. Burrell LM, Risvanis J, Kubota E, Dean RG, MacDonald PS, Lu S, Tikellis C, Grant SL, Lew RA, Smith AI, et al. Myocardial infarction increases ACE2 expression in rat and humans. *Eur Heart J*. 2005;26:369-375; discussion 322-364. doi: 10.1093/eurheartj/ehi114

132. Qi YF, Zhang J, Wang L, Shenoy V, Krause E, Oh SP, Pepine CJ, Katovich MJ, Raizada MK. Angiotensin-converting enzyme 2 inhibits high-mobility group box 1 and attenuates cardiac dysfunction post-myocardial ischemia. *Journal of molecular medicine*. 2016;94:37-49. doi: 10.1007/s00109-015-1356-1

133. Wang Y, Qian C, Roks AJ, Westermann D, Schumacher SM, Escher F, Schoemaker RG, Reudelhuber TL, van Gilst WH, Schultheiss HP, et al. Circulating rather than cardiac angiotensin-(1-7) stimulates cardioprotection after myocardial infarction. *Circulation Heart failure*. 2010;3:286-293. doi: 10.1161/CIRCHEARTFAILURE.109.905968

134. Wang W, Patel VB, Parajuli N, Fan D, Basu R, Wang Z, Ramprasath T, Kassiri Z, Penninger JM, Oudit GY. Heterozygote loss of ACE2 is sufficient to increase the susceptibility to heart disease. *J Mol Med (Berl)*. 2014;92:847-858. doi: 10.1007/s00109-014-1149-y

135. Patel VB, Shah S, Verma S, Oudit GY. Epicardial adipose tissue as a metabolic transducer: role in heart failure and coronary artery disease. *Heart failure reviews*. 2017;22:889-902. doi: 10.1007/s10741-017-9644-1

136. Packer M. Epicardial Adipose Tissue May Mediate Deleterious Effects of Obesity and Inflammation on the Myocardium. *Journal of the American College of Cardiology*. 2018;71:2360-2372. doi: 10.1016/j.jacc.2018.03.509

137. Patel VB, Basu R, Oudit GY. ACE2/Ang 1-7 axis: A critical regulator of epicardial adipose tissue inflammation and cardiac dysfunction in obesity. *Adipocyte*. 2016;5:306-311. doi: 10.1080/21623945.2015.1131881

138. Mori J, Patel VB, Abo Alrob O, Basu R, Altamimi T, Desaulniers J, Wagg CS, Kassiri Z, Lopaschuk GD, Oudit GY. Angiotensin 1-7 ameliorates diabetic cardiomyopathy and diastolic dysfunction in db/db mice by reducing lipotoxicity and inflammation. *Circulation Heart failure*. 2014;7:327-339. doi: 10.1161/CIRCHEARTFAILURE.113.000672

139. Mori J, Patel VB, Ramprasath T, Alrob OA, DesAulniers J, Scholey JW, Lopaschuk GD, Oudit GY. Angiotensin 1-7 mediates renoprotection against diabetic nephropathy by reducing oxidative stress, inflammation, and lipotoxicity. *American journal of physiology Renal physiology*. 2014;306:F812-821. doi: 10.1152/ajprenal.00655.2013

140. Morimoto H, Mori J, Nakajima H, Kawabe Y, Tsuma Y, Fukuhara S, Kodo K, Ikoma K, Matoba S, Oudit GY, et al. Angiotensin 1-7 stimulates brown adipose tissue and reduces diet-induced obesity. *American journal of physiology Endocrinology and metabolism*. 2018;314:E131-E138. doi: 10.1152/ajpendo.00192.2017

141. Kawabe Y, Mori J, Morimoto H, Yamaguchi M, Miyagaki S, Ota T, Tsuma Y, Fukuhara S, Nakajima H, Oudit GY, et al. ACE2 exerts anti-obesity effect via stimulating brown adipose tissue and induction of browning in white adipose tissue. *Am J Physiol Endocrinol Metab*. 2019;317:E1140-E1149. doi: 10.1152/ajpendo.00311.2019

142. Diez-Freire C, Vazquez J, Correa de Adjounian MF, Ferrari MF, Yuan L, Silver X, Torres R, Raizada MK. ACE2 gene transfer attenuates hypertension-linked pathophysiological changes in the SHR. *Physiological genomics*. 2006;27:12-19. doi: 10.1152/physiolgenomics.00312.2005

143. Rentzsch B, Todiras M, Iliescu R, Popova E, Campos LA, Oliveira ML, Baltatu OC, Santos RA, Bader M. Transgenic angiotensin-converting enzyme 2 overexpression in vessels of SHRSP rats reduces blood pressure and improves endothelial function. *Hypertension*. 2008;52:967-973. doi: 10.1161/HYPERTENSIONAHA.108.114322

144. Wysocki J, Ye M, Rodriguez E, Gonzalez-Pacheco FR, Barrios C, Evora K, Schuster M, Loibner H, Brosnihan KB, Ferrario CM, et al. Targeting the degradation of angiotensin II with recombinant angiotensin-converting enzyme 2: prevention of angiotensin II-dependent hypertension. *Hypertension*. 2010;55:90-98. doi: 10.1161/HYPERTENSIONAHA.109.138420

145. Mukerjee S, Gao H, Xu J, Sato R, Zsombok A, Lazartigues E. ACE2 and ADAM17 Interaction Regulates the Activity of Presympathetic Neurons. *Hypertension*. 2019;74:1181-1191. doi: 10.1161/HYPERTENSIONAHA.119.13133

146. Xia H, Sriramula S, Chhabra KH, Lazartigues E. Brain angiotensin-converting enzyme type 2 shedding contributes to the development of neurogenic hypertension. *Circulation research*. 2013;113:1087-1096. doi: 10.1161/CIRCRESAHA.113.301811

147. Thomas MC, Pickering RJ, Tsorotes D, Koitka A, Sheehy K, Bernardi S, Toffoli B, Nguyen-Huu TP, Head GA, Fu Y, et al. Genetic Ace2 deficiency accentuates vascular inflammation and atherosclerosis in the ApoE knockout mouse. *Circ Res*. 2010;107:888-897. doi: 10.1161/CIRCRESAHA.110.219279

148. Chen Y, Wang X, Yang C, Su X, Yang W, Dai Y, Han H, Jiang J, Lu L, Wang H, et al. Decreased circulating catestatin levels are associated with coronary artery disease: The emerging anti-inflammatory role. *Atherosclerosis*. 2019;281:78-88. doi: 10.1016/j.atherosclerosis.2018.12.025

149. Oudit GY, Liu GC, Zhong J, Basu R, Chow FL, Zhou J, Loibner H, Janzek E, Schuster M, Penninger JM, et al. Human recombinant ACE2 reduces the progression of diabetic nephropathy. *Diabetes*. 2010;59:529-538. doi: 10.2337/db09-1218

150. Patel VB, Bodiga S, Basu R, Das SK, Wang W, Wang Z, Lo J, Grant MB, Zhong J, Kassiri Z, et al. Loss of angiotensin-converting enzyme-2 exacerbates diabetic cardiovascular complications and leads to systolic and vascular dysfunction: a critical role of the angiotensin II/AT1 receptor axis. *Circ Res*. 2012;110:1322-1335. doi: 10.1161/CIRCRESAHA.112.268029

151. Tikellis C, Pickering R, Tsorotes D, Du XJ, Kiriazis H, Nguyen-Huu TP, Head GA, Cooper ME, Thomas MC. Interaction of diabetes and ACE2 in the pathogenesis of cardiovascular disease in experimental diabetes. *Clinical science*. 2012;123:519-529. doi: 10.1042/CS20110668

152. Patel VB, Parajuli N, Oudit GY. Role of angiotensin-converting enzyme 2 (ACE2) in diabetic cardiovascular complications. *Clinical science*. 2014;126:471-482. doi: 10.1042/CS20130344

153. Cooper ME, Johnston CI. Optimizing treatment of hypertension in patients with diabetes. *Jama*. 2000;283:3177-3179. doi: 10.1001/jama.283.24.3177

154. Wong DW, Oudit GY, Reich H, Kassiri Z, Zhou J, Liu QC, Backx PH, Penninger JM, Herzenberg AM, Scholey JW. Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. *The American journal of pathology*. 2007;171:438-451. doi: 10.2353/ajpath.2007.060977

155. Bae EH, Fang F, Williams VR, Konvalinka A, Zhou X, Patel VB, Song X, John R, Oudit GY, Pei Y, et al. Murine recombinant angiotensin-converting enzyme 2 attenuates kidney injury in experimental Alport syndrome. *Kidney international*. 2017;91:1347-1361. doi: 10.1016/j.kint.2016.12.022

156. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shahinfar S, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *The New England journal of medicine*. 2001;345:861-869. doi: 10.1056/NEJMoa011161

157. Tikellis C, Johnston CI, Forbes JM, Burns WC, Burrell LM, Risvanis J, Cooper ME. Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. *Hypertension*. 2003;41:392-397. doi: 10.1161/01.HYP.0000060689.38912.CB

158. Verma A, Shan Z, Lei B, Yuan L, Liu X, Nakagawa T, Grant MB, Lewin AS, Hauswirth WW, Raizada MK, et al. ACE2 and Ang-(1-7) confer protection against development of diabetic retinopathy. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2012;20:28-36. doi: 10.1038/mt.2011.155

159. Dominguez JM, 2nd, Hu P, Caballero S, Moldovan L, Verma A, Oudit GY, Li Q, Grant MB. Adeno-Associated Virus Overexpression of Angiotensin-Converting Enzyme-2 Reverses Diabetic Retinopathy in Type 1 Diabetes in Mice. *The American journal of pathology*. 2016;186:1688-1700. doi: 10.1016/j.ajpath.2016.01.023

160. Busik JV, Tikhonenko M, Bhatwadekar A, Opreanu M, Yakubova N, Caballero S, Player D, Nakagawa T, Afzal A, Kielczewski J, et al. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. *J Exp Med*. 2009;206:2897-2906. doi: 10.1084/jem.20090889

161. Hazra S, Jarajapu YP, Stepps V, Caballero S, Thinschmidt JS, Sautina L, Bengtsson N, Licalzi S, Dominguez J, Kern TS, et al. Long-term type 1 diabetes influences haematopoietic stem cells by reducing vascular repair potential and increasing inflammatory monocyte generation in a murine model. *Diabetologia*. 2013;56:644-653. doi: 10.1007/s00125-012-2781-0

162. Hinojosa AE, Garcia-Bueno B, Leza JC, Madrigal JL. CCL2/MCP-1 modulation of microglial activation and proliferation. *J Neuroinflammation*.8:77. doi: 1742-2094-8-77 [pii]

10.1186/1742-2094-8-77

163. Skuljec J, Sun H, Pul R, Benardais K, Ragancokova D, Moharregh-Khiabani D, Kotsiari A, Trebst C, Stangel M. CCL5 induces a pro-inflammatory profile in microglia in vitro. *Cell Immunol*.270:164-171. doi: S0008-8749(11)00111-0 [pii]

10.1016/j.cellimm.2011.05.001

164. Semple BD, Kossmann T, Morganti-Kossmann MC. Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks. *J Cereb Blood Flow Metab*.30:459-473. doi: jcbfm2009240 [pii]

10.1038/jcbfm.2009.240

165. Tsou CL, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, Mack M, Charo IF. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest*. 2007;117:902-909. doi: 10.1172/JCI29919

166. Huang D, Wujek J, Kidd G, He TT, Cardona A, Sasse ME, Stein EJ, Kish J, Tani M, Charo IF, et al. Chronic expression of monocyte chemoattractant protein-1 in the central nervous system causes delayed encephalopathy and impaired microglial function in mice. *FASEB J*. 2005;19:761-772. doi: 19/7/761 [pii]

10.1096/fj.04-3104com

167. Schilling M, Strecker JK, Ringelstein EB, Schabitz WR, Kiefer R. The role of CC chemokine receptor 2 on microglia activation and blood-borne cell recruitment after transient focal cerebral ischemia in mice. *Brain Res*. 2009;1289:79-84. doi: S0006-8993(09)01270-0 [pii]

10.1016/j.brainres.2009.06.054

168. Oudit GY, Kassiri Z, Jiang C, Liu PP, Poutanen SM, Penninger JM, Butany J. SARS-coronavirus modulation of myocardial ACE2 expression and inflammation in patients with SARS. *Eur J Clin Invest*. 2009;39:618-625. doi: 10.1111/j.1365-2362.2009.02153.x

169. Jarajapu YP, Bhatwadekar AD, Caballero S, Hazra S, Shenoy V, Medina R, Kent D, Stitt AW, Thut C, Finney EM, et al. Activation of the ACE2/angiotensin-(1-7)/Mas receptor axis enhances the reparative function of dysfunctional diabetic endothelial progenitors. *Diabetes*. 2013;62:1258-1269. doi: 10.2337/db12-0808

170. Xie X, Chen J, Wang X, Zhang F, Liu Y. Age- and gender-related difference of ACE2 expression in rat lung. *Life sciences*. 2006;78:2166-2171. doi: 10.1016/j.lfs.2005.09.038

171. Rey-Parra GJ, Vadivel A, Coltan L, Hall A, Eaton F, Schuster M, Loibner H, Penninger JM, Kassiri Z, Oudit GY, et al. Angiotensin converting enzyme 2 abrogates bleomycin-induced lung injury. *Journal of molecular medicine*. 2012;90:637-647. doi: 10.1007/s00109-012-0859-2

172. Yilin Z, Yandong N, Faguang J. Role of angiotensin-converting enzyme (ACE) and ACE2 in a rat model of smoke inhalation induced acute respiratory distress syndrome. *Burns*. 2015;41:1468-1477. doi: 10.1016/j.burns.2015.04.010

173. Treml B, Neu N, Kleinsasser A, Gritsch C, Finsterwalder T, Geiger R, Schuster M, Janzek E, Loibner H, Penninger J, et al. Recombinant angiotensin-converting enzyme 2 improves pulmonary blood flow and oxygenation in lipopolysaccharide-induced lung injury in piglets. *Crit Care Med*. 2010;38:596-601. doi: 10.1097/CCM.0b013e3181c03009

174. Rigatto K, Casali KR, Shenoy V, Katovich MJ, Raizada MK. Diminazene aceturate improves autonomic modulation in pulmonary hypertension. *European journal of pharmacology*. 2013;713:89-93. doi: 10.1016/j.ejphar.2013.04.017

175. Shenoy V, Kwon KC, Rathinasabapathy A, Lin S, Jin G, Song C, Shil P, Nair A, Qi Y, Li Q, et al. Oral delivery of Angiotensin-converting enzyme 2 and Angiotensin-(1-7) bioencapsulated in plant cells attenuates pulmonary hypertension. *Hypertension*. 2014;64:1248-1259. doi: 10.1161/HYPERTENSIONAHA.114.03871

176. Hemnes AR, Rathinasabapathy A, Austin EA, Brittain EL, Carrier EJ, Chen X, Fessel JP, Fike CD, Fong P, Fortune N, et al. A potential therapeutic role for angiotensin-converting enzyme 2 in human pulmonary arterial hypertension. *Eur Respir J*. 2018;51. doi: 10.1183/13993003.02638-2017

177. Sama IE, Ravera A, Santema BT, van Goor H, Ter Maaten JM, Cleland JGF, Rienstra M, Friedrich AW, Samani NJ, Ng LL, et al. Circulating plasma concentrations of angiotensin-converting enzyme 2 in men and women with heart failure and effects of renin-angiotensin-aldosterone inhibitors. *Eur Heart J*. 2020;41:1810-1817. doi: 10.1093/eurheartj/ehaa373

178. Narula S, Yusuf S, Chong M, Ramasundarahettige C, Rangarajan S, Bangdiwala SI, van Eikels M, Leineweber K, Wu A, Pigeyre M, et al. Plasma ACE2 and risk of death or cardiometabolic diseases: a case-cohort analysis. *Lancet*. 2020;396:968-976. doi: 10.1016/S0140-6736(20)31964-4

179. Chirinos JA, Cohen JB, Zhao L, Hanff T, Sweitzer N, Fang J, Corrales-Medina V, Anmar R, Morley M, Zamani P, et al. Clinical and Proteomic Correlates of Plasma ACE2

(Angiotensin-Converting Enzyme 2) in Human Heart Failure. *Hypertension*. 2020;76:1526-1536. doi: 10.1161/HYPERTENSIONAHA.120.15829

180. Ramchand J, Patel SK, Srivastava PM, Farouque O, Burrell LM. Elevated plasma angiotensin converting enzyme 2 activity is an independent predictor of major adverse cardiac events in patients with obstructive coronary artery disease. *PLoS One*. 2018;13:e0198144. doi: 10.1371/journal.pone.0198144

181. Ramchand J, Patel SK, Kearney LG, Matalanis G, Farouque O, Srivastava PM, Burrell LM. Plasma ACE2 Activity Predicts Mortality in Aortic Stenosis and Is Associated With Severe Myocardial Fibrosis. *JACC Cardiovasc Imaging*. 2020;13:655-664. doi: 10.1016/j.jcmg.2019.09.005

182. Wallentin L, Lindback J, Eriksson N, Hijazi Z, Eikelboom JW, Ezekowitz MD, Granger CB, Lopes RD, Yusuf S, Oldgren J, et al. Angiotensin-converting enzyme 2 (ACE2) levels in relation to risk factors for COVID-19 in two large cohorts of patients with atrial fibrillation. *Eur Heart J*. 2020;41:4037-4046. doi: 10.1093/eurheartj/ehaa697

183. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation*. 1991;83:1849-1865. doi: 10.1161/01.cir.83.6.1849

184. Dzau VJ, Colucci WS, Hollenberg NK, Williams GH. Relation of the renin-angiotensin-aldosterone system to clinical state in congestive heart failure. *Circulation*. 1981;63:645-651. doi: 10.1161/01.cir.63.3.645

185. Swedberg K, Eneroth P, Kjekshus J, Wilhelmsen L. Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality.

CONSENSUS Trial Study Group. *Circulation*. 1990;82:1730-1736. doi: 10.1161/01.cir.82.5.1730

186. Burnett H, Earley A, Voors AA, Senni M, McMurray JJ, Deschaseaux C, Cope S. Thirty Years of Evidence on the Efficacy of Drug Treatments for Chronic Heart Failure With Reduced Ejection Fraction: A Network Meta-Analysis. *Circ Heart Fail*. 2017;10. doi: 10.1161/CIRCHEARTFAILURE.116.003529

187. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Colvin MM, Drazner MH, Filippatos GS, Fonarow GC, Givertz MM, et al. 2017 ACC/AHA/HFSA Focused Update of the 2013 ACCF/AHA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines and the Heart Failure Society of America. *J Am Coll Cardiol*. 2017;70:776-803. doi: 10.1016/j.jacc.2017.04.025

188. Rossignol P, Hernandez AF, Solomon SD, Zannad F. Heart failure drug treatment. *Lancet*. 2019;393:1034-1044. doi: 10.1016/S0140-6736(18)31808-7

189. Basu R, Poglitsch M, Yogasundaram H, Thomas J, Rowe BH, Oudit GY. Roles of Angiotensin Peptides and Recombinant Human ACE2 in Heart Failure. *J Am Coll Cardiol* 2017;69 805-819.

190. Haschke M, Schuster M, Poglitsch M, Loibner H, Salzberg M, Bruggisser M, Penninger J, Krahenbuhl S. Pharmacokinetics and pharmacodynamics of recombinant human angiotensin-converting enzyme 2 in healthy human subjects. *Clin Pharmacokinet*. 2013;52:783-792. doi: 10.1007/s40262-013-0072-7

191. Khan A, Benthin C, Zeno B, Albertson TE, Boyd J, Christie JD, Hall R, Poirier G, Ronco JJ, Tidswell M, et al. A pilot clinical trial of recombinant human angiotensin-converting

enzyme 2 in acute respiratory distress syndrome. *Crit Care*. 2017;21:234. doi: 10.1186/s13054-017-1823-x

192. H A. A new look at the statistical model identification. *IEEE Transactions on Automic Control* 1974;19:716-723.

193. Gupte M, Thatcher SE, Boustany-Kari CM, Shoemaker R, Yiannikouris F, Zhang X, Karounos M, Cassis LA. Angiotensin converting enzyme 2 contributes to sex differences in the development of obesity hypertension in C57BL/6 mice. *Arterioscler Thromb Vasc Biol*. 2012;32:1392-1399. doi: 10.1161/ATVBAHA.112.248559

194. Der Sarkissian S, Grobe JL, Yuan L, Narielwala DR, Walter GA, Katovich MJ, Raizada MK. Cardiac overexpression of angiotensin converting enzyme 2 protects the heart from ischemia-induced pathophysiology. *Hypertension*. 2008;51:712-718. doi: 10.1161/HYPERTENSIONAHA.107.100693

195. Epelman S, Tang WH, Chen SY, Van Lente F, Francis GS, Sen S. Detection of soluble angiotensin-converting enzyme 2 in heart failure: insights into the endogenous counter-regulatory pathway of the renin-angiotensin-aldosterone system. *Journal of the American College of Cardiology*. 2008;52:750-754. doi: 10.1016/j.jacc.2008.02.088

196. Epelman S, Shrestha K, Troughton RW, Francis GS, Sen S, Klein AL, Tang WH. Soluble angiotensin-converting enzyme 2 in human heart failure: relation with myocardial function and clinical outcomes. *J Card Fail*. 2009;15:565-571. doi: 10.1016/j.cardfail.2009.01.014

197. Ferreira JP, Verdonschot J, Collier T, Wang P, Pizard A, Bar C, Bjorkman J, Boccanelli A, Butler J, Clark A, et al. Proteomic Bioprofiles and Mechanistic Pathways of

Progression to Heart Failure. *Circ Heart Fail*. 2019;12:e005897. doi: 10.1161/CIRCHEARTFAILURE.118.005897

198. Ferrario CM, Jessup J, Chappell MC, Averill DB, Brosnihan KB, Tallant EA, Diz DI, Gallagher PE. Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation*. 2005;111:2605-2610. doi: 10.1161/CIRCULATIONAHA.104.510461

199. Jessup JA, Gallagher PE, Averill DB, Brosnihan KB, Tallant EA, Chappell MC, Ferrario CM. Effect of angiotensin II blockade on a new congenic model of hypertension derived from transgenic Ren-2 rats. *Am J Physiol Heart Circ Physiol*. 2006;291:H2166-2172. doi: 10.1152/ajpheart.00061.2006

200. Liu Y, Yang Y, Zhang C, Huang F, Wang F, Yuan J, Wang Z, Li J, Li J, Feng C, et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. *Sci China Life Sci*. 2020;63:364-374. doi: 10.1007/s11427-020-1643-8

201. Gheblawi M, Wang K, Viveiros A, Nguyen Q, Zhong JC, Turner AJ, Raizada MK, Grant MB, Oudit GY. Angiotensin Converting Enzyme 2: SARS-CoV-2 Receptor and Regulator of the Renin-Angiotensin System. *Circ Res*. 2020. doi: 10.1161/CIRCRESAHA.120.317015

202. Chappell MC, Pirro NT, Sykes A, Ferrario CM. Metabolism of angiotensin-(1-7) by angiotensin-converting enzyme. *Hypertension*. 1998;31:362-367. doi: 10.1161/01.hyp.31.1.362

203. Serfozo P, Wysocki J, Gulua G, Schulze A, Ye M, Liu P, Jin J, Bader M, Myohanen T, Garcia-Horsman JA, et al. 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;75:173-182. doi: 10.1161/HYPERTENSIONAHA.119.14071

204. Pavo N, Wurm R, Goliasch G, Novak JF, Strunk G, Gyongyosi M, Poglitsch M, Saemann MD, Hulsmann M. Renin-Angiotensin System Fingerprints of Heart Failure With Reduced Ejection Fraction. *J Am Coll Cardiol*. 2016;68:2912-2914. doi: 10.1016/j.jacc.2016.10.017

205. Pavo N, Goliasch G, Wurm R, Novak J, Strunk G, Gyongyosi M, Poglitsch M, Saemann MD, Hulsmann M. Low- and High-renin Heart Failure Phenotypes with Clinical Implications. *Clin Chem*. 2018;64:597-608. doi: 10.1373/clinchem.2017.278705

206. Li M, Liu K, Michalicek J, Angus JA, Hunt JE, Dell'Italia LJ, Feneley MP, Graham RM, Husain A. Involvement of chymase-mediated angiotensin II generation in blood pressure regulation. *J Clin Invest*. 2004;114:112-120. doi: 10.1172/JCI20805

207. Psotka MA, Fiuzat M, Carson PE, Kao DP, Cerkvenik J, Schaber DE, Verta P, Kazmierski RT, Shinnar M, Stockbridge N, et al. Design of a "Lean" Case Report Form for Heart Failure Therapeutic Development. *JACC Heart Fail*. 2019;7:913-921. doi: 10.1016/j.jchf.2019.07.001

208. Zile MR, Claggett BL, Prescott MF, McMurray JJ, Packer M, Rouleau JL, Swedberg K, Desai AS, Gong J, Shi VC, et al. Prognostic Implications of Changes in N-Terminal Pro-B-Type Natriuretic Peptide in Patients With Heart Failure. *J Am Coll Cardiol*. 2016;68:2425-2436. doi: 10.1016/j.jacc.2016.09.931

209. Vardeny O, Miller R, Solomon SD. Combined neprilysin and renin-angiotensin system inhibition for the treatment of heart failure. *JACC Heart Fail*. 2014;2:663-670. doi: 10.1016/j.jchf.2014.09.001

210. Schinzari F, Tesauro M, Veneziani A, Mores N, Di Daniele N, Cardillo C. Favorable Vascular Actions of Angiotensin-(1-7) in Human Obesity. *Hypertension*. 2018;71:185-191. doi: 10.1161/HYPERTENSIONAHA.117.10280

211. Sweitzer N. Angiotensin (1-7) Treatment to Improve Cognitive Functioning in Heart Failure Patients. NCT03159988 CgI; 2017.

212. Januzzi JL, Jr., Ibrahim NE. Renin-Angiotensin System Blockade in Heart Failure: More to the Picture Than Meets the Eye. *J Am Coll Cardiol*. 2017;69:820-822. doi: 10.1016/j.jacc.2016.10.083

213. Savergnini SQ, Beiman M, Lautner RQ, de Paula-Carvalho V, Allahdadi K, Pessoa DC, Costa-Fraga FP, Fraga-Silva RA, Cojocaru G, Cohen Y, et al. Vascular relaxation, antihypertensive effect, and cardioprotection of a novel peptide agonist of the MAS receptor. *Hypertension*. 2010;56:112-120. doi: 10.1161/HYPERTENSIONAHA.110.152942

214. Mendes AC, Ferreira AJ, Pinheiro SV, Santos RA. Chronic infusion of angiotensin-(1-7) reduces heart angiotensin II levels in rats. *Regul Pept*. 2005;125:29-34. doi: 10.1016/j.regpep.2004.07.023

215. Triposkiadis F, Butler J, Abboud FM, Armstrong PW, Adamopoulos S, Atherton JJ, Backs J, Bauersachs J, Burkhoff D, Bonow RO, et al. The continuous heart failure spectrum: moving beyond an ejection fraction classification. *Eur Heart J*. 2019;40:2155-2163. doi: 10.1093/eurheartj/ehz158

216. Wang K, Gheblawi M, Oudit GY. Angiotensin Converting Enzyme 2: A Double-Edged Sword. *Circulation*. 2020;142:426-428. doi: 10.1161/CIRCULATIONAHA.120.047049

217. Oudit GY, Pfeffer MA. Plasma angiotensin-converting enzyme 2: novel biomarker in heart failure with implications for COVID-19. *Eur Heart J*. 2020;41:1818-1820. doi: 10.1093/eurheartj/ehaa414

218. Bell JH, Herrera AH, Li Y, Walcheck B. Role of ADAM17 in the ectodomain shedding of TNF-alpha and its receptors by neutrophils and macrophages. *J Leukoc Biol*. 2007;82:173-176. doi: 10.1189/jlb.0307193

219. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature*. 1997;385:729-733. doi: 10.1038/385729a0

220. Bavishi C, Bonow RO, Trivedi V, Abbott JD, Messerli FH, Bhatt DL. Special Article - Acute myocardial injury in patients hospitalized with COVID-19 infection: A review. *Prog Cardiovasc Dis*. 2020;63:682-689. doi: 10.1016/j.pcad.2020.05.013

221. Lala A, Johnson KW, Januzzi JL, Russak AJ, Paranjpe I, Richter F, Zhao S, Somani S, Van Vleck T, Vaid A, et al. Prevalence and Impact of Myocardial Injury in Patients Hospitalized With COVID-19 Infection. *J Am Coll Cardiol*. 2020;76:533-546. doi: 10.1016/j.jacc.2020.06.007

222. Lindner D, Fitzek A, Brauninger H, Aleshcheva G, Edler C, Meissner K, Scherschel K, Kirchhof P, Escher F, Schultheiss HP, et al. Association of Cardiac Infection With SARS-CoV-2 in Confirmed COVID-19 Autopsy Cases. *JAMA Cardiol*. 2020;5:1281-1285. doi: 10.1001/jamacardio.2020.3551

223. Zoufaly A, Poglitsch M, Aberle JH, Hoepler W, Seitz T, Traugott M, Grieb A, Pawelka E, Laferl H, Wenisch C, et al. Human recombinant soluble ACE2 in severe COVID-19. *Lancet Respir Med*. 2020;8:1154-1158. doi: 10.1016/S2213-2600(20)30418-5

224. Force ADT, Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS. Acute respiratory distress syndrome: the Berlin Definition. *JAMA*. 2012;307:2526-2533. doi: 10.1001/jama.2012.5669

225. Khwaja A. KDIGO clinical practice guidelines for acute kidney injury. *Nephron Clin Pract*. 2012;120:c179-184. doi: 10.1159/000339789

226. Yeung ML, Teng JLL, Jia L, Zhang C, Huang C, Cai JP, Zhou R, Chan KH, Zhao H, Zhu L, et al. Soluble ACE2-mediated cell entry of SARS-CoV-2 via interaction with proteins related to the renin-angiotensin system. *Cell*. 2021. doi: 10.1016/j.cell.2021.02.053

227. Takayanagi T, Forrester SJ, Kawai T, Obama T, Tsuji T, Elliott KJ, Nuti E, Rossello A, Kwok HF, Scalia R, et al. Vascular ADAM17 as a Novel Therapeutic Target in Mediating Cardiovascular Hypertrophy and Perivascular Fibrosis Induced by Angiotensin II. *Hypertension*. 2016;68:949-955. doi: 10.1161/HYPERTENSIONAHA.116.07620

228. Shen M, Hu M, Fedak PWM, Oudit GY, Kassiri Z. Cell-Specific Functions of ADAM17 Regulate the Progression of Thoracic Aortic Aneurysm. *Circ Res*. 2018;123:372-388. doi: 10.1161/CIRCRESAHA.118.313181

229. Rydyznski Moderbacher C, Ramirez SI, Dan JM, Grifoni A, Hastie KM, Weiskopf D, Belanger S, Abbott RK, Kim C, Choi J, et al. Antigen-Specific Adaptive Immunity to SARS-CoV-2 in Acute COVID-19 and Associations with Age and Disease Severity. *Cell*. 2020;183:996-1012 e1019. doi: 10.1016/j.cell.2020.09.038

230. Tsujimoto M, Yip YK, Vilcek J. Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor. *J Immunol*. 1986;136:2441-2444.

231. Aggarwal BB, Eessalu TE, Hass PE. Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. *Nature*. 1985;318:665-667. doi: 10.1038/318665a0

232. Gerard L, Lecocq M, Bouzin C, Hoton D, Schmit G, Pereira JP, Montiel V, Plante-Bordeneuve T, Laterre PF, Pilette C. Increased Angiotensin-Converting Enzyme 2 and Loss of Alveolar Type II Cells in COVID-19 Related ARDS. *Am J Respir Crit Care Med*. 2021. doi: 10.1164/rccm.202012-4461OC

233. Ackermann M, Verleden SE, Kuehnel M, Haverich A, Welte T, Laenger F, Vanstapel A, Werlein C, Stark H, Tzankov A, et al. Pulmonary Vascular Endothelialitis, Thrombosis, and Angiogenesis in Covid-19. *N Engl J Med*. 2020;383:120-128. doi: 10.1056/NEJMoa2015432

234. Sakamoto A, Kawakami R, Kawai K, Gianatti A, Pellegrini D, Kutys R, Guo L, Mori M, Cornelissen A, Sato Y, et al. ACE2 (Angiotensin-Converting Enzyme 2) and TMPRSS2 (Transmembrane Serine Protease 2) Expression and Localization of SARS-CoV-2 Infection in the Human Heart. *Arterioscler Thromb Vasc Biol*. 2021;41:542-544. doi: 10.1161/ATVBAHA.120.315229

235. Bois MC, Boire NA, Layman AJ, Aubry MC, Alexander MP, Roden AC, Hagen CE, Quinton RA, Larsen C, Erben Y, et al. COVID-19-Associated Nonocclusive Fibrin Microthrombi in the Heart. *Circulation*. 2021;143:230-243. doi: 10.1161/CIRCULATIONAHA.120.050754

236. Kuriakose J, Montezano AC, Touyz RM. ACE2/Ang-(1-7)/Mas1 axis and the vascular system: vasoprotection to COVID-19-associated vascular disease. *Clin Sci (Lond)*. 2021;135:387-407. doi: 10.1042/CS20200480

237. Klok FA, Kruip M, van der Meer NJM, Arbous MS, Gommers D, Kant KM, Kaptein FHJ, van Paassen J, Stals MAM, Huisman MV, et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res*. 2020;191:145-147. doi: 10.1016/j.thromres.2020.04.013

238. Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, Mehra MR, Schuepbach RA, Ruschitzka F, Moch H. Endothelial cell infection and endotheliitis in COVID-19. *Lancet*. 2020;395:1417-1418. doi: 10.1016/S0140-6736(20)30937-5

239. Pellegrini D, Kawakami R, Guagliumi G, Sakamoto A, Kawai K, Gianatti A, Nasr A, Kutys R, Guo L, Cornelissen A, et al. Microthrombi as a Major Cause of Cardiac Injury in COVID-19: A Pathologic Study. *Circulation*. 2021;143:1031-1042. doi: 10.1161/CIRCULATIONAHA.120.051828

240. Zhao X, Wang K, Zuo P, Liu Y, Zhang M, Xie S, Zhang H, Chen X, Liu C. Early decrease in blood platelet count is associated with poor prognosis in COVID-19 patients-indications for predictive, preventive, and personalized medical approach. *EPMA J*. 2020:1-7. doi: 10.1007/s13167-020-00208-z

241. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020;395:1054-1062. doi: 10.1016/S0140-6736(20)30566-3

242. Zaid Y, Puhm F, Allaeys I, Naya A, Oudghiri M, Khalki L, Limami Y, Zaid N, Sadki K, Ben El Haj R, et al. Platelets Can Associate with SARS-Cov-2 RNA and Are Hyperactivated in COVID-19. *Circ Res*. 2020. doi: 10.1161/CIRCRESAHA.120.317703

243. Files DC, Gibbs KW, Schaich CL, Collins SP, Gwathmey TM, Casey JD, Self WH, Chappell MC. A pilot study to assess the circulating renin-angiotensin system in COVID-19 acute respiratory failure. *Am J Physiol Lung Cell Mol Physiol*. 2021;321:L213-L218. doi: 10.1152/ajplung.00129.2021

244. Ng KK, Vane JR. Conversion of angiotensin I to angiotensin II. *Nature*. 1967;216:762-766. doi: 10.1038/216762a0

245. Esther CR, Marino EM, Howard TE, Machaud A, Corvol P, Capecchi MR, Bernstein KE. The critical role of tissue angiotensin-converting enzyme as revealed by gene targeting in mice. *J Clin Invest*. 1997;99:2375-2385. doi: 10.1172/JCI119419

246. Sharma RK, Stevens BR, Obukhov AG, Grant MB, Oudit GY, Li Q, Richards EM, Pepine CJ, Raizada MK. ACE2 (Angiotensin-Converting Enzyme 2) in Cardiopulmonary Diseases: Ramifications for the Control of SARS-CoV-2. *Hypertension*. 2020;76:651-661. doi: 10.1161/HYPERTENSIONAHA.120.15595

247. Danser AHJ, Epstein M, Batlle D. Renin-Angiotensin System Blockers and the COVID-19 Pandemic: At Present There Is No Evidence to Abandon Renin-Angiotensin System Blockers. *Hypertension*. 2020;75:1382-1385. doi: 10.1161/HYPERTENSIONAHA.120.15082

248. Wang K, Basu R, Poglitsch M, Bakal JA, Oudit GY. Elevated Angiotensin 1-7/Angiotensin II Ratio Predicts Favorable Outcomes in Patients With Heart Failure. *Circ Heart Fail*. 2020;13:e006939. doi: 10.1161/CIRCHEARTFAILURE.120.006939

249. Lew RA, Warner FJ, Hanchapola I, Yarski MA, Ramchand J, Burrell LM, Smith AI. Angiotensin-converting enzyme 2 catalytic activity in human plasma is masked by an endogenous inhibitor. *Exp Physiol*. 2008;93:685-693. doi: 10.1113/expphysiol.2007.040352

250. Rice GI, Jones AL, Grant PJ, Carter AM, Turner AJ, Hooper NM. Circulating activities of angiotensin-converting enzyme, its homolog, angiotensin-converting enzyme 2, and neprilysin in a family study. *Hypertension*. 2006;48:914-920. doi: 10.1161/01.HYP.0000244543.91937.79

251. Atlas SA. The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. *J Manag Care Pharm*. 2007;13:9-20. doi: 10.18553/jmcp.2007.13.s8-b.9

252. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292:C82-97. doi: 10.1152/ajpcell.00287.2006

253. Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AJ, Boomsma F, van Gilst WH. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation*. 2002;105:1548-1550. doi: 10.1161/01.cir.0000013847.07035.b9

254. Grobe JL, Mecca AP, Lingis M, Shenoy V, Bolton TA, Machado JM, Speth RC, Raizada MK, Katovich MJ. Prevention of angiotensin II-induced cardiac remodeling by angiotensin-(1-7). *Am J Physiol Heart Circ Physiol*. 2007;292:H736-742. doi: 10.1152/ajpheart.00937.2006

255. 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). *Physiol Rev*. 2018;98:505-553. doi: 10.1152/physrev.00023.2016

256. Seva Pessoa B, Becher PM, Van Veghel R, De Vries R, Tempel D, Sneep S, Van Beusekom H, Van Der Velden VH, Westermann D, Danser AH, et al. Effect of a stable Angiotensin-(1-7) analogue on progenitor cell recruitment and cardiovascular function post myocardial infarction. *J Am Heart Assoc*. 2015;4. doi: 10.1161/JAHA.114.001510

257. Paz Ocaranza M, Riquelme JA, Garcia L, Jalil JE, Chiong M, Santos RAS, Lavandero S. Counter-regulatory renin-angiotensin system in cardiovascular disease. *Nat Rev Cardiol*. 2020;17:116-129. doi: 10.1038/s41569-019-0244-8

258. Park BM, Li W, Kim SH. Cardio-protective effects of angiotensin-(1-5) via mas receptor in rats against ischemic-perfusion injury. *Peptides*. 2021;139:170516. doi: 10.1016/j.peptides.2021.170516

259. de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev*. 2000;52:415-472.

260. Qiu Z, Zhao J, Huang F, Bao L, Chen Y, Yang K, Cui W, Jin W. Myocardial fibrosis reversion via rhACE2-electrospun fibrous patch for ventricular remodeling prevention. *NPJ Regen Med*. 2021;6:44. doi: 10.1038/s41536-021-00154-y

261. Heidenreich PA, Bozkurt B, Aguilar D, Allen LA, Byun JJ, Colvin MM, Deswal A, Drazner MH, Dunlay SM, Evers LR, et al. 2022 AHA/ACC/HFSA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. *Circulation*. 2022;145:e895-e1032. doi: 10.1161/CIR.0000000000001063

262. Authors/Task Force M, McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Bohm M, Burri H, Butler J, Celutkiene J, et al. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: Developed by the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). With the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail*. 2022;24:4-131. doi: 10.1002/ejhf.2333

263. Deng Q, Hu B, Zhang Y, Wang H, Zhou X, Hu W, Cheng Y, Yan J, Ping H, Zhou Q. Suspected myocardial injury in patients with COVID-19: Evidence from front-line clinical observation in Wuhan, China. *Int J Cardiol*. 2020. doi: 10.1016/j.ijcard.2020.03.087

264. Clemente-Casares X, Hosseinzadeh S, Barbu I, Dick SA, Macklin JA, Wang Y, Momen A, Kantores C, Aronoff L, Farno M, et al. A CD103(+) Conventional Dendritic Cell Surveillance System Prevents Development of Overt Heart Failure during Subclinical Viral Myocarditis. *Immunity*. 2017;47:974-989 e978. doi: 10.1016/j.immuni.2017.10.011

265. Sagar S, Liu PP, Cooper LT, Jr. Myocarditis. *Lancet*. 2012;379:738-747. doi: 10.1016/S0140-6736(11)60648-X

266. Marchant DJ, Boyd JH, Lin DC, Granville DJ, Garmaroudi FS, McManus BM. Inflammation in myocardial diseases. *Circ Res*. 2012;110:126-144. doi: 10.1161/CIRCRESAHA.111.243170

267. Marchant DJ, McManus BM. Regulating viral myocarditis: allografted regulatory T cells decrease immune infiltration and viral load. *Circulation*. 2010;121:2609-2611. doi: 10.1161/CIRCULATIONAHA.110.960054

268. Marchant D, Dou Y, Luo H, Garmaroudi FS, McDonough JE, Si X, Walker E, Luo Z, Arner A, Hegele RG, et al. Bosentan enhances viral load via endothelin-1 receptor type-A-mediated p38 mitogen-activated protein kinase activation while improving cardiac function during coxsackievirus-induced myocarditis. *Circ Res*. 2009;104:813-821. doi: 10.1161/CIRCRESAHA.108.191171

269. Chapman AR, Bularga A, Mills NL. High-Sensitivity Cardiac Troponin Can Be an Ally in the Fight Against COVID-19. *Circulation*. 2020;141:1733-1735. doi: 10.1161/CIRCULATIONAHA.120.047008

270. Freaney PM, Shah SJ, Khan SS. COVID-19 and Heart Failure With Preserved Ejection Fraction. *Jama*. 2020. doi: 10.1001/jama.2020.17445

271. Madjid M, Safavi-Naeini P, Solomon SD, Vardeny O. Potential Effects of Coronaviruses on the Cardiovascular System: A Review. *JAMA Cardiol*. 2020;5:831-840. doi: 10.1001/jamacardio.2020.1286

272. Lindner D, Fitzek A, Brauninger H, Aleshcheva G, Edler C, Meissner K, Scherschel K, Kirchhof P, Escher F, Schultheiss HP, et al. Association of Cardiac Infection With SARS-CoV-2 in Confirmed COVID-19 Autopsy Cases. *JAMA Cardiol*. 2020. doi: 10.1001/jamacardio.2020.3551

273. Lowenstein CJ, Solomon SD. Severe COVID-19 is a Microvascular Disease. *Circulation*. 2020. doi: 10.1161/CIRCULATIONAHA.120.050354

274. Puntmann VO, Carerj ML, Wieters I, Fahim M, Arendt C, Hoffmann J, Shchendrygina A, Escher F, Vasa-Nicotera M, Zeiher AM, et al. Outcomes of Cardiovascular Magnetic Resonance Imaging in Patients Recently Recovered From Coronavirus Disease 2019 (COVID-19). *JAMA Cardiol*. 2020. doi: 10.1001/jamacardio.2020.3557

275. Hirano T, Murakami M. COVID-19: A New Virus, but a Familiar Receptor and Cytokine Release Syndrome. *Immunity*. 2020. doi: 10.1016/j.immuni.2020.04.003

276. Shen M, Morton J, Davidge ST, Kassiri Z. Loss of smooth muscle cell disintegrin and metalloproteinase 17 transiently suppresses angiotensin II-induced hypertension and end-organ damage. *J Mol Cell Cardiol*. 2017;103:11-21. doi: 10.1016/j.yjmcc.2016.12.001

277. Fan D, Takawale A, Shen M, Samokhvalov V, Basu R, Patel V, Wang X, Fernandez-Patron C, Seubert JM, Oudit GY, et al. A Disintegrin and Metalloprotease-17 Regulates Pressure Overload-Induced Myocardial Hypertrophy and Dysfunction Through Proteolytic Processing of Integrin beta1. *Hypertension*. 2016;68:937-948. doi: 10.1161/HYPERTENSIONAHA.116.07566

278. Fan D, Takawale A, Shen M, Wang W, Wang X, Basu R, Oudit GY, Kassiri Z. Cardiomyocyte A Disintegrin And Metalloproteinase 17 (ADAM17) Is Essential in Post-Myocardial Infarction Repair by Regulating Angiogenesis. *Circ Heart Fail*. 2015;8:970-979. doi: 10.1161/CIRCHEARTFAILURE.114.002029

279. Pedersen KB, Chodavarapu H, Porretta C, Robinson LK, Lazartigues E. Dynamics of ADAM17-Mediated Shedding of ACE2 Applied to Pancreatic Islets of Male db/db Mice. *Endocrinology*. 2015;156:4411-4425. doi: 10.1210/en.2015-1556

280. Xu J, Xu X, Jiang L, Dua K, Hansbro PM, Liu G. SARS-CoV-2 induces transcriptional signatures in human lung epithelial cells that promote lung fibrosis. *Respir Res*. 2020;21:182. doi: 10.1186/s12931-020-01445-6

281. Wu Z, Hu R, Zhang C, Ren W, Yu A, Zhou X. Elevation of plasma angiotensin II level is a potential pathogenesis for the critically ill COVID-19 patients. *Crit Care*. 2020;24:290. doi: 10.1186/s13054-020-03015-0

282. 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;77:e29-e31. doi: 10.1161/HYPERTENSIONAHA.120.16724

283. Xie Y, Xu E, Bowe B, Al-Aly Z. Long-term cardiovascular outcomes of COVID-19. *Nat Med*. 2022;28:583-590. doi: 10.1038/s41591-022-01689-3

284. Al-Aly Z, Xie Y, Bowe B. High-dimensional characterization of post-acute sequelae of COVID-19. *Nature*. 2021;594:259-264. doi: 10.1038/s41586-021-03553-9

285. Nalbandian A, Sehgal K, Gupta A, Madhavan MV, McGroder C, Stevens JS, Cook JR, Nordvig AS, Shalev D, Sehrawat TS, et al. Post-acute COVID-19 syndrome. *Nat Med*. 2021;27:601-615. doi: 10.1038/s41591-021-01283-z

286. Sudre CH, Murray B, Varsavsky T, Graham MS, Penfold RS, Bowyer RC, Pujol JC, Klaser K, Antonelli M, Canas LS, et al. Attributes and predictors of long COVID. *Nat Med*. 2021;27:626-631. doi: 10.1038/s41591-021-01292-y

287. Daugherty SE, Guo Y, Heath K, Dasmarinas MC, Jubilo KG, Samranvedhya J, Lipsitch M, Cohen K. Risk of clinical sequelae after the acute phase of SARS-CoV-2 infection: retrospective cohort study. *BMJ*. 2021;373:n1098. doi: 10.1136/bmj.n1098