

# Myocardial Angiotensin Metabolism in End-Stage Heart Failure



Noemi Pavo, MD, PhD,<sup>a</sup> Suriya Prausmüller, MD,<sup>a</sup> Georg Spinka, MD,<sup>a</sup> Georg Goliasch, MD, PhD,<sup>a</sup> Philipp E. Bartko, MD, PhD,<sup>a</sup> Raphael Wurm, MD,<sup>b</sup> Henrike Arfsten, MD,<sup>a</sup> Guido Strunk, MSc, PhD,<sup>c,d,e</sup> Marko Poglitsch, MSc, PhD,<sup>f</sup> Oliver Domenig, MSc, PhD,<sup>f</sup> Julia Mascherbauer, MD,<sup>g</sup> Keziban Uyanik-Ünal, MD,<sup>h</sup> Christian Hengstenberg, MD,<sup>a</sup> Andreas Zuckermann, MD,<sup>h</sup> Martin Hülsmann, MD<sup>a</sup>

## ABSTRACT

**BACKGROUND** The myocardium exhibits an adaptive tissue-specific renin-angiotensin system (RAS), and local dysbalance may circumvent the desired effects of pharmacologic RAS inhibition, a mainstay of heart failure with reduced ejection fraction (HFrEF) therapy.

**OBJECTIVES** This study sought to investigate human myocardial tissue RAS regulation of the failing heart in the light of current therapy.

**METHODS** Fifty-two end-stage HFrEF patients undergoing heart transplantation (no RAS inhibitor: n = 9; angiotensin-converting enzyme [ACE] inhibitor: n = 28; angiotensin receptor blocker [ARB]: n = 8; angiotensin receptor neprilysin-inhibitor [ARNi]: n = 7) were enrolled. Myocardial angiotensin metabolites and enzymatic activities involved in the metabolism of the key angiotensin peptides angiotensin 1-8 (AngII) and Ang1-7 were determined in left ventricular samples by mass spectrometry. Circulating angiotensin concentrations were assessed for a subgroup of patients.

**RESULTS** AngII and Ang2-8 (AngIII) were the dominant peptides in the failing heart, while other metabolites, especially Ang1-7, were below the detection limit. Patients receiving an ARB component (i.e., ARB or ARNi) had significantly higher levels of cardiac AngII and AngIII (AngII: 242 [interquartile range (IQR): 145.7 to 409.9] fmol/g vs 63.0 [IQR: 19.9 to 124.1] fmol/g; p < 0.001; and AngIII: 87.4 [IQR: 46.5 to 165.3] fmol/g vs 23.0 [IQR: <5.0 to 59.3] fmol/g; p = 0.002). Myocardial AngII concentrations were strongly related to circulating AngII levels. Myocardial RAS enzyme regulation was independent from the class of RAS inhibitor used, particularly, a comparable myocardial neprilysin activity was observed for patients with or without ARNi. Tissue chymase, but not ACE, is the main enzyme for cardiac AngII generation, whereas AngII is metabolized to Ang1-7 by prolyl carboxypeptidase but not to ACE2. There was no trace of cardiac ACE2 activity.

**CONCLUSIONS** The failing heart contains considerable levels of classical RAS metabolites, whereas AngIII might be an unrecognized mediator of detrimental effects on cardiovascular structure. The results underline the importance of pharmacologic interventions reducing circulating AngII actions, yet offer room for cardiac tissue-specific RAS drugs aiming to limit myocardial AngII/AngIII peptide accumulation and actions. (J Am Coll Cardiol 2021;77:1731-43) © 2021 by the American College of Cardiology Foundation.

**H**ear failure (HF) remains the leading cause of death and disability in industrialized nations. Activation of the renin-angiotensin system (RAS) is characteristic for HF with reduced ejection fraction (HFrEF) and is responsible for continued disease progression despite advances in treatment. RAS controls the homeostasis of the cardiovascular and renal systems by regulating



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From the <sup>a</sup>Clinical Division of Cardiology, Department of Internal Medicine II, Medical University of Vienna, Vienna, Austria; <sup>b</sup>Department of Neurology, Medical University of Vienna, Vienna, Austria; <sup>c</sup>Complexity Research, Vienna, Austria; <sup>d</sup>Department of Statistics, Complexity Research, FH Campus Vienna, Vienna, Austria; <sup>e</sup>Department of Entrepreneurship and Economic Education, Faculty of Business and Economics, Technical University Dortmund, Dortmund, Germany; <sup>f</sup>Attoquant Diagnostics, Vienna, Austria; <sup>g</sup>Karl Landsteiner University of Health Sciences, Department of Internal Medicine 3, University Hospital St. Pölten, Krems, Austria; and the <sup>h</sup>Clinical Division of Cardiac Surgery, Department of Surgery, Medical University of Vienna, Vienna, Austria. The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the [Author Center](#).

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## ABBREVIATIONS AND ACRONYMS

**ACE** = angiotensin-converting enzyme

**Ang** = angiotensin

**AngI** = angiotensin 1-10

**AngII** = angiotensin 1-8

**AngIII** = angiotensin 2-8

**ARB** = angiotensin receptor blocker

**ARC** = active renin concentration

**ARNi** = angiotensin receptor neprilysin-inhibitor

**AT1R** = AngII type 1 receptor

**AT2R** = AngII type 2 receptor

**HF** = heart failure

**HFrEF** = heart failure with reduced ejection fraction

**HTx** = heart transplantation

**IQR** = interquartile range

**MasR** = Mas receptor

**MRA** = mineralocorticoid receptor antagonist

**NEP** = neprilysin

**NT-proBNP** = N-terminal pro-B-type natriuretic peptide

**NYHA** = New York Heart Association

**PCP** = prolyl carboxypeptidase

**PEP** = prolyl endopeptidase

**RAS** = renin-angiotensin system

**SARS-CoV-2** = severe acute respiratory syndrome-coronavirus-2

extracellular fluid status and vasoactivity. It is a peptidergic system resulting in the formation of several angiotensin metabolites (1). The renin-dependent formation of angiotensin 1-10 (AngI), a peptide consisting of 10 amino acids, from angiotensinogen is the rate-limiting step of the RAS cascade (2). AngI is then further processed to Ang1-8 (AngII) by angiotensin-converting enzyme (ACE). By binding to its receptor, the AngII type 1 receptor (AT1R), AngII exerts plentiful detrimental effects as vasoconstriction, inflammation, cell growth, apoptosis, and fibrogenesis (1). This classical axis of RAS, termed ACE-AngII-AT1R, is thought to be crucially involved in the continuing deterioration of cardiac function and worsening of clinical state of HF patients (3). In contrast, AngII actions mediated by the AT2R seem to be beneficial, resulting in vasorelaxation and antihypertrophic and antifibrotic effects (4). Ang2-8 (AngIII), which is derived from AngII, has a similar receptor affinity toward AT1R and AT2R, and a number of studies have suggested that AngIII and AngII are equipotent at eliciting undesirable cardiovascular effects and pressure response (5,6). The peptides Ang1-9 and Ang1-7, which arise from alternate cleavage of AngI and AngII by ACE2, a homolog of ACE, similarly have a physiologically opposing role (2,7). The cardioprotective effects of especially Ang1-7 and the Mas receptor (MasR) are well studied (2,8), and the pathway was consequently named the ACE2-Ang1-7-MasR, or alternate RAS axis. The main determinant of circulating

AngII levels is ACE, while ACE2 has been proposed as the crucial enzyme for determining Ang1-7 levels (1). An alternate ACE-independent synthesis of AngII can be mediated by chymase, a product of mast cells (1). Ang1-7 has multiple synthetic pathways: AngI can be directly converted to Ang1-7 by neprilysin (NEP) and prolyl endopeptidase (PEP), and alternatively it can be generated from AngII by ACE2 or prolyl carboxypeptidase (PCP) (1,9,10). More downstream peptides have equally been described, although their physiological role is largely unknown. A dysbalance between AngII and Ang1-7 may result in acceleration of cardiovascular disease progression.

Systemic blockade of the RAS by ACE inhibitor, AT1R blocker (ARB), or angiotensin receptor neprilysin-inhibitor (ARNi) therapy is a cornerstone in the treatment of patients with HFrEF (11,12). Currently, attempts are undertaken to additionally

exploit the therapeutic potential of the alternate RAS by intravenous administration of recombinant human ACE2 (13). Although the clinical benefits of RAS inhibitor therapy have been extensively proven, their exact effect on RAS are rather little known, leaving open questions. Circulating AngII may remain elevated in patients optimally treated for HF (14). The attempts of a direct upstream blockade of renin by aliskiren were not overwhelmingly successful (15,16), nor has it ever been shown that the high-renin phenotype would profit more from extended RAS inhibition. Although RAS seems to predominantly function as a systemic entity, many tissues including the myocardium are capable of producing individual components of RAS with autocrine, paracrine, and endocrine effects (1). The local RAS is thought to underlie a partly independent regulation with tissue-specific angiotensin metabolism and cellular signaling capable to shift the balance between organ damage and protection. The myocardium may circumvent the effects of systemic RAS inhibition, a mechanism that was suggested to be responsible for continuing deterioration of organ function despite optimal medical therapy. Only scarce data are available on myocardial tissue RAS regulation and myocardial AngII formation in general, while the effect of state-of-the-art HF therapy including ARNi on myocardial tissue RAS is completely unknown. Understanding HFrEF-related RAS dysregulation and its modulation by current therapy should help us identify alternate strategies to minimize classical RAS and enhance alternate RAS effects.

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The exact determination of angiotensin concentrations has recently become available by mass spectrometry technology. The reliable simultaneous measurement of all known systemic angiotensin peptides provides an exact picture of RAS (17-19). The fingerprints of circulating RAS in stable HFrEF patients showed characteristic AngI, AngII, and Ang1-7 levels for the different RAS inhibitors alongside a general dependence of angiotensin peptide concentrations on plasma renin (20). The importance of the alternate RAS axis in human myocardial tissue samples has been supported by a recent study using the same methodology (19).

The aim of this study was to assess myocardial tissue RAS peptide concentrations and myocardial RAS enzymatic regulation to: 1) characterize myocardial tissue RAS in advanced HF; 2) show the effect of state-of-the-art therapy with different systemic RAS inhibitors; and 3) investigate the

association between myocardial and systemic RAS regulation.

## METHODS

**PATIENT POPULATION.** Between May 2015 and March 2020, we enrolled a total of 52 patients with end-stage HFREF undergoing heart transplantation (HTx) from a prospective registry at the Vienna General Hospital, the university-affiliated tertiary care center of the Medical University of Vienna. Inclusion criteria were end-stage HF, active listing for HTx, and an age >18 years. Etiology of HF, hemodynamics, comorbidities, traditional risk factors, and medical therapy including HF treatment with beta-blocker, RAS inhibitor (ACE inhibitor, ARB, or ARNi), and mineralocorticoid receptor antagonist (MRA) were recorded. Written, informed consent was obtained from all study participants. The study protocol complies with the Declaration of Helsinki and was approved by the local ethics committee of the Medical University of Vienna.

Venous blood samples of all patients were obtained for routine laboratory measurements. Transmural left ventricular myocardial samples were obtained immediately after explantation of the failing heart at the surgical heart transplantation procedure. Samples were snap frozen and stored at  $-80^{\circ}\text{C}$  in liquid nitrogen as well as formalin fixed and paraffin embedded. For a subgroup of patients, additional heparin plasma was stored in a biobank for determination of circulating angiotensin levels. Snap-frozen samples were used for the determination of angiotensin metabolites, enzymatic activities, and investigation of different metabolization patterns depending on background RAS inhibitor therapy. Formalin-fixed and paraffin-embedded tissue was used for immunohistochemistry.

**LABORATORY ANALYSIS.** Routinely available parameters and additionally N-terminal pro-B-type natriuretic peptide (NT-proBNP), active renin concentration (ARC), and plasma aldosterone were determined according to the local laboratory's standard procedure.

**MYOCARDIAL TISSUE ANGIOTENSIN METABOLITE PROFILES (RAS FINGERPRINTS) ASSESSED BY MASS SPECTROMETRY.** Angiotensin metabolites in cardiac tissue were quantified by Attoquant Diagnostics (Vienna, Austria) as described previously (21). Briefly, frozen cardiac tissue segments (50 to 90 mg) were homogenized using pestle and mortar under liquid nitrogen. The frozen tissue powder was dissolved at 100 mg/ml in 6 mol/l aqueous guanidinium chloride

supplemented with 1% (v/v) trifluoroacetic acid (both from Sigma-Aldrich, Munich, Germany) by cooled sonication using a 2-mm microtip (Sonics and Materials, Newton, New Jersey). Stable isotope-labeled internal standards for individual angiotensin metabolites (AngI, AngII, Ang1-7, Ang1-5, AngIII, AngIV) were added to tissue homogenates at 200 pg/ml and stored at  $-80^{\circ}\text{C}$  until liquid chromatography with tandem mass spectrometry analysis. Following C18-based solid-phase extraction, samples were subjected to liquid chromatography with tandem mass spectrometry analysis using a reversed-phase analytical column (Acquity UPLC C18; Waters, Milford, Massachusetts) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters) in MRM mode. Internal standards were used to correct for matrix effects and peptide recovery of the sample preparation procedure for each angiotensin metabolite in each individual sample. Angiotensin peptide concentrations were calculated considering the corresponding response factors determined in appropriate calibration curves, on condition that integrated signals exceeded a signal-to-noise ratio of 10.

## ENZYMATIC ACTIVITIES OF MAIN RAS ENZYMES INVOLVED IN THE METABOLIZATION OF AngI, AngII, AND Ang1-7.

Tissue biopsy was homogenized in phosphate-buffered saline using low-energy sonication. Angiotensin formation rates were determined in tissue homogenates after spiking the samples with AngI, AngII, or Ang1-7 ex vivo, followed by incubation at  $37^{\circ}\text{C}$  in the presence or absence of selected RAS enzyme inhibitors (ACE inhibitor: lisinopril at 10 mmol/l [Sigma-Aldrich]; PEP inhibitor/PCP inhibitor: Z-Pro-prolinal at 20 mmol/l [Sigma-Aldrich]; NEP inhibitor: DL-thiorphan at 100 mmol/l [Sigma-Aldrich]; chymase inhibitor: chymostatin at 10 mmol/l [Sigma-Aldrich]; ACE2 inhibitor: MLN-4760 at 10 mmol/l [Merck-Millipore, Darmstadt, Germany]). Aminopeptidase inhibitor (10 mmol/l; Sigma-Aldrich) was added to all samples. Following incubation, samples were stabilized, spiked with stable isotope-labeled internal standards for angiotensin metabolites (AngI, AngII, Ang1-7) and assayed as described for protease inhibitor-stabilized plasma (see previous). Specific activity of each RAS enzyme was calculated by determining the inhibitor-sensitive fraction (control minus inhibitor) of product formation related to control (pg/ml/h) (AngII or Ang1-7).

Low-energy sonicated tissue biopsies, dissolved in phosphate-buffered saline, were spiked with AngI, AngII, or Ang1-7 ex vivo, followed by a  $37^{\circ}\text{C}$  incubation step in absence of any enzyme inhibitors. Following stabilization, samples were assayed as

described for myocardial tissue angiotensin metabolites (see previous).

**CIRCULATING ANGIOTENSIN METABOLITES PROFILES (RAS FINGERPRINTS) ASSESSED BY MASS SPECTROMETRY.** Drawn blood was mixed immediately at sampling with an appropriated inhibitor cocktail (Attoquant Diagnostics, Vienna, Austria) (20). Following cooled centrifugation, gained plasma was spiked with stable isotope-labeled internal standards for each angiotensin metabolite (AngI, AngII, Ang1-7, Ang1-5, AngIII, AngIV) at a concentration of 200 pg/ml. Liquid chromatography tandem mass spectrometry analysis for tissue angiotensin quantification was conducted in the same way as described for myocardial tissue angiotensin metabolites (see previous).

**IMMUNOHISTOCHEMISTRY.** For histological sections, tissues were embedded in paraffin and cut to 4- to 5- $\mu$ m slices. The organization of collagen and fibrosis was visualized by staining the samples with Picrosirius red and hematoxylin and eosin. Picrosirius red stain kit (#24901-500; Polyscience, Warrington, Pennsylvania) was applied according to the manufacturer's protocol. Briefly, the sections were deparaffinized, rehydrated, and then placed in phosphomolybdic acid for 2 min, followed by incubation in Picrosirius red F3BA stain for 60 min. Then, the sections were placed in 0.1 N hydrochloride acid for 2 min and rapidly dehydrated and mounted with coverslips. Digital images were captured with an Olympus IX83 inverted microscope with both polychromatic and polarized light using the cellSens imaging software (Olympus, Tokyo, Japan).

**STATISTICAL ANALYSIS.** Continuous parameters were presented as median and interquartile range (IQR) and categorical data as counts and percentages. Baseline characteristics, myocardial angiotensin levels, and RAS enzymatic activities for subgroups using different RAS inhibitors were compared by the Kruskal-Wallis test or by the chi-square test. The Spearman correlation coefficient ( $r_s$ ) was calculated to assess the relationship between plasma renin and other continuous parameters. For the association between renin and circulating angiotensins and between circulating and tissue RAS components, linear regression analysis was performed for the log  $\times$  log model with or without constraints or weighting, and best-fit lines were displayed indicating  $R^2$  and the p value. For statistical analysis, numerical values for angiotensin concentrations under the detection limit were considered as the 0.5-fold value of the respective lower limit of quantification (19). For all tests, 2-sided p values  $<0.05$  were considered to indicate statistical significance.

**TABLE 1** Baseline Characteristics of End-Stage Heart Failure Patients (N = 52) Undergoing Heart Transplantation

Basic demographics	
Age, yrs	57 (51-64)
Male	45 (87)
BMI, kg/m <sup>2</sup>	27.0 (24.8-28.3)
Systolic blood pressure, mm Hg	110 (95-120)
Heart rate, beats/min	77 (69-85)
Heart failure etiology	
Ischemic	21 (40)
Nonischemic with dilatative phenotype	29 (58)
Other	2 (4)
Comorbidities	
Known CAD	28 (54)
Atrial fibrillation	17 (33)
Diabetes mellitus	12 (23)
Arterial hypertension	27 (52)
PAD	11 (21)
Dyslipidemia	27 (52)
Medication and device therapy	
Beta-blocker	35 (67)
ACE inhibitor/ARB/ARNi	28 (54)/8 (15)/7 (14)
MRA	36 (69)
Ivabradine	11 (21)
ICD/CRT	38 (73)/10 (19)
LVAD	16 (31)
Laboratory parameters	
Creatinine, mg/dl	1.23 (0.95-1.74)
BUN, mg/dl	25 (15-32)
Albumin, g/l	37.2 (31.9-45.1)
BChE, kU/l	4.68 (3.36-7.14)
AST, U/l	23 (15-28)
ALT, U/l	23 (15-29)
GGT, U/l	66 (28-128)
Total cholesterol, mg/dl	155 (114-179)
CRP, mg/dl	1.08 (0.31-2.90)
Active renin concentration, $\mu$ IU/ml	531 (98-1,953)
NT-proBNP pg/ml	3,498 (1,678-8,298)

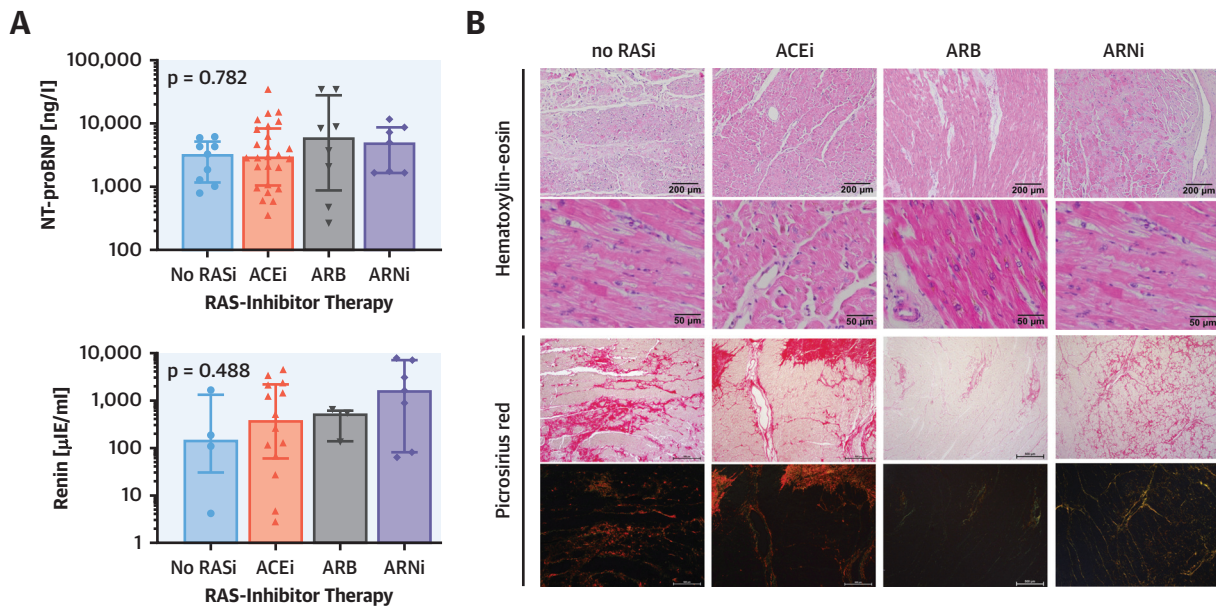
Values are mean (interquartile range) or n (%).

ACE = angiotensin-converting enzyme; ALT = alanine aminotransferase; ARB = angiotensin receptor blocker; ARNi = angiotensin receptor neprilysin-inhibitor; BChE = butyrylcholinesterase; BMI = body mass index; BUN = blood urea nitrogen; CAD = coronary artery disease; CRP = C-reactive protein; CRT = cardiac resynchronization therapy; GGT = gamma-glutamyl transferase; ICD = implantable cardioverter-defibrillator; LVAD = left ventricular assist device; MRA = mineralocorticoid antagonist; NT-proBNP = N-terminal pro-B-type natriuretic peptide; PAD = peripheral artery disease.

## RESULTS

**BASELINE CHARACTERISTICS.** Baseline characteristics of the total study population are listed in **Table 1**. Median age was 57 years (IQR: 51 to 64 years), 45 (87%) patients were male, and 21 (40%) had an ischemic etiology of HF. NT-proBNP was elevated according to the advanced state of disease, with a median of 3,498 pg/ml (IQR: 1,678 to 8,298 pg/ml), and 36 (69%) of patients were in New York Heart Association (NYHA) functional class III or III+. Nine

**FIGURE 1 Neurohumoral Dysregulation and Immunohistochemistry of the Failing Hearts of Patients Undergoing Heart Transplantation According to RAS Inhibition**



**(A)** N-terminal pro-B-type natriuretic peptide (NT-proBNP) and active renin concentration (ARC) for end-stage heart failure patients undergoing heart transplantation. NT-proBNP and ARC levels between groups were compared using the Kruskal-Wallis test. **(A)** Representative immunohistochemistry with hematoxylin-eosin and Picrosirius red staining, here using polychromatic and polarized light, of myocardial tissue samples of the failing hearts. ACEi = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; ARNi = angiotensin receptor neprilysin-inhibitor; RASi = renin-angiotensin system inhibitor.

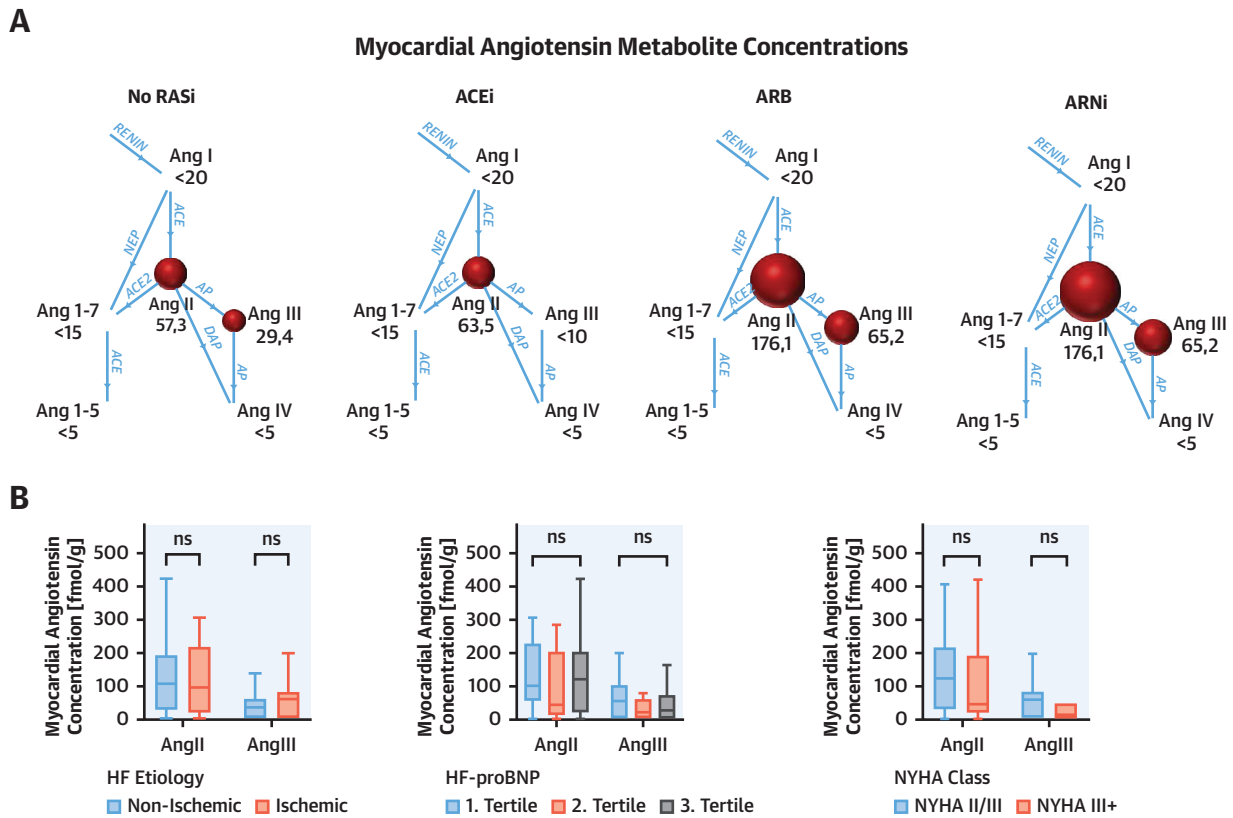
patients were unable to tolerate RAS inhibitors, whereas 28 received an ACE inhibitor, 8 received an ARB, and 7 patients were on an ARNi. There were no substantial differences between the 4 treatment groups regarding age, sex, HF etiology, NYHA functional class, and laboratory findings. Notably, neurohumoral dysregulation reflected by NT-proBNP and ARC was comparable between the different treatment modalities. **Figure 1** displays NT-proBNP and ARC levels together with characteristic myocardial histological images for the respective groups.

**ARB and ARNi are associated with higher myocardial AngII concentrations, and Ang1-7 is not detectable in cardiac tissue.** Angiotensin metabolite concentrations stratified to RAS inhibitor background therapy are displayed in **Figure 2A**, and exact numerical values of peptide concentrations are listed in **Supplemental Table 1**. Only AngII and AngIII were measurable at meaningful concentrations in the failing heart, whereas the concentrations of tissue AngI, Ang1-7, Ang1-5, and Ang3-8 were below the detection limit for almost all samples (AngII was detectable in 47 [90%], AngIII in 32 [62%], AngI and Ang1-7 in 3 [6%], Ang3-8 in 2 [4%],

and Ang1-5 in 1 [2%] samples). Patients receiving either therapy with an ARB component (i.e., ARB or ARNi) had significantly higher levels of both of these angiotensin metabolites compared with patients receiving either no RAS inhibitor or an ACE inhibitor (AngII: 242 fmol/g [IQR: 145.7 to 409.9 fmol/g] vs. 63.0 fmol/g [IQR: 19.9 to 124.1 fmol/g];  $p < 0.001$ ; and AngIII: 87.4 fmol/g [IQR: 46.5 to 165.3 fmol/g] vs. 23.0 fmol/g [IQR: <5.0 to 59.3 fmol/g];  $p = 0.002$ ). Myocardial AngII and AngIII concentrations were independent from HF etiology (AngII: 108.2 fmol/g [IQR: 29.3 to 193.2 fmol/g] vs. 96.5 fmol/g [IQR: 27.2 to 216.8 fmol/g];  $p = 0.823$ ; and AngIII: 37.0 fmol/g [IQR: <5.0 to 62.0 fmol/g] vs. 61.0 fmol/g [IQR: <5.0 to 77.9 fmol/g];  $p = 0.508$  for nonischemic vs ischemic HF), NT-proBNP ( $p = \text{NS}$  for the comparison between NT-proBNP tertiles for both AngII and AngIII), and NYHA functional class ( $p = \text{NS}$  for both AngII and AngIII) (**Figure 2B**). Cardiac AngII and AngIII showed an excellent correlation with each other ( $r_s = 0.87$ ,  $p < 0.001$ ).

**Systemic RAS inhibition does not alter myocardial RAS enzymatic activities or the metabolization of AngI, AngII, and Ang1-7.** **Figure 3A** displays the myocardial activities of RAS enzymes. We observed no

**FIGURE 2** Myocardial Tissue RAS Fingerprints of the Failing Heart



**(A)** Mass spectrometry-based determination of angiotensin metabolites and **(B)** difference in angiotensin II (AngII) and AngIII levels according to heart failure (HF) etiology and severity. For RAS, fingerprint numbers in brackets indicate the specific angiotensin peptides. Size of spheres and numbers beside represent absolute concentrations of angiotensins (fmol/g) (median value) analyzed by mass spectrometry. For the comparisons, AngII and AngIII levels of the different groups are shown as boxplots, and comparisons were made by the Kruskal-Wallis test and p values are indicated in the graph. NYHA = New York Heart Association; other abbreviations as in [Figure 1](#).

differences in the enzymatic activities of tissue chymase, ACE, NEP, PEP, ACE2, and PCP depending on background RAS inhibitor therapy ( $p = \text{NS}$  for comparison between all groups). Tissue chymase was the main enzyme for intracardiac AngII formation from AngI, whereas ACE activity was nondetectable in the myocardium. NEP and PEP had an equally significant role in the conversion of AngI to the beneficial Ang1-7 peptide. PCP, but not ACE2, was responsible for the breakdown of AngII to generate Ang1-7. The enzymatic activities of chymase, NEP, PEP, and PCP were also uninfluenced by intracardiac AngII concentrations ([Figure 3B](#)).

**Relationship between circulating and myocardial tissue RAS: Myocardial AngII is independent from plasma renin but increases with circulating AngII concentrations; myocardial AngIII is characteristic for the tissue site.** For visual comparison, circulating and myocardial RAS fingerprints

are displayed in [Supplemental Figure 1](#). Although the main angiotensins in plasma are AngI and AngII lacking AngIII, the myocardium displays minute amounts of AngI and highest concentrations of AngII and AngIII. The relationship between circulating and myocardial RAS is displayed in [Figure 4](#). Plasma renin concentrations correlated excellently with circulating angiotensin levels as expected ( $r_s = 0.90$ ,  $p < 0.001$  for the sum of angiotensins;  $r_s = 0.89$ ,  $p < 0.001$  for AngI+AngII). Circulating AngII levels were closely related to renin levels for the no RAS inhibitor, ARB, and ARNi groups ( $r_s = 0.86$ ,  $p < 0.001$ ), while ACE inhibitor patients had either no detectable or low levels of circulating AngII due to impaired activity of ACE. In contrast, myocardial angiotensin concentrations were independent from plasma renin levels. Plasma renin concentrations correlated neither with the sum of peptides nor with (AngI+AngII) or AngII

( $p = \text{NS}$  for both). Similarly, none of the myocardial RAS enzymes displayed an association with plasma renin ( $p = \text{NS}$  for all). Nonetheless, circulating and tissue AngII displayed a significant positive correlation between each other ( $r_s = 0.71$ ,  $p < 0.001$ ).

## DISCUSSION

This is the first report on detailed angiotensin profiles and RAS regulation of the myocardium of the failing heart under state-of-the-art HF therapy. Our data demonstrate that among the spectrum of different angiotensin peptides, mainly AngII and AngIII are detectable in the myocardium; myocardial AngII levels significantly depend on circulating AngII levels, yet cardiac concentrations are generally higher; RAS blockade by ARB or ARNi results in higher myocardial AngII and AngIII concentrations compared with no RAS inhibitor or ACE inhibitor; the generation of AngIII seems tissue specific; cardiac NEP might be involved in myocardial Ang1-7 synthesis, given that substrate is present; and therapy with currently used RAS inhibitors does not result in altered myocardial RAS enzyme activities (**Central Illustration**). The results underline the importance of pharmacologic interventions targeting systemic RAS aiming to reduce circulating AngII actions, yet also imply that current medical therapy does not necessarily affect myocardial tissue RAS regulation, which shows unique properties, thereby offering room for extended therapeutic interventions.

**INTRODUCING MYOCARDIAL TISSUE RAS.** Initially RAS was thought to act in an exclusively systemic way, which was challenged by the discovery that most RAS components can be synthesized locally at tissue sites, including the heart (22). The myocardium not only is equipped with renin, ACE, and AngII binding sites (23), but also expresses renin, ACE, and AngII, suggesting the presence of a local RAS with adaptive autocrine or paracrine effects. It seems that the circulating RAS is stimulated acutely and may be quickly turned off after cardiovascular compensation, although tissue RAS exerts long-term actions affecting cardiovascular structure and function (24). It has been proposed that cardiac AngII and ACE play an integral role in myocardial tissue repair and fibrosis after myocardial injury (23). Moreover, ACE inhibitor and ARB exert tissue protective effects preventing fibrosis not only in the heart, but also in other injured tissues in which circulating RAS is not necessarily activated (23). Overactivation of intracardiac RAS has been associated with cardiac hypertrophy, HF, and myocardial injury due to coronary

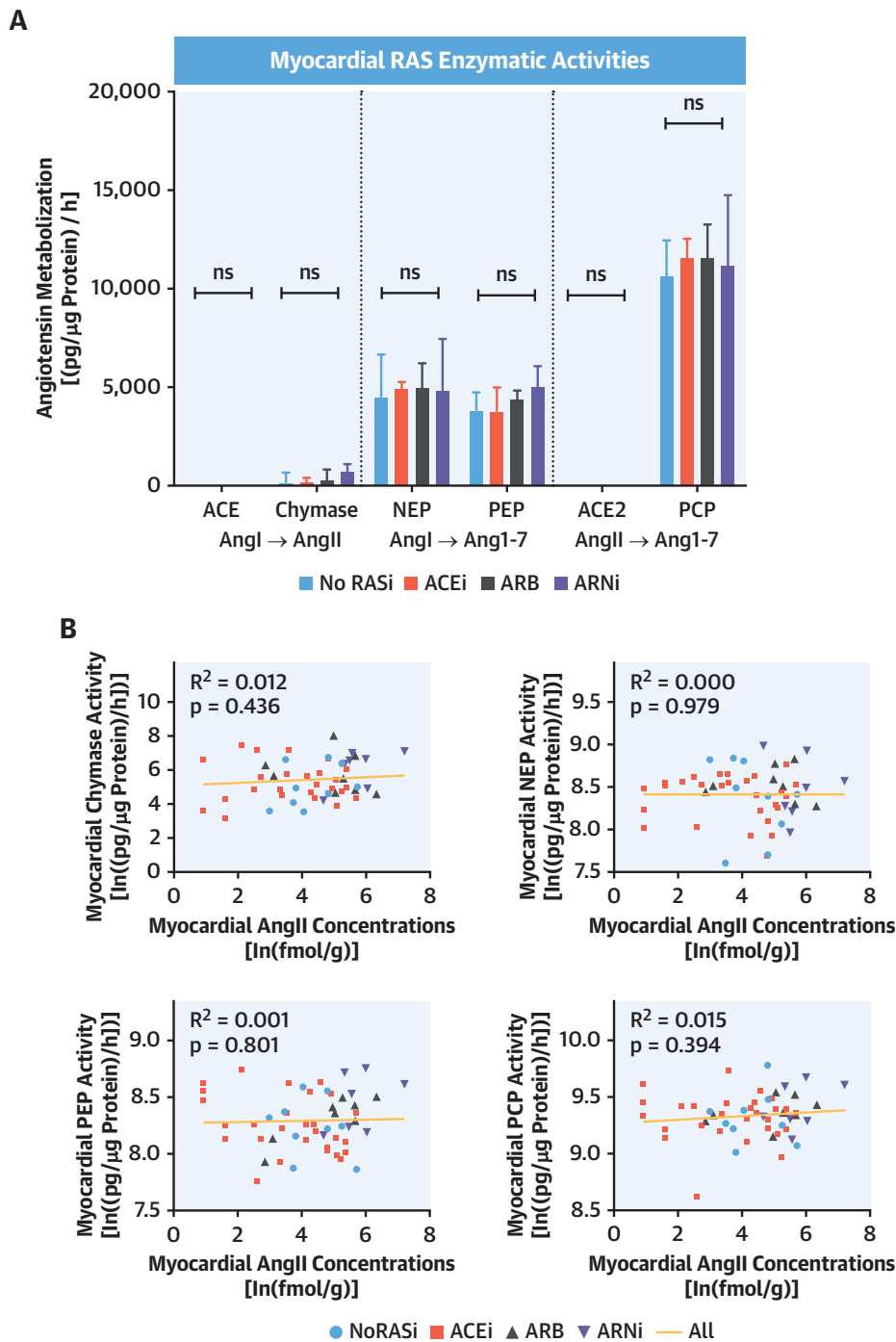
disease (25). Also, acute hemodynamic responses to RAS inhibitors may be predicted by pretreatment plasma renin activity, whereas long-term responses may not. ACE inhibitors have also been shown to be beneficial for pathologic states in absence of an activation of circulating RAS (26). Especially, ACE inhibitors are beneficial in HFrEF patients with normal plasma renin activity (27), which is particularly important because the majority of HFrEF patients display low to normal RAS activation conflictive to the expected neurohumoral dysregulation and renin feedback due to RAS-blocker treatment (20).

HFrEF treatment with enzyme inhibitors and receptor blockers of RAS affect the angiotensin balance in a complex manner (28). There are several studies investigating the behavior of circulating RAS in HF, and indeed, ACE inhibitor, ARB, and ARNi result in characteristic RAS fingerprints of circulating RAS (20). In contrast, cardiac organ-specific regulation under state-of-the-art therapy still remains unclear, though it could considerably affect therapy success of RAS inhibitors.

## MYOCARDIAL TISSUE RAS PEPTIDES IN HF: AngII AND AngIII ARE THE DOMINANT CARDIAC ANGIOTENSINS.

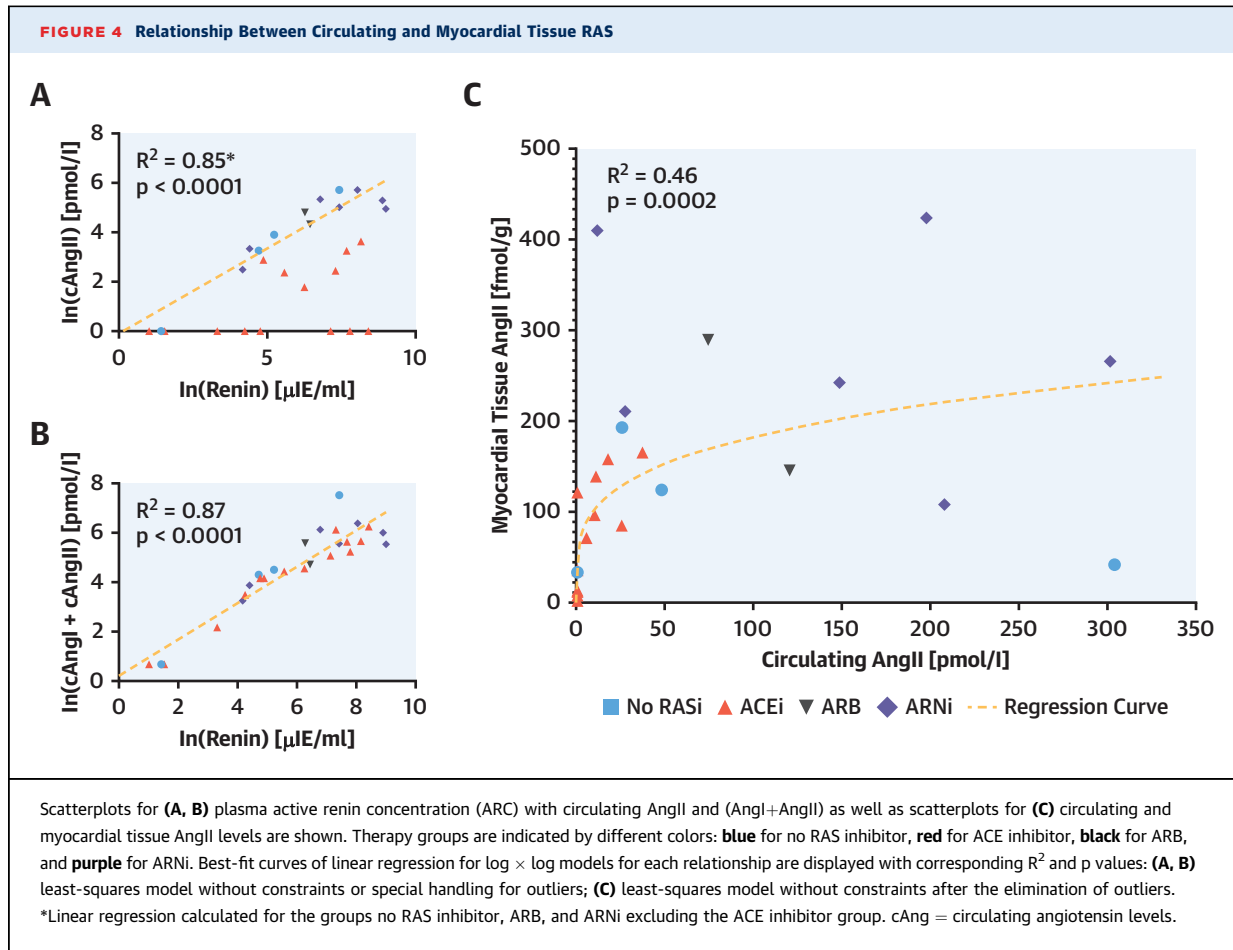
Tissue RAS may be activated in patients with HF, leading to an alternate ACE-independent synthesis of AngII mediated by tissue chymase (1). Indeed, myocardial tissue chymase activity was elevated in failing human hearts compared to controls and correlated with tissue AngII concentrations (19). Tissue chymase was also the main enzyme involved in AngII production in right ventricular samples of donor hearts after HTx (29). In the dilatative phenotype of HF, cardiac AngII was described to be lower in patients receiving an ACE inhibitor (19). At the same time, neither systemic treatment with an ACE inhibitor nor addition of an ACE inhibitor to cardiac tissue homogenates did alter myocardial AngII formation rates in right ventricular samples of transplanted patients (29). Our data show indeed high AngII levels in myocardial samples of failing hearts, although AngII concentrations were independent from the etiology and neurohumoral activation. Alongside AngII, also AngIII levels were detectable at considerable concentrations in the failing hearts, but there is no evidence for the presence of the other angiotensin metabolites, particularly nondetectable levels of beneficial Ang1-7. RAS inhibitor treatment with an ARB component (i.e., ARB or ARNi) in general was associated with increased levels of myocardial AngII and AngIII compared with no RAS inhibitor and ACE inhibitor, in both ischemic and nonischemic HF.

**FIGURE 3** Myocardial Tissue RAS Regulation of the Failing Heart



**(A)** Mass spectrometry-based determination of the enzymatic activities for the main RAS enzymes involved in the metabolism of Ang1-7, AngI, and AngII (i.e., ACE, chymase, [NEP], prolyl endopeptidase [PEP], ACE2, and prolyl carboxypeptidase [PCP]) according to background RAS inhibitor therapy (no RAS inhibitor: n = 9; ACE inhibitor: n = 28; ARB inhibitor: n = 8; and ARNi: n = 7). **(B)** Scatterplots to illustrate the relationship between myocardial AngII levels and enzymatic activities. Enzymatic activities for the respective reaction are shown as boxplots, and comparisons were made by the Kruskal-Wallis test. For the scatterplots, AngII levels and enzymatic activities were log-transformed. Linear regression lines were displayed. Spearman's correlation coefficient and the respective p values are indicated in the figures. Abbreviations as in Figure 1.



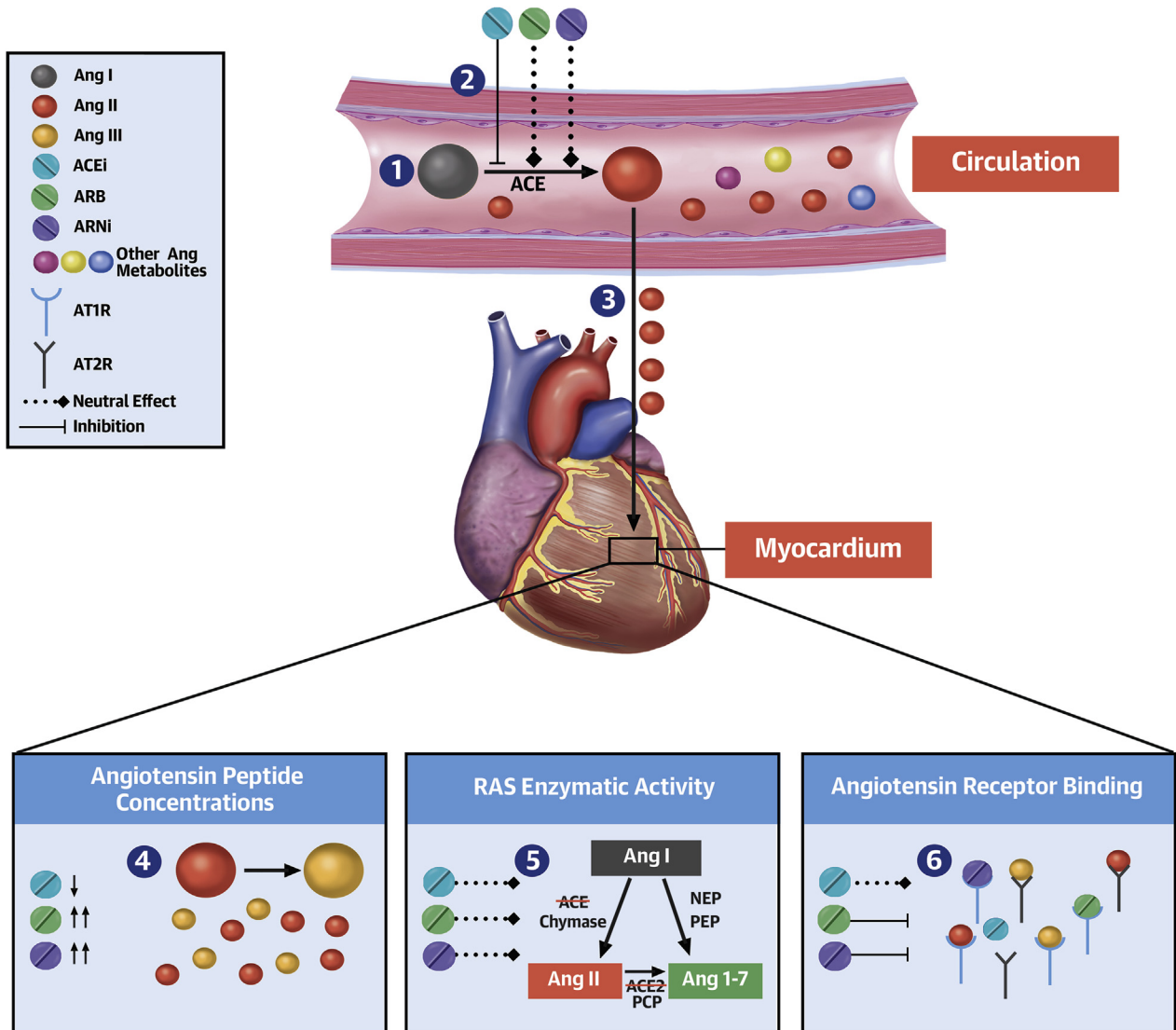


**MYOCARDIAL TISSUE RAS ENZYMES IN HF: NEP, TISSUE CHYMASE, PCP, AND PEP REMAIN UNINFLUENCED BY RAS INHIBITOR THERAPY.** Tissue chymase was the dominant enzyme for the generation of AngII in myocardial tissue, whereas there was no trace of ACE activity. NEP and PEP play both a significant role in the formation of Ang1-7 from AngI. PCP but not ACE2 was the main enzyme to directly shift the balance to enhance the alternate RAS by mediating the breakdown of AngII to Ang1-7. Given that in the myocardium only AngII and AngIII are present, the activity of PCP could be especially crucial, whereas the physiological role of tissue chymase, NEP, and PEP for AngII and Ang1-7 formation remain unclear, as the availability of the substrate AngI is uncertain. In contrast to cardiac angiotensin patterns, we could not observe any difference in local cardiac tissue RAS enzyme regulation depending on the RAS blocker therapy used, especially similar activities of tissue chymase. This suggests that elevated levels of myocardial AngII in ARB or ARNi patients may not solely be explained by altered tissue

chymase activity. Most importantly, also the activity of cardiac NEP remained uninfluenced by the mode of RAS inhibition, especially the use of an ARNi. It seems that while currently used RAS inhibitors result in typically altered profiles of the circulating RAS, they do not succeed to significantly influence myocardial AngI, AngII, and Ang1-7 metabolism patterns.

**CIRCULATING VERSUS MYOCARDIAL TISSUE RAS IN HF: AngII IS SUPPLIED FROM THE CIRCULATION, AngIII IS GENERATED IN A TISSUE-SPECIFIC MANNER.** Circulating RAS patterns were characteristic for patients with different RAS inhibitor therapy with a suppressed AngII-to-AngI ratio for patients receiving an ACE inhibitor and allowing higher plasma levels of AngII with an ARB or ARNi, owing to undisturbed formation of AngII from AngI by ACE, as described previously (20). Although plasma renin levels correlated excellently with the sum of circulating angiotensin levels, we could not observe any significant dependence of myocardial tissue RAS regulation (i.e., angiotensin levels and enzymatic

**CENTRAL ILLUSTRATION** The Influence of Current State-of-the-Art RAS Blockade on Myocardial Angiotensin Metabolites and RAS Enzyme Regulation



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1. The predominant angiotensin metabolites of the circulation are apparently angiotensin (Ang) I and AngII, while AngIII is largely undetectable. 2. The use of current heart failure therapy (i.e., angiotensin-converting enzyme [ACE] inhibitor, angiotensin receptor blocker [ARB], and angiotensin receptor neprilysin-inhibitor [ARNi]) results in characteristic circulating AngI/AngII ratios, whereas AngII levels are markedly reduced with ACE inhibitor (inhibiting AngII formation) and remain uninfluenced by ARB/ARNi (which allow undisturbed AngII formation but aim to block AngII actions at the AT1R receptor level). 3. Myocardial AngII correlates with circulating AngII. 4. In contrast to circulating RAS, the myocardium generally lacks AngI but has high concentrations of AngII and AngIII, suggesting accumulation of AngII and tissue specific conversion of AngII into AngIII. ARB/ARNi result in generally higher cardiac AngII/AngIII concentrations compared to ACE inhibitor patients. 5. There is no trace of ACE or ACE2 activity in the myocardium. Intriguingly, renin-angiotensin system (RAS)-blocker therapy does not affect other myocardial RAS enzyme activities, i.e. tissue chymase, neprilysin, prolyl carboxypeptidase, or prolyl endopeptidase. 6. AngIII shows similar properties and receptor affinities to AngII type 1 receptor and AngII type 2 receptor as AngII. AT1R should be blocked by ARB/ARNi.

activities) on active plasma renin levels. These findings suggest that the regulation of myocardial tissue RAS is independent from direct effects of plasma renin.

The comparison of circulating and myocardial RAS patterns suggests not only that myocardial AngII levels highly depend on circulating AngII, resulting in elevated cardiac AngII concentrations in patients receiving RAS blockade other than ACE inhibitor, but also that AngII accumulates within the myocardium, showing higher concentrations locally than within plasma. Tissue chymase may contribute to locally elevated AngII levels. The absence of AngIII in plasma and the strong correlation between myocardial AngII and AngIII imply a tissue-specific formation of AngIII from AngII. There seems to be a saturation of cardiac AngII with increasing plasma AngII levels, suggesting that cardiac AngII is not freely distributed in the myocardium, but rather is associated to molecular structures as receptors, which also limit cardiac AngII concentrations. AngIII has a similar receptor affinity toward AT1R and AT2R as AngII, and a number of studies have suggested that AngIII and AngII are equipotent at eliciting undesirable cardiovascular effects and pressure response (5,6). AngIII is not yet appreciated sufficiently as a major RAS player, although certain local AngII effects could be attributed to AngIII as claimed by the “AngIII hypothesis” (30). Aminopeptidase A is thought to be responsible for the conversion of AngIII from AngII. Specific aminopeptidase A inhibitors seem to attenuate central AngII actions and are currently tested as antihypertensive agent (31). The inhibition of aminopeptidase A also prevented cardiac dysfunction after myocardial infarction in rodents (32). Concerning high intracardiac levels of AngII and AngIII, it may be that these peptides accumulate at the angiotensin receptor sites, which was also suggested by experiments investigating the RAS of the central nervous system (33). However, the pathophysiologic relevance of this finding in the presence of an ARB or ARNi remains uncertain, as ARBs block AT1R, yet probably also up-regulate angiotensin receptors, and the exact localization of the peptides regarding cellular compartments needs to be investigated. Even beneficial effects could be projected by enhanced AngII and AngIII binding on other receptor subtypes as AT2R.

The nondetectable concentrations of Ang1-7 and other angiotensin metabolites suggest either that these peptides are subjected to a high turnover rate or might not be retained by the myocardium and enter the circulation, or that alternate RAS might be

significantly down-regulated in cardiac tissue in HF or that this axis has limited role in cardiac tissue generally. Importantly, an ARNi does not influence Ang1-7 concentrations significantly in the myocardium, although production can be regarded as strongly NEP dependent.

**ACE2, THE POPULAR RECEPTOR.** ACE2 has gained much attention as a receptor of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), facilitating viral entry into its host cells, predominantly alveolar cells. COVID-19 (coronavirus disease 2019) patients with cardiovascular disease display an especially high mortality rate (34). Besides the presence of the comorbidity itself, also the frequent use of RAS blockers in these patients has been proposed to be associated with a more severe disease course through a putative up-regulation of ACE2. A considerable proportion of patients with COVID-19 also develop cardiac injury, which is linked to an increased in-hospital mortality (35). Similarly, cardiac ACE2 has been proposed to be involved in SARS-CoV-2-related myocardial injury (36). Additionally, AngII actions have been shown to be responsible for the exacerbation of CoV-related lung injury, which again could favor RAS blockade, a concept that should be equally applicable for the myocardium (37). Our data do not support the hypothesis of RAS inhibitor-mediated cardiac tissue RAS alteration and ACE2 up-regulation, but show considerable levels of AngII in the myocardium. Both findings rather encourage the continuation of RAS inhibitor therapy in severe HF in order to minimize cardiac complications.

**STUDY LIMITATIONS.** Statistical analysis of myocardial tissue RAS would naturally benefit from larger sample sizes, which is, however, limited by the relatively small number of patients receiving HTx and the costs of the method. Hearts were not perfused with saline prior to performing biopsy so that minute amounts of blood could be trapped. Myocardial specimens were homogenized in toto, yet characterization and separation of cell types or compartments (e.g., membrane preparations) would allow more exact conclusions about the source of angiotensins and generating enzymes.

## CONCLUSIONS

The RAS should be regarded as both a circulating and cellular organized hierarchical angiotensin network linked by characteristic enzymatic reactions. Myocardial RAS enzyme regulation is mainly independent from that of the circulating RAS, including plasma renin and current RAS inhibitor therapy.

Especially, treatment with ARNi does not alter myocardial NEP activity. The failing heart is characterized by high activity of classical RAS reflected by elevated concentrations of the AngII and AngIII peptides. AngII seems to be supplied from the circulation and might also be generated locally, whereas AngIII is converted from AngII in a tissue-specific manner. Both peptides accumulate within the myocardium compared with the circulation probably by binding to the ATR receptors. AngIII is possibly involved in mediating detrimental effects on cardiovascular structure at the same extent as AngII, while Ang1-7 and other metabolites remain undetectable. Advanced HF patients receiving an ARB or ARNi display elevated levels of cardiac AngII/AngIII compared with no RAS inhibitor or ACE inhibitor, probably due to elevated circulating AngII concentrations by ongoing ACE activity, while AngII actions may be blocked by ARB on the receptor level. Tissue chymase might be the main enzyme responsible for cardiac AngII formation. The role of PCP could be crucial, as it is directly involved in the breakdown of AngII to form Ang1-7. In contrast to circulating RAS, ACE and ACE2 have no role in cardiac tissue-specific angiotensin metabolism. Future cardiac tissue-specific RAS drugs should aim to further limit not only AngII, but also AngIII actions; inhibit tissue chymase activity; favor AngII/Ang1-7 conversion by enhancing PCP activity; and inhibit formation and enhance breakdown of AngIII, all of which presently remain uninfluenced by HF treatment.

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**ADDRESS FOR CORRESPONDENCE:** Dr. Noemi Pavo, Department of Cardiology, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria. E-mail: [noemi.pavo@meduniwien.ac.at](mailto:noemi.pavo@meduniwien.ac.at).

#### PERSPECTIVES

##### COMPETENCY IN MEDICAL KNOWLEDGE:

Currently used systemic inhibitors of the RAS do not affect RAS enzyme regulation in the myocardium, nor is cardiac neprilysin activity influenced by ARNi.

##### TRANSLATIONAL OUTLOOK:

Future studies should examine the effects of cardiac-specific RAS inhibitor drugs on myocardial tissue levels of angiotensin peptides AngII and AngIII in patients with advanced heart failure.

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**KEY WORDS** angiotensin, angiotensin receptor neprilysin inhibition, ARNI, heart failure, RAS, renin, renin-angiotensin system

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**APPENDIX** For a supplemental figure and table, please see the online version of this paper.