SHORT TEHSIS FOR THE DEGREE FOR DOCTOR OF PHILOSOPHY (PhD)

Role of the circulating angiotensin-converting enzyme 2 in cardiovascular pathologies

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The Examination takes place at Division of Clinical Laboratory Science, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, March 28, 2017 11:00

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1. Introduction and literature review

Cardiovascular diseases are the leading cause of death both in developing and developed countries. According to World Health Organization data in 2008, about 17.3 million people died in cardiovascular disease and till 2030, the estimated value will be more than 23.3 million.

Today, the prevalence of heart failure in Hungary 1.6%, which is approximately 160 thousand heart failure patients, the incidence varies between 30-40 thousand.

Heart failure (HF) is a complex clinical syndrome that can create structural or functional cardiac disorder that damages the left ventricular systolic and / or diastolic function.

Dazu and Braunwald formed image from cardiovascular continuum given the first overview about the pathophyiology of heart failure. The cascade of cardiovascular disease starts with risk factors like hypertension, vascular injury, tissue injury, cardiac and vascular remodeling. They are followed by the appearance of organ disorders.

A subset of these risk factors leading to coronary calcification and myocardial infarction. Despite aggressive coronary revascularization systolic heart failure occurs with the serious consequences of left ventricular systolic function damage (HFrEF).

In a significant proportion of patients, the same risk factors and the existence of the same symptoms of heart failure leading to left ventricle diastolic function damage (HFpEF). It is still unclear to what could be the precise moment in the development of cardiovascular disease chain, where the two types of heart failure pathophysiology splits.

Since cardiovascular disease is still the leading cause of death and we do not even have sepcific biomarkers, which could be comletely useful in the diagnosis and disease monitoring, therefore we examined the association between the cardiac failure and the angiotensin converting enzyme 2 (ACE2) activity in large population.

1.1. Pathophysiology of the two types of heart failure

Based on our current knowledge, heart failure associated with reduced ejection fraction (heart failure with reduced ejection fraction - HFrEF), also known as the systolic heart failure, considered as a heart syndrome, which starts with the direct damage of the heart muscle cells.

In contrast, heart failure with preserved ejection fraction (heart failure with preserved ejection fraction - HFpEF), also known as diastolic heart failure is a systemic metabolic syndrome, the cardiovascular risk factor whose responsible for the formation.

We can therefore say that we are talking about two different syndrome where one starts from the heart and leads to peripheral devices (HFrEF) on the other peripheries to the starting point and the heart leads (HFpEF).

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1.2. Pathophysiology of HFrEF

In systolic heart failure, the heart muscle cell death will trigger the left ventricular remodeling. Various forms of cardiac necrosis may appear, for example. autophagy, apoptosis or necrosis, which has the common leading cause: the poresence of oxidative stress in myocytes. The root causes are usually ischemia, infection or toxic hazards. Whatever the cause, the most important consequences:

Activation of the sympathetic nervous system, RAAS activation, disregulation of the vasoactive agents, predominance of the role of vasoconstrictor molecules.

Fibrotic areas are formed, which causes the left ventricular eccentric remodelling. In advanced systolic heart failure both systemic and coronary endothelial dysfunction is present, which further undermining the function of the left ventricle.

1.3. Pathophysiology of HFpEF

In HFpEF the cardiovascular risk factors and preferentially obesity generate a systemic inflammation in the body. Because of the proinflammatory status coronary microvessel endothelial cells are produced reactive oxygen species (ROS), which reduce the usefulness of nitric oxide (NO), whereby the activity of cardiac protein kinase G (PKG).

The low PKG activity induce concentric left ventricular remodeling and by titin hypophosporylation enhanced the cardiomyocyte passive tension. The rigid myocardial cells and hypertrophy are both caused left ventricular dysfunction disfunction. The most important co-morbidities are overweight / obesity, high blood pressure, diabetes, chronic obstructive pulmonary disease, anemia, chronic kidney disease. Co-occurrence all these disease, may increase the chance of the occurrence of systemic inflammation.

1.4. Pharmaceutic treatment

Nowadays the aim of the heart failure therapy not only to suppress signs and symptoms, but also to reduce or prevent of disease progression, reduce the number of hospitalizations and to prolong the survival of disease. Come into the focus of treatment beta-receptor blockers, ACE inhibitors acting on RAAS, ARBs and aldosterone antagonists and diuretics. Based on guidelines the combination of these drugs are the basis of the currently valid heart failure treatment.

Subsequent studies or analyzes of previous studies over the past few years, drew attention to the way that which significantly reduces mortality / morbidity in HFrEF, its effectiveness is far behind in HFpEF. It added to the assumption that RAAS activation was probably not crucial in the pathophysiology of HFpEF.

1.5. B-type natriuretic propeptide in heart failure

Cardiology diagnosis use various biomarkers, such as B-type natriuretic peptide (BNP) or the precursor N-terminal proBNP, which are elevated during classical systolic heart failure. On the other hand, the diastolic heart failure patients had significantly lower NT-proBNP levels than in systolic heart failure. Among diastolic heart filure every third patients has normal BNP levels, so it can not be exclude the diastolic patients based on this parameter so left ventricular ejection fraction remains the best option is to distinguish between the two groups.

1.6. The role of the renin-angiotensin-aldosterone system (RAAS) and ACE2

The renin–angiotensin system (RAS) is a central regulator of cardiovascular and renal functions and plays an important role in the pathophysiology of heart failure. Angiotensin converting enzyme 2 (ACE2) is a recently discovered homologue of ACE. It is a monocarboxypeptidase generating Ang-(1-9) from Ang-I and Ang-(1-7) from Ang-II. Ang-(1-7) is a biologically active metabolite of the RAS acting through the G-protein-coupled Mas receptor. Ang-(1-7) is capable of reducing myocardial oxidative stress and pathological remodeling. Mas receptor can hetero-oligomerize with AT1R acting as a physiological antagonist of AngII.

In the heart, ACE2 is expressed in various cell types including fibroblasts, cardiomyocytes and endothelial cells. Although ACE2 is a plasma membrane-bound ectoenzyme, a soluble active form of the protein was also found in plasma and urine. Tumor necrosis factor alpha converting enzyme (TACE/ADAM17) is the sheddase responsible for the ectodomain cleavage and shedding of ACE2.

Opposite to the AngI-ACE-AngII-AT1R pathway ACE2 may provide a vasoprotective/antiproliferative mechanism resulting in the counter-regulation of the RAS. In accordance, previous animal data have shown that transgenic ACE2 overexpression attenuates hypertension. Suppression of ACE2 expression again established it as a negative regulator of the RAS in blood pressure control. Moreover, ACE2 polymorphisms were related to hypertension in different human populations. Nonetheless, the expression and activity of ACE2 in human hypertension has not been addressed directly yet.

In contrast to hypertension, ACE2 has already been studied in animal and human HF suggesting a protective role for this enzyme. Targeted disruption of ACE2 in mice results in severe cardiac contractility defect, increased plasma and heart AngII levels leading to cardiac dysfunction. Absence of ACE2 causes stress activation of the myocardial NADPH oxidase system and leads to severe adverse myocardial remodeling and dysfunction. It was suggested that myocardial ACE2 gene expression is increased in patients with left ventricular dysfunction and TACE is also upregulated in HF. Loss of ACE2 worsened the pathological remodeling and resulted in a rapid progression to systolic dysfunction and HF.

Epelman et al. showed that increased soluble ACE2 activity is associated with more advanced HF and that elevated ACE2 activity could predict adverse cardiac events. Lehmann et al. recently observed higher soluble ACE2 activity in HF-patients experiencing ventricular arrhythmias and appropriate defibrillator-intervention. Whether these considerable correlations make soluble ACE2 activity suitable as a novel biomarker of heart failure is still not settled.

2. Aims

The aim of my scientific research were the followings:

- to detect the circulating ACE2 activity changes in systolic heart failure patients with improved left ventricular pumping function,
- to describe the serum ACE2 activity in hipertensive, diastolic and systolic heart failure patients,
- to identify relationship between serum ACE2 and ejection fraction,
- to validate the association between serum ACE2 and NT-proBNP changes,
- to explore the clinical applicability of serum ACE2 activity as a biomarker for left ventricular systolic dysfunction in human heart failure.

3. Patients and methods

3.1. Study population

There were four study groups. 45 healthy individuals without any cardiovascular pathology or medication, with normal cardiac morphology and with left ventricular EF above 50% were enrolled.

A second group of 239 hypertensive patients (systolic blood pressure above 140 mmHg and/or diastolic blood pressure above 90 mmHg at the time of the diagnosis of the disease) was established without any sign and symptom of HF. This group was characterized by preserved ejection fraction (above 50%) besides optimal antihypertensive therapy according to the European guidelines.

A third group of 141 patients with severe left ventricular systolic dysfunction with reduced ejection fraction (HFrEF) with indication of cardiac resynchronization therapy (CRT) were also enrolled into the study (HFrEF - CRT before). Patients were selected for CRT according to the current ESC guideline related to pharmaceutical and device therapy of systolic heart failure. Till the date 65 patients fulfilled the first visit between 6 and 9 months (HFrEF - CRT after). Significant mitral regurgitation was present in 22 patients, enabling dP/dt measurements.

Using a left ventricular ejection fraction (EF) cutoff of 50% 47 HF-patients showed evidence of left ventricular diastolic dysfunction with preserved systolic function (HFpEF) was also enrolled to the study.

Clinical assessment comprised age, sex, blood pressure, presence of hypercholesterolemia, diabetes mellitus and atrial fibrillation, besides to other minor parameters. Each visit included echocardiographic measurements and blood sample collection for biochemical measurements. Biochemical analyses comprised of serum ACE activity and concentration, serum ACE2 activity, amino-terminal pro-B-type natriuretic peptide (NT-proBNP) concentration measurements. Medical reports and medication history were obtained from all patients. Examinations were performed at the enrollment (healthy group) and at regular visits at the

outpatient ward of the Department of Cardiology, University of Debrecen (hypertensive, HFrEF, HFpEF groups).

3.2. Ethical approval

All of the studies were approved by the Ethical Committee of the Medical and Health Science Center at the University of Debrecen (UDMHSC REC/IEC: 3261-2010) and the Hungarian Medical Research Council. All of teh individuals involved gave their written informed consent.

3.3. Echocardiographic measurements

Transthoracic echocardiography was performed using Accuson Sequoia (Siemens AG, Germany) or Vivid E9 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) ultrasound machines. Cardiologists with specialized echocardiography training (blinded to the biomarker analyses) interpreted the echocardiograms. Left ventricular ejection fraction (EF) was measured using Simpson's biplane method of disks. Cut-off for normal (preserved) EF was >50%. In case of patients with signs or symptoms of HF and with preserved EF, complementary echocardiographic studies for assessment of diastolic dysfunction consisted of blood flow and tissue Doppler measurements.

3.4. Blood sample collection

Standard aseptic technique was used for blood sample collection. Native blood samples were incubated for 60 minutes at room temperature. Serum fractions were separated by centrifugation (1,500 *g*, 15 min) and kept in a freezer (-20 ºC) until the measurements.

3.5. Measurement of serum angiotensin converting enzyme (ACE) activity

Assessment of ACE activity was based on the spectrophotometric measurement of FAPGG hydrolysis [28] [29]. The reaction mixture (200 µL) contained 50 µL of serum, 0.5 mM FAPGG **(**N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine) (Sigma, St. Louis, MO, USA) substrate, 300 mM sodium chloride, and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 8.2. Measurement of ACE activity is based on the change in the absorption at 340 nm when FAPGG hydrolyzed to furylacryloyl-L-phenylalaline (FAP) and glycylglycine (GG). The reaction was performed in 96-well plates (Greiner Bio-One, Frickenhauser, Germany). Changes in FAPGG absorbance were detected using a microplate reader (NOVOstar; BMG Labtech GmbH, Offenburg, Germany). Hydrolysis of FAPGG by ACE was recorded in every 5 minutes at 37 C°. Optical density values were plotted as a function of reaction time and fitted by linear regression. The fit and the data were accepted when r >0.9. ACE activity was calculated by the equation:

ACE activity= -(S/k)*D,

where S is the rate of decrease in optical density (1/min), k is the change in optical density upon the complete cleavage of 1 nmol of FAPGG, and D is the dilution of the serum. One unit (U) of ACE activity represents 1 nmol of FAPGG hydrolysis per minute at 37 °C.

3.6. Measurement of serum ACE2 activity

ACE2 activity measurement was performed using a specific quenched fluorescent substrate as previously described with some modification. The reaction mixture (200 µl) contained 20 µl serum, 80 µl buffer and 100 µl (50 µM) ACE2-specific fluorescent substrate (7-methoxycoumarin-4-yl)acetyl-Ala-Pro-Lys(2,4-dinitrophenyl)-OH [Mca-APK(Dnp)] (EZ Biolab, Carmel, USA). ACE2 activity was assessed by the change in fluorescence intensity upon the enzymatic cleavage of K(Dnp) from the Mca-APK(Dnp).

The reaction buffer contained protease inhibitors (10 μM Bestatin-hidrochloride, 10 μM Z-prolyl-prolinal, (Enzo Life Science, Exeter, UK), 5 μM Amastatin-hidrochloride, 10 μM Captopril in a buffer of 500 mM NaCI, 100 μM $ZnCI_2$**,** 75 mM TRIS HCI, pH 6.5. All chemicals were from Sigma (St. Louis, MO, USA) if not stated otherwise.
The reaction was performed in black 96-well microtiter plates (Greiner Bio-One, Frickenhauser, Germany). The assay was monitored continuously by measuring the increase in fluorescence (excitation wavelength: 320 nm, emission wavelength: 405 nm) upon substrate hydrolysis using a fluorescence microplate reader (NOVOstar; BMG Labtech GmbH, Offenburg, Germany). Initial enzyme activities were determined from the linear rate of fluorescence increase over the 0-120 min time course. The increase in fluorescence was plotted as a function of reaction time and fitted with a linear regression.
ACE2 activity was calculated by the equation:

ACE2 activity=(S/k)*D,

Where *S* is the rate of increase in fluorescence intensity, *k* is the change in fluorescence intensity upon the complete cleavage of 0.1 nmol of Mca-APK(Dnp), and *D* is the dilution of the serum. 1 unit (U) corresponds to 0.1 nmol Mca-APK(Dnp) cleavage in 1 hour at 37 °C. The specificity of the ACE2 enzyme activity assay was tested by the specific human ACE2 inhibitor DX600, which resulted in a complete inhibition of Mca-APK(Dnp) cleavage. Fits were accepted when r >0.95.

3.7. Measurement of serum ACE concentration

ACE concentration was determined using a Human ACE enzyme-linked immunosorbent assay (ELISA) Development System (catalog No. DY929; R&D System, Inc, Minneapolis, USA) according to the manufacturer's instruction, with minor modifications. In brief, enzyme–linked immunosorbent assay plates were coated with capture antibody diluted to a working concentration of 80 ng/well in Dulbecco's modified phosphate-buffered saline solution (DPBS) (Invitrogen Corp, Carlsbad, CA, USA) overnight at room temperature. The remaining binding

sites were blocked with bovine serum albumin (Sigma, St. Louis, MO, USA), 10 mg/mL, dissolved in DPBS. Human serum samples were diluted 100-fold in the same buffer (10 mg/mL of bovine serum albumin in DPBS) and incubated with the immobilized primary antibodies for 2 hours. Capture antibody-bound ACE was labeled using a biotinylated detection antibody, 20 ng/well for 2 hours. Streptavidin-conjugated horseradish-peroxidase (200-fold-diluted stock from the kit) was added to the wells and incubated for 20 minutes. The immunocomplexes were detected with a chromogenic substrate solution containing 0.3 mg/mL TMB (3,3,5',5'-tetramethylbenzidine), 0.1 μM H_2O_2 and 50 mM acetic acid (incubation time was about 20 minutes). Reaction was terminated by addition of 0.5 M HCl and was evaluated by measuring absorbance at 450 nm. ACE concentration was calculated using a calibration curve. The ACE concentration in the samples were measured at least three times to achieve a standard deviation of at most 15%. Serum ACE concentration was given as ng/mL of serum.

3.8. Amino-terminal pro-B-type natriuretic peptide (NT-proBNP) measurements

NT-proBNP levels were measured in serum using a commercially available kit (Elecsys proBNPII., Roche Ltd., Mannheim, Germany) according to the manufacturer's instructions.

3.9. Statistical analysis

Results are expressed as mean ± S.E.M. for all groups. Patient's characteristics were tested by one-way analysis of variance (ANOVA). Most of the groups did not passed the D'Agostino and Pearson omnibus normality test when tested for serum ACE2 activity or NT-proBNP concentration and therefore nonparametric evaluation was performed. Statistical difference in these cases was tested by one-way analysis of variance (ANOVA) on ranks (Kruskal-Wallis test). Linear regression analysis was performed to correlate serum ACE2 activity with

echocardiographic parameters and correlation was considered to be significant when $r^2>0.1$ and $P<0.05$. Receiver operating characteristic (ROC) curves were generated to test the diagnostic value of serum ACE2 activity and NT-proBNP concentration. To predict the relationship between ACE2 activities and different biomedical circumstances like gender, obesity, cardiovascular comorbidities (diabetes mellitus, dyslipidemia, atrial fibrillation) and cardiovascular medications logistic regression analyses were performed. Parameters with a p value of <0,05 were considered to be meaningful predictors of changes in ACE2 activities. All statistical analyses were performed by GraphPad Prism, version 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

4. Results

4.1. ACE2 activity positively correlates with the clinical status of systolic heart failure patients

Serum ACE2 activity strongly correlated with the clinical condition of patients with severe heart failure (HF - CRT before: NYHAII: 32.8±2.5 UF/mL, NYHAIII: 43.0±2.4 UF/mL, NYHAIV: 72.3±6.3 UF/mL).

The positive correlation between ACE2 and NYHA classes remained unchanged after pacemaker implantation (HF - CRT after: NYHAI: 25.8±2.5 UF/mL, NYHAII: 35.4±2.3 UF/mL, NYHAIII: 45.9±6.0 UF/mL), while the clinical status improved in most of the HF-patients.

A remarkable elevation of ACE2 activity was present in CRT patients before pacemaker implantation (HF – CRT before) compared to healthy people (healthy: 16.2±0.8 UF/mL, HF - CRT before: 42.3±1.7 UF/mL). A robust reduction in serum ACE2 activity was found in parallel to the improving cardiac performance and function called reverse remodeling (HF - CRT after: to 34.3±1.9 UF/mL; $P<0.001$).

4.2. Effects of biventricular pacing on echocardiographic parameters

Systolic heart failure (HF) patients had significantly enlarged initial left ventricular dimensions (ESD, EDD) before cardiac resynchronization therapy (HF - CRT before) compared to healthy individuals. Severe reduction of left ventricular pumping function was observed as represented by reduced EF and dP/dt in the CRT group. A significant decrease of ESD and EDD (ESD: from 56,3±1,2 mm to 51.9±1.4 mm; $P<0.001$, EDD: from 67,2±1,2 mm to 63.9±1.2 mm; $P<0.01$) was found following 6 to 9 months of biventricular pacing (HF – CRT after). Pumping

function also improved to a great extent in this group (EF: from 28,3±0,7 % to 34.2±1.1 %; P<0.001; dP/dt: from 498,2±27,1 Hgmm/sec to 695.4±54.0 mmHg/s; P<0.001).

4.3. Association of serum ACE2 with the progression of the cardiovascular disease

Serum ACE2 activity was the lowest in the healthy group (16.2±0.8 U/ml) which was significantly increased in hypertensive patients (24.8±0.8 U/ml) and further increased when hypertension was accompanied by HFrEF, representing progression of cardiovascular disease toward systolic dysfunction (42.1±2.2 U/ml). HFrEF patients without hypertension also had higher serum ACE2 activities (HFrEF-Hypertension: 49.0±4.5 U/ml). In contrast, patients with hypertension and HF with preserved systolic function (HFpEF) had similar serum ACE2 activities (HFpEF+Hypertension: 24.6±1.9 U/ml) than that of hypertensive patients without HF.

4.4. ACE2 activity correlates with the deterioration of left ventricular systolic function

Serum ACE2 activity correlated with the clinical status of HF. Accordingly, we performed a detailed study to identify serum ACE2 as a biomarker of human heart failure. Serum ACE2 activities were plotted as the function of systolic ejection fraction in all study groups. There was no correlation between serum ACE2 activity and systolic heart function in patient populations with normal (preserved) EF. However, serum ACE2 activity correlated negatively with the EF in the HFrEF group ($P<0.001$, $r^2=0.21$).

4.5. There is no correlation between serum ACE2 activity and the severity of diastolic dysfunction

There was no correlation between serum ACE2 activity and left ventricular diastolic parameters (E/A and E/e') of patients with heart failure with preserved ejection fraction (HFpEF). E/A values were determined in HFpEF patients without atrial fibrillation. E/e' values were determined in patients with sufficient acoustic window for accurate echocardiographic measurement.

4.6. Serum amino-terminal pro-B-type natriuretic peptide (NT-proBNP) concentration parallels cardiovascular disease development

The most often used biochemical marker for cardiovascular disease progression is the amino-terminal pro-B-type natriuretic peptide (NT-proBNP). NT-proBNP levels were increased in hypertension (healthy: 6.5±4.8 pmol/l vs. hypertensive: 32.5±69.4 pmol/l) and further increased in HF, irrespectively of the form of HF (HFrEF: 432±55 pmol/L, HFpEF: 98±18 pmol/L).

There was no correlation between EF and NT-proBNP in individuals with normal left ventricular function (healthy, hypertensive and HFpEF). EF negatively correlated to NT-proBNP levels in systolic heart failure patients (HFrEF: $P<0.001$, $r^2=0.23$).

4.7. Correlation of ACE2 activity with NT-proBNP

There was no correlation between ACE2 activity and NT-proBNP in individuals with normal left ventricular systolic function (healthy and hypertensive). Serum ACE2 activities positively correlated to NT-proBNP levels in HF patients before CRT (HFrEF: $P<0.001$, $r^2=0.23$).

4.8. Comparison of the prognostic value for serum ACE2 activity and amino-terminal pro-B-type natriuretic peptide (NT-proBNP) concentration to differentiate HFrEF and HFpEF from hypertension

Receiver operating characteristic (ROC) curves were generated to test the diagnostic value of serum ACE2 activity or NT-proBNP to differentiate between patients with heart failure and hypertension without heart failure. The clinical applicability of serum ACE2 activity as a biomarker for left ventricular systolic dysfunction in human heart failure was also addressed. Receiver operating characteristic (ROC) curve was generated, in which the clinical applicability of serum ACE2 activity was tested to discriminate HF patients from hypertensive patients. Area under the curves was 0.77 for HFrEF and 0.51 for HFpEF patients. Similar values for NT-proBNP were 0.93 for HFrEF and 0.78 for HFpEF patients.

4.9. Effects of comorbidities on serum ACE2 activity in cardiovascular patients

Effects of comorbidities, such as dyslipidemia, atrial fibrillation, diabetes were tested on serum ACE2 activities in the different cardiovascular patient's populations. There was a general tendency toward males having higher serum ACE2 activities, which was statistically significant among hypertensive and HFrEF patients. None of the other comorbidities had any effects on serum ACE2 activity.

4.10. Logistic regression analyses for confounding variables such as gender, elevated BMI values, cardiovascular comorbidities and cardiovascular drug therapy in hypertensive patients

This observation was particularly confirmed by logistic regression analyses. Serum activities of ACE2 differ according to gender in hypertensive patients ($p<0,01$) however assessed for confounding variables such as high BMI (>25), cardiovascular drug therapy and cardiovascular comorbidities ACE2 activities proved to have no predictive value ($p=0,11$ for high BMI, $p= 0,33$ for ACEi, $p=0,77$ for angiotensin receptor blockers (ARB), $p=0,45$ for aldosterone antagonists (AA), $p=0,99$ for diuretics, $p=0,08$ for beta blockers (BB), $p=0,75$ for calcium-channel blockers (CCB) $p=0,17$ for statins, $p=0,09$ for dyslipidemia, $p=0,29$ for diabetes mellitus and $p=0,33$ for atrial fibrillation).

4.11. Correlation of serum ACE activity and concentration to left ventricular systolic function

ACE activity of the cardiovascular patients (hypertensive: 14.5±0.9 U/mL, HFrEF: 14,22±1,6 U/mL and HFpEF: 23,3±2,8 U/mL) were significantly lower than that in healthy individuals (healthy: 33.6±1.7 U/mL), most probably representing successful ACE inhibitory therapy. Patients with severe systolic or diastolic heart failure had significantly higher enzyme concentration than patients with preserved EF (HFpEF: 100,9±5,1 ng/mL; HFrEF: 101,7 ±3,5 ng/mL vs. healthy: 159.0±9.3 ng/mL, hypertensive: 149.6±6.9 ng/mL; $P<0.001$).

5. Discussion

Cardiovascular disease is the leading cause of death in most developed countries. Cardiovascular disease usually starts with hypertension, dyslipidemia and diabetes. These may progress to heart failure (HF), which has two distinct forms. HF with reduced ejection fraction (HFrEF) is characterized by systolic dysfunction, while HF with preserved ejection fraction (HFpEF) is characterized by diastolic dysfunction.

We have extended the view that serum ACE2 activity correlates with the worsening of HF earlier showing its correlation with the improvement of EF upon biventricular pacing in case of long QRS morphology. Moreover, serum ACE2 activity was found to be elevated in hypertensive patients, opening up new perspectives in the field. Here we continued these efforts. Serum ACE2 activity was measured in various stages of the cardiovascular continuum, with a particular attention to the transition of hypertension to HF. The most important finding was that serum ACE2 activity does not change upon progression of hypertension to HFpEF, contrasting progression of hypertension to HFrEF (2-fold increase). These findings suggested that serum ACE2 activity is a selective biomarker of systolic dysfunction. This idea was tested in detail. ROC analysis confirmed that serum ACE2 activity has no predictive value for HFpEF among hypertensive patients, while it was fairly predictive to identify hypertensive patients with HFrEF. Amino-terminal pro-B-type natriuretic peptide (NT-proBNP, a widely used biomarker for HF) levels were 6-fold increased in hypertensive patients, and further increased in HFpEF (3-fold) and in HFrEF (12-fold), suggesting that natriuretic peptide release is being activated in both HFrEF and HFpEF. Although NT-proBNP was found to be a superior biomarker identifying HFrEF in hypertensive patients when compared to serum ACE2 activity, it was inferior in selectivity, since it was also predictive to HFpEF among the same patients. It appears therefore that NT-proBNP is a rather general biomarker for HF, irrespectively of the form of HF, while serum ACE2 activity is being selective for HFrEF.

One of the limitations of clinical studies dealing with biomarkers is that their level is being influenced by various comorbidities. For example, serum ACE2 activity appears to be gender dependent, which factor is usually overlooked in the clinical studies. Here we made an effort to specifically test the effects of comorbidities. There was no effect of diabetes, atrial fibrillation

and dyslipidemia on the serum ACE2 activity in the different cardiovascular populations. No correlation existed between ACE2 activities and GFR as well as CRP values and in these cohorts of heart failure patients. In contrast, males had significantly higher serum ACE2 activity than females conforming earlier reports. It was the most significant in the HFrEF group, where serum ACE2 activity was about 50% higher in male subjects. Nonetheless, increasing serum ACE2 activities in both genders paralleled the progression of cardiovascular disease to HFrEF, and therefore differences can not be explained by the different gender ratios (such as male dominance in HFrEF and female dominance in HFpEF). In a recent study it was shown that ACE2 activity directly correlated with male gender, diabetes and older age as the classical CV risk factors in chronic kidney disease patients with different clinical stages. That patient population with a high cardiovascular risk, namely diabetic chronic kidney disease patients represented also an increased circulating ACE2 activity and elevation of ACE2 activity correlated with disease progression; further supporting the role of ACE2 as a potential cardiovascular biomarker.

Our data showed highly elevated serum ACE2 activities in HFrEF and moderate elevation in hypertensive patients (when compared to healthy individuals). This suggests that ACE2 expression is either increased as a counter regulatory mechanism to the dysregulation of RAAS, or on the contrary, tissue ACE2 is being released into the circulation (a process called ACE2 shedding) providing a significant step in the pathomechanism of the disease. According to this latter hypothesis, ACE2 shedding plays an important role in the development of HF: release of ACE2 into the circulation limits the availability of ACE2 and promotes angiotensin II accumulation in the tissues. This is supported by the recognition of endogenous ACE inhibitors, in particular by serum albumin in human. Based on these data we proposed that angiotensin II formation may be a rate-limiting step and local angiotensin II levels are determined by its elimination. This is strongly supported by the observation of a positive feedback in the RAAS in mice whereby activation of angiotensin II type 1 receptor (AT1R) increases ADAM17 (an enzyme responsible for the cleavage of ACE2 into the circulation) expression resulting in shedding of ACE2. A similar but circulating leukocyte (i.e. monocyte) related regulatory mechanism was proposed recently in chronic kidney disease patients with high cardiovascular risk. In this human study an adverse relation of monocytic ACE and ACE2 induced a severe pro-atherogenic condition.

Here we extended the potential role of ACE2 shedding in cardiovascular disease. We showed that serum ACE2 activity is already increased at the initiation phase of the cardiovascular continuum (hypertension) and then further increase when hypertension progresses toward systolic dysfunction, but not when diastolic dysfunction develops with maintained systolic function. Moreover we found similar ACE concentrations in case of both kinds of heart failure while the activity of ACE changed according to the rate of ACE inhibitory drug use in the different study groups. Important to note, that these observations are in accordance with the clinical success of RAAS inhibition. RAAS inhibition is one of the primary treatment options to reduce blood pressure or to treat HFrEF according to recent clinical guidelines. On the other hand, there is no elevated ACE2 shedding in HFpEF patients (over hypertensive patients). In accordance, RAAS inhibition is not particularly effective in HFpEF over the antihypertensive effects, especially when compared to HFrEF.

In summary, here we have shown that serum ACE2 activity increases in parallel with the progression of cardiovascular disease. It is elevated in hypertension, then further elevates when systolic dysfunction develops. However, it is not being affected by the development of diastolic HF in hypertensive patients. All of these observations suggest that:

-changes in serum ACE2 activity may be related to the pathomechanism of cardiovascular disease progression,

different therapeutic responses to RAAS inhibition in HFrEF and HFpEF are related to ACE2 dysregulation,

-serum ACE2 activity is a biomarker which can be used to differentiate between HFrEF and HFpEF patients.

6. List of Publications

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Registry number: DEENK/236/2016.PL
Subject: PhD Publikációs Lista

Candidate: Katalin Úri
Neptun ID: HE4QYF
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Úri, K.**, Fagyas, M., Kertész, A. B., Borbély, A., Jenei, C., Bene, O., Csanádi, Z., Paulus, W. J., Édes, I., Papp, Z., Tóth, A., Lizanecz, E.: Circulating ACE2 activity correlates with cardiovascular disease development.
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List of other publications

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