Short thesis for the degree of Doctor of Philosophy (PhD)

Regulation of tissue and circulating angiotensin-converting enzymes in cardiovascular diseases

Viktor Bánhegyi M.D.

Supervisor: Prof. Attila Tóth Ph.D. D.Sc.

UNIVERSITY OF DEBRECEN

KÁLMÁN LAKI DOCTORAL SCHOOL

Debrecen, 2022

Regulation of tissue and circulating angiotensin-converting enzymes in cardiovascular diseases

Dissertation to obtain a doctoral (Ph.D.) degree in the discipline of Theoretical Medicine

Written by: Viktor Bánhegyi M.D. General Practitioner, Resident Cardiothoracic Surgeon

Prepared in the framework of the Kálmán Laki Doctoral School of the University of Debrecen (Cardiovascular Diseases Programme)

Supervisor: Prof. Attila Tóth, Ph.D. D.Sc.

Reviewers:

Prof. Róbert Halmosi M.D. Ph.D. D.Sc.
Béla Nagy M.D. Ph.D.

Evaluation Committee:

Head:	Prof. Csongor Kiss M.D. Ph.D. D.Sc.
Members:	Prof. Róbert Halmosi M.D. Ph.D. D.Sc.
	Prof. István Szokodi M.D. Ph.D. D.Sc.
	Béla Nagy M.D. Ph.D.
	András Balla M.D. Ph.D.

Date of the thesis defence: University of Debrecen, Faculty of Medicine,
Lecture Hall of Building "A",
Department of Internal Medicine,
29.03.2023. 1-3 PM.

1. Introduction

The renin-angiotensin-aldosterone system (RAAS) plays a key role in the development of cardiovascular disease. Abnormal activation of the RAAS and the resulting neurohumoral dysregulation are at the origin of many cardiovascular diseases of public health significance. Our pharmaco-clinical efforts to date to modulate RAAS are clearly a step forward, with angiotensin converting enzyme (ACE) inhibitor and angiotensin II type 1 receptor I blocker (ARB) drugs improving the life expectancy of our cardiovascular patients in the medium and long term. ACE inhibitors and ARBs are among the first-line recommendations as single or combination therapeutic modalities in cardiovascular disease.

In recent years, research on the ACE – angiotensin II (ATII) – angiotensin II type 1 receptor I (ATRI) axis, which underlies cardiovascular pathophysiology, has also brought to the fore the importance of angiotensin converting enzyme 2 (ACE2). In addition, pharmacological exploitation of the regulatory potential of natriuretic peptides in combination with neprilysin (NEP) inhibitors and ARBs also has clear beneficial effects on the life expectancy of our patients with cardiovascular disease.

The coronavirus pandemic (COVID-19) triggered by SARS-CoV-2, which started in 2019 and unfortunately continues to cause serious health problems today, is also linked to the RAAS system. In our experiments, we investigated the distribution of ACE/ACE2 enzymes in human tissues and the mechanisms of their regulation in cardiovascular and COVID-19 patients.

2. Literature review

2.1. The human renin-angiotensin-aldosterone system (RAAS)

2.1.1. The angiotensin converting enzyme (ACE) → angiotensin II (ATII) → ATII receptor type 1 (ATRI) axis

The RAAS plays an indisputable role in the salt and fluid balance of our body and in maintaining proper tissue perfusion. One of its most important members, ACE, was discovered in the mid-1950s. It was the first to isolate a molecule that plays a key role in the cardiovascular system and the first ACE inhibitor therapy was developed.

RAAS is a cascade process, the initial step of which is the conversion of angiotensinogen (ANG), synthesized in the liver, to angiotensin I (ATI) decapeptide. This process is catalysed by renin from renal juxtaglomerular cells. The main physiological triggers for renin release are decreased renal perfusion and sodium chloride concentrations as well as sympathetic nervous system predominance. ACE, a peptidyl dipeptidase enzyme and rate-limiting factor in the cascade process, is responsible for the cleavage of the functionally inactive ATI, ATII, into octapeptides. ATII, acting on ATRI, is responsible for the acute and chronic pathophysiological mechanisms of RAAS. Acute effects of ATII include enhancement of sodium reabsorption (thereby increasing blood volume), vasoconstriction of precapillary blood vessels, increased aldosterone secretion and thirst sensation. The chronic effects of ATII include smooth muscle cell proliferation, the initiation of inflammatory and pro-fibrotic processes (myocardial remodelling). The end effector molecule of RAAS is aldosterone produced in the *zona glomerulosa* of the adrenal cortex. This mineralocorticoid molecule regulates fluid balance by acting primarily in the nucleus of the renal epithelial cells - in accordance with the above - by balancing sodium and potassium ion homeostasis.

2.1.2. Angiotensin converting enzyme 2 (ACE2) → Angiotensin (1-7) (Ang1-7) → Mas receptor (MasR) axis

Research in recent years has shown that, in addition to the classical pathway of RAAS, a counter-regulatory (vasodilatory and reno-protective) cascade can be discovered. ATII can be considered as the starting point of this axis. ATII is cleaved by ACE2 into Ang1-7 heptapeptide. The ACE2 molecule is the main function of the ACE isoenzyme catalysing the above mentioned ATII → Ang1-7 conversion and is also responsible for the ATI → angiotensin 1-9 conversion. The Ang1-7 molecule acts on the MASR to counteract the pathophysiological effect of the ACE – ATII – ATRI axis. The signalling cascade results in the opening of anti-proliferative, vasodilatory (antihypertensive), anti-inflammatory and parasympathetic pathways. Another prominent molecule within this subsystem is neprilysin (NEP). The main function of NEP also known as membrane metallo endopeptidase/neutral endopeptidase/CD 10 is to convert physiologically beneficial (equivalent to the effects of Ang 1-7) atrial and B-type natriuretic peptides (ANP, BNP) to inactive peptides and to degrade vasoconstrictor ATII and endothelin I. NEP can also catalyse AT I→ Ang1-7 to a limited extent. Exploitation and potentiation of this beneficial physiological system and efforts to inhibit NEP have also become clinical therapies, and their efficacy has been demonstrated in daily clinical practice.

2.2. Origin of angiotensin-converting enzymes, sources of circulating ACE and ACE2

As to the origin of circulating ACE, it has been a textbook dogma for decades that enzymatically active ACE present in the circulation is secreted from the endothelial surface of the lung parenchymal capillaries into the human bloodstream. This picture is nuanced by the authoritative and extensive analysis of the Human Protein Atlas online database. Founded in 2003 and updated every calendar year, the Human Protein Atlas is a Swedish-initiated *open access* database that uses modern molecular biology methods (mass spectrometry,

immunohistochemistry, proteomics, *"OMICs"*) to map the proteins that make up the human body on a systemic level. Based on a summary of their analyses and measurements, ACE is most abundant in the gastrointestinal system at both protein and mRNA levels, followed by tissue expression in the excretory system and genitalia, and significantly less abundant in lung tissue.

For ACE2, it follows a similar trend. In terms of both mRNA and protein expression profiles, ACE2 shows significant tissue expression in the gastrointestinal and excretory organ systems and in myocardial tissue itself. In terms of lung tissue, only low expression of ACE2 is observed.

2.3. The relationship between RAAS and SARS-CoV-2

The SARS-CoV-2 virus pandemic, which broke out in the second half of 2019 and is still causing serious public health problems, is closely linked to the RAAS system. The global spread of the pathogen has resulted in millions of deaths and we have faced huge challenges in both economic and health terms in recent years. The coronaviruses (Coronaviridae) are a family of envelope-lipid (peplonous), positive-stranded RNA viruses in birds, mammals and amphibians with high infection potential and mutation rates. The outer surface of the lipid envelope of coronaviruses has relatively large (20 nm) spines/spikes, from which the virus family derives its name, due to its morphology resembling the Sun's corona. The infectivity of coronaviruses is driven by the maintenance of genetic diversity through RNA-based recombination. A scientific observation of great relevance to my research is that the cell surface attachment and internalization of coronaviruses is mediated by the extracellularly present ACE2 molecule.

The disease caused by the viral infection develops a similar clinical picture to influenza (fever, loss of sense of smell and taste, cough, sore throat) in humans, but compared to influenza, the mortality rates associated with coronavirus infection are clearly higher. The

higher mortality is likely to be due to an abnormally increased immune response. Complaints following a viral infection (also) affect human lung tissue. In the case of active coronavirus infection, it has been demonstrated by imaging modalities that the pathogen destroys human lung tissue and develops a clinical picture similar to ARDS through cell surface attachment, requiring invasive (CPAP, BIPAP) ventilation in a significant proportion of cases. In addition to problems affecting the general and respiratory systems, coronavirus infection also has cardiovascular implications.

3. Objectives

3.1. Analysis of tissue and circulating ACE in human lung tissue and serum

Our aim was to investigate, in a population of patients who underwent thoracic surgery, whether ACE activity and expression genotypically (II, ID, DD polymorphisms) determined in the circulation – in line with textbook knowledge – originate from lung tissue? We also aimed to investigate the endogenous regulation of human tissue ACE. To answer our scientific questions, the development of an adequate and valid ACE extraction protocol was warranted.

3.2. Investigation of the relationship between angiotensin converting enzymes (ACE, ACE2) and SARS-CoV-2

We aimed to determine the activity and expression of ACE2, a cell surface receptor for the SARS-CoV-2 pathogen, and to compare these values in groups of patients with cardiovascular disease. We planned to investigate the effect of ACE inhibitor therapy on ACE2 activity and expression levels in cardiovascular patients (in blood and tissue samples).

4. Materials and methods

4.1. Human heart and lung tissue bank

The study of ACE in human lung tissue and circulation was carried out in collaboration with the Department of Thoracic Surgery at the University of Debrecen. Lung tissue samples are from patients who underwent thoracic surgery (2015-2017, mostly diagnosed with adenocarcinoma, n=108). Resected tissues represent pieces of tissue distal to the tumour-infiltrated resection edge, showing physiological structure. In addition to the tissue samples, blood samples (whole blood, serum) were also examined for each patient. The ethical approval of the clinical data collection has been granted by the Hungarian Medical Research Council in accordance with the research ethics principles. The research ethics authorisation number is the following: 20753-7/2018/EÜIG.

In collaboration with the Semmelweis University Heart and Vascular Centre, heart and serum samples from additional heart transplant patients were used to investigate the association between ACE2 and the coronavirus pandemic. Research ethics approval number: ETT TUKEB 7891/2012/EKU (119/PI/12.)

For all the samples mentioned above, storage was done by deep freezing at -80 Celsius degrees, and all patients selected gave written informed consent to the use of blood and tissue samples for research purposes, in line with the principles of the Declaration of Helsinki.

4.2. Determination of the insertion/deletion polymorphism of ACE

Genotyping of patients' peripheral blood was performed using a commercially available DNA isolation kit (FlexiGene; Qiagen GmbH, Hilden, Germany) using PCR technique. Using the *"forward"* (CTGGAGACCACTCCCACTCTTTCT) and *"reverse"* (GATGTGGCCATCACATTCGTCAGAT) primers for amplification, we performed the

experiments as described earlier by our group. After amplification, PCR products were subjected to electrophoresis separation on 3% polyacrylamide gel and *SybrSafe* staining.

4.3. Tissue processing for determination of ACE activity and expression

Human lung and heart samples were processed under liquid nitrogen in a mortar with the aid of a mortar pestle. To each gram (wet weight) of small pieces of tissue thus obtained, 5 ml of 100 mM TRIS-HCl, pH 7.0 buffer was added (ice-cold). The samples were then homogenized (Bio-Gen PRO200, PRO Scientific, Oxford, CT, USA) and centrifuged (16,100 g for 5 min). The resulting supernatants were deep frozen until biochemical measurements were performed.

4.4. Protein determination and protein normalisation

The different protein content of the supernatants from each tissue justified the use of protein determination and normalization according to *bicinchoninic acid* (BCA) assay. Using a Biuret reaction methodology interpolated to a standard bovine serum albumin (BSA) dilution series, we determined the protein content of our samples. Using appropriate dilutions, our samples were uniformly diluted to 1 mg/ml.

4.5. Determination of ACE activity

Tissue and circulating ACE activity measurements were performed by our team as previously published. The measurements were performed using *quenched* fluorescent Abz-FRK(Dnp)P-OH ACE substrate with kinetic assay. The test mixture consists of 0.06 V/V% Trition X-100 containing 100 mM TRIS-HCl, pH 7.0, 50 mM NaCl, 10 µM $ZnCl_2$, 10 µM Abz-FRK(Dnp)P-OH plus any amount of tissue or serum normalized to protein using BCA assay. The activity was determined at 37 Celsius degrees using a fluorescence plate reader (NovoStar, BMG Labtech, Ortenberg, Germany) with excitation at 340 nm and emission at 405 nm. Each measurement was accepted as valuable if the goodness of fit (r^2) of the resulting

lines after linear regression was better than 0.9. The specific determination of ACE activity values was determined by comparing the absolute increase in fluorescence intensity values with the reference curve of the calibration Abz fluorophore intensity increase.

4.6. Determination of ACE2 activity

Circulating and tissue ACE2 activities were determined using Mca-APK(Dnp) *quenched* fluorescent substrate as previously published by our group. The composition of the assay mixture (200 µl) was 20 µl serum/tissue sample, 80 µl sample dilution buffer (75 mM TRIS HCI, pH 6.5 and protease inhibitor cocktail) and 100 µl (50 µM) ACE2 specific fluorescent substrate [Mca-APK(Dnp)]. The measurement mixture (added to sample dilution buffer) contained protease inhibitors consisting of 10 µM Bestatin-HCl, 10 µM Z-prolyl-prolinal (Enzo Life Science, Exeter, UK), 5 µM Amastatin-HCl, 10 µM Captopril. ACE2 specific activity values were determined with 1.25 µM final dilution of MLN-4760 (specific ACE2 inhibitor).

The measurements were performed on a 96-well microplate (Greiner Bio-One, Frickenhauser, Germany) at 340 nm excitation and 405 nm emission using the NOVOStar fluorescence *Plate Reader* already mentioned for ACE measurements. The nominal value of ACE2 enzyme activities was determined by linear regression of fluorescence intensity values after comparison with the reference curve of the intensity increase of the Mca-APK(Dnp) fluorescent substrate ($r^2 > 0.9$) at 37.0 Celsius degrees.

4.7. Determination of ACE and ACE2 expression (concentration)

ACE and ACE2 proteins were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the vendor's guidelines (DY929 and DY933-05, R&D Systems, McKinley Place, MN, USA). After *overnight* incubation with an appropriate concentration of *capture* antibody, the remaining binding sites were blocked with 10 mg/ml of

bovine serum albumin dissolved in Dulbecco's phosphate buffer saline. The samples (lung, heart and serum) were diluted in the same solution at the dilution rate in the mid-range of the standard, which is preferable for the evaluation. After loading the samples, incubation and washing for one hour, the immobilized primary antibodies were loaded onto the *microplate* (Greiner Bio, PS Microplate, Microlon®, No. 655061). After two hours incubation and subsequent washing, biotin-labelled detection antibodies were used. After a further two-hour incubation and washing, the signals were amplified using a 200-fold dilution of streptavidin-horseradish peroxidase solution for 20 minutes. Immunocomplexes were detected using chromogenic substrate (0.3 mg/mL 3,3',5,5'-tetramethylbenzidine, 0.1 mM H_2O_2 and 50 mM acetic acid. The chromogenic colour reaction was stopped with 0.5 mM hydrochloric acid solution. The resulting product was detected at 450 nanometres using a NOVOStar Fluorescence *Plate Reader*. The specific amount of ACE or ACE2 was quantified after comparison with the standard calibration curve (ng/ml).

4.8. Chemicals

The chemicals used in the experimental work (unless specifically indicated) were those supplied by Sigma Aldrich.

4.9. Statistical analysis

The Kolmogorov-Smirnoff test was used to test the distribution function of our data. A nonparametric Mann-Whitney test was performed to compare two groups with non-normal distributions. For simultaneous comparison of several non-normally distributed cohorts, a Kruskal-Wallis test was performed in combination with a post hoc Dunn's test. Correlation analyses were performed using Spearman's test. Statistical analysis was done using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). Individual statistical results were considered significant at $p<0.05$.

5. Results

5.1. Tissue ACE extraction protocol

The first main focus of our research was the introduction of an efficient tissue extraction (membrane permeabilization) protocol. Different detergents (Triton X-100, Triton X-114, SDS) were tested in the concentration range 0.06-5.0 V/V% *applied*. Among our detergents, we first discarded SDS due to its ACE activity inhibition. No significant difference in spectrum and efficacy was observed between Triton X-100 and -114 and therefore Triton X-100, which is commonly used in laboratories, was used in our experimental work. Triton X-100 treatment showed a 250% increase in tissue ACE extraction even at the lowest applied concentration. ACE extraction in buffer solution alone was considered as 100% *("baseline")* extraction. The highest concentration of Triton X-100 at 5.0 V/V% showed a fivefold improvement in enzyme extraction compared to digestion in TRIS HCl buffer. In terms of ACE activity values, the extraction efficiency was different from the previous ones. The activity values showed a strong increase at low concentrations, with maximum values at a final detergent concentration of 0.3 V/V% (approximately 250%). Above this value, the specific ACE activity values showed a decreasing trend, suggesting that the higher concentrations of Triton X-100 used may enhance ACE extraction, but is also thought to inhibit ACE activity. After tissue processing, ACE activity and expression were determined from supernatants. Residual tissue pellets were subjected to two additional detergent treatments on a consecutive basis. Our aim was to test the extraction efficiency. It can be reported that after digestion in general TRIS HCl buffer, a loss of 20-25% ACE activity was realizable even after the 3rd re-digestion. After application of 0.6 V/V% Triton X-100 treatment, the loss was clearly minimized (residual activity < 5%).

5.2. Genotype-dependent (I/D) lung tissue and circulating ACE expression

Genotype-dependent increases in ACE expression levels were observed in serum samples from patients undergoing thoracic surgery (including patients taking ACE inhibitors). It can be reported that ACE expression in patients with the DD genotype (258 ± 109 ng/ml) was significantly higher compared to similar serum samples from patients with genotype II (166 ± 143 ng/ml), suggesting a dominant genetic role of the I/D polymorphism in circulating ACE expression. In matched patients, ACE I/D polymorphisms in lung tissue did not confirm those observed in the circulation (tissue ACE concentrations: II: 1423 ± 1276 ng/mg, ID: 1040 ± 712 ng/mg, DD: 930 ± 1273 ng/mg). In patients not taking an ACE inhibitor, significantly higher circulating (serum) ACE activity levels were observed in the presence of the D allele (II: 3.1 ± 1.4 U/mL, ID: 4.0 ± 1.4 U/mL, DD: 5.0 ± 2.5 U/mL). After measuring supernatants at high dilutions to eliminate the effect of ACE inhibitors, significantly higher ACE activity values (independent of the presence of ACE inhibitor treatment) were measured in the cohort of patients with genotype DD compared to genotype II patients (II: 8.4 ± 4.9 U/mL, ID: 8.9 ± 4.2 U/mL, DD: 10.3 ± 3.9 U/mL). When lung samples from matched patients were compared, no significant association was observed between tissue ACE I/D polymorphism and ACE activity (II: 37 ± 18 U/mL, ID: 37 ± 18 U/mL, DD: 39 ± 15 U/mL, and II: 156 ± 161 U/mL, ID: 115 ± 68 U/mL, DD: 108 ± 121 U/mL).

5.2.1. Correlation between lung tissue and circulating ACE activity and expression levels

Surprisingly, no significant correlation was found between the amount of ACE expressed in lung tissue and the amount of ACE present in the circulation (p=0.06). We also failed to detect a significant relationship between lung tissue and circulating ACE activity levels (p=0.24). In contrast to the above, a highly significant correlation was observed when comparing myocardial tissue and serum ACE activities (p=0.0054).

The expression or activity values observed in serum samples or lung tissue (from the same source) of each patient showed a strong linear correlation (p<0.01).

5.2.2. The importance of endogenous inhibition in human lung tissue

In the present study, in addition to investigating the circulatory and tissue correlations, the effect of dilution of supernatants was also investigated. We found a significant increase in tissue and circulating activity values (specific activity) normalized to ACE volume when dilution was used. At 400-fold dilution, significantly lower specific ACE activity was measured in the circulation compared to lung tissue (0.06 ± 0.004 U/ng vs. 0.13 ± 0.009 U/ng, p<0.05). The percentage of ACE inhibition was 53 ± 2% in the group of patients not receiving ACE inhibitor treatment and 83 ± 3% in the group of patients taking ACE inhibitors, demonstrating a significant difference and efficacy of ACE inhibitor treatment. No similar significant difference in lung tissue ACE inhibition was detected. No significant differences were found in age- and sex-dependence of circulating or pulmonary tissue ACE activities.

5.3. Association of ACE2 activity with cardiovascular disease and cardiovascular risk factors

A clear and marked increase in circulating ACE2 activity was detected during pathological processes in different stages of the cardiovascular continuum. Compared to a group of healthy individuals, we observed a significant increase in ACE2 activity (+32%) in the hypertensive patient group and a drastically higher ACE2 activity (+424%) in the population of patients with end-stage heart failure. In the group of patients characterised by hypertension, we found significantly higher ACE2 activity in males compared to females and in overweight and obese patients compared to patients with physiological body mass index. We also found significantly higher ACE2 activity in patients older than 60 years compared to patients younger than 60 years.

In the group of end-stage heart failure patients, the relationship between circulating and tissue ACE2 activity and cardiovascular risk factors showed a different picture. In this patient group, we were able to demonstrate a significant difference in circulating ACE2 in favour of the male sex when comparing males and females. At the tissue level, we observed significantly lower left ventricular ACE2 activity and expression in a group of obese patients compared to patients with physiological body mass index. With respect to age, we also observed significantly lower left ventricular ACE2 activity above 60 years of age compared with patients under 60 years of age. A similar trend was observed for ACE2 expression.

In the group of patients with lung disease, significantly higher circulating ACE2 levels were found in the male sex compared to female patients. No other significant differences in ACE2 activity or expression were detected at the tissue level.

5.3.1. Correlation of left ventricular ACE2 activity and expression with circulating ACE2 levels

We found a clear significant positive correlation between circulating and left ventricular ACE2 activity and expression ($p<0.01$). The validity of our results was supported by a significant correlation between activity and expression values in myocardium. Circulating and myocardial tissue ACE2 activity and expression levels were not significantly affected by ACE inhibitor therapy.

5.3.2. Correlation analysis of pulmonary tissue ACE2 activity and expression levels with circulating ACE2 levels

Lung tissue ACE2 enzyme activity and expression levels showed no significant relationship with circulating ACE2 activities. Following the same logic applied above, lung tissue ACE2 activities showed a strong correlation with lung tissue ACE2 expression,

confirming the validity of our results. Circulating and lung tissue ACE2 activity and expression levels were not significantly affected by ACE inhibitor therapy.

5.3.3. Effect of RAAS inhibitor therapy on circulating ACE2 activity in hypertensive patients

Circulating ACE2 activity values in hypertensive patients were found to be slightly higher with ACE inhibitor or ARB treatment, while without RAAS inhibitor therapy they overlapped with those in healthy patients. Similar ACE2 activity values were measured following different ACE inhibitor therapy modalities in hypertensive patients (enalapril: 22 ± 14, n=59; perindopril: 23 ± 15, n=167; ramipril: 21 ± 15 mU/L, n=113). No difference in ACE2 activity was detected after more than 12 months of ACEi therapy (23 ± 16 mU/L, n=223) or less (22 ± 14 mU/L, n=52).

5.3.4. Study of the effect of ACE inhibitor therapy on ACE and ACE2 activity levels

Regarding the use of ACE inhibitor therapy, a negative correlation was found between the degree of ACE inhibition as well as systolic and diastolic blood pressure. Maximum ACE inhibition efficacy was observed for both systolic and diastolic blood pressure values in the range of 94-96% inhibition. In our experiments, we found no significant effect or relationship between the efficacy of ACE inhibition and any change in circulating ACE2 activity levels.

6. Discussion

6.1. Genotype-dependent circulating and tissue ACE assay

The widely accepted localization of circulating human ACE declared as a basic textbook theorem is the endothelial cells of the lung capillaries. All of these pulmonary capillary endothelial cells express ACE, whereas in other organs the proportion of cells involved in its expression is about 20%. Therefore, I/D regulation - of the organ(system)

responsible for the source of ACE - can also be assumed Along the lines of the above introductory ideas and following the construction of novel metabolic and cellular (protein and mRNA) human expression databases, other organs and organ systems (small intestine, kidney, heart, central nervous system, urogenital system) have been highlighted as sources of circulating human ACE. Our experimental work has been inspired by these emerging insights and observations.

With regard to the factors determining circulating ACE levels, the ACE inversion/deletion polymorphism (ACE I/D) is a notable one. This genotypic feature shows a strong association with cardiovascular disease and heart failure. Hence, I/D regulation of the organ(system) responsible for the source of ACE is also suspected. In addition to genetic determinants, the role of physiological factors may be elucidated. The inhibitory role of the nitric oxide molecule on ACE expression and the facilitating role of nitric oxide inhibitors can be mentioned. Overall, in addition to the passive determination of the number of metabolically active (ACE-producing) cells, the biochemical and oxidative milieu also contributes to the regulation of ACE expression.

Based on the preliminary knowledge outlined above, we chose human lung tissue (as a potential source) and blood samples from the same patients as the subject of our research to investigate the source of circulating human ACE. Lung samples and related blood samples (serum) were used to determine ACE activity and expression as previously published by our group. When circulating ACE levels were determined, we found significantly higher ACE activity and expression in genotype DD compared to genotype II patients, while an intermediate value was measured in genotype ID, confirming the previous literature data. At the lung tissue level, we were unable to confirm this genotypic specificity, suggesting that the source of genotypically defined ACE present in the circulation is different from the lung tissue.

When investigating an alternative source of circulating ACE, we observed a significant positive correlation between myocardial tissue and circulating ACE, suggesting that human myocardial tissue contributes significantly to circulating genotypically determined ACE activity and expression.

A role for tumour necrosis factor alpha converting enzyme (TACE or ADAMTS17) in tissue ACE release (secretion) is suggested, but this is not yet clearly established. The site of somatic ACE cleavage can be localized to the Arginine1203/Serine1204 peptide position.

Previously published findings suggest that ACE expression is reduced in lung cancer patients. In the mid-1970s, a negative correlation was also reported in a limited number of patients. The present study suggests that, if the number of capillaries is also reduced, circulating ACE also shows reduced expression in these patients.

Our results suggest that the source of circulating ACE is not exclusively and predominantly the pulmonary capillary system. Our experimental work raises the possibility of the human myocardium as an alternative source. Considering registries of complex databases (e.g. Human Protein Atlas) based on additional molecular biology techniques (Western blot, ELISA, mRNA sequencing, immunohistochemistry), the apical epithelial layer of the renal proximal tubules, the mucosal layer of the small intestine, the syncytial trophoblast of the placenta, some regions of the central nervous system and the M1, pro-inflammatory subtype of macrophages that form the effector of the immune system could also be identified as sources of circulating ACE. Furthermore, one of the findings successfully published by our group in recent years is that the relatively high levels of ACE accumulating in pro-inflammatory macrophages in sarcoidosis can be interpreted as a biomarker. In general, the exact role of this ACE expression beyond the classical lung tissue localization is still unclear and requires further research.

Our own tissue samples from a rat model, relevant for ACE production in our experimental work, also support these observations. With our findings we do not intend to ignore the classical textbook knowledge known so far, but to draw attention to the fact that the genotypically defined human ACE source present in the circulation presents a more colourful picture and that the role of the human lung is by no means exclusive.

The endogenous regulation of ACE has been known since the late 1970s. Subsequent experiments on human heart samples and serum samples confirmed the phenomenon of endogenous ACE inhibition. It was further demonstrated that dilution as an adequate biochemical technique also confirms the existence of this phenomenon in rat tissues. Over the past years, our group has demonstrated the role of human serum albumin in the background of endogenous inhibition. In the present study, following the same (dilution) methodology as before, we also demonstrated the presence of endogenous inhibition at both circulating and tissue ACE levels. At the tissue level, similar levels of ACE inhibition were observed regardless of the presence of ACE inhibitor therapy. In lung tissue, the expression of human serum albumin was low, suggesting the presence of an alternative inhibition process still present in the background. Our results are in agreement with previous scientific findings reporting endogenous inhibition levels of at least 85% in rat lung tissue.

Significant differences in tissue and circulating specific ACE activities are found in the present work. This significant difference may be due to post-translational modifications affecting the function and efficacy of metabolically active ACE. Our results are supported by a previous similar observation that demonstrated the importance of cell- and tissue-specific glycosylation patterns of ACE (structural mapping, *fingerprinting*). It is also important to mention the importance of the additional molecule responsible for the alternative ANGI → ANGII conversion mechanism, chymase. This molecule is currently known to be more active than ACE in myocardial tissue, whereby vasoconstrictive and pro-inflammatory ANGII levels

are not exclusively regulated by ACE/ACE2 but rather by the chymase/ACE2 ratio. In the present work, chymase activity and/or expression was not determined.

6.2. The importance of ACE2 in the context of the coronavirus pandemic

The outbreak of the coronavirus pandemic in 2019 opened up a unique research opportunity for our team in our long-standing ACE2 research.

It can be said that in groups of patients with aortic stenosis, hypertension and heart failure (HFrEF), ACE2 levels are higher and can be interpreted as a biomarker. This association, and the close relationship between SARS-CoV-2 and ACE2, supports the higher mortality rates in cardiovascular patients with coronavirus infection. The cardiovascular complaints observed in post COVID19 symptoms (palpitation, reduced exercise capacity, myocardial inflammation) also raise the importance of the SARS-CoV-2 and ACE2 – RAAS relation. Following the above ideas, our group aimed to determine the activity and expression of circulating ACE2 in different groups of cardiovascular patients. In addition, we felt it relevant to investigate the effects of ACE inhibitor therapy on ACE2 levels, thereby inferring the potential dangers of ACE inhibitors in patients with coronavirus and reflecting on ESC guidelines in this direction.

In the present study, the levels and activity of the circulating and tissue ACE2 molecule, which serves as a cell surface receptor for coronavirus, are influenced by cardiovascular risk factors and other pathological processes such as hypertension, old age, obesity and heart failure. Our work aimed to investigate the role of circulating and tissue ACE2 as a potential biomarker for mortality due to coronavirus infection and the use of ACE inhibitor therapy (in the case of coronavirus infection) using a reliable and valid methodological framework. In addition, the results shed light on the role of ACE2 in different stages of the cardiovascular continuum (healthy, hypertensive, end-stage heart failure groups of individuals). In several similar studies, circulating ACE2 levels were determined using

molecular biology, quantitative PCR methodology, which is not suitable for determining true circulating ACE2 concentration values.

The prominent role of ACE2 in the regulation of blood pressure was first highlighted by animal models. In a spontaneously hypertensive rat model, the locus of the *ace2* gene is located close to the genetic regulatory region of hypertension and circulating ACE2 levels were shown to be higher in this model. In a matched model system, deletion of the *ace2* gene led to blood pressure regulation problems as well as functional and structural heart problems. Our group's experiments in human samples over the past years have demonstrated an inverse relationship between circulating ACE2 activity and deteriorating functional stages of heart failure with reduced ejection fraction (HFrEF). The present results suggest that the (relative) increase in ACE2 activity in essential hypertension is relatively small compared to the dramatic increase in ACE2 activity in end-stage heart failure, in agreement with our previous observations and the literature.

Based on the preliminary results of our working group, an analysis of ACE2 activity values in 110 patients with critical coronavirus infection showed that this group of patients had significantly higher ACE2 activity values compared to patients with classical (non-COVID) sepsis. ACE2 was also interpreted as a biomarker for increased 30-day mortality. Increasing ACE2 activity values can be said to correlate with mortality and to have a predictive function for vulnerability and susceptibility to coronavirus.

Our results show that in a group of hypertensive patients on RAAS inhibitor treatment (ACEi, ARB), we observed 50% higher ACE2 activity compared to a healthy control group. With the onset of the coronavirus pandemic, the question arises whether the use of RAAS inhibitors may contribute directly or indirectly to the increase in coronavirus mortality rates. This idea was based on 3 preliminary observations: a higher incidence of hypertensive patients among patients with coronavirus; the use of RAAS inhibitor therapy in animal

preclinical models, which resulted in elevated ACE2 activity levels; and thirdly, the pathophysiological background itself, in which the cell surface ACE2 receptor serves as a gateway for the SARS-CoV-2 pathogen. My experimental work sought to nuance this issue. We used a novel dilution method, previously published by our group, to determine the efficacy of the ACE inhibitor therapy used. This method allowed us to analyse the relationship between circulating ACE2 activity and the efficacy of ACE inhibitor therapy. On the one hand, the omission of ACE inhibitor therapy was (obviously) reflected in higher blood pressure values, but on the other hand, the efficacy of ACE inhibition did not affect circulating ACE2 levels. Based on our previous and current experimental observations, circulating ACE2 activity levels correlate well with human cardiac ACE2 expression (a biomarker function, even for monitoring cardiac coronavirus involvement). No similar relationship is observed for lung tissue. Previous preclinical research also supports these observations. The lack of a link between circulating and lung tissue ACE2 can be explained by several theoretical-practical factors. On the one hand, ACE2 expression in the human heart is nearly four times higher than ACE2 expression in lung tissue, and it is possible that the ACE2 release mechanism in myocardial tissue may be more effective than its lung tissue counterpart. The active involvement of alternative organs/organ systems (e.g., gastrointestinal system) in ACE2 secretion is also an additional possibility. Interestingly, nearly 70% of patients infected with coronavirus report gastrointestinal complaints during the active phase of the disease.

Circulating ACE2 activity levels were consistently higher in males compared to females, confirming previous similar scientific observations. It can also be said that this non-dependent difference in ACE2 activity was observed independently of the pathology, which may indicate an individual, non-specific secretion mechanism. Interestingly, at the level of tissue expression, we found no significant, non-dependent differences in left ventricle or lung

values. In the hypertensive patient group, higher body mass index was associated with higher circulating ACE2 activity values. In hypertensive patients, an age-dependent increase in ACE2 activity was observed, complementing previous similar observations.

Our (indirect) findings on the association between ACE2 and SARS-CoV-2 support the ESC's 2020 *"position statement"* according to which the abandonment of antihypertensive drugs in active coronavirus disease is not clinically or scientifically justified (missing evidence).

7. New scientific results in the doctoral dissertation

Based on our experimental results, we formulate the following new findings:

a) Genotypically determined human ACE activity and expression levels in the circulation do not correlate with ACE activity and expression levels in lung tissue.Circulating ACE activity shows a clear and strong correlation with myocardial ACE activity.

b) Circulating ACE activity shows a strong correlation with myocardial ACE activity in samples of heart failure patients.

c) Circulating and lung tissue human ACE shows endogenous inhibition.

d) Circulating ACE2 is associated (increased) with progressive stages of the cardiovascular continuum and may play a role in the higher cardiovascular mortality observed following coronavirus infection.

8. Summary

Our experimental results presented in this thesis suggest that genotypically determined human ACE activity and expression in the circulation is not exclusively derived from lung tissue. Our own results and observations from databases based on modern molecular biology techniques suggest that ACE (or ACE2) is present in the heart, gastrointestinal, excretory and urogenital tracts in high amounts, thus highlighting the diversity of production and metabolism of these molecules in the human body.

Human circulating ACE2 may be interpreted as a biomarker in progressive stages of the cardiovascular continuum and in patients with coronavirus infection and cardiac/cardiovascular involvement, and may therefore explain the higher mortality rates in patients with coronavirus infections and cardiovascular disease. Circulating ACE2 is also associated with human sex and cardiovascular risk factors (BMI, blood pressure, age). Discontinuation of ACE inhibitor therapy (the degree of ACE inhibition) does not affect circulating ACE2 levels, so discontinuation of these drugs in active coronavirus disease is not justified.

9. List of own publications

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/1/2022.PL
Subject: PhD Publication List

Candidate: Viktor Bánhegyi
Doctoral School: Kálmán Laki Doctoral School
MTMT ID: 10057399

List of publications related to the dissertation

1. Fagyas, M.*, **Bánhegyi, V.***, Úri, K., Enyedi, A., Lizanecz, E., Mányiné Siket, I., Mártha, L., Fülöp, G. Á., Radovits, T., Pólos, M., Merkely, B., Kovács, Á., Szilvássy, Z., Ungvári, Z., Édes, I., Csanádi, Z., Boczán, J., Takács, I., Szabó, G., Balla, J., Balla, G., Seferović, P. M., Papp, Z., Tóth, A.: Changes in the SARS-CoV-2 cellular receptor ACE2 levels in cardiovascular patients: a potential biomarker for the stratification of COVID-19 patients.
GeroScience. [Epub ahead of print], 2021.
* These authors contributed equally this work.
IF: 7.713 (2020)

2. **Bánhegyi, V.**, Enyedi, A., Fülöp, G. Á., Oláh, A., Mányiné Siket, I., Váradi, C., Bottyán, K., Lódi, M., Csongrádi, A., Umar, M. A. J., Fagyas, M., Czuriga, D., Édes, I., Pólos, M., Merkely, B., Csanádi, Z., Papp, Z., Szabó, G., Radovits, T., Takács, I., Tóth, A.: Human Tissue Angiotensin Converting Enzyme (ACE) Activity Is Regulated by Genetic Polymorphisms, Posttranslational Modifications, Endogenous Inhibitors and Secretion in the Serum, Lungs and Heart.
Cells. 10 (7), 1-13, 2021.
DOI: http://dx.doi.org/10.3390/cells10071708
IF: 6.6 (2020)

List of other publications

3. Fagyas, M., Kertész, A. B., Mányiné Siket, I., **Bánhegyi, V.**, Kracskó, B., Szegedi, A., Szokol, M., Vajda, G., Rácz, I., Gulyás, H., Szkibák, N., Rácz, V., Csanádi, Z., Papp, Z., Tóth, A., Sipka, S. i.: Level of the SARS-CoV-2 receptor ACE2 activity is highly elevated in old-aged patients with aortic stenosis: implications for ACE2 as a biomarker for the severity of COVID-19.
GeroScience. 43 (1), 19-29, 2021.
DOI: https://doi.org/10.1007/s11357-020-00300-2
IF: 7.713 (2020)

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

4. Fülöp, G. Á., Oláh, A., Csípő, T., Kovács, Á., Pórszász, R., Veress, R., Horváth, B., Nagy, L., Bódi, B., Fagyas, M., Helgadottir, S. L., **Bánhegyi, V.**, Juhász, B., Bombicz, M., Priksz, D., Nánási, P. P. i., Merkely, B., Édes, I., Csanádi, Z., Papp, Z., Radovits, T., Tóth, A.: Omecamtiv mecarbil evokes diastolic dysfunction and leads to periodic electromechanical alternans.
Basic Res. Cardiol. 116 (1), 2021.
DOI: http://dx.doi.org/10.1007/s00395-021-00866-8
IF: 17.165 (2020)

5. Lódi, M., **Bánhegyi, V.**, Bódi, B., Gyöngyösi, A., Kovács, Á., Árokszállási, A., Hamdani, N., Fagyas, M., Ėdes, I., Csanádi, Z., Czuriga, I., Kisvárday, Z., Lekli, I., Bai, P., Tóth, A., Papp, Z., Czuriga, D.: Prophylactic, single-drug cardioprotection in a comparative, experimental study of doxorubicin-induced cardiomyopathy.
J. Transl. Med. 18 (1), 1-48, 2020.
IF: 5.531

6. Santer, D., Nagel, F., Gonçalves, I. F., Kaun, C., Wojta, J., Fagyas, M., Kršśák, M., Balogh, Á., Papp, Z., Tóth, A., **Bánhegyi, V.**, Trescher, K., Kiss, A., Podesser, B. K.: Tenascin-C aggravates ventricular dilatation and angiotensin-converting enzyme activity after myocardial infarction in mice.
ESC Heart Failure. 7 (5), 2113-2122, 2020.
DOI: http://dx.doi.org/10.1002/ehf2.12794
IF: 4.411

7. Nagy, L., Gődény, I., Nánási, P. P. i., Leskó, Á., Balogh, L., **Bánhegyi, V.**, Bódi, B., Csípő, T., Csongrádi, A., Fülöp, G. Á., Kovács, Á., Lódi, M., Papp, Z.: A szív pozitív inotróp támogatása a miozin-aktivátor hatású omecamtiv-mecarbil segítségével.
Cardiol. Hung. 47 (1), 69-76, 2017.

Total IF of journals (all publications): 49,133
Total IF of journals (publications related to the dissertation): 14,313

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

05 January, 2022

10. Acknowledgements

I would like to thank first of all my supervisor **Professor Dr. Attila Tóth**, whose professional advice and support I could always count on during my Ph.D. studies. I thank him for setting an example in terms of conscious experiment design, quality scientific publication and professional dedication.

I would like to thank with all due respect the head of our department, **Professor Dr. Zoltán Papp**, for providing me with the equipment and professional-material background for my research work, and for supporting me in my scientific work and scholarship applications.

I would like to thank my departmental colleagues **Dr. Miklós Fagyas, Dr. Dániel Czuriga,** and **Dr. Viktória Csató**, as well as **Dr. Levente Bodoki** for their professional help, advice and collaboration.

Special thanks to the selfless help of **Ivetta Siket Mányiné** laboratory assistant, without whom this work could not have been carried out.

Among our collaboration partners, I owe special thanks to the colleagues of the Department of Thoracic Surgery of the University of Debrecen, **Dr. Mária Lódi, Dr. Alíz Varga, Professor Dr. Attila Bácsi, Prof. Dr. med. Bruno Podesser and Dr. Attila Kiss.**

I would like to thank all the former and current members of the **Division of Clinical Physiology of the University of Debrecen** for their willing help and support in my research.

Thank you to my family, my mother, my wife for your dedicated support.

I dedicate my work to my son Medárd and my daughter Otília.

The dissertation was fundedby the grants GINOP-2.3.2-15-2016-00043, GINOP-2.3.2-15-2016-00050 and EFOP-3.6.3-VEKOP-16-2017-00009. The project was implemented with the support of the European Union and co-financed by the Government of Hungary.