Final communiqué of the NKFIH project PD116212, entitled: Role of free fatty acids in endogenous ACE-inhibition

Content

1. Prevention of cardiovascular diseases with functional foods (manuscript has been prepared for submission)

Cardiovascular diseases are the leading causes of mortality all over the world, more people die annually from cardiovascular diseases than from any other causes(1). Therapeutic inhibition of angiotensin converting enzyme (ACE) by ACE-inhibitor drugs can decrease cardiovascular mortality up to 23% (4), which points to essential role of ACE in cardiovascular pathology. Previously we have reported, that human serum albumin (HSA) endogenously inhibits the circulating ACE activity (2,3) and lack of this inhibition may lead to cardiovascular disease development (4). This hypothesis is supported by numerous studies (5–9), which have concluded, that low normal HSA concentration is an independent risk factor for cardiovascular diseases.

Comparison between HSA concentration and endogenous ACE inhibition of 161 cardiovascular patients has showed, that the level of endogenous ACE inhibition linearly relates to HSA concentration (Fig. 1). Higher HSA level in the circulation may reduce the production of the vasoconstrictor angiotensin II by inhibiting ACE, thus albumin can directly lower blood pressure. Other workgroups have observed, that albumin infusion can generate hypotension(10,11), furthermore, high normal HSA level may prevent the development of hypertension (7).

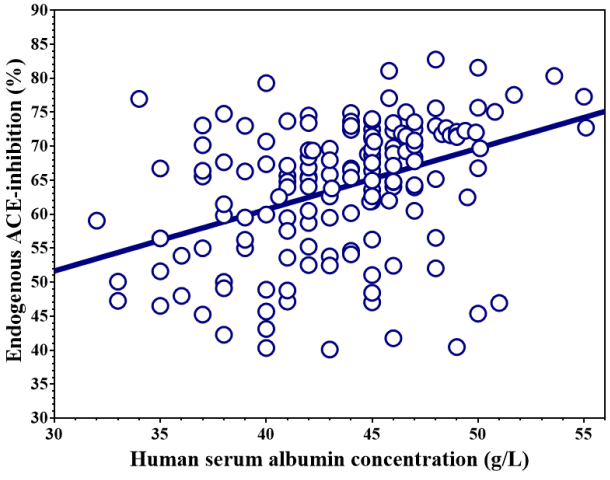


Figure 1. Level of endogenous ACE inhibition is connected to HSA concentration in the circulation, *in vivo*

Elimination of water insoluble molecules (such as free fatty acids, FFA) from the HSA by active charcoal treatment dramatically decreased the ACE-inhibitor property of albumin compared to albumin purified by dialysis (Fig. 2, left side). Difference between purified HSAs promised to be more significant at physiologic HSA concentration (35-52 g/L). Presence of water insoluble molecules on the HSA ensures 89.6±0.5% ACE-inhibition at 45 g/L HSA concentration compared to 59.4±1.4%, in which case the water insoluble molecules were eliminated. The total free fatty acid concentration did not show significant relationship with the level of endogenous ACE-inhibition (Fig. 2, right side) measured from serum samples of individuals. This indicated that FFAs may not influence the HSA-mediated endogenous ACE-inhibition, or different type of FFAs may have different effect on the level of HSA-mediated endogenous ACE-inhibition.

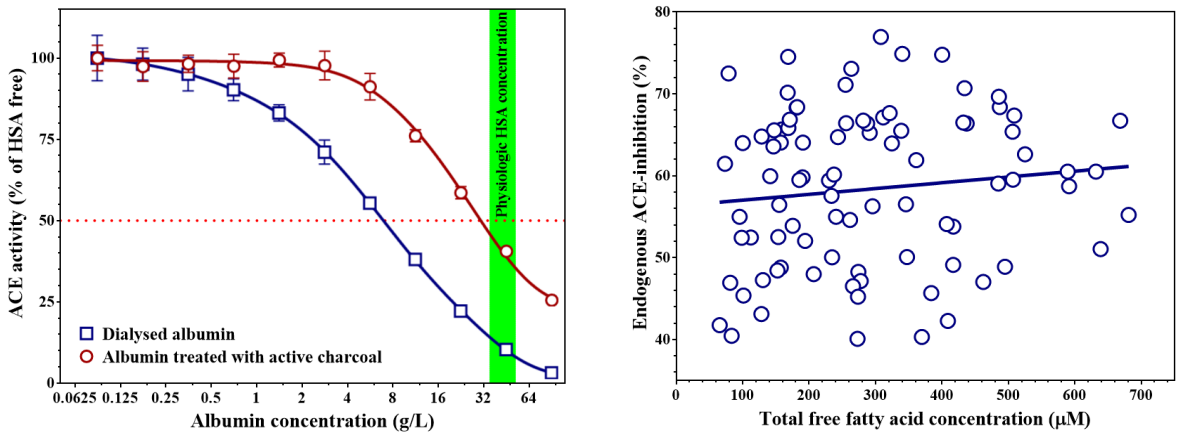


Figure 2. Effect of FFA elimination on HSA-mediated ACE inhibition (left) and lack of association between the level of endogenous ACE inhibition and total FFA concentration, *in vivo*

For testing these hypothesis, we added different type of saturated fatty acids to the fatty acid free HSA (Fig. 3). We found, that binding of FFAs to HSA significantly increased the effectiveness of ACE-inhibitor property of albumin furthermore, the effect on ACE-inhibition highly depended on the length of saturated fatty acid (Fig. 3, left side).

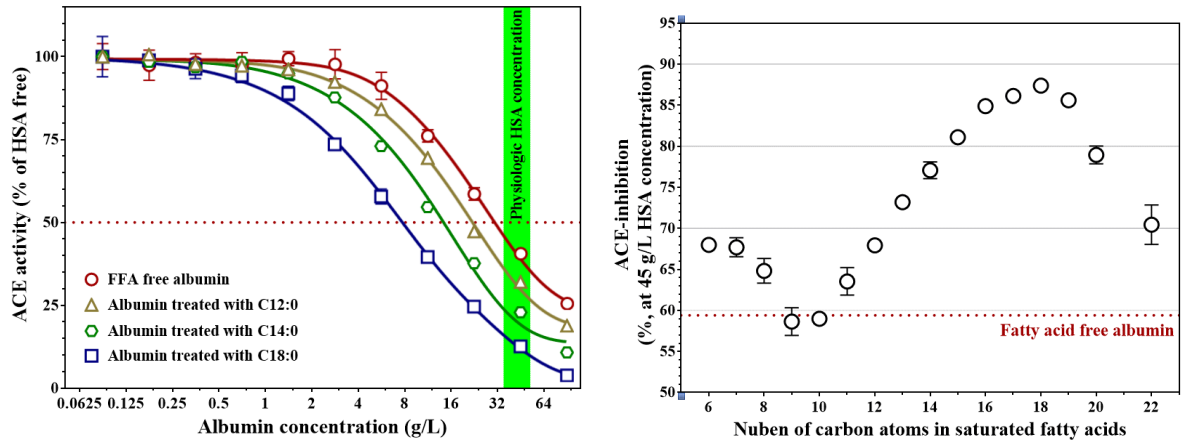


Figure 3. Treatment of FFA free HSA with individual FFAs increases the endogenous ACE inhibitor property of albumin (left) and the level of ACE inhibition at 45 g/L HSA concentration depends on the length of saturated FFA

We determined the level of ACE-inhibition of different FFA-HSA complexes at physiological HSA concentration (Fig. 3, right side). There are some naturally occurring saturated FFAs, which can dramatically increase the ACE-inhibitor property of albumin. Surprisingly, the presence of capric acid (C10:0) on HSA can not modify the HSA evoked ACE inhibition.

Among naturally occurring saturated fatty acids, stearic (C18:0) and palmitic acids (C16:0) can increase the HSA-mediated ACE-inhibition in the highest extent in our experiments. Several previous evidences have shown that diet full of saturated fats has detrimental effect to cardiovascular health. This could be put down to the fact that some saturated fatty acids can increase LDL cholesterol concentration in the serum (12–15). Latest evidence does not support the notion that dietary saturated fatty acids cause heart disease (16). Palmitic and stearic acids are usually present in high concentration in the circulation, thus these fatty acids exert significant influence on HSA mediated endogenous ACE-inhibition, *in vivo*. It is important to note, that palmitic and stearic free fatty acids are released primarily from adipose tissue and do not originate from diet (17). Furthermore, in contrast with circulating esterified fatty acids, the proportion of palmitic and stearic acids among other FFAs may not be influenced by the composition of dietary fat (18). Consequently, procedures which facilitate lipolysis in adipose tissue could be considered as beneficial cardiovascular factors as they increase fatty acid concentration and consequently endogenous ACE-inhibition.

Regular physical activity is a mainstay of cardiovascular prevention (19,20). Cross-sectional studies have shown, that training enhances whole-body lipolysis and FFA release (21) during strenuous exercise and at rest as well (22). Hereby physical activity may increase the level of endogenous ACE inhibition which may partly explain the beneficial effect of physical activity on cardiovascular health.

The presence of a double-bond in FFAs alters the effectiveness of ACE-inhibition mediated by HSA (Fig. 4, left side). Furthermore, the efficacy of fatty acids of the same length are different if a double-bond in trans position is present (Fig. 4, right side).

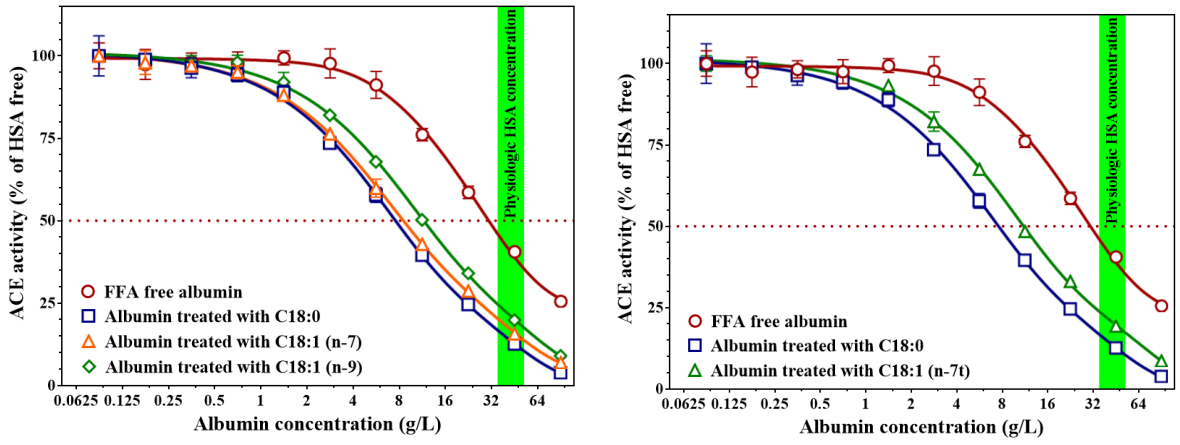


Figure 4. Effect of double-bond (left) and cis-trans position of double bond (right) on ACE-inhibition

HSA treated with linolenic acid (C18:3(n-3)) showed the highest efficacy among the examined ω-3 fatty acids (Fig. 5, left side). Long-chain polyunsaturated fatty acids seemed to be less effective. The ω-6 γ-linolenic acid had the highest ACE-inhibitor modifying effect

among the examined fatty acids (Fig 5, right side). It is interesting to note, that HSA treated with α- and γ-linolenic acid showed partial antagonist effect on ACE activity.

The latest American (23) and European (19) guidelines recommend the increased consumption of omega-3 polyunsaturated fatty acids, such as eicosapentaenoic acid [C20:5 (n-3)] and docosahexaenoic acid [C22:6 (n-3)] for cardiovascular prevention. Whereas the most recent Cochrane Database systematic review (24) has showed, that eicosapentaenoic acid and docosahexaenoic acid have little or no effect on cardiovascular mortality or cardiovascular health. Only alpha-linolenic acid [C18:3 (n-3)] may slightly reduce cardiovascular risk, mortality and the onset of arrhythmia (24). These findings are in harmony with our findings, because alpha-linolenic acid increases the endogenous ACE inhibition at physiological HSA concentration much more effectively than docosahexaenoic or eicosapentaenoic acid. Furthermore, dietary administration of alpha-linolenic acid can decrease either systolic or diastolic blood pressure (25,26) similarly to ACE-inhibitor medications. Increased endogenous ACE-inhibition may explain this blood pressure lowering effect, as albumin binds alpha-linolenic acid.

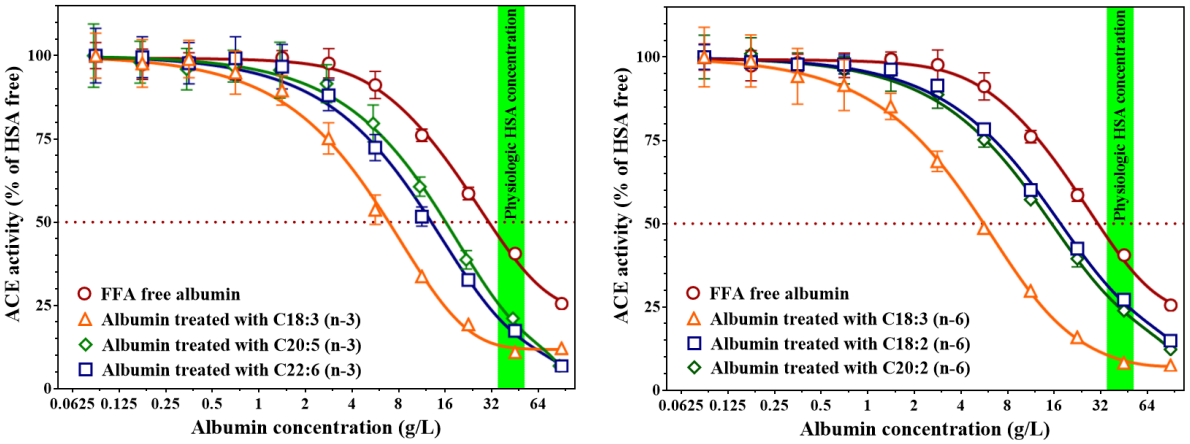


Figure 5. Effect of omega-3 (left) and omega-6 (right) fatty acids on endogenous ACE-inhibition

HSA is an important carrier protein in the blood-stream, it binds several water soluble, water insoluble molecules and a wide range of drugs (27). Our experiments have disclosed the role of free fatty acids in the modulation of endogenous ACE-inhibition mediated by HSA. Albumin has 7 common fatty acid binding sites with high or moderate affinity (28) and FFA binding to albumin induces a dramatic conformational change in the protein (29). This structural change may help the albumin molecule to bind ACE with higher affinity, namely the ACE inhibitor efficacy of albumin increases. This assumption may be supported by the facts, that tyrosine residue (Tyr-138) of fatty acid site 1 has essential role either in fatty acid binding (29) or in the development of endogenous ACE inhibition (30), moreover another potential ACE-biding site of albumin (Ala-213 – Arg-218) (31) participates in the formation of FFA binding site II (28). There are two ways to increase the level of endogenous ACE-inhibition in our body. Mobilizing stearic or palmitic acid (or other beneficial FFAs) from adipose tissues by physical activity may effectively increase the HSA-mediated endogenous ACE inhibition in the circulation. By the other hand, eating foods containing large concentration of beneficial FFAs can directly improve the endogenous ACE inhibition. These foods can be considered as cardio-

protective foods. Our experiments highlighted the significant impact of alpha-linolenic acid on the development of considerable endogenous ACE-inhibition. Flaxseed is an important source of alpha-linolenic acid. A previous human study showed, that dietary supplementation with flaxseed can significantly decrease the systolic and diastolic blood pressure of hypertensive patients (32). Moreover, a recent randomised-controlled trial (FLAX-PAD) has revealed, that 1-year-long diet supplementation with flaxseed significantly decreases the incidence of arrhythmias compared to the placebo group (33). ACE-inhibitor treatment has anti-arrhythmic (34) and antihypertensive effects as well. In FLAX-PAD trial the observed anti-arrhythmic effect and the previously described antihypertensive effect may be due to the increased endogenous ACE-inhibition as alpha-linoleic acid binds to albumin.

In consequence, the level of endogenous ACE-inhibition is highly determined by the presence of free fatty acids on the surface of albumin. Increasing endogenous ACE-inhibition by physical activity or by eating foods containing large amount of beneficial fatty acids may prevent the development and progression of cardiovascular diseases.

2. Diagnostic applicability of endogenous ACE-inhibition (35)

Sarcoidosis is an idiopathic multisystem granulomatous disease that most commonly affects the lungs, lymph nodes and skin (36). There are no gold standard laboratory tests to facilitate earlier diagnosis, albeit several new biomarkers have been discovered in the last decades. Soluble interleukin-2 receptor (SIL-2R) (37), chitotriosidase (38), serum amyloid A (SAA) (39) and other markers were tested as possible diagnostic marker, but only serum angiotensin-converting enzyme (ACE) seems to have diagnostic utility (40).

Majority of the commercially available ACE activity diagnostic tests propose using 1:5 or 1:10 ratios of serum and substrate solution for the measurements. Endogenous inhibition of ACE by albumin is still present at those dilutions; consequently, ACE activity may be underestimated with these tests and sarcoidosis remains underdiagnosed.

We described an optimized fluorescent kinetic assay for ACE activity measurement, in which the inhibitory effect of albumin was eliminated. Genotype-independent and I/D polymorphism-dependent reference intervals for ACE activity were established.

Reversible endogenous inhibition of ACE by albumin can be eliminated by the appropriate dilution of serum. ACE activity of three serum samples obtained from healthy individuals with different ACE I/D genotypes was measured in serial dilutions (Fig. 6). Calculated ACE activity values showed an exponential increase with the dilution in each genotype, reaching the maximum at 35-fold dilution. ACE activities measured at 35-fold dilution did not differ statistically from those values measured at 70-fold dilution in all samples.

Data of 201 individuals were involved in the determination of genotype-dependent and genotype-independent ACE activity reference values. ACE I/D genotype and ACE activity at 35-fold dilution were determined.

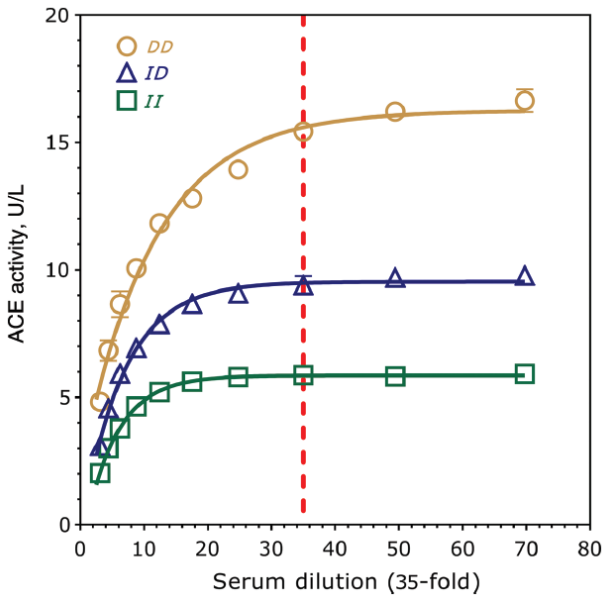
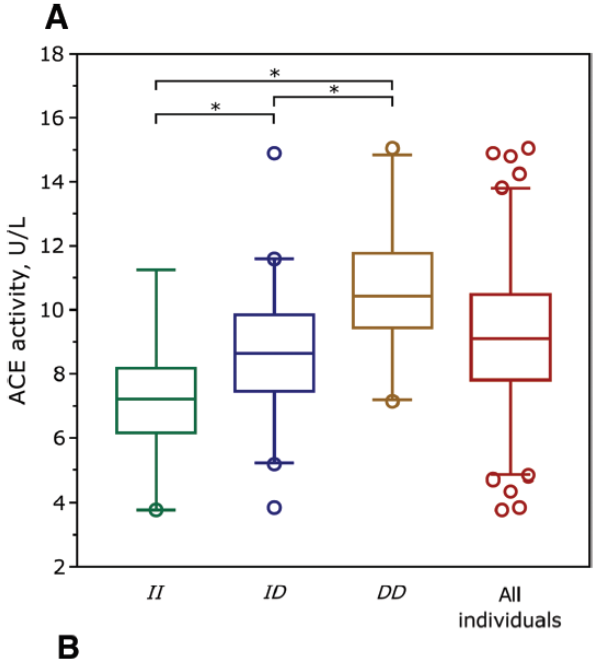


Figure 6. Endogenous ACE-inhibition mediated by albumin can be eliminated by the 35-fold dilution of serum

The ACE I/D genotype significantly influenced ACE activity, as individuals with II genotype showed lower ACE activity than individuals with DD genotype, while subjects with ID genotype showed a mid-range activity (Fig. 7A). Normal reference intervals for each group were calculated and summarized on Figure 7B.



B

Group	n	95% Reference interval, U/L
II	39	3.76 – 11.25
ID	90	5.22 – 11.59
DD	72	7.19 – 14.84
Genotype independent	201	4.85 – 13.79

Figure 7: ACE I/D genotype influences the serum ACE activity. Serum ACE activity distribution (A) and normal reference ranges (B) are shown according to ACE I/D genotype. The boxes indicate the interquartile range with median; whiskers are the 2.5th and 97.5th percentiles. Outliers are plotted as individual points (empty cycles). Significant differences among genotype groups are labelled by asterisks (*).

By this way, we have further optimized a fluorescent kinetic ACE activity method, when the assay is not interfered with endogenous ACE inhibitor albumin. Using our new genotype-dependent reference intervals and cut-off values, this test might be an alternative to invasive biopsy for confirming the diagnosis of sarcoidosis in almost half of patients.

<u>Clinical and economic application:</u> Our optimized ACE activity method has already been introduced into routine clinical laboratory diagnostics in the University of Debrecen. This test is available not only for local specialists (41)***, but for each specialist in Hungary*** (42). ***The Hungarian National Health Insurance finances the test.***

3. Diagnostic evaluation of developed ACE activity measurement method (manuscript has been prepared for submission)

Ninety-nine patients were involved in a clinical study to evaluate the diagnostic accuracy of our optimized ACE activity assay in sarcoidosis and to compare its effectiveness to other commercially available ACE activity measurement assays. Furthermore, we measured sIL-2R, lysozyme and SAA concentration to evaluate the diagnostic applicability of these analytes in sarcoidosis. The patients underwent diagnostic mediastinoscopy or video-assisted thoracoscopic surgery for biopsy sampling intended to verify the lack or the presence of sarcoidosis by histopathology. Results of histopathology was compared to the concentrations and activities of analytes, measured from blood samples of patients.

First, diagnostic accuracy of different ACE activity measurement techniques was determined. Our fluorescent kinetic ACE activity assay (Fig. 8/A.; AUC= 0.8131) has the highest, the colorimetric-based assay has the worse (Fig. 8/C.; ACEcolor® AUC= 0.7806), and the absorbency-based method (Fig. 8/B.; Infinity ACE®, AUC= 0.8071) has medium accuracy among the examined methods.

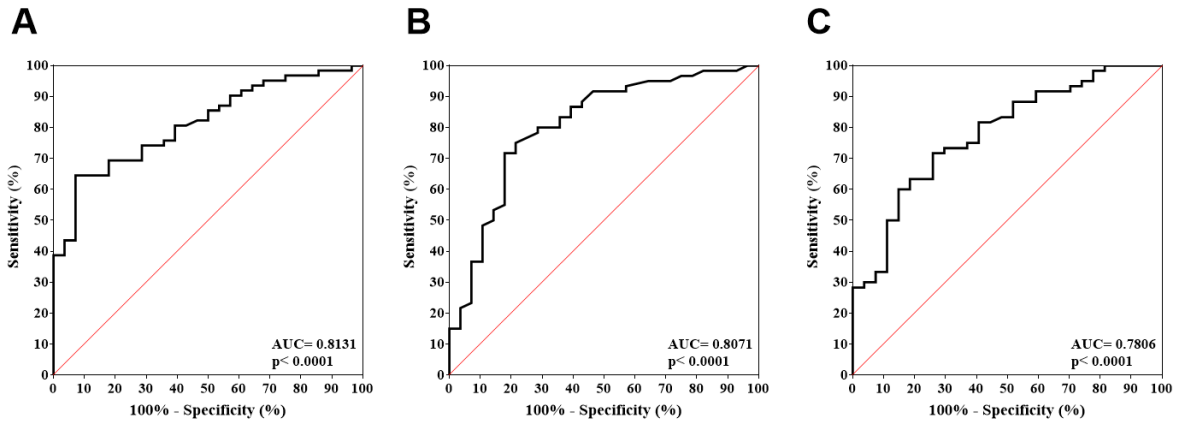


Figure 8: Receiver operating characteristic curves of examined ACE activity measurement techniques. A: our optimized fluorescent kinetic assay, B: Infinity ACE®, C: ACEcolor®. AUC: area under the curve

Our method has a great advantage over the competitors, because previously we have determined the insertion/deletion genotype-dependent ACE activity reference ranges for this assay. Applying these genotype-dependent reference intervals, we can further increase the diagnostic accuracy of this test (Table 1.)

	Applying genotype-independent reference interval	Applying genotype-dependent reference intervals
Sensitivity (%)	29	48
Specificity (%)	100	93
Positive predictive value (%)	100	94
Negative predictive value (%)	39	45

Table 1: Diagnostic accuracy of our fluorescent kinetic assay applying ACE genotype-independent and dependent reference intervals

We have tested the role of other biomarkers in the laboratory diagnostic of sarcoidosis. Diagnostic accuracy of soluble interleukin 2-receptor (Fig. 9/A), lysozyme (Fig 9./B) and serum amyloid A (Fig. 9/C) were determined using the study population. Only soluble interleukin 2-receptor concentration could discriminate between sarcoidotic and control patients (p=0.0101), but diagnostic accuracy of this test was weaker than that of ACE.

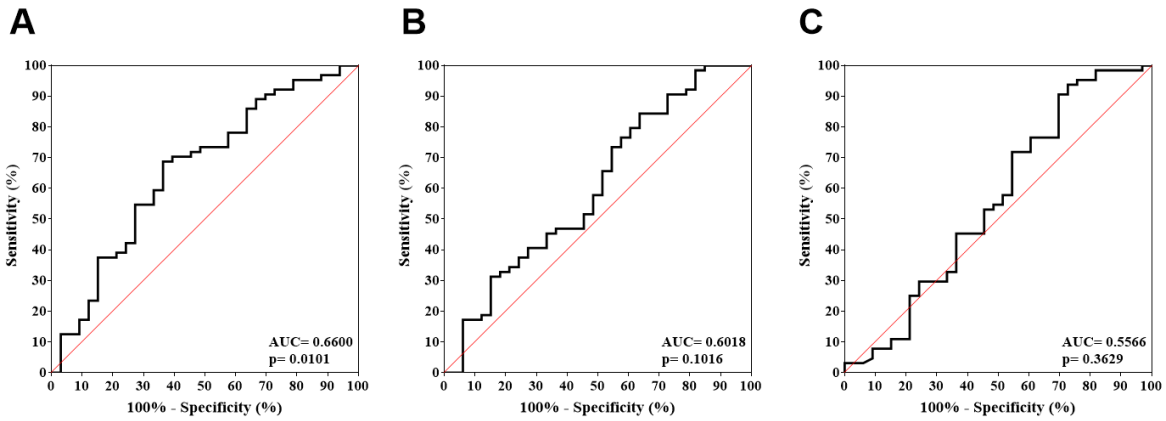


Figure 9: Receiver operating characteristic curves of soluble interleukin 2-receptor (A), lysozyme (B) and serum amyloid A (C). AUC: area under the curve

In summary, ACE activity measurement assay developed by our workgroup has a great diagnostic accuracy in the laboratory diagnosis of sarcoidosis. Using our genotype-dependent reference intervals and cut-off values, this test might be an alternative to invasive biopsy for confirming the diagnosis of sarcoidosis in almost half of the patients.

4. Effective endogenous ACE-inhibition ensures high impact of angiotensin II-degrading enzymes upon the development of cardiovascular diseases (43)

Endogenous regulation of ACE suggests new perspectives in the renin-angiotensin-aldosterone system. In particular, the high level of ACE inhibition by serum albumin suggested a prominent role for AngII-degrading enzymes, such as ACE2 in cardiovascular disease development and progression. In accordance, ACE2 has been related to hypertension and systolic heart function in human (44).

An effort was made to correlate serum ACE2 activities with the stages of the cardiovascular disease continuum. Serum ACE and ACE2 activities were determined in healthy individuals and in patients with hypertension (without heart failure (HF)), HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF). Patients were

recruited for clinical, echocardiographic and biomarker analyses as part of a single-centre, prospective study at the Institute of Cardiology University of Debrecen.

Serum ACE2 activity was the lowest in the healthy group (16.2±0.8 U/mL) which was significantly increased in hypertensive patients and further increased when hypertension was accompanied by HFrEF, representing progression of cardiovascular disease toward systolic dysfunction (Fig. 10). HFrEF patients without hypertension also had higher serum ACE2 activities. In contrast, patients with hypertension and HF with preserved systolic function (HFpEF) had similar serum ACE2 activities to that of hypertensive patients without HF.

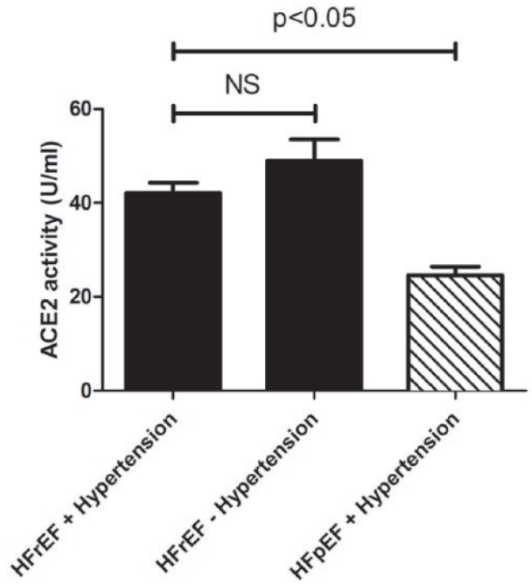


Figure 10. Serum ACE2 activity parallels cardiovascular disease development.

This suggests that ACE2 expression is either increased as a counter-regulatory mechanism to the dysregulation of the 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.

Serum ACE2 activities were plotted as a function of the EF in hypertensive HFpEF and hypertensive HFrEF patients (Fig. 11). There was no correlation between serum ACE2 activity and HF in patient populations with normal (preserved) EF. However, serum ACE2 activity correlated negatively with the EF in the HFrEF group.

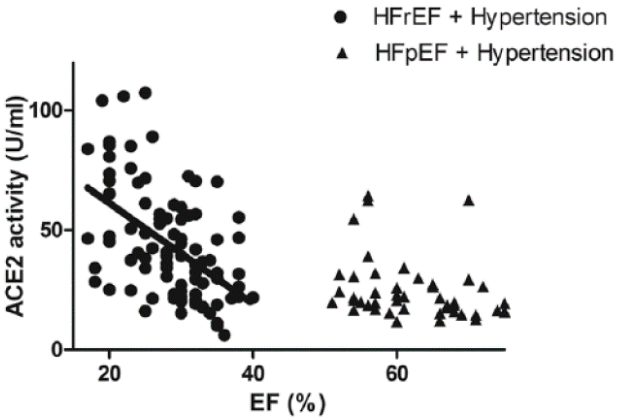


Figure 11. Serum ACE2 activity correlates with the severity of systolic dysfunction.

Clinical and economic applicability: The clinical applicability of serum ACE2 activity as a biomarker for left ventricular systolic dysfunction in human HF was also tested. Area under the ROC curves was 0.77 for HFrEF (Fig. 12/A) and 0.51 for HFpEF patients (Fig. 12/B). These data show, that ACE2 can discriminate only between HFrEF and hypertension and cannot between HFpEF and hypertension. Similar values for NT-proBNP were 0.93 for HFrEF (Fig. 12/C) and 0.78 for HFpEF (Fig. 12/D) patients, indicating increased NT-proBNP concentration of both HFrEF and HFpEF patients. Compared to NT-proBNP, serum ACE2 is a biomarker which can be used to differentiate between HFrEF and HFpEF.

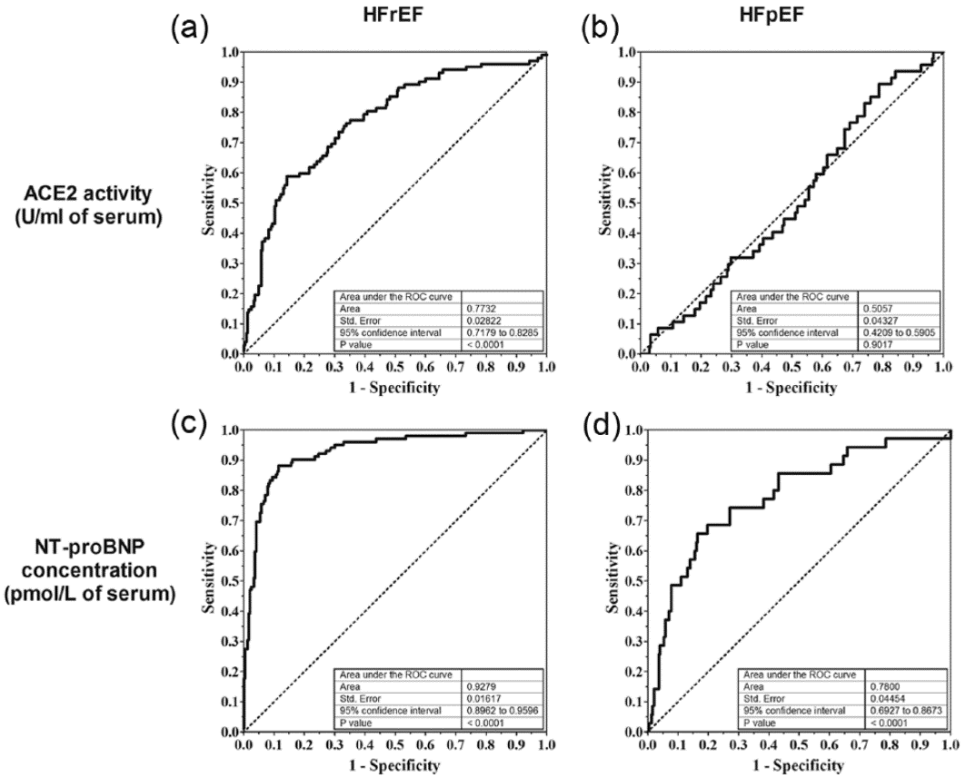


Figure 12. Comparison of the prognostic value for serum ACE2 activity and amino-terminal pro-B-type natriuretic peptide (NT-proBNP) concentration to differentiate HFrEF and HFpEF from hypertension.

5. Ineffective endogenous ACE-inhibition or decreased angiotensin II degradation by ACE2 may lead to diastolic dysfunction of heart (45)

We investigated the influence of hypertension on cardiac contraction and relaxation in transgenic renin overexpressing rats (carrying mouse Ren-2 renin gene, mRen2). Blood pressure was measured, cardiac contractility was characterized by echocardiography, cellular force measurements, and biochemical assays were applied to reveal molecular mechanisms. Sprague-Dawley (SD) rats were used as controls.

We found that transgenic mRen2 rats have an increased plasma renin activity (Fig. 13), which may directly activate the circulating RAAS. Moreover, we found a decrease in cardiac ACE2 activities responsible for angiotensin II breakdown, besides to similar ACE activities in the mRen2 rats. Therefore, in line with former results (46), these findings suggest that cardiac elimination of angiotensin II is lower in mRen2 animals, potentially increasing local RAAS activity.

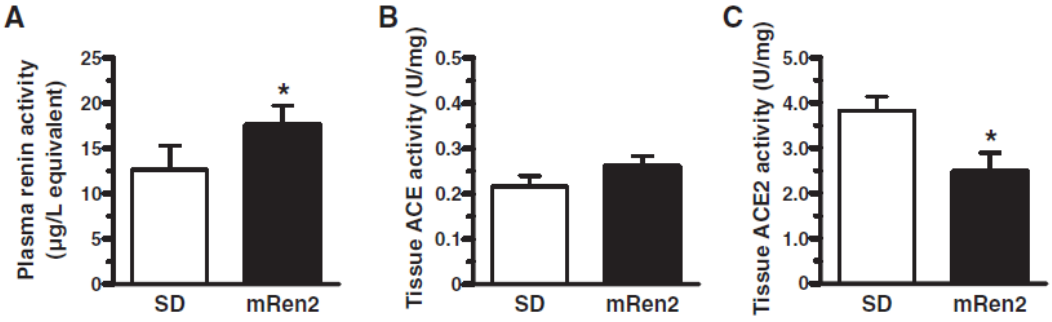


Figure 13. Plasma renin (A), myocardial angiotensin-converting enzyme (ACE, B) and ACE2 (C) activities.

We measured elevated blood pressure in mRen2 rats, resulting in increased left ventricular weight/body weight ratio. Transgenic renin expression had no effect on the systolic parameters, such as left ventricular ejection fraction, cardiomyocyte Ca^{2+}-activated force, and Ca^{2+} sensitivity of force production. In contrast, diastolic dysfunction was observed in mRen2 compared with SD rats: early and late left ventricular diastolic filling ratio (E/A) was lower, LV isovolumetric relaxation time was longer, cardiomyocyte passive tension was higher, and lung weight/body weight ratio was increased, as was left atrial weight/body weight ratio.

The main intracellular determinant of passive tension of the contractile machinery is the titin molecule, spanning the half sarcomere (47). Stiffness is modulated by titin isoform composition, degradation, oxidative modifications, and phosphorylation by various protein kinases (30) especially within the elastic I-band region at N2-Bus and PEVK (proline, glutamate, valine, and lysine reach motif) domains. In our experiments, hyperphosphorylation of titin at Ser-12742 within the PEVK domain and a twofold overexpression of protein kinase C-α in mRen2 rats were detected.

In this study, genetic stimulation of RAAS signalling resulted in hypertension selectively associated with left ventricular diastolic dysfunction, characteristic for human HFpEF. Our work suggests that hyperphosphorylation of Ser-12742 in the PEVK element of titin is a common feature of RAAS-mediated (including ineffective endogenous ACE-inhibition) hypertension with isolated diastolic dysfunction and clinical HFpEF.

6. Further experiments without concrete results

I planned to test the effects of specific FFA substitution on the cardiovascular status and on endogenous ACE-inhibition in a hypertensive animal model (mRen2 transgenic rat). The animals were fed with alpha-linolenic acid completed chow. Unfortunately, the breed was infected with mycoplasma pneumonia, and some rats died during the experiments. Consequently, the study was terminated before the end of the experiments. The study has not been repeated, because it has come to my attention that a human study was executed with a similar protocol. Prof. Grant Pierce organised a randomised, double-blinded study, in which the diet of the treated arm was supplemented with flaxseed (containing alpha-linolenic acid). The supplementation resulted in beneficial cardiovascular effects (decreased blood pressure, antiarrhythmic effect etc.). I plan to measure the endogenous ACE inhibition from human samples of this study in a collaboration with prof. Grant Pierce.

7. References

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