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Title: Endogenous regulation of angiotensin converting enzyme by posttranslational modifications and its involvement in cardiovascular disease development

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I. Introduction

Cardiovascular diseases remain the leading causes of death in developed countries. The involvement of the angiotensin-converting enzyme (ACE) in the development and maintenance of cardiovascular diseases is critical, as inhibition of the enzyme's activity significantly reduces mortality and morbidity from these diseases. The research focused on the physiological and pathophysiological regulation of ACE and the role of post-translational modifications in disease development. Unfortunately, the coronavirus pandemic made it difficult to carry out the planned research work, however, the patient selection and research methodology described and implemented in the research plan provided a unique opportunity for our research team to answer questions of significant cardiovascular relevance that arose in the context of the COVID-19 pandemic quickly and efficiently, without compromising the original work plan. This resulted in a number of research directions and publications that, although not originally planned, were closely related to and built on the original idea. As a result the project has so far resulted in 12 publications with a cumulative impact factor of 88.831.

II. Endogenous regulation of angiotensin converting enzymes

The renin-angiotensin-aldosterone system (RAAS) plays a crucial role in the fluid and salt homeostasis. One of the key biochemical steps within the RAAS is conversion of the inactive angiotensin I decapeptide (AngI) to active angiotensin II (AngII) octapeptide by angiotensin converting enzyme (ACE). It is important to note that AngII generation by ACE is reversed by its isoform ACE2 (which eliminates AngII). We tested the links between ACE activity and its genotype-specific expression, endogenous inhibitors and ACE secretion in clinical samples. ACE activity and ACE concentration were measured in human sera and tissue (lung and heart) samples obtained from patients undergoing lung surgery or heart transplantation.

We found, that patients with the DD genotype had significantly higher circulating ACE concentrations and activities than patients with the ACE II genotype, while patients with the ACE ID genotype showed intermediate values confirming earlier reports. However, we did not

find any correlation of lung tissue ACE expression or activity with the ACE I/D genotype. This finding suggests that ACE expression in the lungs is independent of ACE I/D genotype, and consequently, the genotype-dependent serum ACE secretion must have an alternative source of ACE. Furthermore, there were comparable ACE inhibitory levels in patients with and without ACE inhibitory medications, suggesting a negligible effect of the drug on tissue ACE activities. The concentration of human serum albumin is too low in the lung tissue samples to provide significant ACE inhibition, and thus, this implicates an alternative mechanism for ACE inhibition. Specific ACE activities were significantly higher in human lung tissues than that in the sera of the same patients. This difference suggests that ACE processing is different in these tissues, resulting in different post-translational modifications. This is confirmed by the fact that serum ACE protein is much more highly sialylated than lung ACE. Moreover, our data suggest that circulating ACE activity correlates with left ventricular ACE, but not with lung ACE in human. {More details and the manuscript are available at the link.}

In order to better understand the endogenous regulation of ACE and its role in cardiovascular disease, we also examined posttranslational modifications of renal and urinary ACEs. High expression of ACE (even higher than in lung endothelial cells) was found within the brush border of the proximal tubular cells of the kidney. It is important to mention, that local ACE conformation of endothelial cells and epithelial cells is different: as human kidney (epithelial) ACE may have at least six N-glycosylated sites (residues 9, 25, 82, 117, 480, 913), while serum (endothelial) ACE has mainly three glycosylation sites on Asn480, 666, and 685. Our data revealed, that both lung and epithelial ACEs are fully glycosylated on Asn117 and Asn648, however, the structure of putative glycans on Asn117 is different, as glycan of epithelial ACE exhibits more branches.

We also examined the structure of ACE in the urine of healthy volunteers. Unexpectedly, we found dramatic differences in the structure of ACE in association of genders that may be explained by sex-specific differences in kidney ACE glycosylation and/or sialylation. This provides novel insights into the sex differences observed in some ACE-related diseases. Furthermore, significant structural differences were found of ACE in kidney homogenates compared to lung homogenates, which may be attributed to differential glycosylation in these two organs of certain sites in the ACE protein (including Asn45, Asn117 and Asn731). {The article is correctly under revision: Urinary ACE phenotyping as a research and diagnostic tool: identification of sex-dependent ACE immunoreactivity; Manuscript ID= PONE-D-22-18619R1}

We have also studied the regulation of ACEs in pathological conditions. Myocardial infarction was induced by the permanent ligation of the left anterior descending artery of 12-week-old male Sprague-Dawley rats. We focused on left ventricular alterations and its prevention by repeated remote ischemic conditioning. Functional and biochemical measurements in left ventricular cardiac tissues and cardiomyocytes were performed at the age of 18 weeks *in vivo* and *ex vivo*. Angiotensin-converting enzyme activity was about five times higher in the anterior left ventricular wall at six weeks than that in sham animals. Angiotensin-converting enzyme 2 activity roughly doubled in post-ischemic left ventricular walls. These increases in ACE and ACE2 activities were effectively mitigated by repeated remote ischemic conditioning. The underlying mechanisms have not yet been identified, based on the apparent close relationship between ACE and ACE2 activities and their expressions in lung and heart tissues, a reduction in the expression levels of these enzymes cannot be excluded upon repeated

remote ischemic conditioning. We have found that post-ischemic left ventricular remodeling involves region-specific alterations in ACE and ACE2 activities together with changes in cardiomyocyte myofilament protein phosphorylation and function and repeated remote ischemic conditioning has the potential to prevent these alterations and to improve left ventricular performance following myocardial infarction. {More details and the manuscript are available at the link.}

III. Regulation of ACE and ACE2 in COVID-19

Early diagnosis and effective clinical monitoring of COVID-19 are essential to prevent severe consequences or death. We were the first, who reported on elevated circulating ACE2 and soluble E-selectin levels in severe COVID-19. In a 69-year-old male patient (followed up for 2 months), we have shown that serum ACE2 activity increases dramatically with the severity of the COVID-19 infection. Based on the observed differences, we have raised the possibility that serum ACE2 levels may predict the progression of COVID-19 and thus serve as a prognostic marker of the disease. Our results also do not exclude the hypothesis that elevated serum ACE2 protein levels may be a natural line of defence against coronavirus. {More details and the manuscript are available at the link.}

To test our hypothesis, we retrospectively analysed clinical and laboratory data from COVID-19 patients in parallel with our existing cardiovascular patient populations. This study recruited 176 COVID-19 positive subjects to analyse serum ACE2 activity upon hospital admission and during hospital treatment. ACE2 activity was significantly higher in critically ill than in severe COVID-19 subjects and in non-COVID-19 severe sepsis regardless of comorbidities. Serum ACE2 activities were determined not only in baseline samples but were followed in a subgroup of recruited critically ill and severe patients to study the kinetics of ACE2 activity depending on COVID-19 severity. We found that, when compared to baseline levels, ACE2 activities were further elevated during the hospital treatment of critically ill patients, in contrast to severely ill study participants in whom alterations did not reach statistical significance during hospital stay.

Serum ACE2 was analysed as to whether this biomarker showed any association with disease outcome. In this context, most (both critically ill and severe) patients who finally died of COVID-19 showed significant ACE2 elevations before death, while no significant change in ACE2 activities were observed in survivors before discharge from the hospital. A logistic regression analysis was performed to test whether ACE2 can independently predict the severity of the disease. Higher initial ACE2 activity had a significantly higher odds ratio for a more severe outcome (OD: 1.032, 95% CI [1.005-1.061], P = 0.019). We found, that COVID-19 patients with highly elevated ACE2 levels (≥45.4 mU/L) had a greater risk for 30-day mortality when compared to those with lower ACE2 activity. In conclusion, serum ACE2 activity at hospital admission correlates with COVID-19 severity and predicts mortality, independently of pulmonary function (Horowitz index). It appears that serum ACE2 is a non-specific biomarker in systemic inflammation, since it is also elevated in severe sepsis. {More details and the manuscript are available at the link.}

Based on the data available at the beginning of the coronavirus pandemic, there appeared to be a significant association between cardiovascular disease with increased ACE2 expression and COVID-19 mortality/morbidity. Patients with severe aortic stenosis may represent a prime

population of patients to study ACE2 levels in relation to COVID-19 mortality. We found markedly increased circulating ACE2 activities in the sera of aorta stenosis patients and the serum ACE2 activity was 4-fold higher in the patients with severe aorta stenosis than that in hypertensive patients, suggesting that hypertension itself is probably not the primary determinant of these elevated ACE2 activities. Circulating ACE2 activity did not correlate with systolic or diastolic blood pressure, or left ventricular wall thickness in the severe AS patients, suggesting that ACE2 dysregulation is not the result of hypertension, per se. Our results showed, that the elevation in ACE2 activity can be the combined effect of reduced left ventricular systolic function, elevated pulmonary pressure, and age in this patient population. Among these factors, pulmonary congestion and age seem to be dominant. Our data suggest that circulating ACE2 may reflect susceptibility for SARS-CoV-2 infections. Moreover, we proposed that circulating ACE2 activity can be considered as a cardiovascular biomarker with potential implications for SARS-CoV-2 infections. {More details and the manuscript are available at the link.}

The clinical data and serum/tissue samples from the patients enrolled in the project (FK 128809) provided a unique opportunity to quickly answer the questions that arose at the beginning of the COVID-19 pandemic. Taking advantage of this opportunity we conducted a study to provide a thorough evaluation of ACE2 levels and expression in human sera and tissue samples (heart and lung) in relation to risk factors for COVID-19 mortality, including cardiovascular diseases (hypertension, heart failure), advanced age, obesity and male sex. We also addressed the effects of cardiovascular disease severity and renin-angiotensin-aldosterone system inhibitor medications on ACE2 levels.

We found, that circulating ACE2 activity correlated with the severity of cardiovascular disease. It was slightly elevated in hypertension and dramatically increased at the occurrence of heart failure. Moreover, circulating ACE2 activity strongly correlated with the left ventricular ejection fraction. Circulating ACE2 activity was higher in males than in females, and was elevated in overweight and obese patients; moreover, circulating ACE2 activity was also increased in elderly hypertensive individuals. Both left ventricular ACE2 activity and expression positively correlated with the serum ACE2 activities of the same patients, but neither lung tissue ACE2 activity nor lung tissue ACE2 expression correlated with circulating ACE2 activities in the same patients with pulmonary disease. This study has suggested that ACE2 dysregulation associates with the severity of cardiovascular disease, which can be a pathological step in the worsening of the patient's cardiovascular condition. This pathological pathway may be particularly important in COVID-19, since ACE2 dysregulation may explain the higher mortality among elderly and overweight cardiovascular patients, implicating circulating ACE2 as a biomarker of COVID-19 mortality. {More details and the manuscript are available at the link.}

COVID-19 also evokes cardiovascular complications, including acute coronary syndromes and cardiomyopathy, besides to systemic inflammation (especially in children). Recently, it has been reported that SARS-CoV-2 infection causes development of autoantibodies in patients with severe COVID-19. A set of targets has been identified recently for autoantibodies, including rheumatoid disease-related antigens and interferons, which the latter were also implicated in COVID-19 mortality by suppressing immune responses. Nevertheless, it is likely that autoantibodies in COVID-19 may be also directed against other targets, such as lungs and heart.

Using tissue and blood samples collected during the course of the proposal, we wanted to find out whether anti-myocardial autoantibodies are produced during COVID-19 and whether these have an impact on survival or disease severity. The majority of severe COVID-19 patients develop anti-cardiac autoantibodies. Some of these anti-cardiac autoantibodies are of IgM type, suggesting a COVID-19 associated activation of novel autoantibody production. Moreover, frequent appearance of IgG type of anti-cardiac autoantibodies suggest the reactivation of resident anti-cardiac autoantibodies. These findings suggest that acute phase of COVID-19 may be complicated by cardiac autoimmune reactions. Autoantibodies were produced against proteins of different sizes in myocardium, and there was no significant effect of anti-cardiac autoantibodies on acute disease mortality in our study. Age or gender had no impact on the prevalence of autoantibody production.

Anti-cardiac autoantibody development in COVID-19 patients was compared to two severe cardiac patients' populations. Anti-cardiac autoantibody production was very low in patients with severe aortic stenosis, undergoing transcatheter valve implantation. In contrast, patients with advanced heart failure with dilated cardiomyopathy had similar occurrence of IgG and lower occurrence of IgM anti-cardiac immunoglobulins. This suggests that cardiac failure (similarly to severe COVID-19) results in the release of immunogenic cardiac proteins, which may lead to reactivation of anti-cardiac autoantibodies. {More details and the manuscript are available at the link.}

The degree of tissue damage in COVID-19 may play a major role in the development of myocardial autoantibodies, therefore we wanted to further investigate the activity of lactate dehydrogenase (LDH) isoenzymes in serum (as a marker of tissue damage) in hospitalized COVID-19 subjects in connection with the disease severity and worse clinical outcome.

We found, that no characteristic profile of LDH isoenzymes can be detected in COVID-19 pneumonia, however, elevated calculated LDH-3 and LDH-4 activities are associated with unfavourable clinical outcomes. Based on these data, there must be a direct link between increased LDH activity and SARS-CoV-2 induced lung or heart injuries, but a more widespread tissue damage can simply overwhelm the relative activities of LDH isoenzymes. {More details and the manuscript are available at the link.}

IV. ACE regulation in diseases, focusing on clinical relevance and utility

Sarcoidosis is a systemic granulomatous disease affecting predominantly thoracic organs (lymph nodes and the lungs). Establishing the diagnosis of sarcoidosis often requires biopsy and histopathologic evaluation, which significantly increases disease burden, especially in cases with only thoracic involvement, when the patients lack an easily accessible and safe anatomical site for biopsy procurement. Serum biomarkers play a limited role in diagnosing sarcoidosis, because not a single molecule is known to be specific and sensitive enough for sarcoidosis.

During project implementation we have developed an optimized ACE activity measuring method, which is not influenced by the presence of endogenous ACE inhibitors (e.g. albumin). We also processed samples and data from patients who were investigated for suspected sarcoidosis, as their serum ACE activity was elevated during the course of the disease. This in turn may contribute to understanding changes in ACE regulation. Our results

have shown that our validated and published method has at least as good diagnostic performance in sarcoidosis as commercially available kits, but can be further enhanced by using the ACE I/D genotype and our established genotype-dependent reference ranges.

We have found that the measurement of other biomarkers (e.g. soluble interleukin-2 receptor, lysozyme, serum amyloid A) is less helpful in diagnosing sarcoidosis, with the possible exception of serum chitotriosidase (CTO) activity. The parameter derived from ACE and CTO activities, the 'double product', can be used in the diagnosis of sarcoidosis with a sensitivity and positive predictive value above 90% and a specificity and negative predictive value around 80%, and 'double product ' may be a real alternative to biopsy for confirming the diagnosis of patients with pulmonary manifestation of sarcoidosis. {More details and the manuscript are available at the link.}

A major limitation for CTO activity as a biomarker relates to point mutations and polymorphisms of its coding gene, which influence CTO activity. We determined the effects of Dup24 polymorphism (a frequent CTO gene polymorphism) on circulating CTO activity and concentration in healthy individuals and sarcoidosis patients.

We concluded, that the presence of frequent polymorphisms and rare mutations (null alleles) that diminish or abolish CTO activity may hamper the use of CTO as a diagnostic tool in sarcoidosis. These mutations have also effects on currently available CTO concentration measuring techniques, thus CTO activity measurement cannot be replaced by them. Only genotyping of the CTO gene is available to avoid misinterpreting laboratory findings today, furthermore it can assist in recognition of mutations with low allele frequencies in a given population. We also presented a case for a healthy young male, who did not have serum CTO activity although he was heterozygous for Dup24 polymorphism (had just a single null allele by Dup24). DNA sequencing has revealed that this Hungarian male carried a rare mutation in exon 9 of the CTO gene (Del29). Presence of the Del29 mutation in the Hungarian native population is particularly interesting in light of the fact, that this mutation has been considered unique for Cypriot population to date. {More details and the manuscript are available at the link.}

We also wanted to know if there are diseases other than sarcoidosis that could significantly affect the endogenous regulation of ACE and thereby increase ACE levels, consequently increasing cardiovascular risk. To this aim, we followed patients with rheumatoid arthritis (RA) or ankylosing spondylitis (AS) for one year, monitoring changes in their circulating ACE and ACE2 activity in response to treatment with a tumor necrosis factor α (TNF-α) inhibitor.

We found that one-year anti-TNF-α treatment significantly increased ACE concentration in the mixed cohort, as well as in the rheumatoid arthritis and ankylosing spondylitis subset. TNF-α inhibition also stimulated ACE2 activity in the RA+AS cohort but not in RA or AS. ACE/ACE2 ratios significantly increased in the mixed cohort and in RA, but not in AS. Interestingly, ACE levels and ACE/ACE2 ratios were higher in RA vs. AS, while ACE2 activity values were higher in AS vs. RA at most time points. Moreover, baseline, 6- and 12-month ACE levels, as well as ACE/ACE2 ratios variably correlated with disease duration, CRP, rheumatoid factor level and various parameters of vascular pathophysiology.

In conclusion, anti-TNF-α treatment may increase ACE and ACE2 in the sera of RA and AS patients, which may reflect the shedding and redistribution of ACE and ACE2 from the tissue to the blood. Baseline ACE and ACE2 may be associated with disease duration, markers of inflammation (CRP), autoimmunity (rheumatoid factor) and vascular pathophysiology (flow-mediated vasodilation, common carotid intima-media thickness). The effects of TNF-α inhibition on ACE and ACE2 release may reflect, in part, the beneficial effects of biologics on vascular pathology. {More details and the manuscript are available at the link.}

V. Other research results relevant to the project

Omecamtiv mecarbil is a promising drug candidate to improve cardiac contractility (inotropy) by selectively activating cardiac myosin. In a clinical dose-ranging study, it prolonged systolic time, increased stroke volume, decreased left ventricular dimension and reduced heart rate. It has also been reported to cause a decrease in levels of the heart failure biomarker N-terminal pro-brain natriuretic peptide. Using the methods we have developed in the framework of this project, together with our collaboration partners we aimed to test the cellular effects of omecamtiv mecarbil in permeabilized human left ventricular cardiomyocytes and intact canine cardiomyocytes *in vitro*, and to study the *in vivo* effects in the rat.

We identified two previously unrecognized features of omecamtiv mecarbil that overlap with its positive inotropic effect. The first appears to be an on-target side effect that severely impairs the diastolic filling of the heart. The second is a periodic electromechanical alternation (present at higher omecamtiv mecarbil doses) in which normal beats alternate with diminished cardiac contractions on a beat-to-beat basis, a feature similar to pulsus alternans. Further clinical investigations are therefore required to address hypothetical alterations in diastolic function upon administration of omecamtiv mecarbil. In particular, based on our results, we proposed that echocardiographic indices of isovolumetric relaxation time, left atrial size and Tei index should be considered for future patient selection and personalized omecamtiv mecarbil treatments. {More details and the manuscript are available at the link.}

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