

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

**Angiotensin-converting enzyme:
the impact of drug therapy on laboratory diagnostics and
the measurement of enzyme activity in urine**

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1. Introduction and literature review

Sarcoidosis is a systemic granulomatous inflammatory disease of unknown etiology that often represents a diagnostic challenge in everyday clinical practice because of its heterogeneous clinical presentation and variable course. The diagnosis is generally based on the combined evaluation of the clinical picture, radiological abnormalities, and histopathological findings. However, the invasive nature of biopsy, which places a burden on patients, creates a substantial demand for laboratory biomarkers that can support diagnostic decision-making and are suitable for monitoring disease activity.

The circulating activity of angiotensin-converting enzyme (ACE) may be elevated in granulomatous processes, including sarcoidosis. In clinical practice, determination of ACE activity is the most widely used biomarker in the diagnostic workup of sarcoidosis. At the same time, ACE is a central component of the renin-angiotensin-aldosterone system (RAAS) and a major therapeutic target in the treatment of cardiovascular diseases. Consequently, a proportion of patients investigated for suspected sarcoidosis may receive concomitant angiotensin-converting enzyme inhibitor (ACE inhibitor) therapy, which directly affects the interpretability of ACE activity values. The clinical relevance of this problem is further increased by the fact that information on medication use in routine practice often relies solely on patient self-report.

The applicability of enzyme activity-based biomarkers, however, may be limited not only by exogenous but also by endogenous inhibitory effects. In serum, ACE activity is influenced by human serum albumin in a reversible, dilution-dependent manner. In urine, similarly, the long-recognized presence of small-molecular-weight endogenous inhibitors practically masks urinary ACE (uACE) activity. The clinical relevance of this interference is underscored by literature data suggesting that uACE activity may serve as a potential biomarker in pathological conditions associated with proximal tubular epithelial injury.

ACE is therefore a diagnostic biomarker whose interpretation may be influenced simultaneously by exogenous and endogenous inhibitors. In the course of my research, I attempted to develop laboratory methods capable of objectively identifying or eliminating these interferences, thereby supporting the correct interpretation of the obtained results and assisting clinical decision-making.

1.1. The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system is one of the principal neurohormonal systems responsible for maintaining salt and water homeostasis and regulating blood pressure. Its first component, renin, was identified by Tigerstedt and Bergman in rabbit renal cortex in 1898, thereby laying the foundation for subsequent research. Angiotensin II was independently identified by Page et al. and Braun-Menéndez et al. in 1940. This was followed by the discovery of aldosterone by Simpson et al. in 1953, while Skeggs et al. reported the discovery of angiotensin I, angiotensin-converting enzyme, and angiotensinogen between 1954 and 1957. The functional interrelationship of these components became clear by the mid-1960s.

The renin-angiotensin-aldosterone system is a dynamically regulated enzymatic cascade. Its activation may occur in response to reduced renal perfusion associated with decreased circulating volume, reflected by reduced pressure sensed in the afferent arteriole wall, to low sodium chloride concentration detected by the macula densa, or to β 1-adrenergic receptor activation induced by increased sympathetic tone.

The starting point of the system is angiotensinogen, a high-molecular-weight α 2-globulin-type plasma protein continuously produced by the liver. Proteolytic cleavage of angiotensinogen is carried out by renin synthesized by the granular cells of the juxtaglomerular apparatus in response to the above stimuli. After entering the circulation, renin releases a ten-amino-acid peptide, angiotensin I (Ang I), from the N-terminal end of angiotensinogen.

Ang I itself has little biological activity. This decapeptide is converted into angiotensin II (Ang II) by angiotensin-converting enzyme (ACE), which is abundantly expressed on the surface of the vascular endothelium of the pulmonary circulation and removes two amino acids from Ang I. Ang II is the major effector molecule of the RAAS and exerts pronounced physiological effects despite its short half-life.

Its effects are mediated predominantly through the AT_1 receptor. Activation of this receptor induces vasoconstriction, stimulates aldosterone and vasopressin release, increases sodium and water retention, and activates proliferative and profibrotic signaling pathways. In contrast, the AT_2 receptor initiates opposing processes, including vasodilation, enhanced nitric-oxide production, and antiproliferative effects. The functional balance between these two receptors is a major determinant of cardiovascular homeostasis.

Ang II is further catabolized through additional enzymatic cleavage steps. Aminopeptidase A converts Ang II to angiotensin III, which retains part of the biological

activity of the principal effector molecule. Subsequently, aminopeptidase M generates angiotensin IV, whose role is less dominant than that of the classical RAAS pathway.

The complexity of the system is further increased by the counter-regulatory axis defined by angiotensin-converting enzyme 2 (ACE2). ACE2 is capable of cleaving both Ang I and Ang II. By catabolizing Ang II, it generates angiotensin 1-7, which exerts its effects through the Mas receptor. The angiotensin 1-7 - Mas axis counteracts the unfavorable processes mediated by the AT₁ receptor by activating vasoprotective, antifibrotic, and antioxidant mechanisms. This balancing system is particularly important in pathological conditions in which the classical ACE-Ang II-AT₁ pathway becomes predominant.

Overall, the RAAS is not a linear hormonal pathway but a multidirectional regulatory network of interacting components, with angiotensin-converting enzyme as one of its central elements.

1.2. Angiotensin-converting enzyme

Angiotensin-converting enzyme (ACE; kininase II; peptidyl dipeptidase A; CD143; EC 3.4.15.1) is a zinc-dependent metallopeptidase. In addition to its best-known physiological function, the conversion of Ang I to Ang II, it participates in the metabolism of several bioactive peptides. Its substrates include substance P, amyloid- β peptide, Met-enkephalin, Leu-enkephalin, gonadotropin-releasing hormone, neurotensin, and bradykinin. Through the degradation of bradykinin, ACE modulates the effects of the kinin-kallikrein system, reduces vasodilation, and decreases nitric-oxide and prostacyclin production. This dual role involving both the RAAS and the kinin-kallikrein system explains why ACE has become a major focus of research in cardiovascular and renal regulation.

The 21,000 bp gene encoding ACE is located on the long arm of chromosome 17 (17q23). Two isoenzymes are expressed from this 26-exon, 25-intron gene region: testicular ACE and somatic ACE.

Testicular ACE is expressed exclusively in the testes. This 95-105 kDa protein contains a single active center. Based on evidence from animal experiments, it plays an important role in reproduction.

Somatic ACE, present in the circulation and in tissues, is a high-molecular-weight (150-180 kDa), heavily glycosylated protein that is anchored to the cell surface through a short C-terminal intracellular segment and a hydrophobic transmembrane region. Its catalytically active globular portion is located in the extracellular space. A distinctive feature of somatic ACE is

that it contains two functionally non-identical catalytic domains, the N- and C-domains, which are thought to have arisen through gene duplication during evolution. Although the key motifs of the active centers in the two domains are conserved (HEMGH), their substrate specificity and regulation differ. Cleavage of Ang I is more efficient at the active center of the C-domain, whereas bradykinin catabolism occurs with similar efficiency at both active centers. The different regulatory properties of the two domains, including the greater chloride dependence and lower thermal stability of the C-domain active center, are relevant for the interpretation of drug effects and tissue-specific functions.

Somatic ACE exhibits a diverse tissue distribution. It is highly expressed on the surface of the vascular endothelium of numerous organs and has also been detected on the apical membrane of epithelial cells in the kidney and small intestine, as well as in neuroepithelial cells and monocytes. Regarding the kidney, literature data indicate that ACE is one of the membrane proteins of the proximal tubular brush border. This tubular localization is consistent with the hypothesis that ACE may participate in luminal bradykinin inactivation and thereby influence sodium and water reabsorption, as well as the regulation of amino acid and bicarbonate reabsorption.

In addition to its membrane-bound form, ACE is also present in a soluble form in the body. Through proteolytic cleavage of the extracellular portion of the membrane-anchored enzyme, ACE is released into the circulation. A similar mechanism may be assumed in the case of urinary ACE, although under certain conditions the presence of filtered plasma-derived ACE cannot be completely excluded. The relative contribution of these different sources is likely to depend on both renal function and epithelial integrity.

The amount and activity of circulating ACE show substantial interindividual variability. One of the best-known genetic determinants of this variability is the ACE insertion/deletion (I/D) polymorphism, which is associated with the presence or absence of a non-coding repetitive sequence in an intronic region of the ACE gene. Individuals carrying the D allele have higher serum ACE concentration and activity, whereas those carrying the I allele exhibit lower serum ACE concentration and activity.

1.3. Sarcoidosis

Sarcoidosis is a systemic inflammatory disease of unknown etiology in which non-caseating epithelioid cell granulomas develop in various tissues throughout the body. The disease occurs predominantly in young and middle-aged adults; however, over recent decades,

a shift has been observed toward diagnosis at older ages. Its clinical course is variable. In some cases, spontaneous remission occurs, whereas in others, chronic progressive organ damage develops.

The occurrence of sarcoidosis shows marked variability across geographic regions and ethnic groups. In Europe, its prevalence is approximately 40-80 per 100,000 population; however, substantially higher rates have been reported in Northern European populations (152-215 per 100,000 population). It occurs more frequently in women and in African American individuals. These differing epidemiological patterns suggest the combined contribution of genetic predisposition and environmental factors. Sarcoidosis most commonly affects the lungs and intrathoracic lymph nodes. The pulmonary form is associated with dry cough, dyspnea, and chest discomfort, and bilateral hilar lymphadenopathy may appear as a characteristic abnormality on chest radiography.

Extrapulmonary involvement may develop in the skin in the form of erythema nodosum, lupus pernio, or maculopapular lesions; in the eye in the form of uveitis or retinal inflammation; in the heart in the form of conduction disturbances or arrhythmias; in the liver in the form of liver function abnormalities or portal hypertension; or in the nervous system in the form of meningitis, facial nerve palsy, neuropathy, or pituitary insufficiency. Because of this clinical heterogeneity, the disease may mimic numerous infectious and inflammatory disorders from a differential diagnostic perspective.

The diagnosis of sarcoidosis requires a complex approach based on the combined evaluation of clinical manifestations, radiological abnormalities, and histopathological findings. Definitive diagnosis generally requires histological confirmation of non-caseating granulomas, particularly in cases of atypical clinical presentation or isolated pulmonary involvement. Since biopsy is an invasive procedure that imposes a considerable burden on patients, there is a substantial need for laboratory markers that support diagnostic decision-making and are suitable for monitoring disease activity.

1.4. ACE as a diagnostic biomarker

Over the past decades, numerous candidate biomarkers have been investigated by different research groups; however, to date, the World Association for Sarcoidosis and Other Granulomatous Disorders (WASOG), which developed the international diagnostic guidelines for sarcoidosis, has supported only the use of angiotensin-converting enzyme as a biomarker applicable in clinical practice. Epithelioid cells within granulomas produce ACE, which

subsequently enters the circulation. Elevated serum enzyme activity is therefore regarded as an indirect marker of granuloma mass. Accordingly, increased ACE activity supports the diagnosis, whereas a decrease in activity may be used to monitor treatment response and disease activity.

Several methods are available for the determination of ACE activity. The absorbance change occurring during the hydrolysis of the chromogenic substrate FAPGG (N-[3-(2-furyl)acryloyl]-phenylalanyl-glycyl-glycine) can be detected by spectrophotometry. The main advantage of this assay is its simple implementation; however, its sensitivity and specificity are limited. A more modern alternative is the kinetic assay using the fluorescent substrate Abz-FRK(Dnp)P-OH, which is also employed by our research group and provides greater analytical sensitivity. During substrate hydrolysis, the increase in fluorescence signal intensity is proportional to ACE activity.

The establishment of reference ranges must consider the insertion/deletion polymorphism of the ACE gene. For example, based on the survey performed by our research group, the reference ranges established at 35-fold sample dilution are 3.76-11.25 U/L for genotype II, 5.22-11.59 U/L for genotype ID, and 7.19-14.84 U/L for genotype DD, whereas the genotype-independent reference range is 4.85-13.79 U/L.

However, the biomarker role of ACE has also been raised in other disease states. Increased luminal ACE release has been observed in several conditions associated with damage to the proximal tubular epithelium. According to current knowledge, urinary ACE activity may represent a suitable biological marker in certain kidney diseases.

At the same time, the clinical applicability of ACE activity as a biomarker is limited by several factors. In addition to the differing analytical sensitivities of the various measurement principles and the underlying biological variability, the composition of the sample matrix and the presence of exogenous or endogenous interfering substances may all affect the interpretability of the results.

1.5. ACE inhibitors

ACE inhibitors constitute one of the fundamental drug classes in the treatment of cardiovascular diseases, including hypertension, chronic heart failure, ischemic heart disease, and stroke, as well as in the prevention of renal complications associated with diabetes mellitus. They are among the most widely used medications in both the United States and Europe. Their mechanism of action is based on inhibition of the key enzyme of the RAAS, thereby reducing

Ang II formation. As Ang II production decreases, vasoconstriction, aldosterone secretion, and sodium retention are attenuated, leading to a reduction in blood pressure. In addition to the cardiovascular risk-reducing effect of blood pressure lowering, attenuation of proliferative and profibrotic processes also exerts protective effects in treated individuals.

The development of ACE inhibitors can be traced back to the discovery of teprotide, a peptide composed of 9 amino acids, isolated from the venom of the *Bothrops jararaca* viper. Based on this prototype, captopril, the first orally active ACE inhibitor, was developed. Subsequently, several structurally distinct agents were approved, containing sulfhydryl (captopril, zofenopril), dicarboxylate (lisinopril, ramipril, perindopril, enalapril), or phosphonate (fosinopril) groups. Most compounds are administered in prodrug form and exert their effects after metabolic activation. Differences in lipophilicity and tissue affinity among these drugs result in pharmacodynamic differences.

In parallel with the reduction in Ang II formation, ACE inhibition also decreases bradykinin degradation, which may lead to characteristic adverse effects in predisposed individuals. Accumulation of bradykinin and other inflammatory mediators may induce persistent dry cough and angioedema.

Although numerous clinical trials have demonstrated the efficacy of ACE inhibitors in the treatment of various cardiovascular disorders and in reducing the risk of cardiovascular events, the long-term effectiveness of therapy is substantially influenced by patient adherence, which remains a challenge in clinical practice.

From a laboratory diagnostic perspective, it is important to emphasize that ACE inhibitors reduce serum ACE activity and thereby affect the interpretability of ACE activity measurements performed for diagnostic purposes. As the diagnosis of sarcoidosis has increasingly shifted toward older age groups in recent decades, in whom ACE inhibitor treatment prescribed for cardiovascular indications is more common, consideration of drug effects is essential to avoid misleading laboratory results and erroneous clinical conclusions.

1.6. Endogenous ACE inhibition

A fundamental prerequisite for the applicability of enzyme activity-based biomarkers is that the measured activity should reflect the true catalytic capacity of the enzyme and should not be influenced by inhibitory effects arising from the composition of the sample matrix. In the case of ACE, however, both serum and urine contain endogenous components capable of modifying enzyme activity.

In serum, ACE activity is influenced by human serum albumin. This effect is concentration-dependent and gradually disappears upon dilution. The reversible inhibition caused by albumin can be completely eliminated at a 35-fold dilution; therefore, in routine clinical practice, diagnostic ACE activity measurement is recommended at this dilution.

The presence of endogenous inhibitors with molecular weights below 10 kDa in urine was already described in the late 1970s; however, attempts to identify these small molecules were unsuccessful. The procedures developed for their removal proved to be labor- and time-intensive and difficult to reproduce, which considerably limited the clinical applicability of enzyme activity-based determination.

2. Objectives

The objectives of my research were to:

- Investigate whether conventional diagnostic serum ACE activity measurement is suitable for recognizing the effect of ACE inhibitor therapy.
- Modify the fluorescent kinetic assay so that it can objectively detect the effect of ACE inhibitor drugs from a single serum sample.
- Determine the frequency of ACE inhibitor interference in a patient population investigated for suspected sarcoidosis.
- Develop a reliable and efficient method for urinary ACE activity measurement that overcomes the previous limitations caused by endogenous inhibitors.
- Identify the endogenous inhibitors present in urine.
- Examine the preanalytical and analytical characteristics of urinary ACE activity measurement.
- Evaluate the potential applicability of the urinary ACE activity assay both as a diagnostic biomarker and for monitoring the pharmacological effect of ACE inhibitor therapy.

3. Materials and methods

3.1. Study populations

Individuals treated at the University of Debrecen Clinical Centre, as well as staff members of our department, were enrolled in our studies.

The impact of drug therapy on laboratory diagnostics was investigated in 1823 patients with available medical records in the hospital information system who underwent evaluation for suspected sarcoidosis and diagnostic serum ACE activity measurement at the Clinical Centre between 1 June 2014 and 31 December 2021.

For the development and optimization of the urinary enzyme activity assay, as well as for the identification of endogenous urinary ACE inhibitors, five staff members of our department provided samples on a regular basis. In our prospective comparative study investigating urinary enzyme activity, the study population consisted of 33 cardiovascular patients receiving ACE inhibitor therapy, while 20 individuals not receiving ACE inhibitors served as the control group.

The participants provided informed consent during outpatient care or during individual research consultations for medical evaluation, blood or urine sampling, and determination of ACE activity and urinary creatinine concentration.

Data recorded and stored in the medical (UDMed, ver. 5.46, University of Debrecen, Hungary) and laboratory (GLIMS, ver. 8.11, CliniSys, Belgium) information systems used at the University of Debrecen were processed retrospectively.

3.2. Collection and handling of biological samples

Blood samples were collected during outpatient visits using standard aseptic technique. After coagulation of native blood samples, serum was separated from cellular components by centrifugation at 4 °C for 15 minutes at 2000 g. The resulting serum samples were stored at -20 °C until determination of ACE activity and the degree of ACE inhibition.

First-morning midstream urine samples, as well as dilute daytime urine samples, were collected on the day of examination using standard urine sampling procedures. The samples were transported and centrifuged (10 min, 400 g) at 25 °C. All measurements were performed using fresh, sediment-free urine samples.

3.3. Determination of serum ACE activity

For the determination of ACE activity in serum samples, we used the fluorescent kinetic method previously developed by our research group. The method is based on the use of the synthetically produced Abz-FRK-(Dnp)-P-OH substrate (Peptid 2.0, Chantilly, USA), which can be selectively cleaved by serum ACE. Upon ACE-mediated cleavage, energy transfer between the quenched substrate donor (Abz) and acceptor (Dnp) molecules is abolished, the donor molecule becomes excitable, and during relaxation it emits fluorescent light. The increase in fluorescence signal intensity is proportional to serum ACE activity.

In addition to the appropriate amount of serum sample, the reaction mixture contained 15 μ M Abz-FRK(Dnp)P-OH substrate, 50 mM NaCl, and 10 μ M ZnCl₂ in 100 mM TRIS-HCl buffer (pH 7.0). Measurements were performed in a 96-well black plate (cat. no. 655-900, Greiner-Bio One International GmbH, Kremsmünster, Austria). Changes in fluorescence signal intensity were recorded at 1-minute intervals for 90 minutes at 37 °C using a ClarioStar plate reader (BMG Labtech GmbH, Ortenberg, Germany). The excitation wavelength was $\lambda = 340$ nm and the emission wavelength was $\lambda = 405$ nm. After correction of the fluorescence intensity values for both the initial fluorescence signal measured at time zero (arising from the intrinsic colour of the sample) and the reagent blank containing no sample, fluorescence intensity was plotted as a function of time. A linear regression was fitted to the resulting data points. ACE activity, expressed in U/L, was calculated using the following equation:

$$ACE\ activity = S / k \times D$$

where S is the slope of the fitted line, k is the increase in fluorescence intensity resulting from the complete hydrolysis of 1 μ M substrate under standard conditions within 1 minute, and D is the dilution factor of the serum sample.

3.4. Determination of the degree of serum ACE inhibition

To determine the degree of ACE inhibition, ACE activity was measured not only at the 35-fold serum dilution routinely used for diagnostic purposes, but also at additional dilution levels. Serial two-fold dilutions were prepared from serum samples with and without ACE inhibitor treatment.

Due to the need to maintain adequately buffered reaction conditions, the lowest applicable dilution was 4-fold. The ACE activity measured at this dilution represented the maximally inhibited state that could be determined in vitro.

At dilutions greater than 400-fold, a substantial deterioration of the signal-to-noise ratio was observed, resulting in considerable measurement uncertainty. Based on the findings of the experimental series, the 4-fold and 400-fold dilutions were therefore selected for the assay. The degree of inhibition was defined according to the following equation:

$$\text{ACE inhibition (\%)} = 100 - \left(100 \times \frac{\text{ACE activity}_{4\text{-fold dilution}}}{\text{ACE activity}_{400\text{-fold dilution}}} \right)$$

To establish a cut-off value indicative of the presence of an ACE inhibitor drug, the distribution of endogenous ACE inhibition observed in the local population was investigated.

3.5. Determination of urinary ACE activity

Urinary ACE activity was determined according to a modified and optimized protocol of the fluorescent kinetic assay using the Abz-FRK-(Dnp)-P-OH substrate (Peptid 2.0, Chantilly, USA). In each experiment, sample dilutions appropriate for the given measurement were applied.

The reaction mixture of the final urinary ACE activity assay protocol contained 100 mM tris(hydroxymethyl)-aminomethane hydrochloride (TRIS) buffer (pH 7.0), 15 μ M substrate, 50 mM NaCl, 10 μ M ZnCl₂, and urine samples diluted 64-fold and 128-fold. For each sample, a parallel control reaction containing 1 μ M lisinopril (cat. no. L6292-100 mg, Sigma-Aldrich, St. Louis, MO, USA) was also included. Changes in fluorescence signal intensity were recorded at 1-minute intervals for 90 minutes at 37 °C using a ClarioStar plate reader (BMG Labtech GmbH, Ortenberg, Germany). For data analysis, data points between 20 and 80 minutes were used. When activity measured at 64-fold dilution exceeded that measured at 128-fold, the 64-fold results were used to avoid over-dilution bias of dilute urine samples.

In the membrane filtration experiments, endogenous inhibitors were separated from urinary ACE using Vivaspin 20 membrane filter columns (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Native urine samples (6 mL) were diluted with distilled water to a final volume of 20 mL. The columns were centrifuged at 4000 g relative centrifugal force for 45 minutes at 25 °C. Thereafter, each column was washed twice with 20 mL distilled water under identical centrifugation conditions. The collected 60 mL filtrate was reconcentrated to the original volume of 6 mL by vacuum centrifugation. The retentate was recovered from the membrane in 6 mL of 100 mM TRIS buffer (pH 7.0). In comparative measurements, both native urine samples and membrane-purified ACE samples were analyzed at 4-fold dilution.

For experiments aimed at identifying endogenous inhibitory agents, the potential inhibitors were dissolved according to the manufacturers' instructions (urea: cat. no. U6504-1KG, Sigma-Aldrich, St. Louis, MO, USA; uric acid: cat. no. U2625-25G, Sigma-Aldrich, St. Louis, MO, USA; urobilinogen: cat. no. 651-10, Lee Biosolutions, Maryland Heights, MO, USA).

3.6. Determination of the specificity of the urinary ACE activity assay

The specificity of urinary ACE activity measurement was examined using ACE-specific inhibitors (lisinopril: cat. no. L6292-100 mg, Sigma-Aldrich, St. Louis, MO, USA; captopril: cat. no. C4042, Sigma-Aldrich, St. Louis, MO, USA), as well as inhibitors acting on other proteases (amastatin hydrochloride: cat. no. SC-202051, Santa Cruz Biotechnology, Dallas, TX, USA; apstatin: cat. no. BML-PI145-0001, Enzo Biochem Inc., Farmingdale, NY, USA; bestatin hydrochloride: cat. no. B8385, Sigma-Aldrich, St. Louis, MO, USA; MLN-4760: cat. no. 5.30616, Merck KGaA, Darmstadt, Germany; phosphoramidon: cat. no. R7385, Sigma-Aldrich, St. Louis, MO, USA; sacubitrilat: cat. no. HY-17620, MedChemExpress, Monmouth Junction, NJ, USA; Z-prolyl-prolinal: cat. no. BML-PI112, Enzo Biochem Inc., Farmingdale, NY, USA).

3.7. Measurement of urinary ACE activity following immunodepletion

In the immunodepletion experiments, endogenous inhibitors were first removed using membrane filter columns with a 10 kDa pore size. The inhibitor-free retentate was subsequently incubated with an ACE-specific antibody (final concentration: 120 ng/mL; cat. no. 841365, component of the DY929 kit, R&D Systems Inc., Minneapolis, MN, USA), followed by Protein G Sepharose 4 Fast Flow resin (final bead volume: 10%; cat. no. 17-0618-001, Merck KGaA, Darmstadt, Germany). Incubation with the antibody was carried out for 16 hours at room temperature, whereas incubation with the resin was performed for 2 hours at room temperature under continuous shaking. The activity of ACE-depleted and control urine samples was subsequently determined at 4-fold dilution.

3.8. Stability testing of biological samples

The stability of serum ACE activity under the applied storage conditions at -20 °C was tested in 123 samples after a mean interval of 6 years and 4 months from the initial measurement (min: 5 years 1 month; max: 7 years 3 months).

The stability of urinary ACE activity was examined in three independent experiments at three storage temperatures: 25 °C, 4 °C, and -20 °C.

3.9. Determination of urinary creatinine concentration

Urinary creatinine concentration was determined on a Cobas Integra 400 plus clinical chemistry analyzer (Roche Diagnostics GmbH, Mannheim, Germany) using CREJ2 Creatinine Jaffé Gen.2 cartridges (Ref.: 04810716190, Roche Diagnostics GmbH, Mannheim, Germany).

3.10. Ethical approval

The study procedures and the processing of clinical data were approved by the Regional and Institutional Research Ethics Committee of the Clinical Centre, University of Debrecen (DE KK RKEB/IKEB: 5925-2021), and by the Ministry of Human Capacities (15471-6/2023/EÜIG). The studies were conducted in accordance with the principles of the Declaration of Helsinki.

3.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA) applying the following tests: D'Agostino–Pearson test (normality testing), paired t-test (8.A,B), Mann–Whitney U test (5.B, 15.), one-way ANOVA with Holm–Sidak multiple comparisons test (9.A,B,C; 10.A,B,C; 11.A-I; 13.A,B,C), Kruskal–Wallis test with Dunn's multiple comparisons test (12.A,B,C), and Spearman correlation analysis (14.B). Data showing normal distribution are presented as mean ($\pm$ standard deviation), whereas data with non-normal distribution are presented as median (interquartile range). A *p* value <0.05 was considered statistically significant.

4. Results

4.1. The impact of drug therapy on laboratory diagnostics

4.1.1. A dilution-based methodological approach for the objective detection of ACE inhibitor interference

The method is based on the principle that the measured value of serum ACE activity is significantly influenced by both the degree of sample dilution applied during the assay and the presence of any ACE inhibitor drug in the examined sample.

Human serum albumin reversibly blocks ACE activity, and this inhibition can be eliminated during measurement by diluting the sample at least 35-fold. When dilution factors below 35-fold are applied, ACE activity is underestimated because of the physiological inhibition mediated by albumin, whereas above 35-fold dilution the dilution-corrected ACE activity becomes constant and can be measured reliably.

In serum samples containing ACE inhibitor drugs, a further increase in ACE activity can be observed at dilutions exceeding 35-fold. ACE activity values measured at 35-fold dilution often do not distinguish between patients receiving and not receiving ACE inhibitor therapy, particularly when the measured values fall within the reference range. From the ACE activity values measured in parallel at 4-fold and 400-fold serum dilutions (inhibited and near completely uninhibited activity, respectively), the degree of ACE inhibition can be determined accurately. This ratio provides information on the extent of endogenous inhibition mediated by albumin in patients not receiving ACE inhibitors (degree of inhibition = 55%), as well as on the effect of ACE inhibitor drugs in treated patients (degree of inhibition = 97%).

The presence of an ACE inhibitor in a serum sample was indicated by an ACE inhibition level of 80% or greater, based on the maximum endogenous ACE inhibition (~78%) observed in the local population.

4.1.2. Proportion of patients receiving ACE inhibitor therapy among individuals undergoing diagnostic serum ACE activity measurement

By determining serum ACE activity at three different dilutions, we sought to verify that the 1823 individuals evaluated for suspected sarcoidosis were not receiving ACE inhibitor therapy at the time of blood sampling. We assumed that the clinicians had acted with appropriate caution during sample collection and had either switched the patients to angiotensin II receptor blocker therapy or temporarily suspended ACE inhibitor treatment, since the purpose

of the investigation was to support the suspicion of sarcoidosis by demonstrating elevated serum ACE activity.

Contrary to our expectations, biochemical evidence of ACE inhibitor drug effect was found in every sixth patient included in the study (302 of 1823 patients) at the time of diagnostic ACE activity measurement.

4.1.3. Effect of ACE inhibitor drugs on serum ACE activity

ACE inhibitor therapy significantly increased the degree of ACE inhibition (94.9% [91.6-96.7%]) compared with endogenous, albumin-mediated ACE inhibition (52.9% [46.9-59.85%]). Consequently, serum ACE activity measured at the diagnostic 35-fold dilution was significantly lower in the group exhibiting biochemical evidence of ACE inhibitor effect than in patients without biochemical evidence of ACE inhibitor effect (4.41 U/L [2.93–6.72 U/L] vs. 11.32 U/L [8.79–13.92 U/L]; $p < 0.01$).

However, it became evident that the presence of an ACE inhibitor could not be inferred from a single ACE activity measurement, as the ACE activity values of 77.5% ($n=234$) of patients exhibiting biochemical evidence of ACE inhibitor effect overlapped (2.78-27.42 U/L) with those of patients without evidence of ACE inhibitor effect.

The degree of serum dilution applied in the reaction mixture had a substantial impact on the measured ACE activity. This relationship was clearly observable in our study population. Compared with the 4-fold serum dilution, dilution-corrected ACE activity measured at the 35-fold dilution was significantly higher in the group without biochemical evidence of ACE inhibitor effect (+99%; 5.68 U/L [4.31-7.29 U/L] vs. 11.32 U/L [8.79-13.92 U/L]), owing to the elimination of the reversible endogenous inhibitory effect of albumin. In contrast, further dilution to 400-fold had only a negligible effect on ACE activity (+8%; 12.23 U/L [9.20–15.60 U/L]).

Conversely, in samples exhibiting biochemical evidence of ACE inhibitor effect, the 400-fold dilution resulted in a further and even more pronounced increase in ACE activity (+125%). Considering the dilution-corrected changes in serum ACE activity observed in individuals exhibiting biochemical evidence of ACE inhibitor effect, the activity measured at the 400-fold dilution presumably approached the value that would have been observed in the absence of ACE inhibitor therapy.

4.1.4. Frequency of false-negative test results due to ACE inhibitor therapy

Determination of ACE activity supports the diagnosis of sarcoidosis when the value obtained at the diagnostic 35-fold dilution exceeds the reference interval established for the normal population. We therefore investigated how often elevated ACE activity was masked by ACE inhibitor therapy in our study population. For this analysis, ACE activity values measured at the 400-fold dilution were used.

Among the 161 patients whose ACE activity measured at the diagnostic 35-fold dilution fell below the reference interval, two would have had values above the upper reference limit. Similarly, among the 138 patients whose ACE activity measured at this dilution was within the normal range, 60 would have exceeded the upper reference limit. Overall, ACE activity measurement could have provided additional support for the diagnosis of sarcoidosis in at least 62 patients had they not been under biochemical ACE inhibitor effect at the time of sample collection.

4.1.5. Lessons learned from the review of patient documentation

Following a detailed review of the medical records of the 302 patients with biochemically confirmed ACE inhibitor effect, we found that their age was significantly higher than that of patients without biochemical evidence of ACE inhibitor effect, both among women (60.5 ± 11.7 years vs. 48.2 ± 14.0 years; $p < 0.01$) and men (55.4 ± 13.3 years vs. 44.3 ± 15.1 years; $p < 0.01$).

Furthermore, medication data were documented in only 121 of the 302 cases (all including an ACE inhibitor agent), which would have enabled healthcare personnel to recognize ongoing ACE inhibitor therapy. Although unexpectedly low ACE activity results were noted in 50 cases, the underlying ACE inhibitor treatment was recognized in only 3 cases.

We identified patients whose initial sample collection occurred during ACE inhibitor therapy and whose physicians subsequently requested repeat ACE activity measurements. Of the 43 patients, repeat measurements were again performed during ACE inhibitor therapy in 29 cases. In these individuals, the degree of ACE inhibition remained above 80% ($94.1 \pm 3.8\%$ vs. $93.4 \pm 3.9\%$ at the initial visit; $p > 0.05$), and ACE activity did not change substantially during follow-up (4.62 ± 2.1 U/L vs. 5.16 ± 2.5 U/L; $p > 0.05$).

In contrast, no biochemical evidence of ACE inhibitor effect was detected during follow-up examinations in 14 patients (degree of ACE inhibition: $90.1 \pm 5.4\%$ vs. $58.2 \pm 11.5\%$ at the second visit; $p < 0.01$). In these patients, a marked increase in serum ACE activity was observed (6.0 ± 2.4 U/L vs. 11.4 ± 4.2 U/L at the second visit; $p < 0.01$).

4.2. Measurement of enzyme activity in urine

4.2.1. Determination of the molecular weight of endogenous urinary ACE inhibitors

Urine samples were filtered using membrane filter columns with pore sizes of 5, 10, 30, and 50 kDa, and ACE activity was then determined in the respective retentates in order to estimate the molecular weight of endogenous ACE inhibitors.

In native, unfiltered urine, more than 92% greater ACE inhibition was observed compared with the fraction retained by the 10 kDa pore size filter (ACE activity: $7.79 \pm 6.27\%$ vs. $100 \pm 17.43\%$; $p < 0.005$). Use of the 5 kDa membrane filter resulted in a substantial, but not complete, increase in ACE activity ($71.40 \pm 4.03\%$). Complete separation of endogenous ACE inhibitors was achieved using the 10 kDa column, while no further increase in ACE activity was observed with the 30 and 50 kDa filters ($100.00 \pm 17.43\%$, $102.10 \pm 18.87\%$, and $103.10 \pm 18.85\%$).

Successful removal of endogenous urinary ACE inhibitors was verified by re-adding the filtrates. Removal of inhibitors using a 10 kDa pore-size filter increased ACE activity by more than tenfold, and no significant ACE activity was detected in the filtrate. Recombining the <10 kDa filtrate with the 10 kDa retentate led to a marked decrease in ACE activity, and the result approached the activity measured in native urine ($10.00 \pm 5.80\%$ vs. $7.79 \pm 6.27\%$).

The experimental findings suggested that the endogenous inhibitors might be present in the <5 kDa and 5-10 kDa molecular weight ranges. To investigate the possible presence of a urinary ACE inhibitor in the 5-10 kDa range, sequential membrane filtration was performed by separating the <10 kDa urine fraction into <5 kDa and 5-10 kDa subfractions. Native urine was first filtered through a 10 kDa membrane filter, and the resulting <10 kDa filtrate was then further fractionated using a 5 kDa membrane filter column. This procedure yielded three components: the 10 kDa retentate containing urinary ACE, the retentate of the 5 kDa column corresponding to the 5-10 kDa urinary subfraction, and the <5 kDa filtrate. ACE activity was determined in all three urinary components, as well as in mixtures obtained by re-adding the subfractions to the 10 kDa retentate. Neither the <5 kDa nor the 5-10 kDa subfraction showed ACE activity. Addition of the <5 kDa filtrate to the 10 kDa retentate resulted in a marked decrease in ACE activity, closely reproducing the inhibitory effect observed in native urine ($12.06 \pm 6.62\%$ vs. $10.84 \pm 3.55\%$). In contrast, re-addition of the 5-10 kDa subfraction had no effect on ACE activity ($99.62 \pm 6.67\%$ vs. $100.00 \pm 4.73\%$). These findings do not support the presence of an endogenous ACE inhibitor in the 5-10 kDa molecular weight range.

4.2.2. Effect of dilution on urinary ACE activity and pharmacological inhibitability of the enzyme

By removing the inhibitors using membrane filtration, we demonstrated that endogenous ACE inhibition present in urine is reversible. Seeking a simpler alternative to filtration, we investigated whether the inhibitory effect could be abolished by dilution of the urine sample, thereby reducing the local concentration of the inhibitors. Serial dilutions were prepared from native urine, and ACE activity was determined at each dilution. The degree of urine dilution applied in the reaction mixture significantly influenced the measured ACE activity. A gradual increase in dilution-corrected ACE activity was observed up to 128-fold dilution, above which no residual endogenous inhibition could be detected. Further dilution had a negligible effect, with only a +2.34% change in activity observed at 256-fold dilution.

Urinary ACE activity was completely inhibited by lisinopril ($IC_{50} = 2.034 \times 10^{-9}$ M) and captopril ($IC_{50} = 4.497 \times 10^{-9}$ M).

4.2.3. Evaluation of the specificity of the urinary ACE activity assay

The specificity of the developed urinary ACE activity assay was tested using several different peptidase inhibitors. Amastatin hydrochloride, known as an inhibitor of aminopeptidases A and N, did not affect the measured activity. Similarly, apstatin, an aminopeptidase P inhibitor, and bestatin hydrochloride, which inhibits aminopeptidases B and N, caused no change in urinary ACE activity. The ACE2-selective inhibitor MLN-4760 also had no effect on the assay. Sacubitrilat, a neprilysin inhibitor used in clinical practice, did not reduce urinary ACE activity, and no effect was detected in the presence of the prolyl endopeptidase inhibitor Z-prolyl-prolinal either.

In contrast, phosphoramidon disodium salt, another neprilysin inhibitor similar to sacubitrilat, reduced urinary ACE activity by approximately 35% at a concentration of 1 μ M. Phosphoramidon exerted a similar inhibitory effect on serum ACE activity as well.

Finally, urinary ACE immunodepletion was performed using a commercially available ACE-specific antibody. Following immunodepletion, urinary ACE activity decreased to $5.0 \pm 0.6\%$ of the baseline value, whereas the negative control containing no antibody and only Protein G Sepharose resin retained full activity ($107.7 \pm 4.2\%$).

4.2.4. Physiological endogenous inhibitors of urinary ACE

Urea (60.06 g/mol), uric acid (168.11 g/mol), and urobilinogen (592.7 g/mol) were identified as physiological endogenous inhibitors of urinary ACE. The inhibitory effects of

these three small molecules were examined on purified urinary ACE. Within its reference range, urea was responsible for 10-25% inhibition of urinary ACE activity, while at higher concentrations it exerted a more pronounced effect ($IC_{50} = 1.178 \text{ M}$). Uric acid contributed to 30-50% inhibition within the physiological range, whereas at higher concentrations it produced near-complete inhibition ($IC_{50} = 3.611 \times 10^{-3} \text{ M}$). Under normal conditions, urobilinogen reduced ACE activity by 3-20%, but in hyperurobilinogenic states it showed marked inhibitory potential ($IC_{50} = 2.976 \times 10^{-4} \text{ M}$).

Under pathological conditions, any of these molecules alone may be sufficient to abolish urinary ACE activity.

4.2.5. Stability of urinary ACE activity under different storage conditions

To assess the diagnostic applicability of urinary ACE activity measurement, we investigated the stability of the analyte at three different storage temperatures. Urinary ACE activity remained stable for more than one day at 25 °C even without the use of preservatives. When samples were stored at 4 °C in a refrigerator, no statistically significant change in ACE activity was detectable for at least 12 hours. However, after one day an approximately 20% decrease was observed. In contrast to serum samples (baseline ACE activity: $9.0 \pm 4.0 \text{ U/L}$; ACE activity after thawing: $9.8 \pm 4.2 \text{ U/L}$), freezing resulted in complete loss of urinary ACE activity.

4.2.6. Strong correlation between urinary ACE activity and urinary creatinine concentration

Using the dilution-based uACE activity assay, we measured ACE activity in urine samples from 20 individuals not receiving ACE inhibitor therapy. ACE activity varied by nearly an order of magnitude, ranging from 0.18 to 1.10 U/L.

Visual inspection of the urine samples revealed marked differences in color intensity, suggesting substantial variability in sample concentration. To account for these differences, urinary creatinine concentration was determined, which, similarly to ACE activity, varied over a broad range (5.9-24.6 mmol/L).

A strong positive correlation was demonstrated between urinary ACE activity and creatinine concentration (Spearman $r = 0.76$; $p < 0.0001$), indicating that urine concentration plays a major role in the measured ACE activity. Based on these findings, normalization to urinary creatinine concentration is recommended when measuring urinary ACE activity in order to correct for dilution-related effects and to allow more accurate comparison between samples.

4.2.7. Effect of ACE inhibitor therapy on urinary ACE activity

It is well established that ACE inhibitor therapy markedly reduces serum ACE activity. We therefore sought to investigate the effect of ACE inhibitor treatment on urinary ACE activity. To this end, urine samples from 33 patients receiving ACE inhibitors were analyzed. Urinary ACE activity normalized to creatinine concentration was significantly lower in ACE inhibitor-treated patients (n=33) than in the control group not receiving ACE inhibitors (n=20) (6.492 U/mol [1.327–12.700] vs. 36.690 U/mol [29.200–44.300]; $p<0.0001$). These findings suggest that this ratio may serve as a reliable indicator of ACE inhibitor intake and could help identify patients with poor or limited therapy adherence.

5. Discussion

Research into the physiological role of angiotensin-converting enzyme, its diagnostic applicability, and its significance as a therapeutic target spans nearly seven decades. During this period, numerous discoveries of fundamental importance to clinical practice have emerged in this field. Drugs inhibiting ACE activity have provided additional years of healthy life for many individuals suffering from cardiovascular diseases, while measurement of serum ACE activity has contributed to the diagnosis of sarcoidosis in numerous patients. In the field of laboratory diagnostics, however, several unresolved questions remain to this day, both at the preanalytical and analytical levels; disregarding these issues may lead to clinically relevant misinterpretations and potential patient safety concerns.

Our findings provide improved understanding and answers to some of these unresolved questions. They highlight the central role of laboratory medicine in RAAS-related diagnostic and therapeutic applications by proposing cost-effective approaches applicable in two biological matrices and adaptable to multiple purposes in order to support correct interpretation and clinical decision-making. They also demonstrate the presence of interfering factors affecting activity measurements in routine practice and supplement the tools traditionally available to clinicians as supportive solutions in several disorders and clinical scenarios.

The diagnosis of sarcoidosis often represents a challenge in clinical practice for healthcare teams, a difficulty further compounded by the fact that current international WASOG guidelines identify angiotensin-converting enzyme as the only biomarker considered applicable for the diagnosis and follow-up of the disease. At the same time, reducing ACE activity is a central therapeutic goal in the treatment of cardiovascular diseases, and ACE inhibitors are therefore among the most widely used drug classes worldwide. Under these circumstances, falsely normal or falsely low ACE activity values resulting from ACE inhibitor therapy may be misleading for clinicians when sarcoidosis is suspected. This may delay diagnosis and trigger further unnecessary investigations. To prevent this, knowledge of ongoing ACE inhibitor therapy and careful interpretation of the measured ACE activity value are essential. In clinical practice, however, information on ACE inhibitor use still relies primarily on patient self-report, which is often unreliable.

A simple and well-applicable method for examining the effects of reversible enzyme inhibitors in biological samples is the determination of enzyme activity at different sample dilutions. As the sample is progressively diluted, the concentration of the reversible inhibitor

decreases in the reaction mixture, resulting in gradual loss of its inhibitory effect. Ideally, with appropriate dilution, a state can be achieved that approximates the condition in which the patient is not receiving the drug, thereby allowing objective determination of the uninhibited enzyme activity without interrupting therapy.

Since ACE inhibitors are reversible enzyme inhibitors, we applied this approach during the development of our assay. Using the completed assay, we subsequently investigated whether ACE activity measurement had been performed in parallel with ongoing ACE inhibitor therapy in more than 1800 patients evaluated for suspected sarcoidosis at the University of Debrecen in recent years. Surprisingly, our measurements indicated that approximately one in six patients was under the effect of ACE inhibitor therapy at the time of sample collection. Consequently, falsely low ACE activity values were obtained in these individuals when conventional laboratory ACE activity assays were used.

Although ACE activity values that do not exceed, or even fall below, the reference range may serve as warning signs of inadequate sampling conditions in this patient group, our findings suggest that healthcare providers did not always pay sufficient attention to the ACE-lowering effect of ACE inhibitors. We also identified patients in whom ACE activity measurement was repeatedly requested despite the fact that ACE inhibitor therapy had not been discontinued. One possible explanation is that some patients were unable to accurately list their medications, leaving the treating physician unaware that the patient was receiving therapy capable of affecting the laboratory result. Taken together, these findings support the need for an objective laboratory test that, based on a single blood sample and without knowledge of clinical medication data, enables laboratory professionals to correctly interpret measured values. In possession of objective results, laboratory specialists may also provide feedback to the treating physician about the presence of ACE inhibitor interference and ways to avoid it, thereby reducing unnecessary testing, healthcare staff burden, and patient frustration.

In cases where the measurement results support the presence of interference, reporting of patients' ACE activity values is recommended for informational purposes only and should be accompanied by an interpretive comment. In this comment, laboratory professionals should draw the clinician's attention to the high likelihood that the sample contains an ACE inhibitor drug, which significantly reduces ACE activity. Any modification of therapy, however, must be decided by the treating physician familiar with the patient before a subsequent ACE activity determination, since even temporary withdrawal of ACE inhibitors may increase cardiovascular risk, for example through abrupt elevation of blood pressure. It should also be emphasized that

drugs acting on other components of the RAAS, such as angiotensin II receptor blockers, do not affect serum ACE activity and therefore do not interfere with ACE activity measurement.

Our results confirm, although not for the first time in the literature, that at the population level the mean ACE activity of patients receiving ACE inhibitors is lower than that of untreated individuals. At the same time, our observations also demonstrate that, at the individual level, substantial overlap exists between the ACE activity values of patients receiving and not receiving ACE inhibitors. In 77.5% of the individuals studied, ACE activity fell within the same range (2.78-27.42 U/L) regardless of ACE inhibitor use. Consequently, with the conventional assay it is practically impossible to determine which samples with ACE activity values within the reference range originate from patients who would otherwise have high ACE activity but are receiving ACE inhibitor therapy. Detection of ACE inhibitor drugs in serum is generally performed using costly and instrument-intensive methods such as mass spectrometry. Because of the large number of marketed ACE inhibitors, individual methods, calibrators, and controls would be required, rendering routine application practically unfeasible. In contrast, the method we developed is applicable to any ACE inhibitor regardless of the time of administration, since it does not detect the molecule itself but rather measures its effect. The method still relies on ACE activity measurement; accordingly, it can be readily implemented in any laboratory where ACE activity determination is routinely performed, without the need for new instrumentation or the introduction of new analytical procedures.

In our study, the threshold for the degree of ACE inhibition was set at 80%, above which the presence of ACE inhibitor therapy can be assumed. This cutoff was determined on the basis of the degree of endogenous ACE inhibition observed in the local population, which did not exceed 80% in any individual (maximum 78%). The degree of endogenous ACE inhibition, however, may vary between populations; therefore, before applying this cutoff in another laboratory setting, it is advisable to determine the level of endogenous inhibition in a local population not receiving ACE inhibitor therapy.

Previous reports have also drawn attention to the interfering role of ACE inhibitors in sarcoidosis diagnostics. A Belgian study found a prevalence of approximately 10%, while a retrospective study from Iowa reported that ACE activity measurement had been performed during ACE inhibitor therapy in about 8% of patients. It should be noted, however, that these studies relied exclusively on medication data recorded in medical documentation and on extremely low ACE activity values; drug level determination was not performed in every patient. A major strength of our work lies in the biochemical analysis of the entire study

population. The approximately 17% rate found in our study therefore likely reflects the true magnitude of the problem more accurately. In addition, with our developed assay we propose a simple, readily implementable, and routine-compatible method for identifying patients receiving ACE inhibitors.

In contrast to venous blood sampling, urine is a non-invasive, readily accessible biological sample that can be collected without specialized training or equipment. Literature data had suggested the potential clinical applicability of urinary ACE activity measurement, yet historically uACE activity could not be determined using simple and widely accessible techniques. Building on the experience gained during development of the ACE activity assay used in sarcoidosis diagnostics, we aimed to develop a reliable and reproducible method allowing measurement of uACE activity free from the influence of endogenous inhibitory factors. The purpose of this approach was to facilitate broader investigation of the analyte and its future practical application. To overcome the presence of endogenous inhibitors, we developed a dilution-based measurement procedure using a modern fluorescent substrate, enabling accurate quantitative determination of uACE activity. Given that most ACE inhibitors are eliminated primarily via the kidneys, we also investigated whether this method could reflect pharmacological ACE inhibition and thus whether urine, as a practical matrix, might be suitable for therapeutic monitoring.

Although the presence of endogenous factors inhibiting uACE activity had been recognized previously, these factors had not been identified. In our study, we demonstrated that under physiological conditions uACE activity is almost completely inhibited, and that urea, uric acid, and urobilinogen constitute the principal endogenous inhibitors of uACE. Urea is the end product of amino acid metabolism and is typically present in high concentrations in urine. Consideration of its role is becoming increasingly important in clinical interpretation with the spread of high-protein, ketogenic, and paleolithic diets. Furthermore, the inhibitory effect of urea may be particularly relevant in elderly individuals, in whom increased urea excretion may act as a homeostatic mechanism contributing to reduced bradykinin degradation, thereby promoting natriuresis and water excretion. Uric acid is the breakdown product of purine nucleotides. Modern dietary habits and certain medications frequently lead to elevated serum levels; both conditions may result in hyperuricosuria, further enhancing its inhibitory effect. Urobilinogen is a byproduct of bilirubin degradation. It may become particularly relevant in patients with liver disease or hemolytic anemia, in whom its inhibitory potential becomes especially pronounced. Together, these three low-molecular-weight compounds may account

for up to 90% of physiological uACE inhibition, and under pathological conditions their effect may be even greater.

It should be emphasized that all three molecules behave as reversible inhibitors and that their inhibitory effect on ACE activity can be abolished by dilution of the sample. This phenomenon is analogous to the reversible inhibition of serum ACE mediated by albumin. Our results show that at 128-fold dilution uACE activity is no longer under the inhibitory influence of endogenous inhibitors. This dilution can therefore be regarded as corresponding to the uninhibited state. This observation made it possible to apply our simple fluorescence-based measurement procedure, which obviates labor- and cost-intensive membrane filtration steps. The use of 1 μ M lisinopril in control reactions, resulting in complete inhibition of ACE activity, ensures assay specificity. It should be noted, however, that if the original urine sample is already markedly dilute, for example in daytime samples with creatinine concentration <2.5 mmol/L, the optimal measurement point may occur at a lower dilution such as 64-fold. Accordingly, if low or undetectable uACE activity is observed at 128-fold dilution, repeating the measurement at 64-fold dilution is advisable to avoid underestimation caused by excessive dilution. Like the serum assay used in sarcoidosis diagnostics, the developed method can be easily implemented in any clinical laboratory where ACE activity measurement is routinely performed, without the need to acquire additional instruments.

To validate the clinical applicability of the method, we investigated the stability of uACE activity under different storage conditions. Our results showed that ACE activity remains stable at room temperature and at 4 °C for periods exceeding routine laboratory working hours, although after 24 hours at 4 °C an approximately 20% decrease was observed. Notably, unlike serum samples, which retain enzyme activity after freeze-thawing, uACE is highly sensitive to freezing. Even a single freeze-thaw cycle led to complete loss of ACE activity, precluding long-term storage. This pronounced instability likely reflects structural features distinct from those of serum ACE, particularly previously described differences in glycosylation/sialylation patterns near the N-terminal active site. In addition, the low protein concentration of urine may also contribute to instability. These findings underscore the importance of standardized preanalytical conditions for reliable measurement of uACE activity. To preserve enzyme activity, urine samples should preferably be stored at 25 °C and analyzed, whenever possible, on the day of collection.

uACE activity showed a strong correlation with urinary creatinine concentration. Simultaneous determination of creatinine concentration and normalization of ACE activity to

creatinine effectively eliminate the variability caused by differences in urine concentration, thereby allowing more accurate and reliable comparison between samples. This approach is consistent with the practice used for other urinary biomarkers, such as the albumin-to-creatinine ratio, where normalization to creatinine compensates for dilution effects. Nevertheless, creatinine normalization may have important limitations in chronic kidney disease and tubular epithelial injury. In CKD, reduced glomerular filtration rate is generally associated with lower urinary creatinine concentration because of decreased filtration, reduced muscle mass, and impaired tubular function. Similarly, in acute and chronic tubular injury, disturbances in creatinine secretion and impaired concentrating ability may result in lower urinary creatinine levels. These pathological changes may reduce the reliability of normalization to creatinine and, in dilute samples, may lead to overestimation of analyte excretion. Accordingly, in advanced CKD and/or marked tubular dysfunction, creatinine-normalized uACE activity values should be interpreted with caution. Targeted clinical studies are needed to clarify the extent to which creatinine-based normalization can be reliably applied under such conditions and what its limitations may be.

Although, as discussed above, ACE inhibitors are among the most widely used drug classes worldwide, optimization of therapeutic strategy and dosing remains a major clinical challenge, further complicated by frequently inadequate patient adherence. For the reasons outlined earlier, precise determination of serum and urinary ACE inhibitor concentrations by liquid chromatography-tandem mass spectrometry is not practically feasible. In routine clinical practice, treatment efficacy is therefore judged on the basis of patient self-report and blood pressure measurement. However, such assessment may also be distorted by comorbidities, white-coat hypertension, concomitant use of multiple antihypertensive agents, or the use of ACE inhibitors for indications other than blood pressure reduction, such as kidney disease or post-myocardial infarction management. Although the tertiary structure of uACE shows minor differences, its enzymatic properties, including inhibition by lisinopril and captopril ($IC_{50} = 2.03 \times 10^{-9}$ M and $IC_{50} = 4.54 \times 10^{-9}$ M, respectively), closely parallel the characteristics of circulating ACE. These similarities support the applicability of uACE as a reliable surrogate marker of systemic ACE activity in therapeutic monitoring.

Applying our method in a cohort consisting of 20 untreated individuals and 33 patients receiving ACE inhibitor therapy, we demonstrated that the uACE activity/creatinine ratio is significantly lower in treated patients. Outlying values observed within the treated group may reflect missed doses before sampling, further reinforcing the potential of the method for

adherence assessment. Although absolute ACE activity also differed between the groups, the creatinine-normalized ratio is preferable because of its greater robustness against variability caused by urine dilution.

It is important to bear in mind, however, that some ACE inhibitors are eliminated partly through hepatic pathways, for example fosinopril and trandolapril. In such cases, assessment of therapeutic efficacy based on uACE activity requires increased caution, particularly in patients with renal impairment, in whom hepatic clearance may increase compensatorily.

The simple and highly reproducible method developed in this work may therefore have important clinical implications. It may enable routine monitoring of adherence to ACE inhibitor therapy in the outpatient setting, providing clinicians with an objective tool for identifying non-adherent patients and planning targeted interventions. This may be particularly relevant in chronic cardiovascular diseases such as hypertension and heart failure, where long-term ACE inhibitor therapy is essential for optimal disease control.

At the same time, the robust and interference-resistant nature of the method may open new diagnostic perspectives beyond adherence monitoring. Because uACE activity reflects both enzymatic properties and renal excretory mechanisms, the method may serve as a potential biomarker for the diagnosis of tubular kidney injury, nephritis, and renal sarcoidosis, or for the investigation of local RAAS processes. In the long term, these applications may represent the most clinically significant use of our findings.

As a result of our research, two biochemical assays ready for routine clinical application were developed, operating according to the same general principles and relying on the diverse physiological functions of angiotensin-converting enzyme, its altered activity in pathological states, and its susceptibility to iatrogenic modulation. Along the path leading to their development, we identified several preanalytical factors influencing measurement and result interpretation, analytical interferences, and interpretive postanalytical shortcomings and opportunities. We thereby demonstrated the central role of laboratory medicine in RAAS-related diagnostic and therapeutic applications and created laboratory tools that directly support clinical decision-making in multiple settings.

To place our findings into proper context, it is ultimately essential to review the limitations encountered. Awareness of these limitations helps prevent applications that could compromise patient safety and provides a basis for further research in this field.

For the assay used in sarcoidosis diagnostics, serum sample quality is a non-negligible factor. Hemolysis leads to underestimation of basal ACE activity and artificially increases the

measured degree of inhibition. To eliminate this effect, qualitative evaluation of all serum samples using serum indices is recommended. In cases of substantial interference, ACE activity determination should be rejected. In our study, no hemolyzed samples were included, in accordance with these principles. Furthermore, in the case of ACE inhibitors containing free sulfhydryl groups, such as captopril, prolonged storage over weeks or months may lead to drug dimerization and oxidation of the sulfhydryl group, resulting in loss of ACE inhibitory effect. Consequently, in vitro a gradual decrease in the degree of inhibition may be expected in samples containing this type of ACE inhibitor. None of the patients in our study population was receiving such therapy, but the phenomenon should be considered in other patient groups.

The ACE insertion/deletion polymorphism is known to account for approximately 60% of the interindividual variability of serum ACE activity measured under physiological conditions. Previous studies have shown that this polymorphism also influences renal ACE protein expression; however, its effect on uACE activity has not yet been studied in detail. In our cohort of healthy individuals, we observed substantial variability in uACE activity (range 0.15-1.10 U/L), which may in part be explained by I/D genotype differences. Accordingly, consideration of the patient's I/D genotype may be warranted in the future when establishing uACE reference ranges, similarly to current practice in the interpretation of serum ACE activity.

uACE activity is affected by numerous confounding factors, including dietary influences, age, renal function, urine dilution, and endogenous inhibitors, all of which limit its reliability as a stand-alone biomarker of adherence to ACE inhibitor therapy. Rather than being used in isolation, it should be interpreted in context, together with medical history, physical examination, and other laboratory findings, as is the case for other biomarkers.

The precise cellular origin of uACE remains unresolved. Although shedding from the proximal tubule is likely, plasma-derived ACE may also contribute, particularly under pathological conditions. Clarification of this issue will require further studies, including plasma-urine correlation analyses and detailed biochemical characterization.

The loss of uACE activity during freezing necessitates additional biochemical investigations and may limit broader research applicability of the method.

Finally, the relatively small sample size underscores the need for larger, targeted studies to establish physiological reference ranges for uACE activity and to define its diagnostic value in different pathological conditions.

6. Novel findings

In my PhD dissertation and in the publications on which it is based, we identified the following as novel findings:

1. Conventional serum ACE activity measured at the 35-fold dilution used for diagnostic purposes is not suitable on its own for the reliable detection of ACE inhibitor drug effects, because the activity values of patients receiving and not receiving ACE inhibitors show substantial overlap. In contrast, the ACE inhibition ratio calculated from samples measured at 4-fold and 400-fold dilutions objectively indicates the presence of ACE inhibitor effect from a single serum sample, even without clinical medication data.
2. In the investigated cohort ($n = 1823$), biochemical evidence suggestive of ACE inhibitor interference was detected in approximately 16.6% of patients, indicating that this is a frequent interfering factor in diagnostic ACE activity determination and one that may also influence clinical decision-making.
3. The inhibitory effect of endogenous uACE inhibitors present in urine is attributable to small molecules with a molecular weight below 5 kDa. Urea, uric acid, and urobilinogen were identified as physiological endogenous inhibitors of uACE, contributing to inhibition even within the physiological range, while the inhibitory effect may become more pronounced at pathological concentrations.
4. The dilution-based fluorescent kinetic uACE assay enables quantitative determination of uACE activity by eliminating the effect of endogenous inhibitors. We demonstrated that uACE activity may be abolished by freeze-thawing and that it shows a strong correlation with urinary creatinine concentration.
5. The uACE/creatinine ratio decreases significantly during ACE inhibitor therapy and may therefore be applied as a potential non-invasive indicator to support estimation of ACE inhibitor adherence.

7. Summary

Angiotensin-converting enzyme (ACE) holds a distinct role in clinical practice. It represents the most widely used serum biomarker of sarcoidosis, and its urinary activity may serve as a potential indicator of various renal injuries. At the same time, the enzyme is the primary therapeutic target of the reversible ACE inhibitors used in the treatment of cardiovascular diseases, and its enzymatic activity is influenced by endogenous inhibitors in both serum and urine. These exogenous and endogenous effects may lead to misleading interpretation of laboratory results.

Our aim was to develop biochemical methods capable of objectively identifying or eliminating interferences affecting ACE activity measurements, thereby improving the clinical applicability of the biomarkers.

We demonstrated that conventional serum ACE activity measurement at 35-fold dilution is unsuitable for recognizing ACE inhibitor drug effects, as values of treated and untreated patients substantially overlap. We showed that the ACE inhibition ratio calculated from activities measured at 4-fold and 400-fold dilutions objectively indicates ACE inhibitor effect from a single serum sample, even without clinical medication data. Using this method, biochemical evidence of drug interference was detected in approximately 16.6% of 1823 patients investigated for suspected sarcoidosis, suggesting a greater magnitude of the problem than previously assumed.

We further demonstrated that endogenous inhibitors masking urinary ACE activity are reversible, <5 kDa molecules, and identified urea, uric acid and urobilinogen as principal components. Based on these findings, we developed a dilution-based fluorescent kinetic assay enabling quantitative measurement of uACE activity by eliminating endogenous inhibitory effects. We established the preanalytical conditions of measurement, showed that enzymatic activity is abolished by freezing, and substantiated the rationale for normalization to creatinine concentration.

In clinical samples, uACE to creatinine ratio proved significantly lower during ACE inhibitor therapy, indicating its potential as a non-invasive marker of pharmacological drug effect and therapy adherence.

Our results demonstrate that the interpretability of enzyme activity-based biomarkers fundamentally depends on the recognition of measurement conditions and interferences. With appropriate analytical strategies, their diagnostic added value and clinical usefulness can be substantially improved.

8. List of own publications



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PhD Publication List

Candidate: Attila Ádám Szabó

Doctoral School: Kálmán Laki Doctoral School

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List of publications related to the dissertation

1. **Szabó, A. Á.**, Enyedi, E. E., Pintér, T. B., Mányiné Siket, I., Váradi, C., Bányai, E., Tóth, A., Papp, Z., Fagyas, M.: Interference-Free Measurement of Urinary Angiotensin-Converting Enzyme (ACE) Activity: Diagnostic and Therapeutic Monitoring Implications.
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3. Enyedi, E. E., **Szabó, A. Á.**, Pintér, T. B., Mányiné Siket, I., Pluhár, A., Váradi, C., Bányai, E., Tóth, A., Papp, Z., Fagyas, M.: Albumin-Bound Fatty Acids Modulate Endogenous Angiotensin-Converting Enzyme (ACE) Inhibition.
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