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Optimized angiotensin-converting enzyme activity assay for the accurate diagnosis of sarcoidosis

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Abstract

Background: Serum angiotensin-converting enzyme (ACE) activity determination can aid the early diagnosis of sarcoidosis. We aimed to optimize a fluorescent kinetic assay for ACE activity by screening the confounding effects of endogenous ACE inhibitors and interfering factors. Genotype-dependent and genotype-independent reference values of ACE activity were established, and their diagnostic accuracies were validated in a clinical study.

Methods: Internally quenched fluorescent substrate, Abz-FRK(Dnp)P-OH was used for ACE-activity measurements. A total of 201 healthy individuals and 59 presumably sarcoidotic patients were enrolled into this study. ACE activity and insertion/deletion (*I/D*) genotype of the *ACE* gene were determined.

Results: Here we report that serum samples should be diluted at least 35-fold to eliminate the endogenous inhibitor effect of albumin. No significant interferences were detected: up to a triglyceride concentration of 16 mM, a hemoglobin concentration of 0.71 g/L and a bilirubin concentration of 150 μM. Genotype-dependent reference intervals were considered as 3.76–11.25 U/L, 5.22–11.59 U/L, 7.19–14.84 U/L for *II*, *ID* and *DD* genotypes, respectively. *I/D* genotype-independent reference interval was established as 4.85–13.79 U/L. An ACE activity value was considered positive for sarcoidosis when it exceeded the upper limit of the reference interval. The optimized assay with genotype-dependent reference ranges resulted in 42.5% sensitivity, 100% specificity, 100% positive predictive value and 32.4% negative predictive value in the clinical study, whereas the genotype-independent reference range proved to have inferior diagnostic efficiency.

Conclusions: An optimized fluorescent kinetic assay of serum ACE activity combined with *ACE I/D* genotype determination is an alternative to invasive biopsy for confirming the diagnosis of sarcoidosis in a significant percentage of patients.

Keywords: ACE; angiotensin-converting enzyme; genotype; reference interval; sarcoidosis.

Introduction

Sarcoidosis is an idiopathic multisystem granulomatous disease that most commonly affects the lungs, lymph nodes and skin [1]. The diagnosis of sarcoidosis is established when radiologic findings are supported by histologic findings of noncaseating granulomas in the biopsy specimen and other causes of granulomas can be excluded [2]. There are no gold standard laboratory tests to facilitate earlier diagnosis, albeit several new biomarkers have been discovered in the last decades. Soluble interleukin-2 receptor [3], chitotriosidase [4], serum amyloid A [5] and

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other markers were tested as possible diagnostic marker, but only serum angiotensin-converting enzyme (ACE) seems to have diagnostic utility [6].

The metalloproteinase ACE catalyzes angiotensin II production, thus participating in the maintenance of blood pressure and the salt-water homeostasis [7]. ACE is a membrane protein expressed on the surface of many cells, released into the circulation by proteolytic cleavage [8]. Serum ACE originates primarily from the pulmonary microvasculature [9], and its concentration and activity are influenced by the insertion-deletion (*I/D*) polymorphism of the *ACE* gene [10]. Epitheloid cells of the sarcoid granuloma produce ACE [11], which may reflect to the granuloma burden [12], and the granuloma is responsible for the higher serum ACE level in some sarcoidotic patients.

Beside genotype, endogenous ACE inhibition also significantly influences the circulating ACE enzyme activity [13]. Serum albumin (reference range: 35–52 g/L) has been identified as an endogenous ACE inhibitor with an IC_{50} between 5.7 and 9.5 g/L [14]. Majority of the commercially available ACE activity diagnostic tests propose using 1:5 or 1:10 ratios of serum and substrate solution for measurement. Endogenous inhibition of ACE by albumin is still present at those dilutions; consequently, ACE activity may be underestimated with these tests and sarcoidosis is underdiagnosed.

Here, we describe an optimized fluorescent kinetic assay for ACE activity measurement, in which the inhibitory effect of albumin is eliminated and the interfering factors (hemolysis, icterus and lipemia) are determined. Genotype-independent and *I/D* polymorphism-dependent reference intervals for ACE were established, and the accuracy of the optimized ACE activity assay was validated in a clinical study.

Materials and methods

Subjects

Two hundred and one Hungarian adults were enrolled to the control group from the outpatient clinic of the Department of Cardiology, University of Debrecen, or from employees of the department. According to self-assessment, the majority was deemed to be healthy. Mild (type I) treated hypertension was not an exclusion criterion. None of them was treated with ACE inhibitor drugs, and all participants gave a written informed consent.

Fifty-nine patients were involved in a clinical study to evaluate the diagnostic accuracy of the optimized ACE activity assay in sarcoidosis. These patients underwent a diagnostic mediastinoscopy or video-assisted thoracoscopic surgery in the Department of Surgery, University of Debrecen, for biopsy sampling intended to verify the lack or the presence of sarcoidosis by histopathology. Histopathologic examinations were carried out routinely by the Department of Pathology, University of Debrecen. All patients gave their written informed consent.

Serum and DNA samples

Blood samples were obtained by standard aseptic technique into Vacutainer tubes (Cat. No. 368857, 367955, Becton Dickinson, Franklin Lakes, NJ, USA). Sera were separated from native blood after clotting and 15 min, 1500*g* centrifugation at +4 °C. Sera were stored at –20 °C until ACE activity measurement. The degree of hemolysis, icterus and lipemia was estimated with HIL indices measured on a Cobas C6000 analyzer (Roche, Basel, Switzerland), and sampling was repeated if the values exceeded 50, 50 and 200, respectively. In case of patients in clinical study, blood samples were taken immediately before surgery.

EDTA anticoagulated whole blood was taken for genetic determinations and stored at –20 °C until deoxyribonucleic acid (DNA) isolation. The average time span between blood sampling and DNA isolation was 1 month (the range was 2 days to 3 months). Genomic DNA was prepared using NucleoSpin Blood kit (Cat. No: 740951.50; Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instruction, and purified DNA was stored at +4 °C.

ACE activity measurement with the fluorescent kinetic assay

Serum ACE activity was measured using the protocol of Carmona et al. [15] with minor modification. Briefly, reaction mixture contained in 100 mM tris(hydroxymethyl)aminomethane hydrochloride (TRIS) buffer (pH 7.0): 15 μM Abz-FRK(Dnp)P-OH substrate (synthesized by Peptide 2.0, Chantilly, VA, USA), 50 mM NaCl, 10 μM $ZnCl_2$ and serum at 35-fold final dilution. Change in fluorescent intensity of reaction mixtures was measured by NovoStar plate reader (BMG Labtech GmbH, Ortenberg, Germany) in 96-well black plates (Cat. No: 655-900, Greiner Bio-One International GmbH, Kremsmünster, Austria) at 37 °C. Continuous recording was done for 30 min with 1-min measurement intervals, and the excitation and emission wavelengths were 340 and 405 nm, respectively. The activity was expressed in U/L and calculated by the following equation:

$$\text{ACE activity} = S / k * D,$$

where S indicated the slope of the increase in fluorescence with time, k was the fluorescence intensity of 1 nM total hydrolysed substrate and D was the dilution of serum. ACE activity was measured at least twice for each sample to achieve a coefficient of variation of at most 6%. ACE activity values reported as mean.

ACE activity measurement with the 'ACEcolor diagnostic kit'

ACE activity of 40 out of 51 patients involved in the clinical study was measured with ACEcolor diagnostic reagent (Cat. No: 205259,

Fujirebio Inc, Tokyo, Japan) according to the manufacturers instruction. The applied serum dilution was the recommended sixfold, and the absorbance of reaction mixtures was measured by a Hitachi U-2900 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) at 505 nm in disposable cuvettes (Cat. No: 7591 15, Brand GmbH, Wertheim, Germany). The activity was expressed in IU/L and calculated by the suggested equation:

$$\text{ACE activity} = (A - B) * 87.5,$$

where *A* indicated the absorbance of sample mixture, and *B* indicated the absorbance of sample blank mixture. Normal reference range (8.3–21.4 IU/L at 37 °C) under this test method was adopted from the kit booklet.

Interference testing

Hemolysis interference was examined using hemolysate, which was obtained from sodium-citrate anticoagulated whole blood of a healthy volunteer. Blood was washed with physiologic saline five times, and cell cytosol was liberated by freezing and thawing. Cell debris was removed by centrifugation at 16,000*g*, +4 °C, 15 min, and hemoglobin concentration was measured with sodium lauryl sulfate-hemoglobin method in a Sysmex XE-2100 hematology analyzer (Kobe, Japan).

Intralipid (20%, Fresenius-Kabi, Bad Homburg, Germany) was added to normal sera to generate lipemic samples, then triglyceride concentration was measured using a glycerol-blanked triglyceride assay (Cat. No: 05975573 190, Roche, Basel, Switzerland) on a Cobas C6000 analyzer.

Unconjugated bilirubin (Cat. No: 14370, Merck Life Science, Darmstadt, Germany) was added to normal sera to simulate icteric samples, then total bilirubin concentration was measured using azo-bilirubin method (Cat. No: 05168414 190 Roche, Basel, Switzerland) on a Cobas C6000 analyzer.

Significant interference was defined when the change of ACE activity exceeded 10% of the baseline value.

ACE genotyping

I/D genotype of *ACE*-gene was determined on the basis of the protocol described by Rigat et al. [16], and the presence of allele *I* was confirmed using a second polymerase chain reaction (PCR) with the method of Lindpaintner et al. [17]. Amplicons of these reactions were separated and evaluated using a single 3% agarose gel [10], and DNA was stained with SYBR safe gel stain (Cat. No: S33102, Thermo Fisher Scientific, Waltham, MA, USA).

Ethical approval

All studies were approved by the Regional and Institutional Ethics Committee, Clinical Centre, University of Debrecen, (UDCC REC/IEC number: 4375-2015) and by the Medical Research Council of Hungary (33327-1/2015/EKU). The research was in accordance the tenets of the Declaration of Helsinki.

Statistical analysis

χ^2-Test was used to estimate if the genotype distribution in the control group was in Hardy-Weinberg equilibrium [18]. Gaussian distribution of ACE activity values was tested using Shapiro-Wilk test and D'Agostino-Pearson test. Reference intervals were defined as the middle 95% of the reference population (2.5th–97.5th percentiles). Differences in ACE activity among genotype-determined groups were tested by one-way ANOVA and Tukey's multiple comparisons test. Differences in ACE activity values between samples with different concentration of triglyceride or hemoglobin or bilirubin were tested by unpaired t-test. Statistical analysis was performed using GraphPad Prism software, version 7.00 (San Diego, CA, USA). Data are presented as mean ± standard deviation (SD) or median and range; p values <0.05 were considered statistically significant.

Results

Reversible endogenous inhibition of ACE by albumin can be eliminated by the appropriate dilution of serum. ACE activity of three serum samples obtained from healthy individuals with different *ACE I/D* genotypes was measured in serial dilutions (Figure 1). Calculated ACE activity values showed an exponential increase with the dilution in each genotype, reaching the maximum at 35-fold

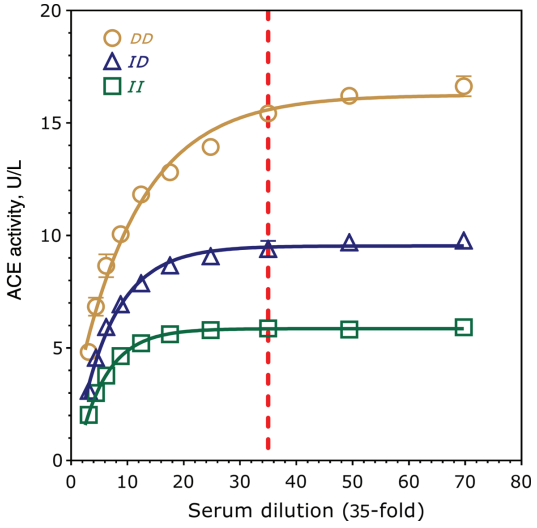


Figure 1: Inhibitory effect of albumin on ACE activity can be eliminated by the 35-fold dilution of serum.
Calculated ACE activity values are plotted as a function of applied serum dilutions. Empty circles, triangles and squares represent data of three healthy individuals with genotype *DD*, *ID* and *II*, respectively. Plots are fitted by means of one phase exponential decay, and symbols denote the means ± SD of two independent determinations. A 35-fold dilution is indicated by dashed red line. Please note, that somewhere the error bars are overlapped by the symbols.

dilution. ACE activities measured at 35-fold dilution did not differ statistically from those values measured at 70-fold dilution in all samples.

Next, hypothetical interferences of triglyceride, hemoglobin and bilirubin on ACE activity were determined (Figure 2). Turbidity of sample did not affect the result of ACE activity reading. The ACE activity was constant for a triglyceride concentration up to 16 mM (Figure 2A). By contrast, hemolysis influenced significantly the results of the fluorescent kinetic assay at hemoglobin concentration of 0.35 g/L or higher (Figure 2B). A hemoglobin concentration of 0.71 g/L led to the underestimation of ACE activity by about 10%. Similarly, jaundice also affects measured ACE activities at total bilirubin concentration of 64 μM or higher (Figure 2C). Samples with bilirubin concentrations higher than 150 μM were associated with an apparent 10% decrease of ACE activity.

Data of 201 individuals were involved in the determination of genotype-dependent and genotype-independent ACE activity reference values. *ACE I/D* genotype and ACE activity at 35-fold dilution were determined and basic clinical data of subjects were collected (Table 1). Genotype distribution of the whole population was in Hardy-Weinberg equilibrium ($\chi^2 = 1.276$ with two degrees of freedom, $p = 0.5283$). ACE activity values showed normal distribution using Shapiro-Wilk test (*II*: 7.30 ± 1.65 U/L, $p = 0.97$, $n = 39$; ID: 8.72 ± 1.70 U/L, $p = 0.30$, $n = 90$, *DD*: 10.6 ± 1.70 U/L, $p = 0.41$, $n = 72$; all subjects: 9.12 ± 2.08 U/L, $p = 0.64$, $n = 201$) and D'Agostino-Pearson test (*II*: $p = 0.94$; *ID*: $p = 0.09$, *DD*: $p = 0.26$; all subjects: $p = 0.49$). The *ACE I/D* genotype significantly influenced ACE activity, as individuals with *II* genotype showed lower ACE activity than individuals with *DD* genotype, while subjects with *ID* genotype showed a mid-range activity (*II*: 7.30 ± 1.65 U/L;

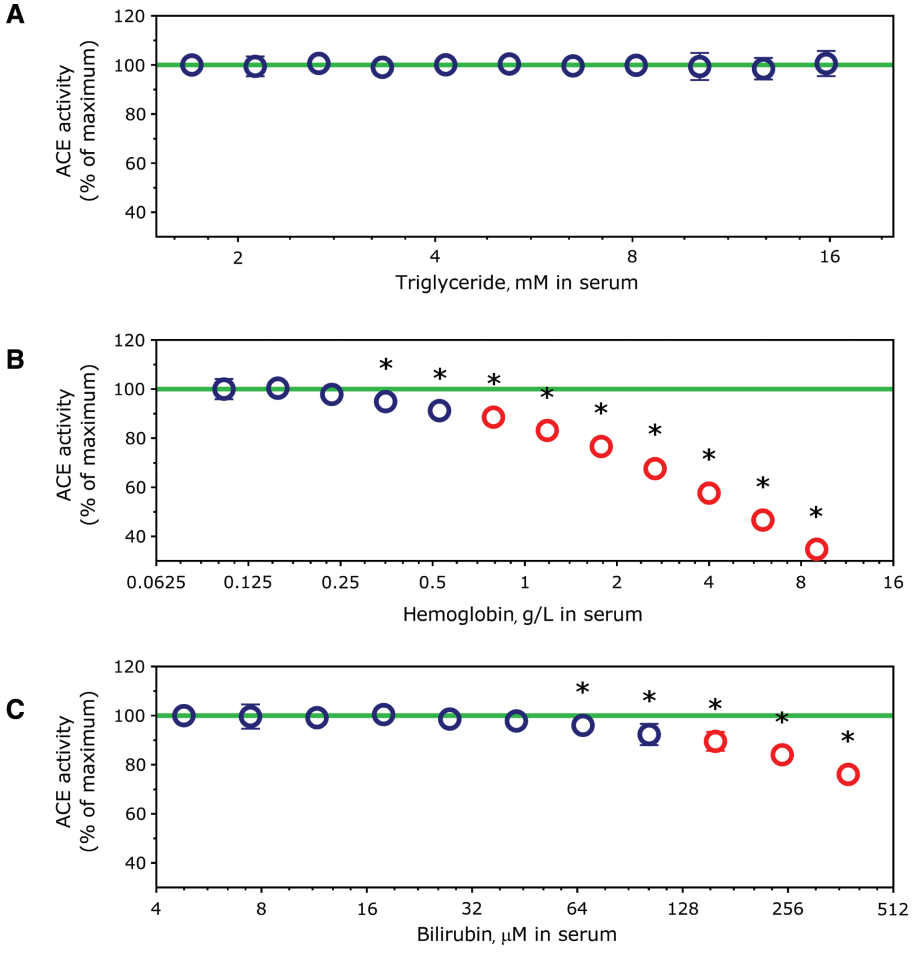


Figure 2: Hemolysis and icterus decrease the ACE activity.
ACE activity values, expressed as % of maximum, are plotted as a function of triglyceride (mM, A), hemoglobin (g/L, B) or bilirubin (μM, C) concentration. Symbols denote the means ± SD of three independent determinations. Significant differences (unpaired t-test) from samples with the lowest triglyceride or hemoglobin or bilirubin concentration are indicated by asterisk (*). More than 10% decrease in ACE activity compared to baseline is indicated by red cycles.

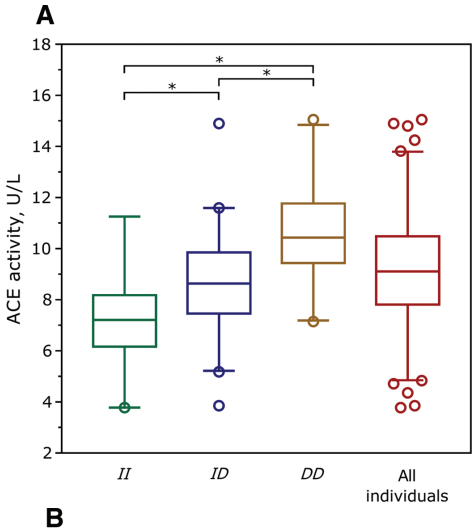
Table 1: Characteristics of control group.

	Female	Male	Total
Number, n	110	91	201
Age, years (mean ± SD)	54.71 ± 14.90	54.85 ± 14.16	54.78 ± 14.52
BMI, kg/m^2	27.29 ± 5.83	29.13 ± 5.03	28.13 ± 5.54
Smoker, n	12	15	27
Systolic blood pressure, mmHg (mean ± SD)	128.35 ± 18.68	128.75 ± 15.48	128.5 ± 17.22
Diastolic blood pressure, mmHg (mean ± SD)	78.42 ± 10.24	80.90 ± 9.90	79.56 ± 10.13
Left ventricular EF, % (mean ± SD)	59.07 ± 6.49	56.20 ± 6.78	57.71 ± 6.76
ACE-activity, U/L (mean ± SD)	9.10 ± 2.06	9.15 ± 2.10	9.12 ± 2.07
II genotype, n	18	21	39
ID genotype, n	44	46	90
DD genotype, n	48	24	72

BMI, body mass index; EF, ejection fraction; ACE, angiotensin-converting enzyme.

ID: 8.72 ± 1.70 U/L; *DD*: 10.6 ± 1.70 U/L, $p < 0.0001$) (Figure 3A). Normal reference intervals for each group were calculated and summarized on Figure 3B.

Diagnostic accuracy of this ACE activity assay was tested in a clinical trial. Fifty-nine patients were enrolled in the study, who all underwent diagnostic mediastinoscopy or thoracoscopy for hilar lymph node or lung tissue biopsy motivated by the symptoms of sarcoidosis. Diagnosis of sarcoidosis was established or ruled out by histopathologic examination of these biopsy specimen. Blood samples were taken from each patient before surgery for genotyping and ACE activity measurement. Patients under steroid therapy were excluded from the study. Eight out of 59 patients were excluded from the evaluation because they took ACE inhibitors and had an artificially low serum ACE activity. Histopathologic examination was specific for sarcoidosis in 40 out of 51 patients, and 11 patients had histologically different diagnoses (lymphoma, carcinoma, anthracosis, sinus histiocytosis, etc.). Concerning the prevalence of *ID* genotype and allele frequencies, there were no statistically significant differences between histologically positive and negative groups for sarcoidosis, and between positive for sarcoidosis and control group (data not shown). Clinical characteristics of patients are listed in Table 2.



B

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

Figure 3: *ACE I/D* genotype influences the serum ACE activity. Serum ACE activity distribution (A) and normal reference ranges (B) are shown according to *ACE I/D* genotype (insertion, *II*, n = 39; heterozygotes, *ID*, n = 90; deletion, *DD*, n = 72; all individuals, n = 201). The boxes indicate the interquartile range with median; whiskers are the 2.5th and 97.5th percentiles. Outliers are plotted as individual points (empty cycles). Significant differences (one-way ANOVA, Tukey's multiple comparisons test) among genotype groups are labeled by asterisks (*).

An ACE activity value was considered positive for sarcoidosis, when it exceeded the upper limit of the reference interval. Nine out of 40 sarcoidotic patients had ACE activity higher than the upper limit of the reference range when genotype-independent reference interval was applied, and 17 out of 40 sarcoidotic patients had ACE activity higher than the upper limit of reference range when genotype-dependent reference interval was used (Table 3). Thereby, the sensitivity of ACE activity determination for confirming the diagnosis of sarcoidosis is greatly improved by genotype-dependent reference intervals (42.5% vs. 22.5%). Eleven out of the 51 patients had true negative results which finally proved to be negative based on both evaluation techniques, and 31 and 23 patients were false negative with genotype-independent and genotype-dependent reference intervals, respectively. Specificity and positive predictive value (PPV) were 100% in both cases, whereas

Table 2: Characteristics of patients enrolled in the clinical study.

Total number of patients, n	59	
Excluded patients because of ACE inhibitor treatment, n	8	
	Positive histology for sarcoidosis	**Negative histology for sarcoidosis**
Number, n	40	11
Age, year (mean ± SD)	39.0 ± 11.0	54.0 ± 15.2
Gender, n (female/male)	18/22	3/8
II genotype, n	4	3
ID genotype, n	24	5
DD genotype, n	12	3
ACE activity (*II*), U/L (mean ± SD)	10.38 ± 3.67	5.73 ± 0.91
ACE activity (*ID*), U/L (mean ± SD)	12.24 ± 4.35	7.38 ± 2.90
ACE activity (*DD*), U/L (mean ± SD)	13.99 ± 4.82	10.97 ± 0.70
Radiographic stage of lung disease		
Stage 0, n	1	0
Stage I, n	24	8
Stage II, n	11	3
Stage III, n	3	0
Stage IV, n	0	0
Left ventricular EF, % (mean ± SD)	60.4 ± 4.8	61.1 ± 5.4
FVC, % (mean ± SD)	96.0 ± 16.5	85.8 ± 19.1
FEV1, % (mean ± SD)	92.7 ± 15.0	80.0 ± 18.8
Tiffeneau-index, % (mean ± SD)	82.82 ± 5.82	79.05 ± 7.13

ACE, angiotensin-converting enzyme; EF, ejection fraction; FVC, forced vital capacity; FEV1, forced expiratory volume (1 s); Tiffeneau-index, FEV1/FVC.

negative predictive value was 26.2% and 32.4% according to genotype-independent and genotype-dependent reference ranges, respectively. The accuracy of the ACE activity assay was higher when the genotype-dependent reference interval was applied (54.9% vs. 39.2%).

We compared the diagnostic accuracy of this fluorescent kinetic assay to a commercial available diagnostic test, 'ACEcolor' (Table 4). Based on 40 out of 51 patient's ACE activity values, the diagnostic accuracy of our test (with genotype-independent decision limit) was similar to the accuracy of 'ACEcolor' test (42.5% vs. 40%, respectively), although 'ACEcolor' identified

Table 3: Diagnostic accuracy of fluorescent kinetic ACE activity assay.

	Evaluation with genotype-independent decision limit	**Evaluation with genotype-dependent decision limits**
True positive, n	9	17
True negative, n	11	11
False positive, n	0	0
False negative, n	31	23
Sensitivity, %	22.5	42.5
Specificity, %	100.0	100.0
PPV, %	100.0	100.0
NPV, %	26.2	32.4
Accuracy, %	39.2	54.9

PPV, positive predictive value; NPV, negative predictive value.

Table 4: Diagnostic accuracy of ACEcolor diagnostic assay and the optimized fluorescent kinetic assay.

	ACEcolor (Fujirebio Inc.)	**Optimized fluorescent kinetic ACE activity assay**	
		Evaluation with genotype-independent decision limit	**Evaluation with genotype-dependent decision limit**
			n=40
True positive, n	6	6	9
True negative, n	10	11	11
False positive, n	1	0	0
False negative, n	23	23	20
Sensitivity, %	20.7	20.7	31.3
Specificity, %	90.9	100	100
PPV, %	85.7	100	100
NPV, %	30.3	32.4	35.5
Accuracy, %	40	42.5	50

PPV, positive predictive value; NPV, negative predictive value.

a non-sarcoidotic sample as false positive. When genotype-dependent decision limits were used, our test was superior to 'ACEcolor' diagnostic test (50% vs. 40%, respectively).

Discussion

Measurement of ACE activity from human serum can provide physicians with essential information for diagnosing some diseases, such as sarcoidosis [19], Gaucher disease [20] and granulomatous infections [21]. ACE activity seems to be a useful biomarker for the diagnosis and monitoring of sarcoidosis [6]. Radiolabeled, colorimetric and fluorometric assays are available to measure serum ACE activity, and recently a rapid, very sensitive fluorescent kinetic assay has been developed using an internally quenched fluorogenic substrate (Abz-FRK(Dnp)P-OH) [22]. Here we further optimized this test to eliminate inhibitory effects of the endogenous ACE inhibitor albumin. Interfering factors were revealed and normal reference ranges were determined for this method.

Albumin-mediated endogenous ACE inhibition is reversible, which means that the degree of inhibition is highly affected by the degree of dilution during measurement. In other words, inhibition can be eliminated by appropriate dilution of the sample. We identified, that albumin-mediated inhibition on serum ACE becomes negligible at 35-fold or higher dilution of sera. The 35-fold serum dilution is applicable in case of all the three *ACE I/D* genotypes; therefore, it is not necessary to know the patient's *I/D* genotype for the measurement, only for the evaluation of results. It is important to note that the 35-fold dilution is not sufficient to eliminate the exogenous interfering effects of ACE inhibitor drugs. Before the ACE activity measurement, patients treated with ACE inhibitors are recommended to be switched to another type of drug, such as angiotensin II receptor blocker.

Manufacturers of ACE activity diagnostic reagents suggest a 5- or 10-fold final dilution of serum despite the fact that Lieberman and Sastre [23] have already called attention to the importance of serum dilution in 1986. These authors examined 6-, 12-, 24- and 48-fold final dilutions, and they suggested 48-fold final dilution (8-fold before assay and 6-fold during measurement) to eliminate the effects of endogenous inhibitors. This is in accordance with our findings because we have found that ACE is partially inhibited at a 24-fold dilution, but apparently uninhibited at 35-fold dilution.

Endogenous interference studies have revealed that icterus and hemolysis may interfere with this fluorescent kinetic activity assay. Considering the slight individual variability of serum ACE activity and the fact that in some sarcoidotic patients ACE activity increases only to a small extent, we suggest 10% decrease in ACE activity due to interference to be considered as significant. Previously, it was reported that bilirubin decreases the apparent ACE activity in spectrophotometric but not in radionuclide assays [24]. Bilirubin can directly bind to ACE and modulate its conformation, thereby influencing ACE shedding from endothelial cells [25] and maybe ACE activity as well. A large amount of hemoglobin is released from red blood cells during hemolysis. Hemoglobin shows strong absorbance at 415-, 540- and 570-nm wavelengths [26]. This assay measures change in fluorescent intensity at 405 nm. In hemolysed samples, the emitted light can partially be absorbed by cell free hemoglobin, which may result in decreased apparent ACE activity readings. On the other hand, it cannot be excluded that hemoglobin exerts some kind of direct effects on ACE. Lipemia does not influence the assay for a triglyceride concentration of 16 mM; thus, fasting blood sampling is not crucial. Relying upon these findings, further dilution of serum samples with hemoglobin concentration of >0.71 g/L or bilirubin concentration of >150 μM is required to eliminate probable effects of interfering factors.

We determined genotype-dependent and genotype-independent ACE activity reference values relying on the data of 201 Hungarian individuals. To our knowledge, this is the first time that reference ranges has been published using this ACE activity assay. Current guidelines of Clinical and Laboratory Standards Institute [27] and European Federation of Clinical Chemistry and Laboratory Medicine [28] recommend the involvement of at least 120 individuals into a reference group. Although our genotype-independent reference group ($n = 201$) fulfils this recommendation, the numbers of individuals are less than 120 in each genotype-dependent reference group, representing a limitation of these reference intervals. The *I/D* genotype distribution in our reference population (*II* = 19.4%; *ID* = 44.8%; *DD* = 72%) was in accordance with the previously published data in Caucasians [29–31], and the mean ACE activity values were also statistically different ($p < 0.0001$) among these genotype groups. Our genotype-dependent reference ranges overlap with each other from the value of 7.19 U/L (the lower reference limit of group *DD*) to the value of 11.25 U/L (the upper reference limit of group *II*). We suggest that *ACE I/D* genotype should only be determined when the measured ACE activity value is higher than 11.25 U/L. Patients with ACE activity higher than 11.25 U/L will especially benefit from

genotyping and applying the genotype-dependent reference intervals. This method may help rationalizing the expenses of genotyping.

It is important to note that this assay results in much lower ACE activity values than other methods [32, 33]. The data presented here confirm that the internally quenched fluorogenic substrate (Abz-FRK(Dnp)P-OH has a slower hydrolysis rate than that for other substrates, such as used in the 'ACEcolor' assay (Table 4). However, the fluorogenic detection of the decomposition of the (Abz-FRK(Dnp)P-OH is more sensitive, making this substrate preferred in this report. It needs also be highlighted that here we showed that ACE is endogenously inhibited by a common ingredient of the samples, namely by albumin. Hence, earlier data are biased by the unknown contribution of albumin inhibition to the measured ACE activities. Here we used an inferior substrate (lower conversion rate) to set up a more sensitive assay and provided a method to eliminate the albumin mediated inhibitory effect.

Diagnostic accuracy of this fluorescent kinetic assay was tested in a clinical study. Sarcoidosis suspect patients underwent diagnostic surgery to collect tissue samples for histopathology, and at the same time ACE activity was also determined from serum samples. Patients with ACE activity values exceeding the upper limit of the reference interval were considered positive for sarcoidosis. Almost twice as much patients proved to be positive for sarcoidosis according to the genotype-dependent reference intervals compared to those evaluated according to the genotype-independent reference range. Applying this ACE activity assay and genotype-dependent reference intervals, the diagnosis of sarcoidosis can be confirmed with 42.5% sensitivity, 100% specificity and 100% PPVs. This ACE activity assay and the applied reference ranges did not result in any false positive cases; therefore, the number of false negative cases became higher and the sensitivity lower. With other words, the false-positive results were eliminated by sacrificing the sensitivity. Consequently, the presence of symptoms consistent with the diagnosis of sarcoidosis and ACE activity values exceeding the upper limit of the reference range should prompt physicians to consider initiation of pharmacological treatment instead of performing surgical biopsy.

Various methods and diagnostic kits can be used to measure ACE activity. Comparison of diagnostic accuracy of the fluorescent kinetic assay presented here to another commercial ACE activity kit revealed a slight superiority of the fluorescent kinetic assay. On the other hand, identification of the *ACE I/D* genotype significantly improved both sensitivity and specificity to detect sarcoidosis irrespectively of the method used to measure the activity.

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

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