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

Optimized usage of serum biomarkers in the diagnostics of sarcoidosis

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The Examination will be held 12:00 p.m., February 23, 2021

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The Defence will be held 13 p.m., February 23, 2021

Live online access will be provided. If You wish to take part in the discusson, please send an e-mail to drenyediattila@gmail.com not later than 12 am on the day before the discussion(February 22, 2021). After the deadline ,for technical reasons,it is no longer possible to join tot he defence.

1. INTRODUCTION

Sarcoidosis is a systemic granulomatous disease affecting predominantly thoracic organs (lymph nodes and the lungs, > 90%), although extrapulmonary manifestation may also be present in half of the cases. Establishing the diagnosis of sarcoidosis often requires biopsy and histopathologic evaluation for supporting clinicoradiological findings. Invasive, diagnostic tissue sampling significantly increases disease burden, especially in cases with only thoracic involvement, when the patients lack an easily accessible and safe anatomical site for biopsy procurement.

Serum biomarkers play a limited role in diagnosing sarcoidosis, because not a single molecule is known to be specific and sensitive enough for sarcoidosis. Several potential biomarkers have been tested for this role in the last decades (e.g. chitotriosidase (CTO), lysozyme (LZM), serum amyloid-A (SAA), soluble interleukin-2 receptor (sIL-2R), etc.), but only angiotensin converting enzyme (ACE) has entered into clinical practice. The metalloproteinase ACE catalyzes angiotensin II production, thus participating in the maintenance of blood pressure and the salt-water homeostasis. ACE is a membrane protein expressed on the surface of many cells, released into the circulation by proteolytic cleavage. Serum ACE originates primarily from the pulmonary microvasculature, and its concentration and activity are influenced by the insertion-deletion (*I/D*) polymorphism of the *ACE* gene. Epitheloid cells of the sarcoid granuloma produce ACE, which may reflect to the granuloma burden, 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. Serum albumin (reference range: 35–52 g/L) has been identified as an endogenous ACE inhibitor with an IC50 between 5.7 and 9.5 g/L. 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.

In our earlier works we described 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. In the first phase of our present research, we validated the accuracy of the optimized ACE activity assay in a clinical study.

In the second phase of our present research we compared the laboratory efficiency of diverse ACE activity measuring techniques, determined the diagnostic accuracy of several, single serum biomarkers, and developed a combined biomarker analysis tool for the diagnosis of sarcoidosis.

2. AIMS

1. Validation of the accuracy of the optimized ACE activity assay in a clinical study.
2. Comparison of the laboratory efficiency of diverse ACE activity measuring techniques.
3. Development of a combined biomarker analysis tool for the diagnosis of sarcoidosis.

3. MATERIALS AND METHODS

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 with the tenets of the Helsinki Declaration. All patients gave a written informed consent.

3.1. First phase

3.1.1. 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.

Fifty-nine patients were involved in the 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.

3.1.2. 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..

3.1.3. ACE activity measurement with the fluorescent kinetic assay

Serum ACE activity was measured using the protocol of Carmona et al. 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 ZnCl2 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: 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.

3.1.4. ACE activity measurement with '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: 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.

3.1.5. ACE genotyping

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

3.1.6. Statistical analysis

$\chi 2$-Test was used to estimate if the genotype distribution in the control group was in Hardy-Weinberg equilibrium. 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 Graph- Pad 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.

3.2. Second phase

3.2.1. Subjects

One hundred and four sarcoidosis suspect patients were involved in our investigations. All patients were subjected to diagnostic mediastinoscopy or video-assisted thoracoscopic surgery in the Department of Surgery, University of Debrecen for biopsy sampling to verify the lack or the presence of sarcoidosis. Histopathologic examinations were carried out routinely by the Department of Pathology, University of Debrecen. Patients under steroid therapy were excluded from the study. All patients gave a written informed consent.

One hundred thirty-three adults were enrolled from the outpatient clinic of the Department of Cardiology, University of Debrecen, or from employees of the department for the determination of reference intervals (CTO activity, LZM and sIL-2R concentrations). According to self-assessment, the majority was deemed to be healthy. Mild (type I) treated hypertension was not an exclusion criterion. Nine out of 104 patients were excluded from evaluation of ACE activity, because they took ACE inhibitors and had an artificially low serum ACE activity. None of the participants was treated with steroid or any sarcoidosis medications, and all participants gave a written informed consent..

3.2.2. Serum and DNA samples

Blood samples were taken 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, 1500g centrifugation at +25 °C. Sera were stored at −20 °C until measurements. In case of patients participating in the clinical study, blood samples were taken immediately before surgery.

EDTA anticoagulated whole blood was taken for genetic determinations and stored at −20 °C until DNA isolation. 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..

3.2.3. Measurement of ACE activity

Serum ACE activity was measured by three different methods. Previously, we described an optimized fluorescent kinetic assay, which is not influenced by the presence of endogenous ACE-inhibitors. Results of this method were compared with those of two commercially available ACE activity measuring assays, namely Infinity ACE (Cat. No:TR85056, Fisher Diagnostics, Middletown, USA) and ACEcolor (Cat. No: 205259, Fujirebio Inc., Tokyo, Japan). Infinity ACE is an UV kinetic assay based on the cleavage of N-[3-(2-furyl)-acryloyl]-L-phenylalanylglycilglycine, while ACEcolor is an end-point

colorimetric method, based on the cleavage of p-hydrocybenzoyl-glycyl-L-histidyl-L-leucine substrate. Tests were performed according to the manufacturers' instructions on a plate reader (fluorescent kinetic assay; NovoStar, BMG Labtech, Germany), or on a clinical chemistry analyzer (Infinity ACE, Cobas c501, Roche, Basel, Switzerland), or on a spectrophotometer (ACEcolor, Hitachi U-2900, Japan)..

3.2.4. Measurement of serum CTO activity

Serum CTO activity was measured as described by Hollak et al. with minor modifications. In brief, CTO activity was determined with an artificial substrate (4-Methylumbelliferyl-β-D-N,N′,N″-triacetyl-chitotrioside; Cat. No: 19715, Cayman Chemicals, Ann Arbor, USA) in a reaction mixture containing 20 μM substrate, 50-fold diluted serum in 100 mM citrate and 200 mM phosphate buffer, at pH 5.2. Measurements were performed in 96-well plates (Cat No: 655-900, Greiner Bio-One GmbH, Germany) at 37 °C. Changes in fluorescent intensity (λex = 340 nm, λem = 450 nm) were measured at 1-min intervals for at least 30 min with a plate reader (NovoStar plate reader; BMG Labtech, Germany). Fluorescent intensity values were plotted as a function of reaction time and fitted by linear regression (GraphPad Prism 7.2). CTO activity was calculated using 4-Methylumbelliferone (Cat. No: M1381, Merck, Germany) calibration curve and expressed in mU/L.

3.2.5. Measurement of serum LZM concentration

Serum LZM concentration was measured by using a commercially available ELISA kit (Cat. No: SEB193Hu, Cloud-Clone Corp., Katy, Texas, USA) according to the manufacturer's instructions. Absorbance values of wells were measured on a plate-reader (NovoStar, BMG Labtech GmbH, Ortenberg, Germany).

3.2.6. Measurement of SAA concentration

SAA concentration was measured with an immunonephelometric assay (Cat. No: OQMP11, Cruinn Diagnostics Limited, Dublin, Ireland) on a BN ProSec analyzer (Siemens Healthcare GmbH, Erlangen, Germany) according to the manufacturer's instructions. Concentration range between 0 mg/L and 6.4 mg/L was used as a reference interval.

3.2.7. Measurement of sIL-2R concentration

Serum sIL-2R concentration was measured using a commercially available ELISA kit (Cat. No: BMS212INST, eBioscience, Bender MedSystems GmbH, Wien, Austria) according to the manufacturer's instructions. Absorbance values of wells were measured on a platereader (NovoStar, BMG Labtech GmbH, Ortenberg, Germany).

3.2.8. Determination of ACE-gene insertion-deletion and chitotriosidase gene duplication polymorphisms

I/D genotype of ACE-gene (rs1799752) was determined on the basis of the protocol described by Rigat et al., and the presence of allele I was confirmed by a second polymerase chain reaction (PCR) with the method of Lindpaintner et al.. Amplicons of these reactions were separated and evaluated using a single 3% agarose gel, and DNA was stained with SYBR safe gel stain (Cat. No: S33102, Thermo Fisher Scientific, Waltham, MA, USA). Twenty four base pair duplications (rs3831317) of CTO gene (CHIT1, chitinase 1) was determined according to the protocol described by Livnat et al.

3.2.9. Statistical analysis

The distribution pattern of data was assessed using D'Agostino Pearson omnibus normality test. All values with normal distributions are shown as mean (± standard deviation), while those with non-normal distributions are expressed as median (range), for which range is the 25-75th percentile. Normally distributed data were compared using unpaired t-test with Welch's correction, while data with non-normal distribution were compared using Mann-Whitney U test. Analysis of nominal values was conducted with two-tailed Fisher's exact test. $\chi 2$-test was used to estimate if the genotype distributions were in Hardy-Weinberg equilibrium. Allele distributions in different groups were compared using two-tailed Fisher's exact test. The statistical studies used to obtain the reference ranges followed the recommendation of the International Federation of Clinical Chemistry (IFCC) reported in the guideline of the Clinical and Laboratory Standards Institute (CLSI). Reference intervals were determined by non-parametric percentile method, outliers were identified according to Tukey. Combined sensitivity and specificity were calculated using the method for simultaneous testing according to Kanchanaraksa. Statistical analysis was performed using Graph-Pad Prism software, version 7.2 (San Diego, CA, USA). P values < 0.05 were considered statistically significant.

4. RESULTS

4.1. First phase

In our earlier works we described 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. We identified, that albumin-mediated inhibition on serum ACE becomes negligible at 35-fold or higher dilution of sera. In order to avoid the interfering effect of bilirubin and hemoglobin, further dilution of the sera is recommended above 0,71 g/l hemoglobin concentration or 150 μM bilirubin concentration. Genotype-independent and *I/D* polymorphism-dependent reference intervals for ACE were established. 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.

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 genotypedependent reference interval was used. 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 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’. 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 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).

4.2. Second phase

Histopathologic examination was specific for sarcoidosis in 69 out of 104 patients, and 35 patients had histologically different diagnoses (sinus histiocytosis, lymphoma, carcinoma, anthracosis, etc.). One hundred and thirty-three healthy individuals were also enrolled into the study as a reference population. Patients with sarcoidosis were significantly younger than patients with negative histology for sarcoidosis or the reference population (40.9 ± 12.3 vs. 50.5 ± 14.0 or 48.3 ± 15.0 years, respectively). We did not observe differences between groups regarding left ventricular systolic function, platelet count, forced vital capacity and Tiffeneau-index. Blood lymphocyte count was statistically lower in sarcoidotic group compared to negative or reference population (1.56 G/L (1.21–1.85 G/L) vs 1.82 G/L (1.39–2.38 G/L) or 1.89 G/L (1.58–2.25 G/L), respectively), albeit blood lymphocyte ratio proved to be lower in both patient groups compared to reference population (23.3% (18.5–28.1%) or 24.3 ± 9.3% vs. 29.2% (24.4–32.7), respectively). Platelet-to-lymphocyte ratio was significantly higher only in sarcoidotic group (160 (125–213)) compared to reference population (130 (113–165)). Serum creatinine concentration did not differ between groups. Forced expiratory volume (1 s) was higher in the sarcoidotic group compared to the negative patient group (93 ± 16% vs 84 ± 19%, respectively).

ACE insertion-deletion polymorphism significantly affects the activity of circulating ACE, just as CTO gene duplication polymorphism affects CTO activity. Genotype distribution of ACE I/D polymorphism and CTO gene duplication polymorphism were in Hardy-Weinberg equilibrium and did not differ among groups. Mean or median serum ACE activity (measured with any methods), sIL-2R concentration and CTO activity were significantly higher between sarcoidotic and non-sarcoidotic patient groups, while we did not observe any difference in CRP, LZM and SAA concentrations between these groups.

We measured ACE activity by three different methods. Previously our workgroup described an optimized fluorescent kinetic assay, the diagnostic accuracy of this technique was compared with two commercially available ACE activity tests (ACEcolor and InfinityACE). An ACE activity value was considered positive for sarcoidosis, when it exceeded the upper limit of the reference interval determined by the manufacturer or other research groups. We compared the area under the receiver operating characteristic (ROC) curves of these tests and we found similar AUC values in each test (fluorescent kinetic assay vs. ACEcolor vs. InfinityACE = 0.823 vs. 0.801 and 0.816, respectively). Nonetheless, there were significant differences among sensitivity, specificity, positive and negative predictive values of these methods. Fluorescent kinetic assay and ACEcolor had low sensitivity (25.0% and 25.4%, respectively) but exceptionally high specificity and positive predictive values (100% and 100%; 96.4%

and 94.1%, respectively). In contrast with this, Infinity ACE test showed approximately 80% sensitivity, specificity and positive predictive values. When data were re-evaluated using genotype-dependent ACE activity reference ranges we found, that inclusion of ACE genotype significantly improved the diagnostic accuracy of optimized fluorescent kinetic assay (sensitivity increased from 25.0 to 45.2% with maintained specificity and positive predictive values), while mild decline was found in the diagnostic accuracy of ACEcolor and InfinityACE tests.

Next, we determined the reference intervals for sIL-2R and LZM concentrations, because these data were not provided by the manufacturers of the kits. We identified 8 outliers in case of sIL-2R (≥0.879 μg/L) and 2 outliers in case of LZM (≥2.477 mg/L) from the reference population. Reference ranges were determined as 0–0.6823 μg/L for sIL-2R concentration and as 0–2.2538 mg/L for LZM concentration.

In view of the reference intervals, we tested the diagnostic accuracies of sIL-2R, LZM and SAA concentrations in our patient population. ROC analyses showed that only sIL-2R concentration could discriminate between sarcoidotic and non-sarcoidotic patients in this study ($p = 0.0117$, $p = 0.0643$, $p = 0.5684$, respectively), although sIL-2R had a low AUC value. The sensitivity (52.2%, 35.8% and 47.8%, respectively) and specificity (73.5%, 48.9% and 44.1%, respectively) of these analytes was low.

Serum CTO activity seemed to be a promising diagnostic test in sarcoidosis diagnostics in the last decades. We set up a method for measuring CTO activity from serum and determined the reference range as well. We identified 10 outliers (≥1423 mU/L) from the reference population and we determined 0–1233 mU/L as a reference range for serum CTO activity. ROC analysis showed, that serum CTO activity can effectively discriminate sarcoidotic patients from non-sarcoidotic patients (AUC = 0.8608, $p < 0.0001$). Using the upper reference limit (1233 mU/L) as decision limit for sarcoidosis, this test had 85.1% sensitivity, 76.5% specificity, 87.7% positive predictive and 72.2% negative predictive value and 82.2% diagnostic accuracy in sarcoidosis.

We were curious to know whether the combination of serum biomarkers with the highest sensitivity and specificity improves the effectiveness of laboratory diagnostics of sarcoidosis. Combination of serum ACE (highest specificity, determined with fluorescent kinetic assay) and CTO activity (highest sensitivity) measurements as a simultaneous testing resulted in improved sensitivity (88.8%) with maintained specificity (76.5%). In order to further improve the diagnostic accuracy, so we derived a new parameter, called ‘double product’ from serum ACE and CTO activities with the multiplication of these values by each other. We determined the reference interval of the ‘double product’, which was 0–10.614 U2/L2. ROC analysis of the double product showed the best so far AUC value, 0.8984. Using the upper limit of the reference interval as a decision limit, the double product had 90.5% sensitivity, 79.3% specificity, 90.5% positive predictive and 79.3% negative predictive values in this study, meaning, that only 13% of patients was misdiagnosed by laboratory tests.

5. DISCUSSION

Measurement of ACE activity from human serum can provide physicians with essential information for diagnosing some diseases, such as sarcoidosis, Gaucher disease and granulomatous infections. ACE activity seems to be a useful biomarker for the diagnosis and monitoring of sarcoidosis. 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). In our work 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. We identified, that albumin-mediated inhibition on serum ACE becomes negligible at 35-fold or higher dilution of sera. In order to avoid the interfering effect of bilirubin and hemoglobin, further dilution of the sera is recommended above 0,71 g/l hemoglobin concentration or 150 μM bilirubin concentration. Genotype-independent and *I/D* polymorphism-dependent reference intervals for ACE were established. Diagnostic accuracy of this ACE activity assay was tested in a clinical trial.

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.

In the second phase of our study we compared the laboratory efficiency of diverse ACE activity measuring techniques, determined the diagnostic accuracy of several, single serum biomarkers, and developed a combined biomarker analysis tool for the diagnosis of sarcoidosis.

Sarcoidosis was defined as a multisystem granulomatous disease of unknown cause by the American Thoracic Society, the European Respiratory Society and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) in 1999. It has been stated, that the diagnosis of sarcoidosis is established when radiologic findings are supported by histologic findings of non-caseating granulomas in the biopsy specimen and other causes of granulomas can be excluded. Moreover, it has been postulated, that once the pathogenesis of sarcoidosis is understood it is likely that a highly accurate diagnostic test will be developed based on the specific mechanisms of the disease. Until that time, specialists in laboratory medicine can support clinicians with the development of new biomarker-based assays or refinements of already available techniques for the laboratory diagnosis of sarcoidosis.

One of the traditional serum biomarkers of sarcoidosis is ACE, which has been considered in this context since 1975 as ACE is secreted by macrophages and epithelioid cells located in the granuloma. Proper evaluation of serum ACE activity requires caution, because many conditions including endogenous ACE-inhibitors, ACE-inhibitor drugs, ACE gene insertion/deletion polymorphism, etc. modify its value. Here we compared the diagnostic accuracy of three different ACE activity measuring techniques. In ROC analyses, we did not find significant differences among these methods. Previously it has been published, that determination of I/D polymorphism of ACE gene and using the genotype-dependent ACE activity reference values increase the sensitivity but decrease the specificity of ACE in sarcoidosis. In contrast here we demonstrated, that our optimized fluorescent kinetic ACE activity method and genotype-dependent reference intervals significantly improved the sensitivity of the test with a maintained, high specificity.

Recently it has been described, that platelet-to-lymphocyte ratio can be used for guiding both the diagnosis of sarcoidosis and the involvement of lung parenchyma in the disease. According to this, we found, that platelet-to-lymphocyte ratio is higher in the sarcoidotic group than in the control group, but ROC analysis revealed worse AUC value (0.584) for the above variable than previously published (0.79).

sIL-2R is a truncated protein that is released from activated T lymphocytes, therefore it is considered a serological marker of T-cell activation. As far as we know, we are the first who performed a study to the diagnostic accuracy of sIL-2R in patients with pulmonary sarcoidosis in comparison with a control group. Our results supported previous finding in that sIL-2R concentration is elevated in patients with sarcoidosis. However, it should be emphasized, that we also measured statistically higher sIL-2R

concentration in the non-sarcoidotic group compared to the reference population. This may be related to other, so far unknown mechanisms in hematologic malignances, other granulomatous diseases and various autoimmune disorders where pathologic processes also increase sIL-2R level in the serum. Accordingly, the majority of our patients in the non-sarcoidotic group suffered from hematologic malignances. ROC analysis showed low AUC value in our study, therefore sIL-2R does not seem to be a good single serum biomarker for diagnosing pulmonary involvement of sarcoidosis.

Macrophages and epithelioid cells forming the granuloma produce lysozyme, which is a low molecular weight bacteriolytic enzyme. Several recent studies have demonstrated that serum lysozyme concentration can be elevated in the ocular involvement of sarcoidosis. Patients involved in our study had only lung or hilar lymph node involvements, which may explain the lack of difference between groups with positive and negative results of biopsy for sarcoidosis. It is important to note, that lysozyme is filtrated by renal glomeruli, thus renal dysfunction may increase its circulating level. Patients and healthy controls involved in our study had normal kidney function, hereby it could not influence the results.

Serum amyloid A is an acute phase reactant, which regulates inflammation through Toll-like receptor-2 in sarcoidosis. Elevated levels of SAA have been found in sarcoidosis, albeit there was no correlation with the presence of pulmonary involvement. In contrast to previous studies, we could not observe higher SAA concentration in biopsy-proven sarcoidosis patients compared to biopsy negative patients.

Since 2004, more and more studies have demonstrated, that plasma CTO activity can be a useful biomarker either for diagnosing sarcoidosis or for the assessment of sarcoidosis severity. CTO is produced by activated macrophages and this enzyme is involved in the elimination of chitin producing microorganisms by degrading chitin. In accordance with previous reports our data showed, that serum CTO activity is a good biomarker for diagnosing hilar lymph node or pulmonary sarcoidosis. It is important to note, that a common mutation of CHIT1 gene significantly modifies CTO activity. In particular, individuals with heterozygous genotype for 24 bp duplication have approximately half as much CTO activity than individuals with homozygous wild genotype, while homozygous mutant individuals have no CTO activity in serum or plasma. This fact makes the evaluation of the results more difficult, mainly in case of heterozygous patients.

Some studies have combined more than one biomarker for staging, for the assessment of disease activity or analysis of therapy response in sarcoidosis. Moreover, there were only few studies, which examined the efficiency of biomarker combinations in the diagnostics of sarcoidosis. Collectively, these investigations provided limited success. For example, Groek-Hakan et al. found that combined analysis of sIL-2R and ACE was not superior than the individual use of these biomarkers in patients with sarcoidosis when associated uveitis. Here we combined the optimized fluorescent ACE activity test with the highly sensitive CTO activity test and derived a new parameter (called ‘double product’) by the

multiplication of these values. This ‘double product’ had the highest diagnostic accuracy in our clinical study with sensitivity and positive predictive values above 90% and with reasonable high specificity and negative predictive values (~80%). The main advantage of the ‘double product’ is that this high diagnostic accuracy can be reached without genetic examination of ACE or CHIT1 gene polymorphisms in patients, hereby laboratory diagnosis of sarcoidosis may become faster and more feasible.

After the examination of several biomarkers in sarcoidosis, we have found that the analysis of a single biomarker is not capable of ensuring convincing, correct laboratory diagnosis to clinicians. ‘Double product’ derived from ACE and CTO activities may be a real alternative to biopsy for confirming the diagnosis of patients with pulmonary manifestation of sarcoidosis.

6. MAIN RESULTS AND CONCLUSIONS

1. In our earlier works we described an optimized fluorescent kinetic assay for ACE activity measurement, in which the inhibitory effect of albumin is eliminated. We identified, that albumin-mediated inhibition on serum ACE becomes negligible at 35-fold or higher dilution of sera.
2. We determined other interfering factors (hemoglobin and bilirubin) as well. In order to avoid the interfering effect of bilirubin and hemoglobin, further dilution of the sera is recommended above 0,71 g/l hemoglobin concentration or 150 μM bilirubin concentration.
3. Genotype-independent and *I/D* polymorphism-dependent reference intervals for ACE were established.
4. Comparison of diagnostic accuracy of the fluorescent kinetic assay presented here to another commercial ACE activity kit revealed a slight superiority of our fluorescent kinetic assay.
5. Comparing the diagnostic accuracy of three different ACE activity measuring techniques, in ROC analyses, we did not find significant differences among these methods
6. .We examined thrombocyte:lymphocyte ratio, sIL-2R, SAA, lyzozime and CTO as potential serum biomarkers in the diagnostics of sarcoidosis, however, they did not seem to be good single serum biomarkers for diagnosing pulmonary involvement of sarcoidosis.
7. We combined the optimized fluorescent ACE activity test with the highly sensitive CTO activity test and derived a new parameter (called ‘double product’) by the multiplication of these values. This ‘double product’ had the highest diagnostic accuracy in our clinical study with sensitivity and positive predictive values above 90% and with reasonable high specificity and negative predictive values (~80%).

After the examination of several biomarkers in sarcoidosis, we have found that the analysis of a single biomarker is not capable of ensuring convincing, correct laboratory diagnosis to clinicians. ‘Double product’ derived from ACE and CTO activities may be a real alternative to biopsy for confirming the diagnosis of patients with pulmonary manifestation of sarcoidosis.

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List of publications related to the dissertation

1. **Enyedi, A.**, Csongrádi, A., Altorjay, I., Beke, G. L., Váradi, C., Enyedi, E. E., Kiss, D. R., Bányai, E., Kalina, E., Kappelmayer, J., Tóth, A., Papp, Z., Takács, I., Fagyas, M.: Combined application of angiotensin converting enzyme and chitotriosidase analysis improves the laboratory diagnosis of sarcoidosis.
   Clin. Chim. Acta. 500, 155-162, 2020.
   DOI: http://dx.doi.org/10.1016/j.cca.2019.10.010
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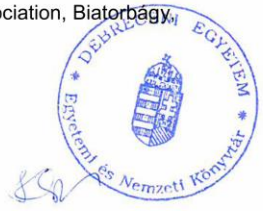
3. **Enyedi, A.**: Treatment of sternoclavicular joint inflammation with NPWT.
   In: Theoretical knowledge and practical applications negative pressure wound therapy. Ed.: Rashed Aref, et.al, Negative pressure therapy for wound healing association, Biatorbágy, 130-135, 2019.



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