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

Optimized application of angiotensin-converting enzyme and chitotriosidase activity in the diagnosis of sarcoidosis

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The Examination will be held at the Department of Internal Medicine Building B, Faculty of Medicine, University of Debrecen, at 11 a.m. on 17th October, 2022.

Head of the **Defense Committee**:	Csongor Kiss, PhD, DSc
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The PhD Defense will be held at the library of Institute of Cardiology, Faculty of Medicine, University of Debrecen, at 2 p.m. on 17th October, 2022.

1. Introduction and literature review

Sarcoidosis is a granulomatous disease of unknown origin affecting several organ systems, but most commonly the lungs which occurs worldwide regardless of age, sex and ethnicity. Nearly 50% of affected individuals are asymptomatic and often a bilateral lymph node enlargement on chest X-ray is a clue to its detection during routine examination. A number of clinical investigations, including invasive tissue sampling, may be necessary to make an accurate diagnosis, which can be a significant burden, especially for patients who do not have granulomas that are easily and safely accessible for sampling.

Although many laboratory tests have been developed since its discovery, a non-invasive, sarcoidosis-specific laboratory diagnostic test is still not available. Serum angiotensin-converting enzyme (ACE) activity is one of the most commonly defined biomarkers, but the specificity and sensitivity of the different tests vary widely. ACE is a zinc-dependent metalloproteinase enzyme, a key enzyme of the renin-angiotensin-aldosterone system (RAAS). It catalyses the conversion of angiotensin I to active angiotensin II, thereby playing a prominent role in the regulation of arterial blood pressure and the salt and water balance of the body. Its activity is significantly affected by the inversion/deletion (I/D) polymorphism of the ACE gene. In sarcoidosis, ACE is produced by granuloma epithelioid cells, so its activity is thought to be proportional to the number of granulomas present in the body.

Chitotriosidase (CTO) is a hydrolase involved in the breakdown of chitin, and its activity increases in sarcoidosis in proportion to ACE. However, the activity of CTO is strongly influenced by polymorphisms in the chitotriosidase gene (CHIT1).

ACE and CTO are two potential biomarkers in the diagnosis of sarcoidosis. Our research has focused on optimising the determination of ACE activity and the identification of polymorphisms in the ACE and CHIT1 genes, which may lead to the development of a laboratory method for the diagnosis of sarcoidosis from blood.

1.1. Sarcoidosis in general

Sarcoidosis is a multisystemic inflammatory disease characterised by non-necrotizing granulomas throughout the body, but mainly in the lungs. Its history dates back to the 19th century, when the English surgeon-dermatologist Jonathan Hutchinson identified the first case in London in 1877. Despite its early discovery, the aetiology of sarcoidosis remains unknown to this day. Its various presentations and multisystemic nature make it very difficult to unravel.

It is hypothesised that, in addition to increased genetic predisposition, certain environmental factors and exposure to antigens, as well as the activation of autoimmune processes may contribute to the development of sarcoidosis.

Describing the epidemiological data on sarcoidosis is also a challenge due to the uncertainty of the number of asymptomatic patients worldwide. Its prevalence varies considerably between geographical areas and ethnic groups, with an incidence of 1-64/100 000 cases per year. The incidence is highest in African-Americans, particularly among women, and in Northern European countries, moderate in Caucasian populations and lowest in Asian countries and Hispanic areas. The prevalence of sarcoidosis also varies by age and sex. It most commonly affects young adults and is predominantly female. More than 80% of cases are between 20 and 50 years of age, but there is a second peak in the incidence of sarcoidosis between 50 and 65 years.

1.2. Manifestations of sarcoidosis

The clinical presentation of sarcoidosis is highly variable depending on the different organs involved. Lung involvement is observed in 90% of patients with sarcoidosis. Common respiratory symptoms include dry cough, dyspnoea and chest discomfort. The most common presentations are bilateral hilar lymphadenopathy and pulmonary infiltration, usually accompanied by a skin lesion or lesion in the eye. Based on the abnormalities on chest radiographs, four stages of pulmonary sarcoidosis are distinguished, the most severe of which is fibrotic remodelling of the tissue.

Pulmonary involvement is not exclusive, extrapulmonary manifestations (10-30%) may occur before, after or even at the same time as the development of lung lesions. Granuloma formation can occur anywhere in the body, with ocular involvement being the second most common (13-79%) after pulmonary involvement, leading to visual impairment or even loss as a result of uveitis and retinitis. The most common non-specific skin lesion (20-35%) is erythema nodosum and lupus pernio that is common on the face, ears and joints. Granulomas in the liver (10-30%) may cause alteration of liver function, enlargement of the liver and, rarely, portal hypertension. Cardiac sarcoidosis (20-27%) can cause serious complications, which can be fatal. Musculoskeletal symptoms (10-25%) are usually joint pain and inflammation, with difficulty in movement. The nervous system is relatively rarely affected, in 10% of cases, and its presence can cause major damage, particularly to the central nervous system. Sarcoidosis

affects the kidneys less frequently (5%). Hypercalcaemia and hypercalciuria caused by altered calcium homeostasis may lead to nephrocalcinosis and occasionally renal failure.

The course of sarcoidosis can be acute, subacute or chronic. For most patients, sarcoidosis is a benign granulomatous disease, but in some cases it can develop into a long-lasting, life-threatening condition. The prognosis of sarcoidosis is greatly influenced by the stage of the disease, its clinical manifestations and the extrapulmonary involvement present. Prognosis also depends on the patient's sex, age and ethnicity. The prognosis for patients with Löfgren's syndrome is markedly favourable, with 80% of patients recovering spontaneously within a few months. Mortality in sarcoidosis is estimated at 1-5%, usually leading to death due to respiratory failure caused by pulmonary fibrosis and cardiac or nervous system involvement.

1.3. Therapy for sarcoidosis

There is currently no specific therapy available for sarcoidosis, although with proper medication the process of pathological granuloma formation can be slowed down and the onset of symptoms can be reduced. Continuous monitoring of the condition of patients with sarcoidosis is of paramount importance, since, in addition to a worsening prognosis, there is also the possibility of spontaneous remission, in which case the patient's condition does not require further drug therapy. First-line treatment is the use of corticosteroids, usually prednisone, which reduces the production of cytokines responsible for persistent granuloma formation, including TNFα and INFγ. Corticosteroids have been shown to be reliable in relieving symptoms and reversing organ dysfunction, but their use is always a high-risk. Intolerable side-effects (e.g. adrenocortical insufficiency, diabetes mellitus, hypertension) and the development of corticosteroid refractory state justify the use of second-line therapies.

Examples of such drugs include antimalarial agents, methotrexate or azathioprine, which are less effective than steroid therapy and are often used as adjunctive agents or in combination. Other agents may also be used, such as TNFα antagonists like infliximab, adalimumab, rituximab, but also mycophenolate mofetil and leflunomide have been shown to be effective in the treatment of sarcoidosis. In patients in chronic stage or in the presence of severe organ failure, transplantation may be the final therapy.

1.4. Diagnosis of sarcoidosis

The early and accurate diagnosis of sarcoidosis is a challenge for clinicians due to the variety of symptoms affecting multiple organ systems and the possible absence of symptoms.

In 50% of cases, the possibility of sarcoidosis is raised during the period of non-complacency by abnormalities on chest X-ray during routine examination. A further difficulty is the lack of a specific method to make a definitive diagnosis of sarcoidosis. As a consequence, three criteria must be fulfilled for a diagnosis to be made: (1) in addition to clear clinical and radiological signs, (2) confirmation of the presence of non-necrotizing granulomas by histopathological examination, and (3) exclusion of alternative diseases.

Due to the significant lung and thoracic lymph node involvement, a chest radiograph is a key radiological finding. High-resolution computed tomography (HRCT) may be helpful in the assessment of parenchymal and mediastinal involvement, and magnetic resonance imaging (MRI) may be necessary to detect extrapulmonary manifestations.

Because of the high chance of spontaneous remission, an exception to tissue sampling may be made for asymptomatic bilateral hilar lymphadenopathy (stage I pulmonary sarcoidosis) and for sarcoidosis-specific syndromes that can be diagnosed by clinical presentation. Sampling is mainly performed from the most easily accessible lesions, e.g. subcutaneous lesions or enlarged lymph nodes. If readily accessible granulomas are not available, examination of the lungs and thoracic lymph nodes is necessary. Tissue specimens suitable for hilar and mediastinal lymph nodes can be obtained during thoracoscopic and mediastinoscopic procedures, which are now increasingly being replaced by minimally invasive endobronchial ultrasound (EBUS) procedures. Bronchoscopic lymph node biopsy can also be performed without EBUS during carinapunctio.

Histochemical analysis of biopsy tissue samples revealed a well-defined, compact epithelioid cellular granuloma characteristic of sarcoidosis, with a concentric arrangement of macrophages, lymphocytes and fibroblasts involved in the inflammatory processes. Macrophages may differentiate into epithelioid cells or fuse into multinucleated giant cells, which together with $CD4^+$ T lymphocytes form the central part of the granuloma. Giant cells often contain inclusion bodies, such as Schaumann or asteroid bodies, in their cytoplasm. The peripheral part is characterised by the presence of $CD8^+$ T lymphocytes and fibroblasts, from which a fibrotic lesion of the granuloma may sometimes be initiated, leading to complete remodelling of the granuloma towards the central part.

An essential part of the diagnosis of sarcoidosis is the exclusion of alternative diseases. Most important is the differentiation between infectious diseases and malignant lesions. By culture and specific microbiological tests, infections caused by pathogens, such as tuberculosis

caused by mycobacteria, or in the case of fungal infections, e.g. cryptococcosis, histoplasmosis, can be excluded. In these cases, the presence of necrotising granulomas may be observed in biopsy specimens. In contrast, 4.4% of carcinomas in regional lymph node specimens show sarcoid-type granulomas, so that the differentiation of lymphoma and other malignant lesions from sarcoidosis requires careful consideration. Further differentiation may also be necessary for pulmonary dust-breathing diseases (e.g. berylliosis, silicosis), immunodeficiency (e.g. chronic granulomatous disease) and inflammation-induced (e.g. eosinophilic granulomatous disease) diseases. Hence, the diagnosis of sarcoidosis is a difficult and complex task, which requires thorough expert judgement and relevant medical investigations in addition to knowledge of clinical signs and patient history.

1.5. Potential biomarkers in the diagnosis of sarcoidosis

Today, there is an increasing demand for reproductively determinable biomarkers for the diagnosis of sarcoidosis that can be obtained by less invasive sampling. However, the discovery of sarcoidosis-specific biomarkers is problematic for several reasons. As a result of the ambiguous aetiology, the pathomechanism of sarcoidosis is not well understood, which makes the characterization of an appropriate biomarker considerably more difficult. In addition, its multisystemic nature and different phenotypic presentation further limit the identification of a unique biomarker. Nevertheless, there are a number of potential biomarkers that can help in the diagnosis of sarcoidosis and may also be useful for monitoring the activity of sarcoidosis, following the effectiveness of therapy and may be useful in prognosis. These biomarkers are mostly proteins, cytokines, chemokines involved in the processes of granuloma formation, released mainly during inflammatory reactions triggered by the activation of $CD4^+$ T cells and macrophages. Some of them are serological markers that can be determined from blood, such as soluble interleukin 2 receptor (sIL-2R), serum amyloid A (SAA), lysozyme, chitotriosidase or angiotensin-converting enzyme, which is the only marker that has been introduced into clinical practice.

1.5.1. Angiotensin-converting enzyme

Increased activity of angiotensin-converting enzyme (ACE) in sarcoidosis was described as early as 1975. ACE is a zinc-dependent metalloproteinase enzyme, a key component of the renin-angiotensin-aldosterone system (RAAS). Its main function is to convert the inactive vasopressor angiotensin I to angiotensin II and to inactivate the vasodilator bradykinin, thereby playing an important role in the regulation of blood pressure and the maintenance of salt and

water balance in the body. Two isoenzymes of ACE are known, encoded by a single gene on the long arm of chromosome 17 (17q23). It is ~21 kb in size, containing 26 exons and 25 introns. The larger isoform, somatic ACE, is a ~140 kDa protein consisting of two active centres (C- and N-terminal), a C-terminal transmembrane region and an intracellular segment. The two active centres are presumably the result of gene duplication. Both catalytic domains have the amino acid motif HEMGH, where the two histidines coordinate the zinc ion. Although their sequences are largely identical, their chloride activation profiles, substrate specificity, inhibitors and physiological functions are also different. ACE belongs to the group of type I transmembrane proteins. ACE secretase cleaves the extracellular part of the transmembrane region, resulting in the release of the active enzyme into the circulation. Somatic ACE is expressed in many tissues, including endothelial cells of blood vessels, lungs, kidneys, small intestine, heart, and is also expressed by some cells of the immune system, macrophages and dendritic cells. The smaller isoenzyme is the testicular or germinal ACE ~70 kDa in size. It has only one active site, corresponding to the C-terminal catalytic domain of soluble ACE. It is expressed exclusively in the testis and its dysfunction leads to reduced fertility, thus it plays a crucial role in reproduction.

The epithelioid cells of the sarcoid granuloma also produce ACE, the activity of which is thought to be proportional to the number of granulomas present in the body. However, the use of ACE as a diagnostic marker is still a controversial issue. ACE activity is elevated in 30-80% of patients with sarcoidosis, with test sensitivity ranging from 22-86% and specificity from 54-95% based on previous studies. Several reasons are known for such variations in test sensitivity and specificity. Although some of the variation may be due to differences in methods, ACE activity is also genetically determined. The inversion/deletion (I/D) polymorphism of the ACE gene affects the ACE activity measured in serum, which can account for up to 50% of the difference in activity between individuals. The presence (I allele) or absence (D allele) of a 287 bp non-coding repetitive sequence wedged in intron 16 significantly determines ACE activity. The I allele is associated with low ACE activity, whereas the D allele is associated with high ACE activity, resulting in the lowest ACE activity in genotype II, the highest in genotype DD, and an intermediate ACE activity in genotype ID. The difference between genotypes II and DD can be up to twofold. The prevalence of I and D alleles varies in different ethnic groups. In Asian populations, the prevalence of the I allele is significantly higher than in Europeans, therefore it is useful to define genotype-dependent reference ranges of ACE activity for the diagnosis of sarcoidosis in this population. Furthermore, it is important

to note that serum ACE activity is significantly reduced during ACE inhibitor therapy, and consequently, when used as a diagnostic test, may give false negative results. However, ACE is also under endogenous inhibition, which may also affect circulating enzyme activity. Serum albumin has a significant inhibitory effect (reference range: 35-52 g/L), with a half-maximal inhibitory concentration (IC_{50} value) of between 5.7 and 9.5 g/L. The low sensitivity may be partly due to the fact that ACE activity may be elevated in other inflammatory diseases such as interstitial lung diseases, tuberculosis, berylliosis, Gaucher's disease and other granulomatous diseases.

1.5.2. Chitotriosidase

Chitotriosidase (CTO) is a member of the 18-member glycosyl hydrolase (chitinases) family, which is involved in the breakdown of chitin and chitin-like substances. The human body lacks an endogenous chitin substrate, therefore the exact physiological functions of CTO are currently unclear. Through its phagocyte-specific expression, it is thought to be involved in innate immune processes, where it possibly has a role in defence against chitin-containing pathogens (fungi, arthropods, crustaceans, nematodes).

The gene coding for CTO (CHIT1) is located on the long arm of chromosome 1 at positions 1q31-q32, which is ~20 kb in size and contains 12 exons. The enzyme occurs in two isoforms. The 50 kDa enzyme is composed of a catalytic domain, a C-terminal chitin-binding region and a connecting section. Exon 11 can be excised during alternative splicing to generate the smaller 39 kDa isoform, which lacks the C-terminal region but is sufficient for enzymatic activity.

The polymorphism of the CHIT1 gene significantly affects the activity of the protein. One of its most common mutations affects exon 10, where a 24 bp duplication (Dup24) activates the cryptic splicing site at the 3' end, causing deletion of an 87 bp region of the transcribed mRNA without a frame shift. 35% of the population is heterozygous for the Dup24 mutation, and a further 6% are homozygous mutants in which CTO activity is completely absent.

Neutrophil granulocytes and macrophages of pulmonary origin produce CTO in response to TNFα, IFNγ and GM-CSF (granulocyte-macrophage colony-stimulating factor) stimulation, which is proportional to serum ACE activity. Highest CTO activity was measured in cases with progressive sarcoidosis, which was significantly reduced by immunosuppressive therapy. Although CTO activity may be elevated in other diseases such as Gaucher disease,

tuberculosis, malaria, and Alzheimer's disease, CTO activity may be a potential biomarker for the diagnosis, monitoring and prognosis of sarcoidosis.

2. Aims

During our experimental work our main goal was to develop a method to accurately and reliably diagnose sarcoidosis without biopsy and histopathology. To this end, our experiments aimed to:

1. optimisation of the fluorescent kinetic ACE activity assay developed by our research team
    - in order to eliminate the endogenous inhibitory effect of albumin.
    - We are going to investigate the effects of the interfering factors, haemolysis, icterus and lipemia, which are the most common interfering factors in laboratory determinations, on ACE activity.
    - We are going to determine the reference ranges of genotype-dependent and independent ACE activity for the I/D polymorphism.
2. In the second part of our experiments, we aimed to investigate the effects of the Dup24 polymorphism on circulating CTO activity and concentration in healthy and sarcoid individuals.
3. We also attempt to identify additional mutations that may also affect CTO activity or concentration.

3. Materials and methods

3.1. Subjects

Two hundred and one Hungarian adults were enrolled from the outpatients clinic of the Department of Cardiology, University of Debrecen or from employees of the department to conduct the experiments and to determine the normal reference ranges. Treated primary hypertension was not an exclusion criterion. The individuals included did not show any symptoms of sarcoidosis. None of the participants were receiving ACE inhibitor or steroid treatment.

Eighty sarcoidosis patients were also included in our studies. These patients underwent thoracic surgery for mediastinal lymph node or lung tissue sampling at the Department of

Surgery, University of Debrecen. Histological diagnosis confirmed the presence of sarcoidosis. Patients treated by steroid or ACE inhibitor were not eligible for the study.

All patients gave written informed consent to participate in the trials.

3.2. Serum and DNA isolation

Blood samples were collected in a routine blood collection procedure under sterile conditions. The serum was separated from the cellular elements by centrifugation at 1500 g for 15 min at 4 ºC after blood clotting. The resulting serum was stored at -20 ºC until measurements were performed.

The degree of haemolysis, icterus and lipemia as parameters indicating the quality of serum samples were characterised by HIL indices. If haemolysis (H index > 50), icterus (I index > 50) or lipemia (L index > 200) were detected in patient samples to an extent that affected the results of the experiments, the sampling was repeated.

EDTA anticoagulated whole blood was collected for genetic testing and stored at -20ºC until DNA isolation. Purified DNA was stored at 4 ºC until use.

3.3. Determination of ACE activity by fluorescent kinetic assay

Serum ACE activity was determined by fluorescent kinetic assay with a minor modification of the protocol of Carmona et al. The synthetically synthesized fluorescent substrate (Abz-FRK-(Dnp)-P-OH) was used for the measurement that can be selectively cleaved by ACE. An increase in the intensity of the emitted fluorescent light was detected. The reaction mixture was prepared in 96-well black plates, and the changes in the fluorescent intensity were monitored at 1-min intervals for 30 min at 37 ºC using a Novostar plate reader. During measurements the excitation wavelength was λ= 340 nm and the emission wavelength was λ= 405 nm. After correction for blank, the fluorescence signal intensity values were plotted as a function of time and a line was fitted to the points. The activity rate in U/L was calculated using 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 μM 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 was reported as mean.

3.4. Interference studies

We used hemolysate from sodium citrate-coagulated venous blood of a healthy participant to investigate the effect of hemolysis-induced interference on ACE activity. The whole blood was washed five times with physiological saline and the cell membrane was ruptured during repeated freeze-thawing. Cell debris was removed by centrifugation at 16000 g for 15 min at 4 ºC. The concentration of free haemoglobin in the supernatant was determined by the sodium-lauryl-sulphate-haemoglobin method.

The effect of lipemia on ACE activity was investigated by adding Intralipid to serum samples. Triglyceride concentrations of the resulting samples were determined using a Cobas C6000 automated glycerol-blank corrected method.

Unconjugated bilirubin was added to normal serum samples at various concentrations to prepare icteric samples. The total bilirubin concentration was determined by the azobilirubin method.

The degree of interference was considered significant when the change in ACE activity exceeded 10% of the baseline activity.

3.5. Analysis of genetic polymorphisms of ACE

The ACE gene I/D genotype was determined using the protocol previously described by Rigat et al. The presence of the I allele was confirmed by a second PCR reaction based on the work of Lindpainter et al. PCR products were run on 3% agarose gel and results were visualized using SYBR safe gel stain.

3.6. Serum CTO activity determination

The determination of serum CTO activity was performed with minor modifications as described by Hollak et al. Serum CTO activity was determined using an artificial substrate (4-methylumbelliferyl-β-D-N,N',N''-triacyl-chitotrioside). The measurements were performed in 96-well plates at 37 ºC. The change in fluorescence intensity was monitored for at least 30 min at 1-min intervals using a Novostar plate reader. The excitation wavelength was λ= 340 nm and the emission wavelength was λ= 405 nm. Fluorescence signal intensity values were determined from the slope of the linear line plotted against time. CTO activity was calculated from 4-Methylumbelliferone calibration curve, activity values were expressed in U/L.

3.7. Determination of serum CTO concentration

Serum CTO concentration was determined using two commercially available ELISA kits. The serum samples were diluted with the serum diluent solution available in the reagent kit according to the CTO activity of the sample. The absorbance values of the samples were measured with a Clariostar plate reader and the concentration was calculated from the standard in the kit.

3.8. Determination of the duplication polymorphism of the CHIT1 gene

The Dup24 polymorphism of the CTO gene (CHIT1, chitinase 1, HGNC:1936) was determined according to the protocol published by Livnat et al. PCR products were run on 3% agarose gel and visualized with SYBR safe gel stain.

3.9. Direct DNA sequencing of the CHIT1 gene

The lack of CTO activity in one member of the control group was not explained by the Dup24 polymorphism. Therefore, the nucleotide sequence of the CHIT1 gene exons of the affected individual was determined by Sanger sequencing. The primer pairs used for sequencing were previously published by Grace et al. DNA sequencing of purified PCR products was performed by Microsynth AG.

3.10. Analysis of the cis-trans location of CHIT1 gene mutations

One subject in the control group carries both the duplication (rs3831317) and deletion (rs536102546) mutations in heterozygous form. The cis-trans location of these mutations was investigated by PCR and the DNA nucleotide sequence of the products was subsequently determined by Sanger sequencing. Two pairs of primers were used to cover the DNA sequence between the mutations. The first pair of PCR primers (forward: 5'-AATCCAGGATCAGAAGGGTGGGC-3'; reverse: 5'-CCTTAGCTCCTGCGGGTACAT-3') resulted in a PCR product when the deletion mutation was present at exon 9. DNA segment amplification was performed using a VeritiTM 96-well Thermal Cycler. Samples were placed at 4 ºC until purification of the PCR product. The second pair of PCR primers (forward: 5'-CTCCAGGCTTCCTCAGACAG-3'; reverse: 5'- CCCGCCCAGTCCCTAGACCAT-3') recognized the Dup24 mutation present in exon 10, and thus resulted in a product only if the Dup24 genotype was present. Samples were kept at 4 ºC until purification of the PCR product. Sequencing of purified PCR products was performed by MicroSynth AG.

3.11. Statistical analysis

The equilibrium distribution of the genotype of the study population according to Hardy-Weinberg was examined by χ^2 test. The distribution patterns of the data were tested using Shapiro-Wilk and D'Agostino-Pearson tests. Reference ranges were defined as the mean 95% (percentiles 2.5-97.5) of the reference population values. Differences in ACE activity values between genotype-dependent groups were tested by one-way ANOVA and Tukey's multiple comparison tests. Data with a normal distribution were compared using Welch's unpaired T-test, while data with a non-normal distribution were compared using Mann-Whitney U test. Statistical analysis was performed using GraphPad Prism 7.0 software. Values showing normal distribution are presented as mean (± standard deviation), while data showing non-normal distribution are presented as interquartile range. Differences between two variables were considered significant when $p<0.05$.

3.12. 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). All experiments were conducted in accordance the tenets of the Declaration of Helsinki.

4. Results

4.1. Removal of the albumin-mediated endogenous inhibitory effect

Reversible endogenous ACE inhibition by serum albumin can be eliminated by appropriate dilution of the serum sample. ACE activity of serum samples from individuals with three different ACE I/D genotypes was determined at different serum dilutions. For all three genotypes, ACE activity values increased with increasing serum dilution, reaching a maximum at 35-fold serum dilution. The ACE activity values determined at 35-fold dilution were not statistically different from the activity values measured at 70-fold dilution for any of the genotypes.

4.2. Examination of the effects of haemolysis, icterus and lipemia on ACE activity

We were also curious about the effect of factors that impair the quality of serum samples, such as increased turbidity caused by lipids or reddish and yellowish discolouration caused by free haemoglobin and bilirubin, on the measurement of ACE activity. Sample turbidity did not

affect the determination, ACE activity was constant up to 16 mM triglyceride concentration. In contrast, the yellowness of the serum sample at 64 μM and higher bilirubin concentrations significantly affected the ACE activity value. Bilirubin concentrations higher than 150 μM resulted in a 10% underestimation of ACE activity. A hemoglobin concentration of 0.35 g/L or higher already significantly affected the fluorescence kinetic determination. The presence of 0.71 g/L hemoglobin caused an approximate 10% decrease in ACE activity compared to the actual ACE activity.

4.3. Determination of I/D genotype-dependent and independent reference ranges of ACE activity

In order to establish genotype-dependent and genotype-independent reference ranges of ACE activity, 201 participants were included in our studies. We collected baseline clinical data from enrolled subjects and determined ACE I/D genotype and ACE activity measured at 35-fold dilution.

The genotype distribution of the whole population was in equilibrium according to the Hardy-Weinberg rule. ACE activity values showed normal distribution by both the Shapiro-Wilk test and the D'Agostino-Pearson test. ACE activity was significantly affected by I/D polymorphism, ACE activity in individuals with genotype II was significantly lower than ACE activity in individuals with genotype DD, while activity in individuals with genotype ID was intermediate between the two values (II: 7.30 ± 1.65 U/L; ID: 8.72 ± 1.70 U/L; DD: 10.6 ± 1.70 U/L; $p<0.0001$).

4.4. Effects of the CHIT1 gene Dup24 polymorphism on CTO activity and concentration

In the second part of our experimental work, we investigated the effects of the CHIT gene Dup24 polymorphism on CTO activity and concentration in 80 patients with histopathologically confirmed sarcoidosis and 133 healthy adults. The Dup24 polymorphism of the CHIT1 gene was in Hardy-Weinberg equilibrium in both the sarcoidosis and control groups. The sarcoidosis patients were significantly younger than their healthy counterparts (sarcoidosis group: 42.2 ± 12.2 years, control group: 48.3 ± 15.0 years). There were no significant differences in left ventricular ejection fraction, platelet count, and creatinine concentration between the two groups, suggesting that the control group had no or at most mild cardiovascular disease. The lymphocyte count and lymphocyte ratio in the blood were significantly lower in the sarcoidosis group compared to the control group (sarcoidosis group: 1.49 G/L (1.23-1.83 G/L, control group: 1.89 G/L (1.58-2.45 G/L); sarcoidosis group: 23.9% ±

6.7%), control group: 29.2% (24.4-32.6%)). The platelet/lymphocyte ratio, used as a marker of sarcoidosis, was significantly higher in the sarcoidosis patients (170 (135-221)) than in the control population (130 (113-165)). The forced vital capacity (FVC) and forced expiratory volume (1 sec; FEV1) were significantly higher in sarcoidosis patients carrying the Dup24 duplication heterozygous form than in individuals with the homozygous wild type. In contrast, the Tiffeneau index was significantly higher in patients homozygous for Dup24 duplication than in individuals carrying the wild type. ACE, CTO activity and "double product" were also found to be significantly higher in the sarcoidosis group compared to the control group (ACE activity= sarcoidosis group: 11.84 U/L (10.1-13.5 U/L), control group: 9.19 U/L (7.09-11.29 U/L), CTO activity= sarcoidosis group: 2882 mU/L (1497-4166 mU/L), control group: 539 mU/L (318-884 mU/L), "double product" = sarcoidosis group: 34.19 U^2/L^2 (17.4-51.5 U^2/L^2), control group: 4.86 U^2/L^2 (2.66-7.60 U^2/L^2).

The CHIT1 gene Dup24 polymorphism significantly affected serum CTO activity. The mean value of serum CTO activity in homozygous wild-type individuals (838.1±856 mU/L, n= 81) was 1.8-fold higher in the control group compared to heterozygotes (471.5±367 mU/L, n= 49, $p<0.001$). CTO activity was not detectable in control individuals with homozygous Dup24 genotype (n= 3). We found a similar pattern between wild-type and heterozygous members of the sarcoidosis patient group, but the mean CTO activity was almost five times higher than in the control group (wild-type = 5125±4802 mU/L, n= 48; heterozygous: 2300±2105 mU/L, n= 29). In sarcoidosis patients with homozygous Dup24 genotype, serum CTO activity was also not measurable.

We next investigated how the Dup24 polymorphism affects the amount of CTO in serum. Our experiments were performed with a commercially available ELISA kit (kit 1). Our results showed that the presence of the polymorphism clearly reduced the amount of CTO in both healthy and sarcoidosis populations. The mean value of CTO concentration in the control group was highest in individuals with homozygous wild genotype (18.9±13.0 µg/L, n= 36), in heterozygous individuals we detected intermediate CTO concentration (7.2±1.9 µg/L, n= 11), while in individuals with homozygous Dup24 genotype CTO concentration was not detectable.

A similar pattern was observed in the sarcoidosis patient group, although the mean CTO concentration was significantly higher compared to the control population (wild type = 157.1±132.4 µg/L, n= 47; heterozygous: 63.16±56.5 µg/L, n= 29). No CTO concentration was measured in the serum of sarcoidosis patients with the homozygous Dup24 genotype (n= 2).

Surprisingly, in our experiments we identified a healthy young person who is a carrier of the Dup24 polymorphism, but who has neither detectable serum CTO activity nor measurable serum CTO concentration.

The CTO activity and concentration values determined showed a significant correlation (slope = 0.979 mU/μg; precision of fit: $r^2 = 0.846$; n= 123). This apparently constant specific activity suggests that the duplication polymorphism affects both CTO activity and concentration, therefore the determination of CTO concentration does not provide additional information for the laboratory diagnosis of sarcoidosis. The effect of the duplication polymorphism on CTO concentration was also confirmed using a second commercially available ELISA kit (kit 2). The concentration values determined were consistent with the results of the previous measurement (slope = 0.968; precision of fit: $r^2 = 0.954$, n= 40; $p<0.001$).

4.5. Identification of the CHIT1 gene Del29 mutation in the Hungarian population

In the next part of our experimental work, we focused on a healthy individual who is only a carrier of the Dup24 duplication polymorphism, but no CTO activity was detectable in his serum sample. Determination of the sequence of exon 10 of the CHIT1 gene confirmed the heterozygous form of the Dup24 duplication polymorphism, but still could not explain the absence of CTO activity.

Therefore, DNA sequence analysis was performed for each exon of the CHIT1 gene, resulting in the heterozygous identification of a 29 base pair long deletion mutation in exon 9 (c.(965_993)del; rs536102546; designated as Del29).

The presence of the Del29 mutation results in the excision of a 10 amino acid long section (322-331) resulting an expression of a truncated CTO protein. If the individual carries both mutations as a compound heterozygous form, the results of these genetic studies may explain the lack of CTO activity. The cis-trans orientation of the mutations was determined by PCR reaction followed by Sanger sequencing of the PCR products. First, we designed a PCR reaction that would yield a product only if the exon 9 Del29 mutation was present. In this case, the PCR product contains the complete exon 10, which can be sequenced to the nucleotide sequence. This PCR product was observed in gel electrophoresis only for the test subject, whereas sequencing confirmed the presence of the wild type allele. The presence of the Dup24 polymorphism was also confirmed by a PCR reaction, the product of which contained the complete exon 9. The second PCR yielded a product for Dup24 heterozygous, homozygous control and the tested individual. The sequencing confirmed the presence of the wild allele in

exon 9 of the test individual, thus confirming the trans location of the Del29 and Dup24 mutations.

5. Discussion

ACE activity can be a potential biomarker for the diagnosis and monitoring of sarcoidosis. ACE activity can be determined using radiolabeled substrates, colorimetric and fluorometric methods, but recently a rapid, sensitive fluorescent kinetic assay has been developed that measures ACE activity using a quenched fluorescent substrate (Abz-FRK(Dnp)P-OH).

The albumin-induced endogenous ACE inhibitory effect is reversible, and the degree of inhibition is greatly influenced by the degree of dilution used in the measurement. Thus, by properly diluting the serum sample, the inhibitory effect can be eliminated. In our experiments, we determined that by diluting the serum sample at least 35-fold, the albumin-mediated inhibitory effect on ACE could be almost completely abolished. The 35-fold serum dilution can be applied to all three ACE I/D genotypes, so to be aware of the I/D genotype is not necessary for measuring activity, but may be useful for evaluating results. It is important to know that 35-fold serum dilution is not sufficient to eliminate the activity-reducing effect caused by ACE inhibitor drugs. Before determining the ACE activity in these patients for diagnostic purposes, it is advisable to temporarily discontinue drug therapy or to change the ACE inhibitor drug to an angiotensin II receptor blocker.

Despite the fact that Lieberman and Sastre drew attention to the importance of serum dilution as early as 1986, the manufacturers of diagnostic reagents currently available for the determination of ACE activity recommend dilution of the serum sample 5 or 10 fold. In their experiments, Lieberman and Sastre concluded that a 48 fold dilution of the serum sample is necessary to eliminate the endogenous inhibitory effect. This finding is consistent with our measured results, which show that ACE is still partially inhibited when 24 fold dilution is used, whereas the inhibitory effect appears to be completely abolished when at 35 fold dilution.

In studies on endogenous interference, it has been described that the presence of haemolysis and bilirubin can cause interference in fluorescence kinetic activity measurements. Taking into account the slight inter-individual variability of ACE activity and the fact that in some patients with sarcoidosis ACE activity increases only slightly, the 10% decrease in ACE activity due to interference is considered to be significant. In a previous study, it was described that bilirubin decreases ACE activity when using spectrophotometric method, while it does not affect

radionucleotide-based determination. Bilirubin is able to directly bind to ACE, thereby altering its conformation, and as a consequence may influence the process of ACE cleavage from endothelial cells and perhaps also ACE activity. During haemolysis, large amounts of haemoglobin are released from red blood cells. Haemoglobin shows strong absorbance at wavelengths of 415 nm, 540 nm and 570 nm. During the fluorescent kinetic assay the fluorescence intensity is measured at 405 nm, hence free hemoglobin in hemolyzed serum samples may partially absorb the emitted light, which may result in an apparent decrease in ACE activity. On the other hand, the possibility that haemoglobin has a direct effect on ACE can not be excluded. Lipemia up to a triglyceride concentration of 16 mM does not affect fluorescent ACE activity measurements, so fasting is not required before blood samping. Based on these findings, further dilution of serum samples at hemoglobin concentrations higher than 0.71 g/L and bilirubin concentrations higher than 150 μM is necessary to eliminate the potential effects of these interfering factors in ACE activity measurements.

Based on the data of two hundred and one Hungarian individuals, we determined the genotype-dependent and genotype-independent reference ranges of ACE activity. To the best of our knowledge, reference ranges for ACE activity based on fluorescence method have not been previously published. Current guidelines of the Clinical and Laboratory Standards Institute and the European Federation of Clinical Chemistry and Laboratory Medicine recommend at least 120 individuals to be selected for the reference group. Although this condition is met for the genotype-independent reference group (n = 201), there are less than 120 individuals in all three genotype-dependent groups, representing a limitation of these reference intervals. The I/D genotype distribution of the reference population (II = 19.4%; ID = 44.8%; DD = 72%) was consistent with previously published data in Caucasians, and the mean values of ACE activity were also significantly different between the three genotypes ($p<0.0001$). Our genotype-dependent reference values overlapped between 7.19 U/L (lower limit of the DD genotype) and 11.25 U/L (upper limit of the II genotype). In our opinion, ACE I/D genotyping is only necessary if the ACE activity value exceeds 11.25 U/L. Individuals with higher activity values have a great advantage in genotyping and applying the genotype-dependent reference intervals.

Diagnosing sarcoidosis is often a challenge for clinicians, as histological examinations may be required to support radiological findings. The invasive procedure required to obtain a biopsy is not therapeutic, it is of diagnostic importance only, therefore particularly burdensome for patients. Currently, the American Thoracic Society does not recommend the use of any blood

tests or biomarkers to diagnose sarcoidosis. However, in order to develop such a reliable, blood test, a number of possible biomarkers have been proposed that may contribute to the unequivocal diagnosis of sarcoidosis without surgery.

Although the difference in ACE activity between patients with sarcoidosis and healthy individuals is generally small, determination of serum ACE activity is recommended as a diagnostic test. Accordingly, the mean ACE activity of sarcoidosis patients was only 1.3 times higher than the ACE activity of the control group as determined by the optimized fluorescence kinetic method. Furthermore, it should be noted that circulating ACE activity is also influenced by the I/D polymorphism of the ACE gene. By defining the genotype and using genotype-dependent reference ranges of ACE activity, the accuracy of ACE activity measurement in sarcoidosis can be further improved.

We have previously demonstrated that the combination of serum ACE and CTO activities can result in a so-called "double product" in sarcoidosis has a high diagnostic accuracy, and the sensitivity and positive predictive value of the test is above 90%. Mapping the genetic background of patients can further increase the accuracy.

The I/D polymorphism in the ACE gene does not cause amino acid changes in the expressed ACE protein because the mutation is located in the intronic region of the gene. The presence of the I allele decreases circulating ACE activity, with a corresponding decrease in the amount of ACE protein. However, this is not the case for the Dup24 mutation, which is a relatively common CTO polymorphism. The Dup24 duplication mutation is located in exon 10 of the CHIT1 gene, the presence of which causes premature termination of protein synthesis. As a result, the synthesized CTO protein is truncated by 29 amino acids and becomes completely inactive. During our experiments we examined the hypothesis whether the amount of circulating CTO determined by ELISA increases the accuracy of CTO determinations in sarcoidosis. Two different commercially available ELISA kits were used in which the capture antibody did not bind to the catalytic domain of the CTO protein (the region affected by the polymorphism). None of the ELISAs were able to detect the inactive protein, so determining the circulating CTO concentration instead of its activity did not improve the diagnostic performance of the assay.

The frequency of the Dup24 allele varies significantly between continents as well as between countries. In our experiments, we determined the frequency of the Dup24 allele in the Hungarian population, which was 21% in line with the results of other European countries. The

above data suggest that there is a possibility that CTO-based tests in patients with sarcoidosis carrying the Dup24 allele may give a false negative result. The use of Dup24 genotyping and the use of genotype-dependent reference values of CTO activity may significantly increase the diagnostic efficacy of CTO (in heterozygotes) to better support the diagnosis of sarcoidosis.

A number of rare mutations are known to affect serum CTO activity, however, these mutations cannot be detected by Dup24 genotyping. A complete lack of CTO activity was observed for the p.Gly354Arg mutation, and mutations in p.Gly102Ser, p.Glu74Lys, or p.Ala442Val were also associated with decreased CTO activity.

In our experiments, we identified a healthy young individual carrying the Dup24 mutation in heterozygous form, but no CTO activity could be determined from his serum sample. DNA sequencing revealed that this Hungarian male carries a rare mutation in exon 9 of the CHIT1 gene, which also results in inactive CTO enzyme expression. In this case, we have successfully demonstrated that the Dup24 and Del29 alleles are present in a compound heterozygous form in the subject, resulting in no measurable CTO activity.

Common polymorphisms or rare mutations that reduce or eliminate CTO activity significantly influence the use of CTO as a biomarker in the diagnosis of sarcoidosis. These mutations affect the current methods for determining the amount of CTO and therefore can not be a substitute for measuring CTO activity. Only genotyping of the CHIT1 gene can help to avoid misinterpretation of laboratory results and detect low allele frequency mutations.

New results presented in the doctoral thesis:

- In our experiments, we have shown that the reversible endogenous inhibitory effect mediated by serum albumin can be eliminated by diluting the serum at least 35-fold.
- We have measured that the determination of fluorescence kinetic ACE activity is not affected by the turbidity of the serum sample and that the interfering effect of haemoglobin and bilirubin can be eliminated by dilution of the serum.
- We were the first to determine the ACE I/D genotype dependent and independent reference ranges for the Hungarian population.
- Based on our observations, determination of circulating CTO concentration in individuals carrying the Dup24 mutation of the CHIT1 gene does not improve the diagnostic performance of the test in sarcoidosis.
- We detected the presence of a Del29 mutation in the CHIT1 gene in a Hungarian individual, which has so far only been described in the Cypriot population. This mutation further complicates the use of CTO definitions as biomarkers in the diagnosis of sarcoidosis.

6. Summary

Sarcoidosis is a granulomatous disease of unknown origin that most commonly affects the lungs. In addition to radiological findings, the diagnosis often requires invasive sampling which can be particularly burdensome for patients. Therefore, nowadays there is a growing demand for appropriately sensitive and specific serum biomarkers.

Angiotensin-converting enzyme (ACE) measurement is currently present in clinical practice, the activity of which may be affected by a number of factors, including endogenous serum albumin-induced inhibition, the presence of interfering factors, and the I/D polymorphism of the ACE gene. During our work, we tried to eliminate these confounding factors by optimizing the fluorescent ACE activity measurement we applied. It was determined that a reversible albumin-mediated inhibitory effect on ACE could be abolished by diluting the serum sample at least 35-fold. We also examined the effects of the most common factors that degrade the quality of serum samples. Lipemia up to a triglyceride concentration of 16 mM did not affect the measurement of fluorescent ACE activity. In contrast, hemoglobin concentrations of 0.71 g/L and bilirubin greater than 150 μM resulted in a 10% underestimation of ACE activity. We also determined the genotype-dependent and genotype-independent reference ranges of ACE activity for the Hungarian population, which can further increase the accuracy of ACE activity measurement in sarcoidosis diagnostics.

Chitotriosidase (CTO) is also a promising serum biomarker in the diagnosis of sarcoidosis. We have previously demonstrated that the so-called "double product" derived from the combination of ACE and CTO activity has a high diagnostic accuracy for sarcoidosis, in addition sensitivity and positive predictive value are above 90%. However, CTO activity is significantly influenced by the polymorphism of the CHIT1 gene. The Dup24 mutation results in an inactive CTO protein. In our work, we examined whether measuring the amount of CTO increases the accuracy of CTO determinations in sarcoidosis, which, however, did not improve the diagnostic performance of the test. We also identified the presence of Del29 mutation in the Hungarian population, causing lack of CTO activity that raises further difficulties in its use in the laboratory diagnosis of sarcoidosis.

List of publications

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List of publications related to the dissertation

1. **Csongrádi, A.**, Altorjay, I., Fülöp, G. Á., Enyedi, A., Enyedi, E. E., Hajnal, P., Takács, I., Tóth, A., Papp, Z., Fagyas, M.: Chitotriosidase gene polymorphisms and mutations limit the determination of chitotriosidase expression in sarcoidosis.
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