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Chitotriosidase gene polymorphisms and mutations limit the determination of chitotriosidase expression in sarcoidosis

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ABSTRACT

Serum chitotriosidase (CTO) activity was proposed as a biomarker in sarcoidosis being potentially useful in diagnostics. Nevertheless, a common duplication polymorphism (c.1049_1072dup24, Dup24) of the CTO gene influences CTO activity and thereby compromises its use in sarcoidosis.

Here we aimed to substitute CTO activity with CTO concentration to prevent the confounding effect of Dup24.

CTO activity, concentration and genetic backgrounds were determined in 80 histopathology proven sarcoidosis patients and 133 healthy individuals.

CTO activities were lower in healthy individuals and sarcoidosis patients heterozygous for Dup24 mutation (472 ± 367 mU/L, n = 49; 2300 ± 2105 mU/L, n = 29) than in homozygous wild types (838 ± 856 mU/L, n = 81; 5125 ± 4802 mU/L, n = 48; $p < 0.001$, respectively). Sera of Dup24 homozygous individuals had no CTO activity. CTO concentrations were also lower in healthy individuals and sarcoidosis patients heterozygous for Dup24 mutation (7.2 ± 1.9 μg/L, n = 11; 63.16 ± 56.5 μg/L, n = 29) than in homozygous wild types (18.9 ± 13.0 μg/L, n = 36; 157.1 ± 132.4 μg/L, n = 47, $p < 0.001$, respectively) suggestive for an interaction between Dup24 mutation and CTO concentration determinations. We also identified a healthy Hungarian male subject without CTO activity carrying a rare mutation (c.(965_993)del), which mutation has been considered unique for Cypriot population to date.

Taken together, CTO concentration determination does not add to the CTO activity measurement when CTO is used as a biomarker in sarcoidosis. Therefore, genotyping of CTO gene should be involved in the interpretation of laboratory findings.

1. Background

Chitotriosidase (CTO, EC 3.2.1.14) enzyme has chitinase activity and it is involved in the defense against chitin-containing organisms such as fungi, nematodes and insects in the human body [1]. This enzyme is primarily produced by macrophages in peripheral tissues and in central organs and by microglia in the central nervous system [2]. It has been observed, that circulating CTO activity is elevated in some acquired and inherited diseases, thereby it can be a useful biomarker for diagnosing and monitoring these disorders, such as malaria [3], Gaucher disease [4] or sarcoidosis [5,6].

Sarcoidosis is a multisystem granulomatous disease which affects individuals worldwide of all ages irrespective of ethnicity or race [7]. It is often recognized accidentally when a routine chest radiographic picture shows bilateral hilar lymphadenopathy. These patients should undergo multiple clinical examinations including invasive tissue sampling and histopathologic evaluation for definite diagnosis [8]. Albeit several laboratory tests have been developed since the first description of sarcoidosis in 1877 [9], a disease-specific non-invasive diagnostic assay is still in demand. Serum angiotensin-converting enzyme (ACE) activity

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is one of the most frequently measured serum biomarkers having high specificity but low sensitivity for sarcoidosis [10]. Recently we have demonstrated, that the combination of ACE with CTO activity (expressed as a 'double product') significantly improves the diagnostic accuracy since sensitivity of this combination exceeds 90% for the diagnosis of sarcoidosis [11].

A major limitation for CTO activity as a biomarker relates to point mutations and polymorphisms of its coding gene, which influence CTO activity. One of the most frequent polymorphism is caused by a 24-base-pair duplication in exon 10 of the CTO gene (c.1049_1072dup24; rs3831317; referred to as Dup24). The presence of this mutation results in the activation of a cryptic 3′ splice site leading to an in-frame deletion of 87 nucleotides [12,13]. Consequently, the synthesized enzyme is inactive. The impact of Dup24 polymorphism on CTO concentration has not been clarified in sarcoidosis patients yet.

It is an important question, because sarcoidosis patients with Dup24 polymorphism may provide false negative values upon CTO activity measurements. This can be circumvented by measuring circulating CTO concentration (instead of its activity). Alternatively, the enzyme activity based methods need to be complemented by CTO gene genotyping.

Here we determined the effects of Dup24 polymorphism on circulating CTO activity and concentration in healthy individuals and sarcoidosis patients. Furthermore, we attempted to identify additional mutations of the CTO gene with influence on CTO expression and activity in relation to the diagnostics of sarcoidosis.

2. Methods

2.1. Subjects

Eighty sarcoidosis patients were involved in our investigations. Diagnosis of sarcoidosis was verified by histopathologic examination of mediastinal lymph node or lung biopsy samples. Patients under steroid therapy were excluded from the study. All patients gave a written informed consent.

One hundred thirty-three adults were enrolled as controls having no signs of heart failure (normal ejection fraction) from the outpatient clinic of the Department of Cardiology, University of Debrecen, and from the employees of the department. According to self-assessment, these individuals declared themselves without symptoms of sarcoidosis. None of the participants was treated with steroid or any sarcoidosis medications, and all participants gave a written informed consent.

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, 1500 *g* centrifugation at + 25 °C. Sera were stored at − 20 °C until measurements. In case of sarcoidosis patients, blood samples were taken immediately before biopsy. 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.

2.3. Measurement of serum CTO activity

Serum CTO activity was measured as previously described [11]. Serum CTO activity was expressed as mU/L.

2.4. Measurement of serum CTO concentration

Serum CTO concentration was measured by two commercially available ELISA kits (Kit No. 1: Cat. No: RAB1364, Merck KGaA, Darmstadt, Germany, Kit No. 2: Cat. No: EH105RB, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's information. Serum samples were diluted with sample diluent (kit component) according to the sample's CTO activity as follows: CTO activity 0–1000 mU/L = 80-fold dilution; 1001–2000 mU/L = 160-fold dilution; 2001–3000 mU/L = 240-fold dilution; 3001–4000 mU/L = 320-fold dilution; 4001–6000 mU/L = 400-fold dilution; 6001 – 10000 mU/L = 600-fold dilution; >10000 mU/L = 1000-fold dilution. Absorbance values of wells were measured on a microplate reader (ClarioStar, BMG Labtech GmbH, Ortenberg, Germany).

2.5. Determination of CTO gene duplication polymorphism

Dup24 polymorphism of *CTO gene* (*CHIT1, chitinase 1, HGNC:1936*) was determined according to the protocol described by Livnat et al [14]. Amplicons of the reaction were separated and evaluated using a 3% agarose gel, and DNA was stained with SYBR safe gel stain (Cat. No: S33102, Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Direct DNA sequencing of CTO gene

In one case of control population, lack of CTO activity could not be explained by Dup24 polymorphism. Therefore, the nucleotide sequence of all exons of *CTO gene* were determined with Sanger sequencing. The exons of *CTO gene* were amplified using primer pairs described by Grace et al. [15]. Purified PCR products (MinElute PCR purification kit, Cat. No: 28006, Qiagen GmbH, Germany) were sent to Microsynth AG (Balgach, Switzerland), where the DNA sequencing was performed.

2.7. Examination of cis–trans configuration of CTO gene mutations

A subject carried both duplication (rs3831317) and deletion mutations (rs536102546, designated as Del29) in a heterozygous form. The cis–trans position of these mutations was analyzed with PCR reaction followed by Sanger sequencing of PCR products. We used two PCR primer pairs, which PCR products cover DNA sequence between these mutations. One of the PCR primer pairs (forward: 5′-AATCCAGGATCAGAAGGGTGGGC-3′; reverse: 5′-CCTTAGCTCCTGCGGGTACAT-3′) recognizes the deletion mutation of exon 9 (rs536102546) and gives PCR product when the deletion genotype is present. The DNA segment was amplified using a Veriti™ 96-well Thermal Cycler instrument (Cat. No: 4375786; Thermo Fisher Scientific, Waltham, MA, USA) according to the following protocol: 95 °C for 5 min followed by 34 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min 40 sec. Samples were kept at 72 °C for 5 min then at 4 °C until PCR product purification. The other PCR primer pairs (forward: 5′-CTCCAGGCTTCCTCAGACAG-3′; reserve: 5′-CCCGCCCAGTCCCTAGAC CAT-3′) recognizes Dup24 mutation of exon 10 and gives PCR product when the Dup24 genotype is present. The PCR protocol was the following: 95 °C for 5 min followed by 30 cycles of 95 °C for 30 sec, 63 °C for 30 sec and 72 °C for 1 min 40 sec. Samples were kept at 72 °C for 5 min then at 4 °C until PCR product purification. Fifty μl reaction mixtures contained 10 μl GoTaq colorless buffer and 0.26 μl GoTaq G2 DNA polymerase (Cat. No: M30001, Promega Corp., WI, USA), 1 μl of 10 mM dNTP solution (Cat. No: 18427013, Thermo Fisher Scientific, Waltham, MA, USA), 5% DMSO, 2–2 μl of 10 μM primers and 1 μl DNA template. Purified PCR products were sent to Microsynth AG, where the sequencing was performed.

Ethical approval

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

2.8. 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. χ^2-test was used to estimate if the genotype distributions were in Hardy-Weinberg equilibrium [16]. Allele distributions in different groups were compared using two-tailed Fisher's exact test. Statistical analysis was performed using Graph-Pad Prism software, version 8.0 (San Diego, CA, USA). P values < 0.05 were considered statistically significant.

3. Results

Eighty biopsy-proven sarcoidosis patients and 133 healthy individuals were enrolled into the study (Table 1.). CTO gene Dup24 polymorphism was in Hardy-Weinberg equilibrium both in sarcoidosis and control groups, allele frequencies were not different between groups (wild allele:duplication allele = 0.794:0.206 and 0.793:0.207, respectively). Patients with sarcoidosis were significantly younger than healthy individuals (42.2 ± 12.2 and 48.3 ± 15.0 years, respectively). We did not observe differences between groups regarding left ventricular ejection fraction, platelet count and creatinine concentration, suggesting that individuals of the control group suffered from mild (if any) cardiovascular diseases. Blood lymphocyte counts and ratios were statistically lower in sarcoidosis patients than in the control population (1.49 G/L (1.23–1.83 G/L) vs. 1.89 G/L (1.58–2.45 G/L); 23.9% (±6.7%) vs. 29.2% (24.4–32.6%), respectively). A sarcoidosis marker, the platelet-to-lymphocyte ratio was significantly higher in sarcoidosis patients (170 (135–221)) than in the control population (130 (113–165)). Forced vital capacity and forced expiratory volume (1 s) were significantly higher in heterozygous Dup24 carrier sarcoidosis patients than in those with homozygous wild type genotype for the same gene. Conversely, Tiffeneau-index was significantly higher in homozygous mutant patients than in those of the homozygous wild type genotype for the same gene. ACE and CTO activities and double products were significantly higher in sarcoidosis patients than in the control population (11.84 U/L (10.1–13.5 U/L) vs. 9.19 U/L (±2.1 U/L); 2882 mU/L (1497–4166 mU/L) vs. 539 mU/L (316–884 mU/L); 34.19 U^2/L^2 (17.4–51.5 U^2/L^2) vs. 4.86 U^2/L^2 (2.66–7.60 U^2/L^2), respectively).

Duplication polymorphism of the *CTO gene* significantly decreased serum CTO activity (Fig. 1). The mean value of serum CTO activity (838.1 ± 856 mU/L, n = 81) in individuals with the homozygous wild type genotype was about 1.8-times higher than that in the heterozygous control group (471.5 ± 367 mU/L, n = 49, $p < 0.001$) (Fig. 1/A). We could not detect CTO activity in samples from individuals with homozygous Dup24 genotype (n = 3). A similar pattern was found in cases of sarcoidosis patients carrying the wild alleles, although mean CTO

Table 1
Characteristics of groups.

	Sarcoidosis patients			Control population		
Chitotriosidase Dup24 genotype	Wild/Wild	Wild/Dup24	Dup24/Dup24	Wild/Wild	Wild/Dup24	Dup24/Dup24
Number, *n*	80			133		
	49	29	2	81	49	3
Gender, Female/Male, *n*	40/39			72/61		
	21/28	17/12	2/0	40/41	30/19	2/1
Age, years	42.2 (±12.2)*			48.3 (±15.0) *		
	41.5 (±11.7)	44.8 (±12.4)	24.5 (±5.7)	45.0 (35.0–57.0)[b]	52.1 (±16.3)[a]	50.7 (±1.5)
Left Ventricular Ejection Fraction, %	60 (±5)			60.0 (56.0–64.0)		
	60 (±5)	61 (±4)	61 (±1)	61.0 (56.0–65.0)	59.5 (±5.9)	51.2 (±10.2)
Platelet count, G/L	247 (217–304)			255 (225–299)		
	254 (218–317)	240 (±51)[c]	337 (±2)	260 (228–304)	252 (221–297)	250 (215–284)
Lymphocyte count, G/L	1.49 (1.23–1.83) *			1.89 (1.58–2.45) *		
	1.58 (1.25–1.95)	1.54 (±0.59)	1.76 (±1.38)	1.87 (1.61–2.12)	1.94 (±0.62)	1.66 (±0.32)
Lymphocyte ratio, %	23.9 (±6.7) *			29.2 (24.4–32.6) *		
	24.5 (±6.7)	22.9 (±6.3)	24.0 (±15.4)	29.7 (24.9–33.5)	27.8 (±5.4)	31.9 (±9.9)
Platelet-to-lymphocyte ratio	170 (135–221) *			130 (113–165) *		
	181 (±63)	174 (±61)	272 (±213)	130 (115–164)	130 (101–174)	156 (114–197)
Creatinine, mM	70.8 (±16.7)			73.0 (59.8–83.3)		
	74.4 (±16.9)[b,c]	66.5 (±14.7)[a,c]	44.0 (±2.8)[a,b]	74.5 (63.5–88.3)	71.0 (59.5–82.2)	62.5 (52.0–73.0)
Radiographic stage of lung disease						
Stage 0, %	2	0	0	100	100	100
Stage 1, %	66	46	50	0	0	0
Stage 2, %	32	54	50	0	0	0
Stage 3, %	0	0	0	0	0	0
FVC, %	97 (±17)			ND		
	94 (81–105)[b]	103 (±13)[a]	88 (±11)	ND	ND	ND
FEV1, %	94 (±18)			ND		
	90 (±18)[b]	102 (±13)[a]	84 (±19)	ND	ND	ND
Tiffeneau-index, %	82 (78–86)			ND		
	80 (77–84)[c]	82 (79–86)	94 (88–100)[a]	ND	ND	ND
ACE activity, U/L	11.84 (10.1–13.5) *			9.19 (±2.1) *		
	11.18 (10.1–13.3)	12.56 (±3.2)	8.89 (8.7–9.1)	8.80 (±2.0)[b]	9.89 (±1.9)[a]	8.20 (±4.1)
ACE genotype, II/ID/DD, %	20/55/25			18/47/35		
	16/64/20	21/45/34	100/0/0	19/53/28	18/37/45	33/34/33
CTO activity, mU/L	2882 (1497–4166) *			539 (316–884) *		
	3448 (2662–5402)[b,c]	1520 (919–2866)[a,c]	10 (LOD)[a,b]	637 (447–1055)[b,c]	413 (173–641)[a,c]	10 (LOD)[a,b]
Double product, U^2/L^2	34.19 (17.4–51.5) *			4.86 (2.66–7.60) *		
	41.77 (28.5–55.1)[b,c]	17.94 (12.2–38.2)[a,c]	0.09 (0.08–0.09)[a,b]	5.70 (3.92–9.00)[b,c]	3.98 (1.80–6.46)[a,c]	0.07 (0.05–0.13)[a,b]

FVC = forced vital capacity, FEV1 = forced expiratory volume (1 sec), ACE = angiotensin converting enzyme, CTO = chitotriosidase, ND = data are not available, LOD = limit of detection. *= significant difference between groups; Significantly different value from: [a] = homozygous wild subgroup; [b] = heterozygous subgroup; [c] = homozygous mutant subgroup

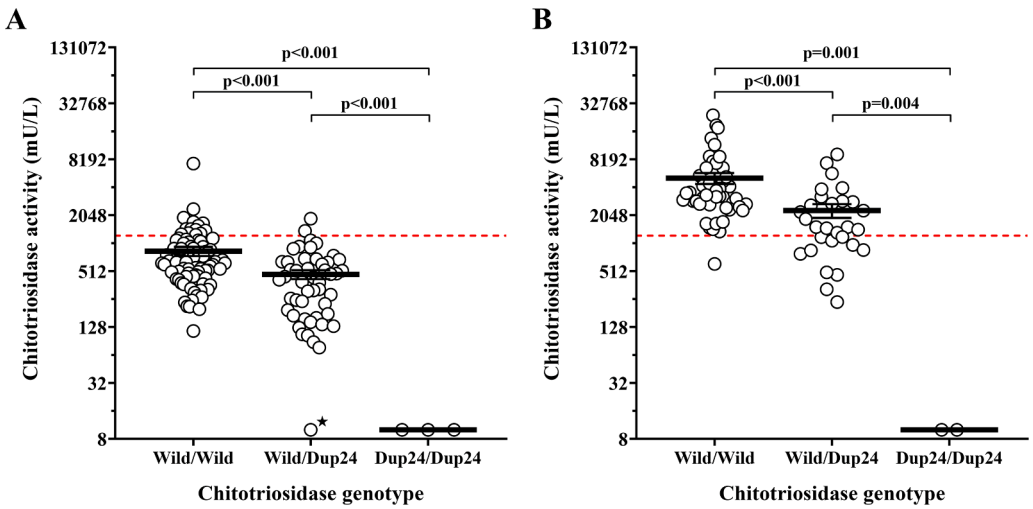


Fig. 1. The Dup24 allele decreases circulating CTO activity in the control population (A) and in sarcoidosis patients (B). Each symbol represents a single individual. Horizontal thick lines indicate means with standard error of means. Dashed lines show the upper reference limit for serum CTO activity (1233 mU/L). The asterisk (*) depicts an individual who carries both the Dup24 and the Del29 CTO alleles.

activity values were approximately 5-times higher in these cases (5125 ± 4802 mU/L, n = 48; 2300 ± 2105 mU/L, n = 29), respectively) (Fig. 1/B). Sarcoidosis patients with homozygous Dup24 genotype (n = 2) had no serum CTO activity in their sera.

Next, we tested the effect of the duplication polymorphism on CTO concentration in sera. Using a commercially available ELISA kit (Kit No. 1), this polymorphism apparently decreased the measured CTO concentrations in both healthy individuals (Fig. 2/A) and sarcoidosis patients (Fig. 2/B). Individuals with homozygous wild type genotype had the highest mean CTO concentration within a subset of the control population (18.9 ± 13.0 μg/L, n = 36), heterozygous subjects had an intermediate mean CTO concentration (7.2 ± 1.9 μg/L, n = 11), while for those with the homozygous Dup24 genotype the CTO concentration could not be detected by the this ELISA kit. A similar pattern was found in sarcoidosis patients, although mean CTO concentrations were significantly higher than in healthy individuals (157.1 ± 132.4 μg/L, n = 47; 63.16 ± 56.5 μg/L, n = 29, respectively). Sarcoidosis patients with homozygous Dup24 genotype had no detectable CTO concentrations in their sera (n = 2). Interestingly, we have identified a healthy individual, who was a carrier for Dup24 polymorphism, but he did not have detectable CTO activity (Fig. 1/A, asterisk) or concentration (Fig. 2/A, asterisk) in his serum.

The measured CTO activity and concentration values showed a remarkable correlation (Fig. 3; slope of linear regression was 0.979 mU/μg; goodness of the fit, $r^2 = 0.846$; n = 123). This apparently constant specific activity suggested that the duplication polymorphism affected both the concentration and activity, and hence the resultant CTO concentrations could not give any extra information to CTO activity values in the evaluation of laboratory tests of sarcoidosis. We confirmed the effects of the duplication polymorphism on CTO concentration by another commercially available ELISA kit (Kit. No. 2) as well. The measured concentration values were in concordance with the previously determined values (Fig. 3/B; slope of linear regression = 0.968; goodness of the fit, $r^2 = 0.954$, n = 40; $p < 0.001$).

Next we set out to determine all exon sequences of the CTO gene in case of the healthy individual, who was a carrier for Dup24 polymorphism but he did not have detectable CTO activity (Fig. 1/A, asterisk). These assays confirmed the presence of Dup24 in heterozygous form (Suppl. Fig. 1). In addition, we identified 29 base pairs deletion in exon 9 of CTO gene in heterozygous form (c.(965_993)del;

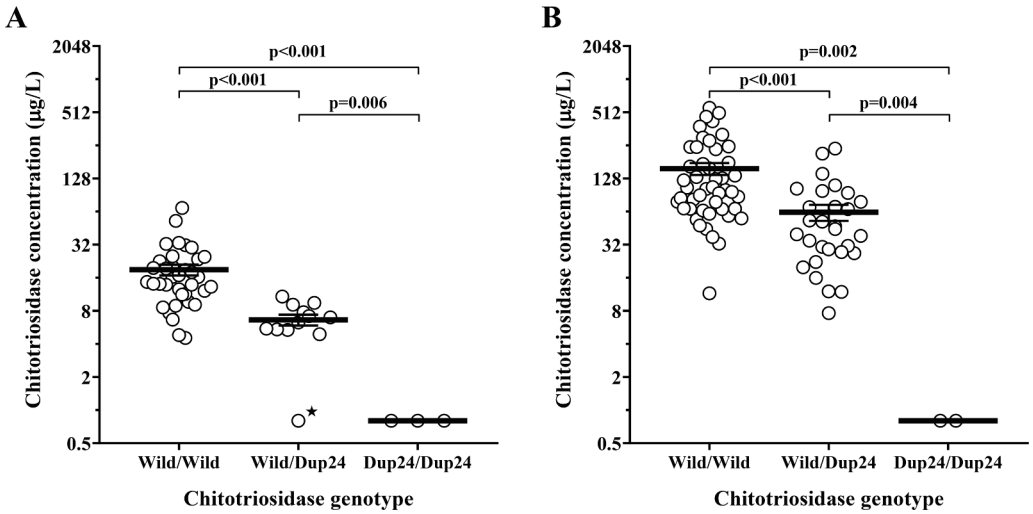


Fig. 2. The Dup24 allele decreases circulating CTO expression in healthy individuals (A) and in sarcoidosis patients (B) determined by a commercially available ELISA kit. Each symbol represents a single individual. Horizontal thick lines indicate means with standard error of means. The asterisk (*) depicts an individual who carries both the Dup24 and the Del29 CTO alleles.

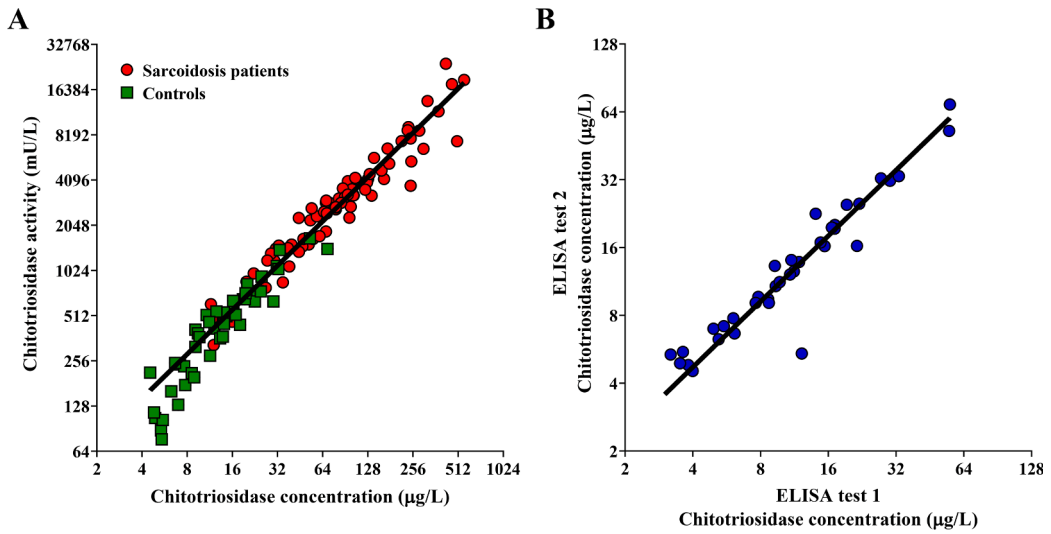
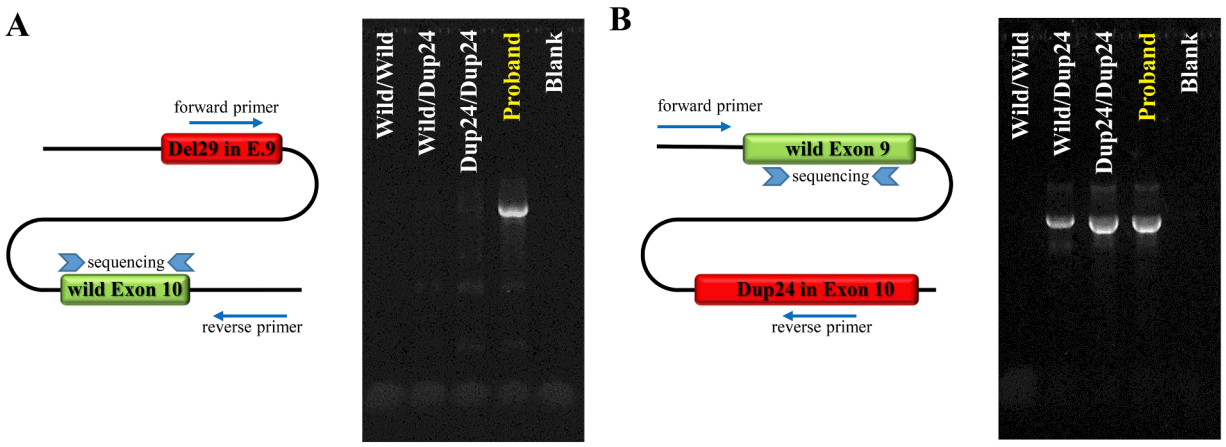


Fig. 3. Chitotriosidase activity is linearly related to chitotriosidase concentration in the serum determined by a commercially available ELISA test (A). Rectangles represent single values for healthy individuals, while circles denote data of sarcoidosis patients. Another commercially available ELISA test resulted similar values in 40 selected serum samples (B).

rs536102546; designated as Del29; Suppl. Fig. 2). This mutation results in the removal of 10 amino acid residues (322–331) from the CTO protein, followed by a frameshift and the generation of a premature stop codon 3 amino acids thereafter [12]. These genetic findings can explain the lack of CTO activity if the proband is compound heterozygous for these mutations. The cis–trans position of these mutations was analyzed by PCR reaction followed by Sanger sequencing of PCR products. First, we set up a PCR reaction, in which the product can only be observed, if the Del29 mutation of exon 9 is present (Fig. 4/A). The PCR product, in this case contained the whole sequence of exon 10, which could be analyzed by sequencing. A PCR product was observed just in case of proband (representative gel, Fig. 4/A), while sequencing confirmed the presence of wild allele in this DNA chain. The second PCR identified the presence of Dup24 polymorphism, and the product contained the whole

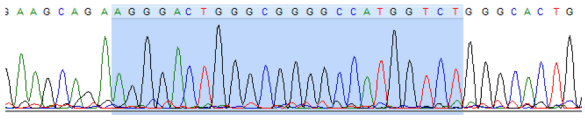


Exon 10

Reference sequence, wild genotype:

GTCAGCTATCTGAAGCAGA**AGGGACTGGGCGGGGCCATGGTCT**GG
GCACTGGACTTAGATGACTTTGCCGGCTTCTCCTGCAACCAGGGCCGA
TACCCCCTCATCCAGACGCTACGGCAGGAACTGA

Electropherogram and sequence of probands exon 10:



Exon 9

Reference sequence, wild genotype:

GTCTGCTCCTGGAAGGGGGCCACCAAACAGAGAATCCAGGATCAGAA
GGTGCCCTACATCTTCCGGGACAACCAGTGGGTGGGCTTTGATGAT
GTGGAGAGCTTCAAAACCAAG

Electropherogram and sequence of probands exon 9:

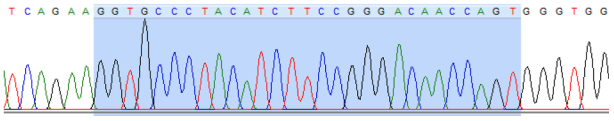


Fig. 4. Trans position of Dup24 and Del29 mutations was confirmed in the proband. Schematic drawings illustrate the hybridization positions of the two sets of primer pairs. Only the proband's DNA sample resulted in a PCR product where a Del29 specific primer pair was used (A, representative gel, product size ~ 1600 bp). PCR products were observed in cases of heterozygous and homozygous controls of Dup24 and in case of the proband when Dup24 specific primer pair was used (B, representative gel, product size ~ 1700 bp). Wild type DNA sequences were found by Sanger sequencing of the PCR products (bottom parts, representative electropherograms).

sequence of exon 9. The second PCR resulted in products in case of Dup24 heterozygous, homozygous controls and the proband, while sequencing confirmed the presence of wild allele in exon 9 of the proband (electropherogram, Fig. 4/B) underlining the trans position of Del29 and Dup24 alleles of the proband.

4. Discussion

Diagnosing sarcoidosis is often challenging for clinicians, as it requires biopsy and histologic analysis for supporting clinical radiology findings [8]. Biopsy is especially burdensome for patients with isolated lung or mediastinal abnormalities, because thoracic surgery is just diagnostic and not curative in these cases. The American Thoracic Society does not recommend any blood test for diagnosing sarcoidosis [17]. In response to this need for a reliable blood test, increasing number of biomarkers are being proposed, which may help to establish sarcoidosis diagnosis without surgery [18].

Serum ACE activity is proposed as a diagnostic test in sarcoidosis, although differences in ACE activities between sarcoidosis patients and healthy individuals is regularly minimal [19,20]. Accordingly, average ACE activity of sarcoidosis patients was only 1.3-fold higher than controls determined with an optimized fluorescent kinetic assay [10]. Of note, circulating ACE activity is influenced by the insertion-deletion polymorphism of the *ACE gene* [21]. Determination of this polymorphism and the application of genotype-dependent ACE activity reference values thus can improve the accuracy of ACE activity measurements in sarcoidosis [10].

We have previously demonstrated, that the combination of serum ACE activity with CTO activity (as 'double product') has high diagnostic accuracy in sarcoidosis with sensitivity and positive predictive values above 90% [11]. Determination of the genetic background of the patients may further improve the accuracy of this 'double product' value.

The existence of insertion/deletion genotype (I/D) in ACE gene does not result in different amino acid sequences in the expressed ACE protein, because I/D polymorphism is located in the intronic region of the ACE gene. Allele I results in decreased circulating ACE activities and ACE concentrations in a proportional manner [21]. This is not the case with Dup24, which is a relatively frequent CTO polymorphism. Dup24 is located in exon 10 of the CTO gene and results in premature termination of translation. Consequently, the synthesized protein lacks 29 amino acids, and it is completely inactive. Here we tested a hypothesis, that detection of the circulating CTO concentration (instead of its activity affected by the polymorphism) by ELISA technique can improve the accuracy of CTO measurements in sarcoidosis. We chose two different commercially available ELISA kits, in which the capture antibody was not directly against the catalytic domain (affected by the polymorphism) of the CTO protein. We found no expression of the inactive CTO with the tested two commercially available ELISA kits. In accordance, measurement of the circulating CTO by these kits did not improved the accuracy of the assay.

It is important to note, that allele frequency of Dup24 shows significant differences among countries and continents. Namely, this polymorphism is completely absent in the Republic of Benin (Africa) [22], present at 20% in Dutch population (Europe) [23], 23% in the USA (North-America) [15], 32% in Brazilian population (South-America) [24], and 58% in Chinese Han population (Asia) [25]. Here we found that dup24 allele frequency is 21% in Hungarian healthy and sarcoidosis population, in accordance with the Dup24 allele frequency in other European countries [23,26]. The above data suggest that patients with sarcoidosis harboring Dup24 allele (representing up to 58%) may be at the risk of having false negative results in CTO based assays. Genotyping of Dup24 and the usage of genotype-dependent CTO reference values (for heterozygous) can improve the accuracy of CTO measurements potentially leading to a blood test supporting the diagnosis of sarcoidosis.

Furthermore, there are several rare mutations, which are also influencing serum CTO activity but cannot be revealed with Dup24 genotyping. Complete absence of CTO activity was observed in the p.Gly354Arg mutation [27], moreover p.Gly102Ser [15,23], p.Glu74Lys [15] or p.Ala442Val [27] were all associated with reduced CTO activities.

Here we present a case for a healthy young male, who did not have serum CTO activity although he was heterozygous for Dup24 polymorphism. DNA sequencing has revealed that this Hungarian male carried a rare mutation in exon 9 of the CTO gene, which also resulted in a CTO protein without enzymatic activity. We verified in this case, that the complete loss of CTO activity was the consequence of a compound heterozygous state of the proband for Dup24 and Del29 alleles. Presence of the Del29 mutation in the Hungarian native population is particularly interesting in light of the fact, that this mutation has been considered unique for Cypriot population to date [12].

5. Conclusion

The presence of frequent polymorphisms and rare mutations that diminish or abolish CTO activity may hamper the use of CTO as a diagnostic tool in sarcoidosis. These mutations have also effects on currently available CTO concentration measuring techniques, thus CTO activity measurement cannot be replaced by them. Only genotyping of the CTO gene is available to avoid misinterpreting laboratory findings today, furthermore it can assist in recognition of mutations with low allele frequencies in a given population.

CRediT authorship contribution statement

Alexandra Csongrádi: Investigation, Methodology. **István T. Altorjay:** Investigation. **Gábor Á. Fülöp:** Resources. **Attila Enyedi:** Resources. **Enikő E. Enyedi:** Investigation. **Péter Hajnal:** Investigation. **István Takács:** Resources. **Attila Tóth:** Funding acquisition, Writing - original draft, Writing - review & editing. **Zoltán Papp:** Writing - original draft. **Miklós Fagyas:** Conceptualization, Funding acquisition, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

We declare that we have no competing interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2020.11.025.

References

[1] S. Arndt, A. Hobbs, I. Sinclaire, A.B. Lane, Chitotriosidase Deficiency: A Mutation Update in an African Population, JIMD Rep. 10 (2013) 11–16, https://doi.org/10.1007/8904_2012_193.

[2] A.M. Varghese, A. Sharma, P. Mishra, K. Vijayalakshmi, H.C. Harsha, T. N. Sathyaprabha, S.M. Bharath, A. Nalini, P.A. Alladi, T.R. Raju, Chitotriosidase - a putative biomarker for sporadic amyotrophic lateral sclerosis, Clin. Proteomics. 10 (2013) 19, https://doi.org/10.1186/1559-0275-10-19.

[3] R. Barone, J. Simporé, L. Malaguarnera, S. Pignatelli, S. Musumeci, Plasma chitotriosidase activity in acute Plasmodium falciparum malaria, Clin. Chim. Acta. 331 (2003) 79–85, https://doi.org/10.1016/s0009-8981(03)00089-5.
[4] C.E. Hollak, S. van Weely, M.H. van Oers, J.M. Aerts, Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease, J. Clin. Invest. 93 (1994) 1288–1292, https://doi.org/10.1172/JCI117084.
[5] R.G. Boot, C.E.M. Hollak, M. Verhoek, C. Alberts, R.E. Jonkers, J.M. Aerts, Plasma chitotriosidase and CCL18 as surrogate markers for granulomatous macrophages in sarcoidosis, Clin. Chim. Acta. 411 (2010) 31–36, https://doi.org/10.1016/j.cca.2009.09.034.
[6] D. Bennett, P. Cameli, N. Lanzarone, L. Carobene, N. Bianchi, A. Fui, L. Rizzi, L. Bergantini, G. Cillis, M. D'Alessandro, M.A. Mazzei, R.M. Refini, P. Sestini, E. Bargagli, P. Rottoli, Chitotriosidase: a biomarker of activity and severity in patients with sarcoidosis, Respir. Res. 21 (2020) 6, https://doi.org/10.1186/s12931-019-1263-z.
[7] Rashi Jain, Dhananjay Yadav, Nidhi Puranik, R. Guleria, J.-O. Jin, Sarcoidosis: Causes, Diagnosis, Clinical Features, and Treatments, J. Clin. Med. 9 (2020) 1081. doi:10.3390/JCM9041081.
[8] U. Costabel, G.W. Hunninghake, ATS/ERS/WASOG statement on sarcoidosis. Sarcoidosis Statement Committee. American Thoracic Society. European Respiratory Society. World Association for Sarcoidosis and Other Granulomatous Disorders, Eur. Respir. J. 14 (1999) 735–737.
[9] Jonathan Hutchinson, Anomalous disease of the skin of the fingers: Case of livid papillary psoriasis, Illus. Clin. Surg. 1 (1877) 42–43.
[10] A. Csongrádi, A. Enyedi, I. Takács, T. Végh, I.S. Mányiné, Z. Pólik, I.T. Altorjay, J. Balla, G. Balla, I. Édes, J. Kappelmayer, A. Tóth, Z. Papp, M. Fagyas, Optimized angiotensin-converting enzyme activity assay for the accurate diagnosis of sarcoidosis, Clin. Chem. Lab. Med. 56 (2018) 1117–1125, https://doi.org/10.1515/cclm-2017-0837.
[11] A. Enyedi, A. Csongrádi, I.T. Altorjay, G.L. Beke, C. Váradi, E.E. Enyedi, D.R. Kiss, E. Bányai, E. Kalina, J. Kappelmayer, A. Tóth, Z. Papp, I. Takács, M. Fagyas, Combined application of angiotensin converting enzyme and chitotriosidase analysis improves the laboratory diagnosis of sarcoidosis, Clin. Chim. Acta. 500 (2020) 155–162, https://doi.org/10.1016/j.cca.2019.10.010.
[12] G. Mavrikiou, P. Petrou, T. Georgiou, A. Drousiotou, Chitotriosidase deficiency in the Cypriot population: Identification of a novel deletion in the CHIT1 gene, Clin. Biochem. 49 (2016) 885–889, https://doi.org/10.1016/j.clinbiochem.2016.03.013.
[13] R.G. Boot, G.H. Renkema, M. Verhock, A. Strijland, J. Bliek, T.M.A.M.O. De Meulemeester, M.M.A.M. Mannens, J.M.F.G. Aerts, The human chitotriosidase gene - Nature of inherited enzyme deficiency, J. Biol. Chem. 273 (1998) 25680–25685. doi:10.1074/jbc.273.40.25680.
[14] G. Livnat, R. Bar-Yoseph, A. Mory, E. Dagan, N. Elias, R. Gershoni, L. Bentur, Duplication in CHIT1 gene and the risk for Aspergillus lung disease in CF patients, Pediatr. Pulmonol. 49 (2014) 21–27, https://doi.org/10.1002/ppul.22749.
[15] M.E. Grace, M. Balwani, I. Nazarenko, A. Prakash-Cheng, R.J. Desnick, Type 1 Gaucher disease: null and hypomorphic novel chitotriosidase mutations-implications for diagnosis and therapeutic monitoring, Hum. Mutat. 28 (2007) 866–873, https://doi.org/10.1002/humu.20524.
[16] S. Rodriguez, T.R. Gaunt, I.N.M. Day, Hardy-Weinberg Equilibrium Testing of Biological Ascertainment for Mendelian Randomization Studies, Am. J. Epidemiol. 169 (2009) 505–514, https://doi.org/10.1093/aje/kwn359.
[17] E.D. Crouser, L.A. Maier, R.P. Baughman, E. Abston, R.C. Bernstein, R. Blankstein, C.A. Bonham, E.S. Chen, D.A. Culver, W. Drake, M. Drent, A.K. Gerke, M. Ghobrial, P. Govender, N. Hamzeh, W.E. James, M.A. Judson, L. Kellermeyer, S. Knight, L. L. Koth, A.S. Morgenthau, K.C. Patterson, V. Poletti, S.V. Raman, M.H. Tukey, G. E. Westney, K.C. Wilson, Diagnosis and Detection of Sarcoidosis An Official American Thoracic Society Clinical Practice Guideline, Am. J. Respir. Crit. Care Med. 201 (2020) E26–E51, https://doi.org/10.1164/RCCM.202002-0251ST.
[18] M.A. Judson, The diagnosis of sarcoidosis, Curr. Opin. Pulm. Med. 25 (5) (2019) 484–496, https://doi.org/10.1097/MCP.0000000000000596.
[19] S. Kahkouee, K. Samadi, A. Alai, A. Abedini, L. Rezaiian, Serum ACE Level in Sarcoidosis Patients with Typical and Atypical HRCT Manifestation, Polish J. Radiol. 81 (2016) 458–461, https://doi.org/10.12659/PJR.897708.
[20] P. Ungprasert, E.M. Carmona, C.S. Crowson, E.L. Matteson, Diagnostic Utility of Angiotensin-Converting Enzyme in Sarcoidosis: A Population-Based Study, Lung. 194 (2016) 91–95, https://doi.org/10.1007/s00408-015-9826-3.
[21] M. Fagyas, K. Úri, I.M. Siket, A. Daragó, J. Boczán, E. Bányai, I. Édes, Z. Papp, A. Tóth, New perspectives in the renin-angiotensin-aldosterone system (RAAS) III: endogenous inhibition of angiotensin converting enzyme (ACE) provides protection against cardiovascular diseases, PLoS One. 9 (2014), e93719, https://doi.org/10.1371/journal.pone.0093719.
[22] L. Malaguarnera, J. Simporè, D. Prodi, A. Angius, A. Sassu, I. Persico, R. Barone, S. Musumeci, A 24-bp duplication in exon 10 of human chitotriosidase gene from the sub-Saharan to the Mediterranean area: role of parasitic diseases and environmental conditions, Genes Immun. 4 (2003) 570–574, https://doi.org/10.1038/SJ.GENE.6364025.
[23] A. Bussink, M. Verhoek, J. Vreede, K.G. der Vlugt, W. Donker-Koopman, R. Sprenger, C. Hollak, J. Aerts, R. Boot, Common G102S polymorphism in chitotriosidase differentially affects activity towards 4-methylumbelliferyl substrates, FEBS J. 276 (19) (2009), https://doi.org/10.1111/J.1742-4658.2009.07259.X.
[24] T. Adelino, G. Martins, A. Gomes, A. Torres, D. Silva, V. Xavier, J. Guimarães, S. Araújo, R. Fernandes, M. Oliveira, A. Godard, E. Valadares, Biochemical and Molecular Chitotriosidase Profiles in Patients with Gaucher Disease Type 1 in Minas Gerais, Brazil: New Mutation in CHIT1 Gene, JIMD Rep. 9 (2013) 85–91, https://doi.org/10.1007/8904_2012_184.
[25] Y.-H. Chien, J.-H. Chen, W.-L. Hwu, Plasma chitotriosidase activity and malaria., Clin. Chim. Acta. 353 (2005) 215; author reply 217. doi:10.1016/j.cccn.2004.11.013.
[26] P. Irún, P. Alfonso, S. Aznarez, P. Giraldo, M. Pocovi, Chitotriosidase variants in patients with Gaucher disease. Implications for diagnosis and therapeutic monitoring, Clin. Biochem. 46 (18) (2013) 1804–1807, https://doi.org/10.1016/J.CLINBIOCHEM.2013.09.006.
[27] P. Lee, J. Waalen, K. Crain, A. Smargon, E. Beutler, Human chitotriosidase polymorphisms G354R and A442V associated with reduced enzyme activity, Blood Cells, Mol. Dis. 39 (3) (2007) 353–360, https://doi.org/10.1016/j.bcmd.2007.06.013.