Lung (2019) 197:295–302
https://doi.org/10.1007/s00408-019-00219-2

INTERSTITIAL LUNG DISEASE

Identification of Active Sarcoidosis Using Chitotriosidase and Angiotensin-Converting Enzyme

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Received: 13 October 2018 / Accepted: 13 March 2019 / Published online: 19 March 2019

Abstract

Purpose Activity/remission differentiation is a great challenge in the follow-up and treatment of sarcoidosis patients. Angiotensin-converting enzyme (ACE) and high sensitivity C-reactive protein (hs-CRP) were proposed as sarcoidosis biomarkers. More recently, chitotriosidase (CHITO) has been described as a better alternative. This study has the aim to evaluate the association of CHITO activity, ACE, hs-CRP or a combination of these biomarkers and to construct a clinical algorithm to differentiate between sarcoidosis activity/remission status.

Methods Forty-six patients with either active sarcoidosis or sarcoidosis in remission and 21 healthy individuals were included. ACE, hs-CRP, and CHITO were evaluated in serum samples. Comparisons of the laboratory variable means among groups were performed by linear models. The cutoff points of the biomarkers for activity/remission differentiation were calculated using the Youden's index. Biomarker cutoff points and decision tree classifier (DTC) performance were estimated by their leave-one-out cross-validation (LOOCV) accuracy (Acc), sensitivity (Se), and specificity (Sp).

Results A 55% mean Se and a 100% mean Sp were found for CHITO, while an 88% Se and a 47% Sp were found for ACE, and a 66% Se and a 68% Sp for hs-CRP cutoff points for activity/remission differentiation. The DTC algorithm with CHITO, hs-CRP, and ACE information had an LOOCV mean Acc of 82%, Se of 78%, and Sp of 89% for sarcoidosis activity/remission differentiation.

Conclusions The algorithm involving CHITO, hs-CRP, and ACE could be a suitable strategy for differentiation between sarcoidosis activity/remission status.

Keywords Sarcoidosis · Activity · Biomarkers · Chitotriosidase · Angiotensin-converting enzyme

Introduction

Sarcoidosis is a multisystem disease, with predominantly pulmonary involvement, no clear etiology, and a variable prevalence throughout the world [1]. Since the 1980s, some markers have been studied to aid the follow-up and treatment of patients and to differentiate between active sarcoidosis and sarcoidosis that is in remission; however, the latter still poses a great challenge [2–5].

Angiotensin-converting enzyme (ACE) has been studied as a biomarker for active sarcoidosis since 1975, but its use is complicated by the fact that other diseases have been also shown to raise the levels of this enzyme [6–9]. The usefulness of ACE for the diagnosis or follow-up of sarcoidosis is controversial, with values of sensitivity and specificity varying between studies [5, 10, 11].

High-sensitivity C-reactive protein (hs-CRP) is a non-specific acute-phase response biomarker. It is produced by hepatocytes and is used to screen for diseases, monitor therapeutic responses, and detect infections in immunocompromised patients [12]. In sarcoidosis, however, hs-CRP has failed to demonstrate a significant change in most of studies,

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only being associated with constitutional symptoms, such as fatigue and with Lofgren's syndrome [13, 14].

The diagnosis of sarcoidosis requires histopathological demonstration of non-caseating granulomas composed of activated macrophages [15, 16]. These macrophages express an enzyme called chitotriosidase (CHITO), which is involved in the pathogenesis of sarcoidosis [17].

Chitotriosidase is an enzyme of the chitinase family. It degrades a polymer known as chitin, which is found in the cell walls of fungi and the exoskeletons of insects and crustaceans. Pulmonary neutrophils and macrophages secrete the enzyme after the stimulation of Toll-like receptors by interferon-γ (IFN-γ), tumor necrosis factor (TNF), and granulocyte/macrophage colony-stimulating factor (GM-CSF) [18]. Its activity is also high in other diseases, such as Gaucher disease, malaria, leishmaniasis, beta thalassemia, multiple sclerosis, atherosclerosis, Alzheimer's disease, and tuberculosis [19, 20]. Chitotriosidase is a biomarker that can be measured in both blood and bronchoalveolar lavage fluid samples [21].

The aim of this study was to evaluate the association of serum CHITO activity, ACE levels, and high-sensitivity C-reactive protein (hs-CRP) levels, or a combination of these putative biomarkers, with active sarcoidosis and to construct an algorithm to better differentiate between active sarcoidosis and sarcoidosis that is in remission.

Methods

Study Subjects

A cross-sectional study was performed in sarcoidosis patients and healthy controls at the State University of Rio de Janeiro (Brazil) between August 2015 and February 2017. The diagnosis of sarcoidosis was made according to the guidelines of international societies [14]. The active sarcoidosis group consisted of patients with symptoms, but without previous treatment (treatment-naive patients); patients with an established diagnosis, persistent symptoms, and currently under treatment; or patients with symptoms and evidence of disease progression in tomographic images of the chest [11, 12, 15]. The second study group consisted of sarcoidosis patients in remission. These patients had previously been treated for sarcoidosis, but were currently asymptomatic, with demonstrated disease improvement in tomographic images [11, 12, 15]. The third group consisted of healthy, non-smoking individuals, with no prior or current illnesses and not currently taking any medication.

Laboratory Measurements

Peripheral blood was collected from all study subjects and used to measure serum ACE levels, hs-CRP levels, and CHITO activity. The concentration of hs-CRP in serum samples was determined using a latex-enhanced turbidimetric immunoassay (BioSystems S.A., Barcelona, Spain). Data were expressed as mg/dL. Serum ACE concentration was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, USA). Data were expressed as ng/mL. CHITO activity was determined by a fluorometric method [21], using the Chitinase Assay Kit (catalog number CS1030, Sigma Chemical Co, St Louis, MO, USA). CHITO activity was expressed in U/mL.

Statistical Analysis

Sociodemographic data, clinical data, and laboratory measurements were analyzed among the different groups. For continuous numerical variables, Kruskal–Wallis ANOVA by ranks tests was used to test the hypothesis that the different samples in the comparison were drawn from the same distribution or from distributions with the same median. Similarly, for categorical nominal variables, the Fisher's exact test was used to evaluate frequencies among the different groups to test the independence between the groups and these variables. Pairwise comparisons of laboratory variable means among the groups of interest were performed by contrasts/differences obtained after both bi- and multivariate linear models fitted using ordinary least square regressions. To eliminate sample bias, confounding variables were selected by bivariate linear models fitted with ordinary least square regressions by backward elimination and were retained in multivariate models. The cutoff points of the biomarkers for active disease/remission differentiation were calculated from receiver operating characteristic (ROC) curves using the Youden's index. Additionally, a decision tree classifier (DTC) was built using an implementation of Quinlan's C4.5 algorithm [22]. DTC training parameters included the following: (1) two, as the minimum number of observations that must exist in a node for a split to be attempted; (2) one, as the minimum number of observations in any terminal leaf node; (3) ten, as the maximum depth of any node of the final tree, with the root node counted as depth 0; and (4) Gini impurity as a measure of how often a randomly chosen element from the set would be incorrectly labeled if it were randomly labeled according to the distribution of labels in the subset. Performance of sarcoidosis status differentiation by the biomarkers' cutoff points and by the classification tree was

estimated by its leave-one-out cross-validation (LOOCV) accuracy, sensitivity, specificity, positive (PPV) and negative predictive values (NPV), and false positive and negative ratios, with their 95% confidence intervals (CI). All analyses were performed using R software v.3.4.3.

Results

Almost all patients had histopathological evidence of non-caseous granulomatous lesions. Only one patient had a transbronchial biopsy specimen that was insufficient for histological diagnosis. However, bronchoalveolar lavage showed a CD4/CD8 ratio of 8.2 for this patient. Fifty-three patients were selected for the study. Of these, seven were excluded due to severe asthma and corticosteroid use, four were excluded due to autoimmune diseases (two with lupus, one with rheumatoid arthritis, and one with Sjogren's syndrome), and the other two were excluded due to active tuberculosis and leukemia. After exclusions, 46 patients diagnosed with sarcoidosis and 21 healthy individuals were included in the study. Patients with sarcoidosis were divided into two groups based on whether the disease was active ($n=27$) or in remission ($n=19$). The active sarcoidosis group consisted of 12 treatment-naïve patients with symptoms; 15 patients with an established diagnosis and persistent symptoms; and 2 patients still under treatment for the maintenance of symptoms, with evidence of disease progression in tomographic images of the chest [11, 12, 15]. The symptoms found in patients currently undergoing treatment for active disease, included cutaneous lesions ($n=12$), dyspnea ($n=9$), cough ($n=1$), and chest pain ($n=1$). Of the fifteen patients undergoing treatment, thirteen were using prednisone and two were using hydroxychloroquine. However, eight patients required a second drug (methotrexate or hydroxychloroquine) to optimize their treatment.

The median ages of the patients with sarcoidosis were 46 and 56 years (active and in remission, respectively), with a higher ratio of females, non-Caucasians, and individuals with no history of smoking in the sarcoidosis groups compared with the control group (Table 1). Serum ACE levels, hs-CRP levels, and CHITO activity were measured for all subjects.

The mean (95% CI) serum ACE concentrations were 341.18 ng/mL (269.57–412.79) in the control group, 337.866 ng/mL (262.47–413.26) in the sarcoidosis remission group, and 470.96 ng/mL (407.81–534.09) in the active sarcoidosis group. These values were significantly higher in the active sarcoidosis group compared to the control ($P=0.023$) and sarcoidosis remission groups ($P=0.024$). Mean (95% CI) serum chitotriosidase activities were 65.55 U/mL (37.78–168.88) in the control group, 38.096 U/mL (70.54–146.73) in the sarcoidosis remission group, and 297.11 U/mL (205.98–388.24) in the active sarcoidosis group. Again, these values were significantly higher in the active sarcoidosis group compared to the control ($P=0.004$) and sarcoidosis remission groups ($P=0.001$). There were no significant differences in ACE levels or CHITO activity between the control and sarcoidosis remission groups (Fig. 1). Mean values were adjusted to avoid sample bias by gender in the ACE analysis.

The mean (95% CI) serum hs-CRP concentrations were 0.279 mg/dL (0.18–0.74) in the control group, 0.378 mg/dL (0.11–0.86) in the sarcoidosis remission group, and 1.003 mg/dL (0.6–1.41) in the active sarcoidosis group. We find only nonsignificant trend toward increased serum hs-CRP concentration in the active sarcoidosis group ($P=0.056$) compared to the control group (Fig. 1).

For serum ACE concentration, we calculated a cutoff value of 270 ng/mL to identify active sarcoidosis, with an approximate mean sensitivity (95% CI) of 88% (70–97%), a specificity of 47% (24–71%), a PPV of 70% (46–92%), and an NPV of 75% (47–89%). For serum hs-CRP concentration, the cutoff was calculated at 0.4 mg/dL, with an approximate mean sensitivity of 66% (46–83%), a specificity of 68% (43–87%), a PPV of 75% (51–88%), and an NPV of 59% (38–82%). The cutoff value for serum CHITO activity was 120 U/mL, with a mean sensitivity of 55% (35–75%), a specificity of 100% (82%–∞), a PPV of 100% (78%–∞), and an NPV of 61% (40%–∞) (Fig. 2).

All patients were classified into two subgroups according to their radiological stage, which was categorized from 0 to 4. Higher mean levels (95% CI) of CHITO activity were observed in a combined group of patients with radiological stages of 0, 1, or 2 (447.255 U/mL; 273.61–620.9), than in a combined group of patients with stages 3 and 4 (135.417 U/mL; − 45.51–316.34; $P=0.022$). We also observed higher ACE concentrations in stage 0/1/2 patients (532.63 ng/mL; 454.84–610.41) than in stage 3/4 patients (401.553 ng/mL; 320.49–482.62; $P=0.031$). There was no significant differences in hs-CRP levels according to radiological stage (Fig. 3).

In the group of patients with active sarcoidosis, there were no significant differences in CHITO activity, ACE levels, or hs-CRP levels between those with or without treatment. The forced vital capacity (FVC), forced expiratory volume-one second (FEV1), FEV1/FVC, and diffusing capacity of the lung for carbon monoxide (DLCO) values were also not significantly different between patients with active sarcoidosis and those in remission.

By training and pruning a DTC for active disease/remission differentiation, we found an algorithm with a mean LOOCV accuracy of 82.61% (74.24–90.97%), LOOCV sensitivity of 77.78% (65.61–89.94%), and LOOCV specificity of 89.47% (78.59–100%). To assemble this algorithm, the following parameters were included: CHITO activity,

Table 1 Clinical aspects of the study population

	Sarcoidosis, active ($n=27$)	Sarcoidosis, remission ($n=19$)	Healthy ($n=21$)	P value*
Age in years	46 (13.5)	56 (18.5)	39 (20)	0.0018
Gender				
Female	19 (28.4)	12 (17.9)	15 (22.4)	0.8516
Male	8 (11.9)	7 (10.4)	6 (9.0)	
Race				
Caucasian	11 (16.4)	4 (6.0)	16 (23.9)	0.0015
Other	16 (23.9)	15 (22.4)	5 (7.5)	
Smoking status				
Current	1 (1.5)	1 (1.5)	0	0.0418
Never	21 (31.3)	13 (19.4)	21 (31.3)	
Former	5 (7.5)	5 (7.5)	0	
Stage		Current stage		
0	0	8 (11.9)	0	
1	1 (1.5)	4 (6.0)	0	< 0.0001
2	13 (19.4)	0	0	
3	8 (11.9)	4 (6.0)	0	
4	5 (7.5)	3 (4.5)	0	
N.A	0	0	21 (31.3)	
Treatment				
Untreated	12 (17.9)	0	0	< 0.0001
In treatment	15 (22.4)	0	0	
Treated	0	19 (28.4)	0	
N.A	0	0	21 (31.3)	
FVC (%)	85 (20.5)	88 (17.5)	N.A	0.554
FEV_1 (%)	82 (24.0)	86 (13)	N.A	0.3481
FEV_1/FVC	77 (11,.0)	78 (11.01)	N.A	1
DLCO (%)	87 (26.5)	98 (23.75)	N.A	0.1098
Clinical manifestation				
Pulmonary	9 (13.4)	N.A	N.A	< 0.0001
Extrapulmonary	9 (13.4)	N.A	N.A	
Pulmonary + extrapulmonary	4 (6)	N.A	N.A	
Asymptomatic	5 (7.5)	N.A	N.A	
Years after diagnosis	1 (6.75)	7 (8)	N.A	0.0013

Data are given either as median (IQR) or absolute (relative) frequencies for numeric continuous and categorical nominal variables, respectively

N.A. not available; *FVC* forced vital capacity; *FEV1* forced expiratory volume-one second; *DLCO* diffusing capacity of the lung for carbon monoxide

*For categorical nominal variables, P values were calculated using Fisher's exact test. For numeric continuous variables, P values were calculated using Kruskal–Wallis ANOVA by ranks test. Differences were considered significant with * P values < 0.05. N = number of individuals

hs-CRP concentration, and ACE concentration (Fig. 4; Table 2).

Discussion

One of the major challenges in the management of patients with sarcoidosis is identifying whether they have active disease or are in remission. Treatment of the disease is time dependent and involves progressively smaller doses until it is adequately controlled [14–23]. The purpose of this study was to construct an algorithm, using three recognized biomarkers of sarcoidosis, to allow the differentiation between active disease and remission. In addition, we showed that CHITO activity has discriminatory power when its values are high, with a specificity of 100%. Elevated CHITO activity in peripheral blood has been previously described for sarcoidosis diagnosis and should be investigated when the

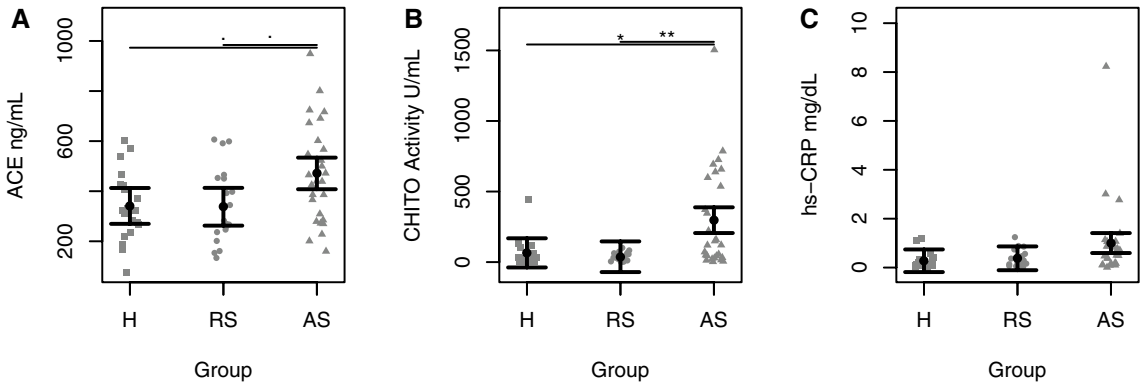

Fig. 1 Biomarker levels according to sarcoidosis status. Patients with sarcoidosis were divided into two groups: those with active disease ($n=27$, AS) and those in remission ($n=19$, RS). A third group of healthy control individuals was also included ($n=21$, H). Serum **a** ACE levels, **b** CHITO activity, and **c** hsCRP levels are represented by gray squares, circles, and triangles, respectively, for groups H, RS, and AS. Black dots and vertical bars represent the linear model estimated adjusted means and 95% confidence intervals (95% CI), respectively. Adjusted means and 95% CI were 341.18 (269.57–412.79), 337.866 (262.47–413.26), and 470.955 (407.81–534.09) ng/mL for ACE; 65.552 (37.78–168.88), 38.096 (70.54–146.73), and 297.111 (205.98–388.24) U/mL for CHITO activity; and 0.279 (0.18–0.74), 0.378 (0.11–0.86), and 1.003 (0.6–1.41) mg/dL for hs-CRP for groups H, RS, and AS, respectively. Serum ACE levels were adjusted by gender. No confounding variables were selected in bivariate analysis of serum CHITO activity or hs-CRP levels. *P* values were determined using the Tukey Honest significant difference post hoc method. $P<0.1$, $*P<0.05$, $**P<0.01$

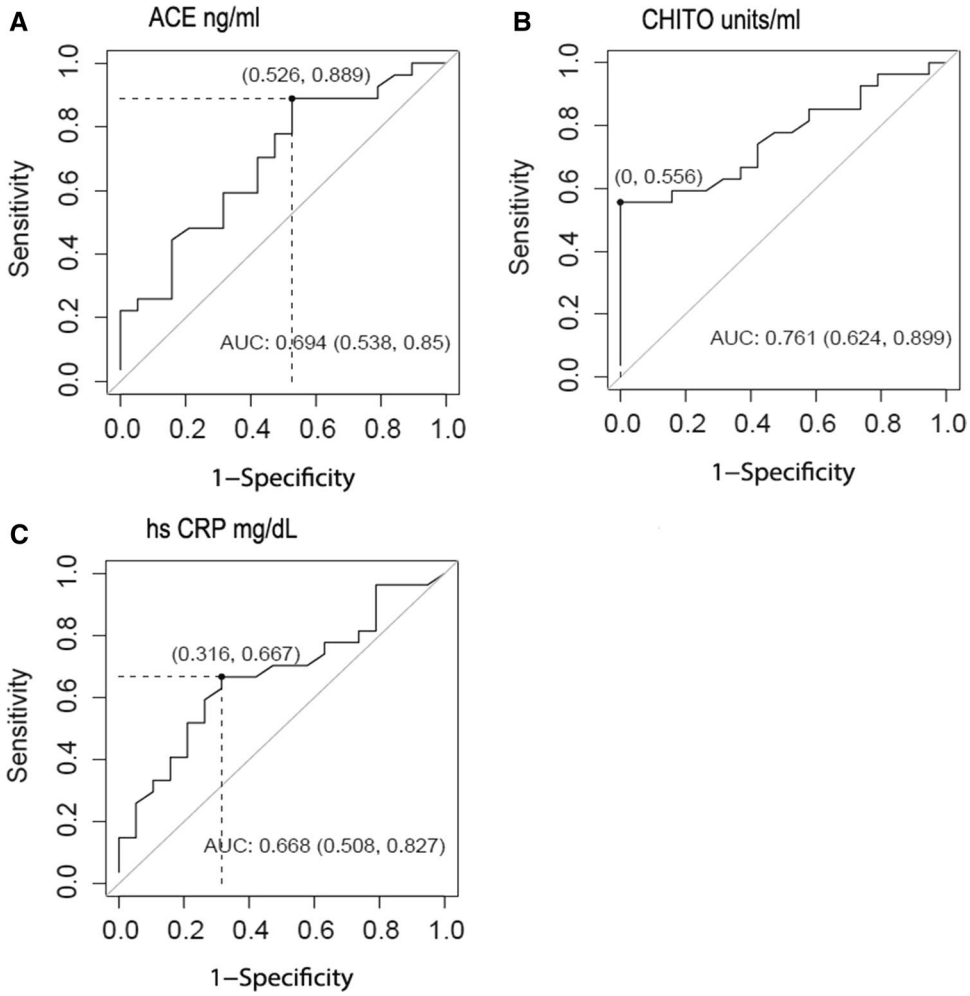

Fig. 2 Selection of optimal discriminant sarcoidosis activity cutoff values calculated from the ROC. ROC curves are represented by solid black lines and optimal sarcoidosis activity discrimination points, as selected by the Youden's index, are represented by solid black dots. Diagonal gray solid lines represent random discrimination. The main results are **a** ACE concentration: sensitivity of 88% and specificity of 47% for a cutoff value of 270 ng/mL; **b** CHITO activity: sensitivity of 55% and specificity of 100% for a cutoff value of 120 U/mL; and **c** hs-CRP concentration: sensitivity of 66% and specificity of 68% for a cutoff value of 0.4 mg/dL. *AUC* area under the curve

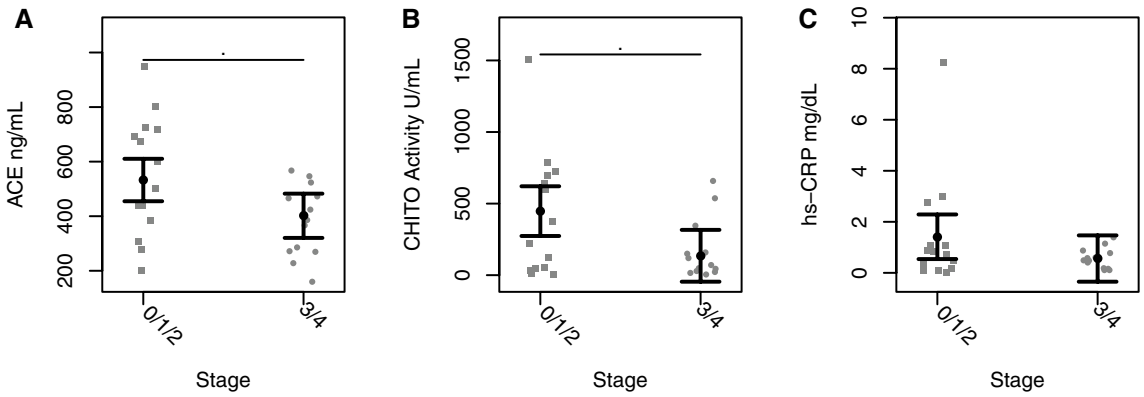

Fig. 3 Biomarker levels in patients with active sarcoidosis according to radiological stage. Patients with active sarcoidosis were divided by radiological stage, as either stage 0/1/2 ($n=14$) or stage 3/4 ($n=13$). Serum **a** ACE levels, **b** CHITO activity, and **c** hs-CRP levels are represented by gray squares, circles, and triangles, respectively. Black dots and vertical bars represent linear model estimated adjusted means and 95% confidence intervals (95% CI). Adjusted means and 95% CI were 532 ng/mL in stage 0/1/2 and 401 ng/mL in stage 3/4 for ACE concentration; 447 U/mL in stage 0/1/2 and 135 U/mL in stage 3/4 for CHITO activity; and 1.4 mg/dL in stage 0/1/2 and 0.56 mg/dL in stage 3/4 for hs-CRP concentration. Serum ACE levels were adjusted for the confounding variables, gender, clinical manifestation, and treatment. Serum CHITO activity levels were adjusted for the confounding variables, race, treatment, and time (in years) after sarcoidosis diagnosis. No confounding variables were selected in bivariate analysis of serum hs-CRP levels. *P* values were calculated using the Tukey Honest Significant Difference post hoc method. $P<0.1$, $*P<0.05$, $**P<0.01$

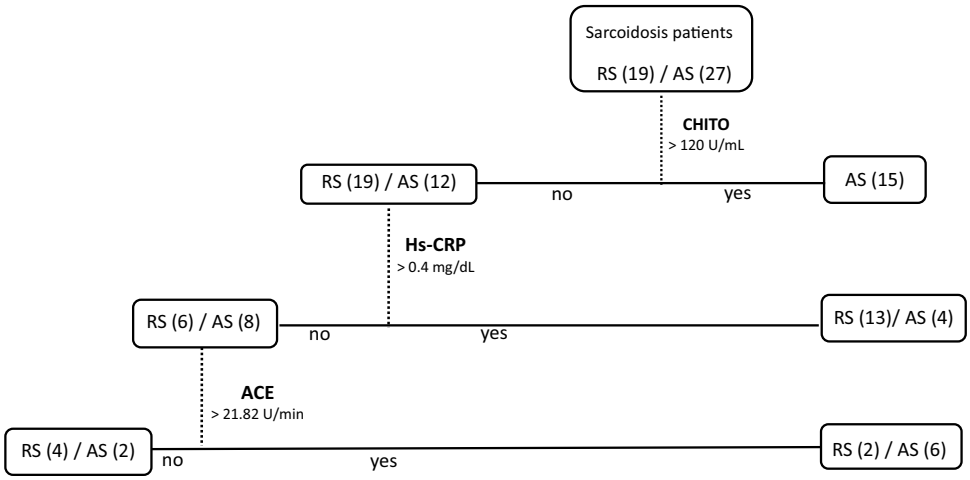

Fig. 4 Graphical representation of decision tree classifier (DTC) for discrimination between active sarcoidosis ($n=27$, AS) and sarcoidosis in remission ($n=19$, RS). Terminal branches (squares) indicate the class (either AS and RS), and the number inside the branches are their true positive and false positive results (black) and false positive and false negative results (gray). A mean LOOCV accuracy of 82% was found for the determination of patients with active sarcoidosis

clinical and epidemiological context is compatible with other clinical conditions [10, 20, 21].

ACE is the most commonly used biomarker for both diagnosis and follow-up [24]. In sarcoidosis patients, the use of corticosteroids and different radiological stages of disease may affect ACE levels. In contrast to other reports, we did not find a difference in ACE levels between treated and treatment-naïve patients.

The highest ACE levels were seen in patients at stages 0, 1, and 2. Some other studies have failed to demonstrate a relationship between serum ACE levels and radiological stages, suggesting that ACE levels may be related to the patient's granuloma burden [4, 5, 7, 10, 25, 26]. ACE levels also depend on genetic variations, such as the I/D polymorphism, where DD genotypes are associated with higher levels, ID with intermediate levels, and II with lower levels [27]. Two published studies have shown mean sensitivity values of 66 and 77% and mean specificity values of 54 and 88% for the differentiation of active sarcoidosis from remission [10, 11]. In our study, ACE levels were higher in patients with active disease than in controls or patients in remission, with a sensitivity of 88% and a specificity of 47%.

CRP is used as a biomarker of inflammation in several diseases. In sarcoidosis, however, only slight elevations in CRP have been reported [14, 28]. We also observed slightly elevated levels of hs-CRP in patients with active sarcoidosis.

Since 2004, studies have shown that CHITO activity is higher in sarcoidosis patients compared to healthy controls and the activity levels increase with the severity and radiological stage of the disease [29]. However, in

Table 2 Assessment of accuracy by the algorithm to identification of activity or remission sarcoidosis

	Prediction[a]
Accuracy (Acc)	0.8261 (0.7424; 0.9097)
Sensitivity (Se)	0.7778 (0.6561; 0.8994)
Specificity (Sp)	0.8947 (0.7859; 1.0036)
Positive predictive value (PPV)	0.913 (0.8231; 1.003)
Negative predictive value (NPV)	0.7391 (0.599; 0.8793)
False positive ratio (FPR)	0.1053 (− 0.0036; 0.2141)
False negative ratio (FNR)	0.2222 (0.1006; 0.3439)

[a]Performance was estimated by its leave-one-out cross-validation (LOOCV) accuracy (Acc), sensitivity (Se), specificity (Sp), positive (PPV) and negative predictive values (NPV), false positive and negative ratios, with their 95% confidence intervals (CI). Performance evaluation of sarcoidosis activity by the classification tree for activity/remission differentiation combining CHITO activity (> 120 U/mL), hs-CRP (> 0.4 mg/dL), and ACE activity (> 21.82 U/min) data. The performance was estimated by its leave-one-out cross-validation (LOOCV) accuracy, sensitivity, specificity, positive and negative predictive values, false positive and negative ratios, with their 95% confidence intervals (CI)

individuals in remission, CHITO activity is reduced [29, 30]. We also found that mean CHITO activity was higher in patients with active disease than in controls or patients in remission. Other studies have also shown a decrease in CHITO levels in sarcoidosis patients, including in those who started treatment with corticosteroids or immunosuppressants [30–32]. In addition, the active sarcoidosis group did not show difference in CHITO activity among treated and untreated patients. This may be due to the fact more than half of the patients undergoing treatment did not have their disease under control and required further optimization of their therapy. CHITO activity may be related to this disease profile. Moreover, we may have found a relationship if we continued to measure CHITO activity during the course of treatment and not only at one time point. However, we did not have the opportunity to do this in these patients. To gain large enough groups to achieve appropriate statistical power, we needed to group the patients into two groups based on radiological stage. Patients classified as stage 0, 1, and 2 had the highest levels of CHITO activity, which is contrary to what has been reported by other authors [10, 30, 33].

A *CHITO* gene polymorphism has been described, consisting of the duplication of 24 bp in exon 10, which introduces a stop codon that causes the deletion of 87 nucleotides in the transcribed RNA. This deletion results in decreased production of the enzyme. Lee et al. [34] estimated the allele frequency of this polymorphism in different populations and reported values varying between 7–64%. In Brazil, this variant is present in 30% of the population [35]. Among the patients in our study classified as having active sarcoidosis, we observed a case with a CHITO activity value of 3.58 U/mL, which may be explained by the presence of this variant. However, genotyping was not performed to confirm this hypothesis.

Our work does have some limitations. For example, this was a single-center study, performed over a short time interval, with a limited sample size, and the influence of immunosuppressive therapy on the activity of biomarkers. Furthermore, we did not perform genotyping to detect possible *CHITO* or *ACE* variants. Biomarkers were also measured at just one time point, thus not allowing the assessment of their changes during treatment.

Many potential biomarkers of sarcoidosis have been studied in isolation for the diagnosis and follow-up of the disease. However, this is the first study using a combined analysis of more than one biomarker to determine disease activity. We achieved high accuracy and specificity, and therefore, this approach may help in the follow-up of these patients.

Funding This study was supported by FAPERJ.

Compliance with Ethical Standards

Conflict of interest None.

Ethical approval This project was approved by the research ethics committee (Protocol No. 1158044).

Informed consent Informed consent was obtained from all participants included in this study.

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