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Use of discriminant analysis in assessing pulmonary function worsening in patients with sarcoidosis by a panel of inflammatory biomarkers

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Abstract
Objectives and design To date, no sufficiently sensitive and specific single marker has been found to predict the clinical course of sarcoidosis. We designed a cohort study to investigate whether a panel of biomarkers measured in bronchoalveolar lavage (BAL) and peripheral blood could help predict pulmonary function worsening during the clinical course of sarcoidosis.
Methods We analyzed 30 individuals with histologically proven sarcoidosis. At baseline, participants underwent pulmonary function tests (PFTs), fiberoptic bronchoscopy and radiological investigations. BAL and blood cellular profiles were obtained from all individuals and six pro-inflammatory molecules were quantified in BAL and serum. PFTs were performed at follow-up visits over a 2-year period. Using discriminant function analysis, a canonical variable was generated to optimize the accuracy of selected variables in predicting pulmonary function worsening and was validated on a subset of nine consecutive individuals with sarcoidosis.
Results A combination of 6 markers from BAL was able to predict pulmonary function worsening in 96 % of patients [95 % confidence interval (CI) 84.4–99.81]. We validated the generated formula on a group of nine patients with sarcoidosis, obtaining 77.8 % correct classification (95 % CI 45.3–93.7).
Conclusions Our results show that a combinational approach could contribute to identifying individuals likely to experience pulmonary function worsening, thus helping to decide the correct therapeutic strategies.

Keywords Biomarker · Discriminant analysis · Disease severity · Pulmonary function worsening · Sarcoidosis

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Introduction

Sarcoidosis is a chronic multi-systemic disorder of unknown aetiology, characterized by an increased degree of immune response at the site of disease. Although any organ

can be affected, the lungs and the lymphatic system are predominantly involved, with an increased recruitment of immunocompetent cells in the lower respiratory tract [1].

The clinical expression and prognosis of sarcoidosis are highly unpredictable: spontaneous remissions occur in nearly 30–75 % of patients but the course of the disease may be chronic or progressive in 10–30 % of cases. At least 10–15 % of patients show permanent sequelae and fatalities occur in 1–5 % of individuals because of progressive respiratory failure or central nervous system or myocardial involvement [2–9].

Sarcoidosis' protean behaviour has prompted many studies with the aim of finding biomarkers able to predict disease progression and response to therapy [10, 11]. Given the high rate of self-limiting cases and spontaneous remissions, it could be crucial to identify individuals who may need a closer follow-up.

To date, despite the many efforts carried out, no definitive predictive indicators have been identified in sarcoidosis and, currently, monitoring serial functional changes remains the best approach to assessing prognosis [1, 9, 12]. Furthermore, the common practice of applying strict cut-off values in diagnostic tests may result in a loss of information and in a risk of a clinical misinterpretation of the results.

In the present study, trying to shift the outlook from the comparison of single variables to the analysis of a combination of biomarkers, we evaluated the effectiveness of a panel of markers in serum and bronchoalveolar lavage (BAL) to predict respiratory function worsening in subjects with sarcoidosis.

Materials and methods

Study population

We enrolled in the study a population of consecutive non-smoking individuals with histologically proven sarcoidosis who were not receiving corticosteroid treatment. A second population of consecutive individuals with sarcoidosis was enrolled as a validation group. Subjects with any co-morbidity were excluded from the evaluation.

All individuals underwent pulmonary function tests (PFTs), fiberoptic bronchoscopy (FBS) and radiological investigations (chest X-ray and high resolution computed tomography, HRCT) at initial examination. Disease stage was evaluated, according to chest X-ray findings, following American Thoracic Society recommendations [1]. Blood samples were collected at diagnosis and stored at −80 °C until analysis.

The study was approved by the local ethics committee (909/CE) and informed signed consent was obtained from all participants.

Sample size

The sample size was calculated on the following assumptions: specificity of the combination of biomarkers = 0.75; sensitivity of the combination of biomarkers = 1; power = 80 %; two-sided alpha level = 0.05. The required sample size was 28, and it was increased by 10 % to limit the missing values.

FBS and BAL processing and determination of biomarker concentrations

All individuals underwent FBS for BAL acquisition as previously described [13]. Briefly, after local anaesthesia, a fiberoptic bronchoscope was wedged into a segmentary bronchus of the middle right lobe or into the lingula and 100 ml of saline solution in 5 × 20 ml aliquots were injected and immediately aspirated by gentle suction. The recovered BAL was filtered through a two-layer gauze to remove mucus and cellular debris.

Following filtration, an aliquot of fluid was removed for total cell counts and cytoslide preparation. Cell counts were performed by light microscopy using a hemocytometer, cytospots were prepared using a Cytospin II (Shandon Instruments, Cheshire, UK), and differential cell counts were obtained by light microscopy after treatment with Diff-Quik stain solutions (Allegiance, McGraw Park, IL, USA). Lymphocyte phenotype evaluation was accomplished by flow-cytometer analysis (Becton–Dickinson, Franklin Lakes, NJ, USA).

The remaining BAL was centrifuged at 300*g* for 15 min at 4 °C to separate supernatants from cell pellets. Supernatants were removed and stored at −80 °C until protein quantification assays were performed.

BAL and serum concentrations of eosinophilic cationic protein (ECP), myeloperoxidase (MPO), tryptase and procollagen III peptide (PIIIP) were quantified by using commercially available RIA kits while soluble interleukin-2 receptor (sIL2R) and tumor necrosis factor-α (TNF-α) were measured by ELISA following the manufacturer's instructions (Pharmacia AB, Uppsala, Sweden, and Endogen, Cambridge, MA, USA, respectively).

Follow-up

All patients underwent a follow-up evaluation every 6 months by physical examination, chest X-ray or chest HRCT, and pulmonary function tests for a 2-year period.

According to previous reports, pulmonary function worsening was defined as a decline of total lung capacity (TLC) and diffusing capacity of the lung for carbon monoxide (DLCO) >10 %, or forced vital capacity (FVC) and forced expired volume in 1 s (FEV_1) >15 % at follow-up visit [14–16].

Statistical analysis

Due to non-normal distribution of the raw data, comparisons or correlations between variables were analysed using non-parametric tests (Mann–Witney U test or Spearman rank test).

Discriminant function analysis was performed to optimize the accuracy of selected variables in predicting pulmonary function worsening. This method allows the generation of an index, negative or positive, able to separate 2 groups. The index is named the canonical variable (CV). CV = 0 is the cut-off point and corresponds to an unclassified individual [17]. In the canonical variable we generated, CV < 0 identified patients with a stable disease and CV > 0 indicated progressive functional worsening. Inclusion of the variables was obtained by the criterion of corrected means using the F statistic. The analysis was performed using the "equal a priori probability" option to assign the subjects to groups. The jack-knifed approach was used to discriminate the patients. By means of this approach, each patient was evaluated by a CV generated after exclusion of the same patient data.

Positive and negative likelihood ratios (LR+ and LR−) obtained using the CV were also calculated as LR+ = sensitivity/(1 − specificity) and LR− = (1 − sensitivity)/specificity, respectively.

Statistical analyses were performed by using Statistica 5.1 software (Stat Soft, Inc., Tulsa, OK, USA).

Results

Baseline demographic characteristics, BAL, and peripheral blood analysis in patients with sarcoidosis

A study population of 30 individuals was enrolled for the generation group and nine subjects were evaluated as the validation subset. The observed clinical and demographic characteristics are shown in Table 1.

At follow-up evaluation, 76 % of the study group and 77.8 % of the validation subset individuals had stable disease, while 24 and 21.2 %, respectively, experienced respiratory function worsening needing corticosteroid treatment according to the recommended therapeutic regimen [1, 12].

BAL fluid and peripheral blood findings in patients with stable sarcoidosis and individuals with progressive disease

The analysed BAL and peripheral blood cellular and molecular profiles are shown in Tables 2 and 3. BAL neutrophil (NEU) and eosinophil (EOS) absolute number, BAL ECP and serum PIIIP levels were significantly higher in individuals with progressive disease than in subjects with stable disease ($p = 0.002$, $p = 0.01$, $p = 0.04$ and $p = 0.02$ respectively).

Correlation analysis

A significant correlation was found between neutrophil percentage and FEV_1 (rho = −0.57, $p = 0.01$), MPO and FVC (rho = −0.46, $p = 0.03$), ECP and DLCO (rho = −0.65, $p = 0.01$), and PIIIP and DLCO (rho = −0.61, $p = 0.02$). A significant correlation was observed between eosinophils and ECP ($r = 0.4$, $p = 0.03$) and between MPO and tryptase ($r = 0.54$, $p = 0.002$). No correlations were found between pulmonary function parameters and serum concentrations of the analysed biomarkers, and between neutrophils, eosinophils and lymphocytes in BAL.

Discriminant function analysis

We generated a canonical variable with the aim of developing a panel of markers able to predict the clinical course of sarcoidosis (stable disease or progression). The formula obtained was: CV = BAL NEU × 0.06 + BAL EOS × 0.37 + BAL ECP × 0.7 + BAL MPO × 0.02 − BAL Tryptase × 0.39 + BAL PIIIP × 7.2 + 3.46.

The CV sensitivity was 1 [95 % confidence interval (CI) 0.6–1], while specificity was 0.96 (95 % CI 0.79–0.99), with a 96.6 % (95 % CI 84.4–99.81) of overall correct allocation rate (Fig. 1). LR+ was >20 and LR− was 0.

We also analysed the allocation rate obtained by each marker as a single variable. The BAL neutrophil and eosinophil percentages precisely allocated 85.7 % and 42.8 % of worsening sarcoidosis (95 % CI 46.9–99.2 and 12.5–78.1) and 95.5 % and 100 % of stable disease (95 % CI 79.7–99.8 and 87.5–100), respectively. Using ECP, a correct classification was obtained in 71.4 % of patients with progressive disease (95 % CI 34.4–94.25) and 100 % of stable disease (95 % CI 87.5–100). Tryptase was not able to identify any of the individuals with disease progression (95 % CI 0–43.7), whereas it exactly allocated 100 % of stable disease (95 % CI 87.5–100). The MPO classification rate was 42.8 % for worsening (95 % CI 12.5–78.1) and 95.5 % for stable disease (95 % CI 79.7–99.8). PIIIP achieved a 28.6 % correct classification for worsening (95 % CI 5.5–65.2) and 95.6 % (95 % CI 81.2–99.8) for stable disease.

The canonical variable formula generated was validated on a group of nine individuals with sarcoidosis (7 with stable and 2 with progressive disease) (Table 1). A correct classification was obtained in seven patients (77.8 %, 95 % CI 45.3–93.7) (Fig. 2). Sensitivity and specificity for the validation group were: 1 (95 % IC 0.34–1) and 0.72 (95 % IC 0.36–0.92), respectively. LR+ was 3.5 and LR− was 0.

Table 1 Clinical characteristics of study populations

Variable	Study group ($n = 30$)			Validation group ($n = 9$)		
	Stable disease ($n = 23$)	Progressive disease ($n = 7$)	Significance	Stable disease ($n = 7$)	Progressive disease ($n = 2$)	Significance p
Age (years)	55.6 ± 13.1	54.2 ± 13.3	0.8	62.2 ± 10.6	49.5 ± 34.7	0.3
M/F	6/17	1/6	0.3	4/3	0/2	0.2
Stage 2/3	18/5	5/2	0.7	6/1	2/0	0.6
FEV_1 (%)	91.2 ± 12.7	86.2 ± 10.2	0.2	92.4 ± 15.2	72.3 ± 12.4	0.2
FVC (%)	91.7 ± 14.3	94 ± 12	0.7	91.5 ± 12.5	86.4 ± 12.6	0.6
TLC (%)	86.1 ± 10.1	84.2 ± 10.4	0.8	89.5 ± 10.1	73.5 ± 30.4	0.2
DL_{CO} (%)	77.9 ± 6.6	73.7 ± 6.4	0.2	77.5 ± 6.7	76.0 ± 8.5	0.8

Values are expressed as mean ± standard deviation

FEV_1 Forced expired volume in one second, *FVC* forced vital capacity, *TLC* total lung capacity, *DL_{CO}* diffusing capacity of the lung for carbon monoxide

Table 2 BAL fluid and peripheral blood cellular profile in patients with stable sarcoidosis and progressive disease

Variable	Stable disease ($n = 23$)	Progressive disease ($n = 7$)	Significance p
BAL total cell count	368.7 ± 194.9	278.0 ± 113.6	0.7
BAL AM (absolute number)	198.8 ± 72.4	160.9 ± 85.9	0.2
BAL neutrophils (absolute number)	10.1 ± 7.3	39.5 ± 30.7	0.002
BAL lymphocytes (absolute number)	124 ± 83.1	89.9 ± 73.3	0.4
BAL eosinophils (absolute number)	0.4 ± 0.8	3.7 ± 4.2	0.01
BAL T-helper (%)	55.1 ± 27.1	64.2 ± 11.0	0.4
BAL T-suppressor (%)	17.3 ± 11.3	20.5 ± 11.1	0.5
BAL H/S (%)	5.2 ± 5.8	4.2 ± 4.6	0.6
Blood T-helper (%)	40.9 ± 13.6	43.4 ± 10.5	0.7
Blood T-suppressor (%)	30.7 ± 13.3	32.6 ± 10.5	0.8
Blood H/S (%)	1.7 ± 1.1	1.4 ± 0.6	0.5

Values are expressed as mean ± standard deviation

AM alveolar macrophages, *H/S* helper/suppressor ratio

We also calculated the classification accuracy of each single variable in the validation group. Neutrophils and eosinophils were able to precisely allocate 50 % and 0 % of the worsening sarcoidosis (95 % CI 9–91 and 0–66) and 71.4 and 85.7 % of stable disease (95 % CI 36–92 and 49–97), respectively. ECP achieved a correct classification in 50 % of individuals with progressive disease (95 % CI 9–91) and 85.7 % of stable disease (95 % CI 49–97). Tryptase did not identify any of the individuals with disease progression (95 % CI 0–66), whereas it was able to exactly discriminate 100 % of stable disease subjects (95 % CI 64–100). The correct classification rate obtained using MPO was 50 % for worsening (95 % CI 9–91) and 71.4 % for stable disease (95 % CI 36–92). PIIIP correctly allocated 0 % of patients with functional worsening (95 % CI 0–66) and 57.1 % (95 % CI 25–84) of individuals with stable disease.

An unsatisfying allocation was obtained using biomarkers from peripheral blood (data not shown).

Discussion

This study shows that a panel of biomarkers from the lower respiratory tract has the ability to predict pulmonary function worsening in patients with sarcoidosis.

Sarcoidosis is a chronic multi-systemic disorder of unknown cause characterized in affected organs by an up-regulation of T lymphocytes and mononuclear cells. Once activated, these cells may secrete several pro-inflammatory molecules likely to play an important role in the inflammatory response typical of this disease.

While respiratory complications are a major cause of morbidity and mortality during the clinical course of sarcoidosis, the extent of lung involvement is unpredictable and biomarkers able to identify subjects likely to progress are lacking. Furthermore, corticosteroids, which are the mainstay of treatment for disease control, have many potential side-effects. Thus, the decision to start therapy should be justified only for patients in whom the benefits outweigh the risks.

Many molecular and cellular markers have, in turn, been suggested as diagnostic and prognostic factors able to identify patients with sarcoidosis and predict their clinical evolution: lymphocytes, neutrophils, eosinophils, natural killer cells, sIL2R, chitotriosidase, the chemokine ligand 18 (CCL18), the anti-endothelial cell antibodies, and Kerbs

Table 3 BAL and peripheral blood biomarkers levels in patients with stable disease and progressive disease

Variable	Stable disease ($n = 23$)	Progressive disease ($n = 7$)	Significance p
BAL ECP (μg/l)	2.2 ± 0.5	3.1 ± 1.5	0.04
BAL MPO (μg/l)	23 ± 34	28.8 ± 16	0.7
BAL tryptase (U/l)	2.3 ± 1.2	3.5 ± 1.7	0.06
BAL PIIIP (U/l)	0.46 ± 0.5	0.35 ± 0.1	0.5
BAL sIL2R (U/l)	533.5 ± 197	416 ± 20	0.1
BAL TNFα (μg/l)	7.14 ± 9.1	15.8 ± 11.5	0.05
Serum ECP (μg/l)	12.4 ± 7.7	25.2 ± 25.7	0.05
Serum MPO (μg/l)	387 ± 160	487 ± 251	0.2
Serum tryptase (U/l)	2.5 ± 1.3	3.6 ± 1.8	0.08
Serum PIIIP (U/l)	0.4 ± 0.2	1.1 ± 0.8	0.02
Serum sIL2R (U/l)	1417 ± 765	1874 ± 1263	0.2
Serum TNFα (μg/l)	48.3 ± 67.2	33.8 ± 34.5	0.6

Values are expressed as mean ± standard deviation

ECP Eosinophil cationic protein, *MPO* myeloperoxidase, *PIIIP* procollagen III peptide, *sIL2R* soluble interleukin-2 receptor, *TNFα* tumor necrosis factor alpha

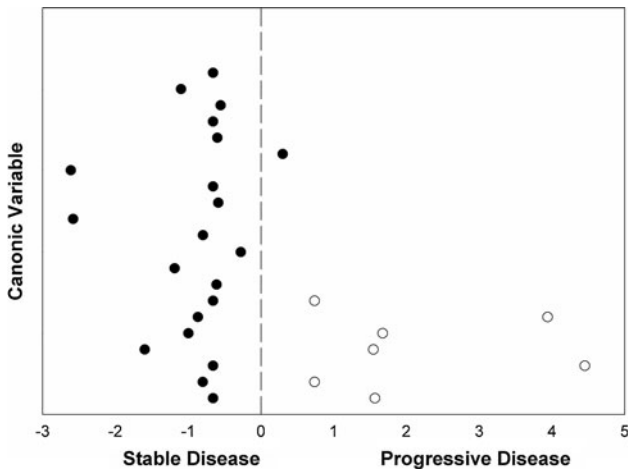

Fig. 1 Discriminant function analysis generated in patients with stable disease (*filled circle*) and progressive disease (*open circle*). The formula was obtained by multiplying the included variable averages by the standardized coefficients CV = BAL NEU × 0.06 + BAL EOS × 0.37 + BAL ECP × 0.7 + BAL MPO × 0.02 − BAL tryptase × 0.39 + BAL PIIIP × 7.2 + 3.46 allowed the correct classification of 95 % (95 % CI 81.2–99.8) of individuals with stable disease (1 misclassified) and 100 % (95 % CI 65.7–100) of subjects with progressive disease. The overall correct classification was 96.6 % (95 % CI 84.4–99.81)

von Lungren 6 antigen (KL-6) [14, 18–25]. All of these mediators have been proven to be clinically useful, but only few of them have been found to possess adequate sensitivity and specificity.

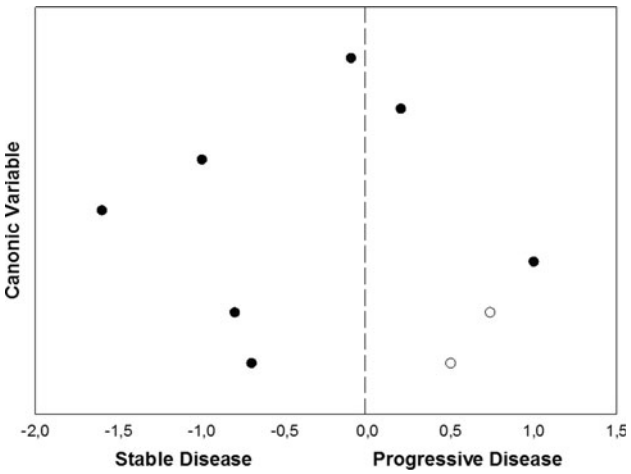

Fig. 2 Discriminant analysis validation in patients with stable disease (*filled circle*) and progressive disease (*open circle*)

With the awareness that no ideal single markers for monitoring sarcoidosis' clinical course and progression are currently available, we designed a cohort study to investigate whether a panel of selected variables obtained from BAL and/or peripheral blood samples could help anticipate respiratory function worsening. Because of the potential misleading effects of smoking on BAL profile, we excluded smokers from the study.

For this purpose we performed a discriminant function analysis, a mathematical method used to determine which variables discriminate between two or more naturally occurring groups. This approach represents one of the most useful techniques for associating the discriminant power of more variables to obtain the maximum classification accuracy [26–29]. This method has already been used in patients with interstitial lung diseases to evaluate its efficacy in discriminating between individuals with sarcoidosis, extrinsic allergic alveolitis and idiopathic pulmonary fibrosis [30, 31]. Recently, a similar strategy was performed by Beirne and co-workers, who in addition to distinguishing patients with systemic sclerosis from subjects with sarcoidosis, were able to identify the presence of lung fibrosis in systemic sclerosis [32]. To date, no studies have been conducted to investigate its usefulness in predicting pulmonary function during the clinical course of sarcoidosis.

In our study we utilized BAL and peripheral blood biomarkers which have already been shown to be involved in the inflammatory response that characterizes sarcoidosis.

A significant increase in BAL neutrophil and eosinophil percentages and absolute numbers at the time of diagnosis has been observed in patients with progressive disease [14, 18], while a decrease in neutrophil numbers was detected in patients with Löfgren syndrome, an acute form of sarcoidosis, associated with a high rate of spontaneous remissions [19]. Lymphocytic alveolitis with increased

CD4/CD8 ratio is the most common finding in sarcoidosis patients but the role of BAL lymphocytes (especially CD4/CD8 ratio) as indicators of an acute presentation and a better prognosis is controversial [19, 33, 34].

It could be possible that, while a high lymphocyte number (especially a high CD4/CD8 ratio) should be considered an important tool to support sarcoidosis diagnosis, high neutrophil and eosinophil numbers could be related to patients' prognosis.

With this as background, we investigated the ability of ECP, MPO and sIL-2R, major products of these cell subpopulations, to predict disease prognosis [14, 18–23, 34–38]. In addition, we measured the concentrations of tryptase, a specific marker of activation of mast cells thought to be involved in the fibroproliferative response and recently found to be increased in patients with progressive disease and therefore a candidate for a role in the immunopathogenesis of sarcoidosis [16, 39]. Finally, we tested the discriminatory power of TNF-α, a product of alveolar macrophages with a pivotal role in granuloma formation, which has been associated with sarcoidosis progression [9, 40], and procollagen III peptide, a marker of collagen synthesis produced by fibroblasts [41, 42].

In attempt to optimize the allocation power while performing discriminant analysis, we first included all measured variables in the model. The best allocation rate was obtained by combining the percentages of neutrophils and eosinophils and the average concentrations of ECP, MPO, tryptase and PIIIP.

A combinational approach, using multivariable logistic regression analysis, has been previously applied by De Smet and colleagues [43] and our group [44] to distinguish subjects with pulmonary sarcoidosis from individuals with other interstitial diseases.

We believe that our 6-component model, although not showing a significantly higher performance, may have a better reliability compared to a single-component model (e.g. BAL neutrophil level). In fact the use of strict cut-off criteria, with its potential shortcoming in being influenced by biological or technical variance, may result in a loss of diagnostic information, especially when the measurements are slightly below or above the threshold. In the combinational approach, the other components may compensate for such minor variance.

As shown in Tables 2 and 3, a significant difference between the variables analysed was observed for neutrophil and eosinophil numbers and ECP concentration, but no single variable was able to identify a disease group or to predict the respiratory function decline with a sufficient accuracy. We believe that this is probably due to the considerable overlap in the marker levels between participant groups.

Interestingly, the concentrations of MPO, tryptase and PIIIP, mediators contributing to the allocation rate, were not significantly different between groups, suggesting that the common practice of restricting the marker selection only to those with levels differing significantly between the subsets of patients evaluated, should probably be revised. In line with this observation, attempting to remove each of the variables included in the formula resulted in a worse outcome, indicating that each of the variables contributed a portion of the final overall information.

We also compared the effectiveness of the marker panel from the lower respiratory tract with that from the peripheral blood combination, observing that all the variables with the maximum allocation rate were obtained from the lower respiratory tract. This finding may generate novel insights into the local inflammatory response profile characterizing sarcoidosis' clinical course and may be a tantalizing approach for tracking its evolution and response to therapy.

We believe that our results, with the inclusion of neutrophils and eosinophils in the canonical formula generated, along with the addition of ECP and MPO, support the hypothesis that these cells may have a role in predicting sarcoidosis' clinical course.

A possible shortcoming of our study could be the limited number of patients. Although the number of individuals evaluated in our study is in line with previous reports analysing combinational approaches [32, 43], the possibility that testing a larger sample size would detect a decrease in discrimination accuracy cannot be ruled out.

In addition, because of the large number of markers involved in sarcoidosis pathogenesis recently described in the literature, we should consider the possibility that other and newer molecules (e.g., chitotriosidase, interleukin-18, KL-6, CCL18) may be combined in discriminant function analysis to obtain better and more reproducible results.

In conclusion, in this study we demonstrate that a combinational approach is significantly more effective in patient allocation than each marker used as a single variable, and propose a novel strategy to trace disease course and response to therapy. These findings, if validated by prospective multicenter studies, may help physicians in their daily practice to identify subjects who need to be carefully monitored and to decide between the different therapeutic options for a better management of sarcoidosis patients.

Conflict of interest All the authors declared no financial or personal relationship with other people or organizations that could have inappropriately influenced their work.

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