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Clinically-useful serum biomarkers for diagnosis and prognosis of sarcoidosis

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ABSTRACT

Introduction: Sarcoidosis is a complex systemic disease with a silent, long-term evolution, and a heterogeneous clinical presentation. The diagnostic approach is complex with no single diagnostic test that may confirm the disease.

Areas Covered: A large list of serum biomarkers has been tested during the last 40 years. In this review, we analyse the potential usefulness in the diagnosis and prognosis of sarcoidosis of serum biomarkers classified according to their corresponding cellular source.

Expert commentary: Diagnosis of sarcoidosis must always be approached as a multistep process based on a case-by-case integration of clinical, radiological, histological and serological data, none of which being pathognomonic. We found sIL-2R, CRP, SAA and chitotriosidase to be the best markers to confirm sarcoidosis (highest sensitivity), while ACE, gammaglobulins and lysozyme may be more useful for discarding sarcoidosis (highest specificity), taking into account that with the use of a higher cut-off we can increase specificity and with a lower cut-off we can increase sensitivity. Other biomarkers (TNF-a and CCL18) could help to identify patients with an enhanced risk of developing pulmonary fibrosis or progressive disease. The future scenario of the serological diagnostic approach of sarcoidosis will be the use of multi-assays including biomarkers from different cellular sources.

KEY WORDS

Sarcoidosis, biomarkers, angiotensin convertase enzyme, cytokines, chitotriosidase, macrophages, T-cells, B-cells

1. Introduction

Sarcoidosis is a systemic disease characterized by the development of non-caseating epitheloid granulomas affecting overwhelmingly thoracic organs (the lungs and lymph nodes) in more than 90% of patients), although extrathoracic involvement has been reported in nearly half the cases (1). The diagnostic approach is complex with no single diagnostic test that may confirm the disease, and with no standardized classification criteria internationally accepted. In clinical practice, a patient is diagnosed with sarcoidosis on the basis of a compatible clinical and/or radiological picture together with histopathologically-proven non-caseating granulomas, always excluding other diseases with similar clinical or histolopathological features (2). Since most patients with sarcoidosis have thoracic involvement, chest radiograph has played a key diagnostic role for decades, now replaced by high-resolution computed tomography (CT) and ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET) in complex cases. Certain highly-specific clinical presentations of sarcoidosis (Löfgren and Heerfordt syndromes) or patients presenting with thoracic stage I (bilateral hilar lymphadenopathy unrelated to infectious or neoplasic diseases) are often diagnosed clinically with sarcoidosis avoiding the need for histopathological confirmation (3). In complex cases, however, pathologic confirmation of noncaseating granulomas is necessary and biopsy should be obtained from the most accessible and safest anatomical site (3).

Since laboratory work-up alone cannot diagnose the disease, serum biomarkers have been always considered of little usefulness in the diagnosis and prognosis of sarcoidosis. A large list of biomarkers has been tested during the last 40 years, although only one (angiotensin-converting enzyme, ACE) is often used in clinical practice. However, the clinical usefulness of ACE measurement is often associated with significant limitations (low specificity test, large interindividual variability in the results, inconsistent correlation between serum levels and disease expression) (4–6).

The non-caseating granuloma is the pathological hallmark of sarcoidosis, formed through the recruitment of different cell types, mainly monocyte-macrophages, T and B cells and epithelioid cells as a consequence of an abnormal autoimmune response involving both adaptive and innate immune systems (7). In this review, we analyse the potential usefulness of serum biomarkers classified according to their corresponding cellular source in the diagnosis and prognosis of sarcoidosis.

2. Monocyte/macrophage-related markers

Macrophages and CD4+ T cells are the key cellular players in the granulomatous reaction and associated inflammation seen in sarcoidosis. Monocyte-macrophage lineage cells are the

architectural basis of sarcoid granulomas and the enhanced local expression of macrophage-derived molecules induces and maintain an inflammatory response further amplified by the recruitment of other immune cells into the targeted tissues (7).

2.1. Angiotensin-converting enzyme (ACE)

Angiotensin convertase enzyme (ACE) is an acid glycoprotein with a molecular weight of 140,000 d that converts a decapeptide (angiotensin I) to an octapeptide (angiotensin II) by cleaving the dipeptide histidine and leucine C-terminal, resulting in a vasoactive molecule that plays a key role in regulating blood pressure and electrolyte balance. ACE may be secreted by monocytes, macrophages and epitheloid cells (4–6) and is involved in the pathogenesis of sarcoidosis as an important modulator of granuloma formation (6).

Serum ACE activity has been used as a diagnostic marker of sarcoidosis since 1975 when Lieberman (8) reported raised serum ACE levels in 15 of 17 patients with active sarcoidosis; in 1981, the Ninth International Conference of Sarcoidosis recommended the measurement of serum concentrations of ACE as a useful diagnostic and prognostic tool. In addition to the classical spectrophotometric assay used by Lieberman, other methods have been reported, including spectrofluorimetric, colorimetric and radio assays, with variable cut-offs that may include either lower (20-30 IU/mL) or higher (70-80 U/L) values, although most of the studies published in the last 10 years are using techniques with cut-offs between 50 and 70 IU/L (**Table 1**). Serum ACE activity is not influenced by human gender, although children and young adults may have higher ACE levels than older people. However, ACE concentrations are genetically influenced, since an insertion (I)/deletion (D) polymorphism in the ACE gene has been associated with significant variations in serum levels (9), with homozygous carriers of the deletion (DD) or insertion (II) express the highest and lowest ACE levels, respectively, whereas heterozygous ID individuals express intermediate ACE levels (10), with significant variations between Caucasians and Asians; unfortunately, the use of a genotype-specific reference range slightly increased the diagnostic sensitivity of the standard serum ACE measurement at the expense of lowering the specificity of the test (11). In addition, serum ACE levels are uniformly low if the patient is receiving ACE-inhibitor medications (6).

Raised serum ACE levels have been reported in 1385 (55%) out of 2529 patients with sarcoidosis included in 16 studies published in the last 20 years (**Table 1**) (12–27) , with a wide range of frequencies including 40% as the lowest (16) and 86% as the highest (18). Raised serum concentrations of ACE have been reported in other diseases related to an enhanced monocyte-macrophage activation, including granulomatous infections (tuberculosis, leprosy), pneumoconiosis (silicosis, berylliosis), deposit metabolic diseases (Gaucher's disease),

endocrine diseases (diabetes mellitus, hyperthyroidism) and liver cirrhosis, while serum ACE levels are reduced in patients with Crohn's disease (4,6). In **Table 2** (15,18,20,25,28–32), we summarize the sensitivity and specificity values of the main studies reported in the last 20 years. Two studies in patients with uveitis with a similar cut-off (51-52 U/L) reported a sensitivity of 54-77% and specificity of 70-88% for the use of serum ACE levels to confirm sarcoidosis in patients presenting with uveitis (25,28); Niederer et al (25) reported that ACE had a very high negative predictive value for sarcoid uveitis (normal levels of serum ACE may obviate further investigations for sarcoidosis in most patients), and that the highest positive predictive value was reported in patients presenting with intermediate uveitis or panuveitis. In unselected sarcoidosis, the two studies with a similar cut-off (17-21 U/L) showed a sensitivity of 68-71% and specificity of 71-75% to distinguish between sarcoidosis and healthy controls (30,31).

Several studies have evaluated the clinical usefulness of measuring serum ACE levels in patients with sarcoidosis (Table 3). With respect to pulmonary involvement, there are more studies reporting a lack of correlation between serum ACE levels and radiological thoracic stages or pulmonary involvement than studies reporting a positive correlation. Some studies have reported increased ACE levels in patients with radiological stage I or in those with bilateral hilar lymphadenopathies -BHL- (14,33), while others reported normal or even reduced levels in patients with erythema nodosum (34) or Lofgren syndrome (35). From a therapeutic view, most studies have reported a reduction of serum ACE levels after treatment with corticosteroids (18,36–40), chloroquine (41) or infliximab (42,43). Although elevated serum ACE levels at diagnosis are not an indication to start systemic treatment (6), it can be helpful in monitoring the therapeutic response after initiation of treatment (25,26). In contrast, the usefulness of serial measurement of serum ACE levels to assess the prognosis and outcome of sarcoidosis is unclear: some studies have reported higher ACE levels in patients with active disease (32,33,44,45) and in those with progressive/chronic disease (22,35,39), although a similar number of studies have reported negative correlations (32,46–51).

2.2. Chitotriosidase

Chitotriosidase is a member of a family of glycosylhydrolases enzymes (also called chitinases) involved in the degradation of chitin, a N-acetylglucosamine polymer secreted by fungi or parasites (4). Chitin-producing microorganisms activate macrophages and neutrophils that secreted the enzyme (52).

Chitotriosidase is a specific marker of macrophage activation and the principal biochemical marker of Gaucher's disease (53), although raised levels have been also reported in patients

with atherosclerosis, b-thalassemia, malaria and visceral leishmaniasis (54–57). Chitotriosidase activity can be determined in serum by a fluorimetric test (58), with cut-offs ranging between 48.8 and 100 nmol/h/mL (32,59,60). Significantly raised levels have been reported in patients with sarcoidosis in comparison with patients with tuberculosis, asbestosis, idiopathic pulmonary fibrosis or systemic sclerosis (61,62). The sensitivity and specifity rates were 48.8% and 88.8% for the lowest cut-off (48.8) (59), respectively, while for a higher cut-off (100) the figures were 82.5% and 70% (32).

With respect to clinical correlations with phenotype and outcome, several studies have reported higher serum levels of chitotriosidase in patients with pulmonary involvement demonstrated by abnormal imaging or functional studies (32,59,63). Two studies reported a reduction of chitotriosidase serum levels after treatment with corticosteroids or after adding an imunosuppresive agent (59,60). In a follow-up study, there was a positive correlation with clinical, radiological and functional worsening, with levels increasing significantly in patients who relapsed during the follow-up (63). Several studies have found a good correlation with active disease (32,60), clinical outcome status (32) and disease progression (59).

2.3. Lysozyme

Lysozyme (or muramidase) is a bacteriolytic enzyme, produced by monocytes and macrophages, discovered in 1922 by Fleming who reported that it had antibacterial activity through cleavage of b1-4 glycoside bonds in bacterial cell walls. Lysozyme is located into the granules of monocytes, macrophages, and polymorphonuclear leukocytes, whence it may be released into biologic fluids. In sarcoidosis, lysozyme is mainly produced by macrophages and epithelioid cells forming the granuloma. Serum levels of lysozyme are not influenced by gender or smoking (64), while renal dysfunction may increase circulating levels because lysozyme is filtered by renal glomeruli. Elevated serum levels have been reported in pulmonary tuberculosis and pneumoconiosis (silicosis, asbestosis, and berylliosis) (18,37,39). In patients with uveitis, two studies have reported significantly raised serum levels of lysozyme in patients with sarcoid uveitis in comparison with those with other underlying diseases (ankylosing spondylitis, Behçet disease, tuberculosis or syphilis) (15,65).

Serum lysozyme levels are elevated in 162 (66.4%) out of 244 patients with sarcoidosis included in 7 studies published in the last 10 years (14,15,17,27,60,66,67), with a very wide range of frequencies including 29% as the lowest (67) and 84% as the highest figure (27). The cut-offs vary according to the technique used, although most of the studies published in the last 10 years have used a cut-off of 10 mg/L.

Some studies have reported a good correlation between raised serum levels of lysozyme and parenchymal pulmonary infiltration (14,48,68). The association between serum lysozyme levels and extrapulmonary sarcoidosis is unclear, one study reported a good correlation with extrathoracic involvement (68) while Nguyen et al described no significant association either with multiple organ involvement or with BHL (14). Two studies reported a reduction of lysozyme levels after treatment with corticosteroids (37,39) (Table 3).

2.4. Neopterin

Neopterin or trihydroxypropylpteridin is a precursor of biopterin, a metabolite of guanosine triphosphate, an essential cofactor in neurotransmitter synthesis. It is released *in vitro* from monocytes/macrophages stimulated by interferon-gamma (4,6). Serum neopterin levels are highly sensitive (95%) but rather less specific (45%) (69), although a recent study have reported inverse figures (80% and 100%, respectively) (70).

The values are higher in patients with advanced chest radiographic stages II-III (51), in those with an acute clinical onset and in those requiring long-term therapy (51), and also correlated with an active disease disclosed by PET (70).

2.5. Cytokines and chemokines

Monocyte-macrophage lineage cells are the origin of an enhanced local expression of a large number of cytokines and chemokines, responsible for the attraction of T-cells at the targeted tissues, including cytokines overwhelmingly involved in promoting Th1/Th17 differentiation (IL-2, IL-8, IL-12, IL-15, IL-18, and Th17-related IL-17 and IL-22) (71), and a large variety of chemokines, such as CCL5 (RANTES), CCL9 (MIG), CCL10 (IP-10) and CCL18 (or PARC) (4,7). Among the molecules evaluated at least in 2 different studies in the last 20 years, the more solid and potentially useful results are reported for CXCL10 levels, that were significantly higher in 5 different studies in comparison with both healthy controls and patients with uveitis caused by other etiologies (47,72–75), as well as for CXCL9 (47,74) and CC16 (76,77). Among cytokines, highly significant levels are reported for IL-18 (78–80), IL-12 (81,82) and TNFa (75,83) in comparison with healthy controls (**Table 4).**

3. T-cell activation markers

The inflammatory mediators produced by the macrophages involved in the granuloma formation trigger the recruitment of additional immune cells, especially CD4+ Th cells, that produce IL-2 to induce T-cell proliferation and the accumulation of effector T cells (4). In the

early phases, the microenvironment at the sites of active disease is characterized by a highly polarized Th1/Th17 profile (7).

3.1. Soluble Interlukin-2 receptor

Activation of CD4+ lymphocytes leads to the expression of IL-2 receptors on the cell surface with shedding of soluble IL-2 receptor (sIL-2R) into the circulation; therefore, serum sIL-2R is considered a reliable serological marker of T-cell activation (5). Serum IL-2R levels can be elevated in several infections (HIV, tuberculosis, leprosy), lymphoproliferative disorders (lymphoma and leukemia) and other inflammatory conditions (idiopathic pulmonary fibrosis, scleroderma, rheumatoid arthritis, systemic lupus erythematosus , Graves' ophthalmopathy, and cardiac and renal allograft rejection) (5,6). Semenzato et al (84) reported in 1987 raised serum levels of IL-2R in patients with sarcoidosis, and further studies have reported raised levels are elevated in 176 (57%) out of 308 patients with sarcoidosis (14,24,29,43,49,85), with a very wide range of frequencies including 30% as the lowest (14) and 100% as the highest figure (85). With respect to diagnostic accuracy, the figures ranged from 63 to 82% for sensitivity, and from 57 to 100% for specificity in studies differentiating between healthy controls and unselected cases of sarcoidosis (30,31,70,86,87), while for patients with ocular sarcoidosis, the figures were 92-98% for sensitivity and 26-94% for specificity (28,29). In the study by Rothkrantz-Kos et al (31), an increase of the cut-off increased both sensitivity and specificity, respectively, while a decrease of the cut-off increased sensitivity but decreased specificity; in the study by Groen-Kahan et al (28) in patients with ocular sarcoidosis, specificity increased from 26% to 64% after increasing cut-off until >4000 pg/mL.

Several studies showed that serum IL-2R levels correlated with radiographic stage of disease (Table 3), but the results were heterogeneous: some studies found higher levels in patients with pulmonary parenchymal involvement (14,48) while other studies reported high levels mainly in patients with no pulmonary involvement including those with stage I (87), BHL (14) or erythema nodosum (87). In contrast, the association with extrapulmonary activity was confirmed by several studies (14,50,87–89). Some studies have reported a good correlation between changes in sIL-2R level and treatment response showing a reduction in serum sIL-2R levels after corticosteroid therapy (90,91) or after treatment with infliximab (42,43), while no therapeutic correlation was informed in another cohort (87). High serum sIL-2R levels have been associated with acute or active disease (51,90), with disease progression over the next 6 months in untreated patients (92), or in patients requiring long-term therapy (51); however, a similar number of studies reported negative correlations (47,48,50,87,93) (Table 3).

3.2. Other cytokines

T helper (Th) cells are characterized by different patterns of cytokine secretion which are used to define their subsets: Th1 cells are characterized by secreting interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF), Th2 cells by secreting IL-4, IL-5 and IL-13, Th9 cells by secreting IL-9 and Th17/22 cells by secreting IL-17 and IL-22 (94). The local cytokine milieu that drives histopathological abnormalities sarcoidosis represents a pivotal immunological characteristic for this disease. Sarcoidosis remains defined as a disease characterized by enhanced expression of interferon-gamma (47), but more recently there has been evidence to suggest that the origin of this may be from lymphocytes of Th1 and / or Th17 lineage (95); recently, a subset of lymphocytes bearing a Th17 phenotype were identified to be a significant contributor to IFNg-expressing cells in sarcoidosis (96). Other studies have tested serum Th cytokine levels (**Table 3**): two studies have reported raised levels of TNF alpha in patients with sarcoidosis in comparison with healthy controls (75,83), with no significant results about the mean serum levels of interferons (alpha and gamma), IL4 and IL-6 (75).

4. B-cell-related markers

Despite the central involvement of cellular immunity in the pathogenesis of sarcoidosis, several studies have suggested an etiopathogenic role of B cells. In the early non-granulomatous lesions (lymphocytic infiltrates), Th infiltration is often accompanied by the presence of plasma cells and immunoglobulin deposits, suggesting a local hyperactivity of the B-cell immune system (97). In fact, patients with active sarcoidosis have an abnormal peripheral B-cell profile including significantly less circulating $CD27^{+}$ memory B cells and increased circulating transitional B cells and Bregs producing IL-10 in comparison with healthy donors or patients with inactive sarcoidosis (98,99). A recent open-label study has treated 10 patients with 2 doses of 1 g of rituximab 2 weeks apart with improvement in FVC and/or 6-min walk in 7 patients at week 24 (100).

4.1. Lymphocyte count

Three studies have reported a lower white cell count in 81/321 (25%) patients, while lymphopenia has been reported in 40/246 (16%) patients (101–103). Several studies have specifically evaluated the clinical significance of lymphopenia that was associated with a worse prognosis of the disease (101,102), including severe internal organ involvement (104), extrathoracic disease (105), higher disease activity (106) or requirement of GC for severe disease (101). In addition, a significant higher frequency of lymphopenia is reported in patients with sarcoid uveitis (102) or myelitis (107) with respect to patients with other etiologies. From

a therapeutic point of view, Crouser et al (108) have proposed that the presence of CD4(+) lymphopenia may identifiy a specific phenotype particularly responsive to anti-TNF agents, after reporting 5 patients with CD4(+) lymphopenia who improved clinically after being treated with infliximab and that showed a significant increase in absolute peripheral blood lymphocyte and CD4(+) T-cell counts.

4.2. Gammaglobulins

Belhomme et al (109) have reported hypergammaglobulinemia (>13.5g/L) in nearly 40% of patients with sarcoidosis, with a median immunoglobulin level significantly higher in some epidemiological and clinical subsets (BAA patients, extrapulmonary involvement, requiring a treatment or relapsing patients), while lower levels were reported in patients presenting with a Löfgren's syndrome; in this study, immunoglobulin levels improved the capacity of a statistical model to predict relapse. In patients with uveitis, the frequency of hypergammaglobulinemia was nearly 5-fold higher in those with sarcoidosis in comparison with patients presenting uveitis of other etiologies (27% vs 6%) (102).

4.3. b2-Microglobulin

Beta2-Microglobulin is a low-molecular-weight protein produced by all cells (except mature red cells) that is considered a good serum biomarker of lymphocytic activation. In 1982, Parrish et al (110) reported raised levels of beta 2-microglobulin in 63% of patients with sarcoidosis, with no correlation with thoracic stages, duration of disease or requirement of corticosteroid treatment, and further studies confirmed higher levels in comparison with healthy controls. In 1987, Selroos et al (111) measured beta2-microglobulin in the serum of 107 patients with sarcoidosis and found increased levels in patients with acute sarcoidosis, normal or slightly raised levels in those with newly-detected sarcoidosis but without erythema nodosum, and normal levels in patients with chronic active sarcoidosis or in those with resolved sarcoidosis.

4.4. BAFF

B cell activating factor (BAFF) is a cytokine that play a major role in the maintenance of B cell homeostasis. Three recent studies have evaluated the clinical usefulness of measuring serum BAFF levels in patients with sarcoidosis, and found higher levels in patients with active sarcoidosis with a strong correlation with serum hypergammaglobulinemia (98) and with the disease severity score (112), and a higher frequency of skin and eye involvement in patients with elevated serum BAFF (113).

5. Acute phase reactant proteins

The systemic release of pro-inflammatory cytokines (TNF, IL-1, IL-6) by activated immune-related cells (leucocytes, macrophages, monocytes, fibroblasts and endothelial cells) may trigger the so-called hepatic acute-phase response. C- reactive protein and amyloid A are the main acute-phase proteins released by the liver, and several studies have reported high levels of these reactants in patients with sarcoidosis.

5.1. CRP

Serum CRP levels are elevated in 202 (48.9%) out of 413 patients with sarcoidosis included in 3 studies (16,107,114), with a quite homogeneous range of frequencies (47-58%), with the cut-off often used being of 10 mg/L. Raised levels have been associated with fatigue (115), Lofgren syndrome (35,114) and lower functional lung tests (114), with no correlation between serum CRP levels and clinical course (47). CRP levels were useful to confirm sarcoidosis in patients presenting with myelitis (107) and may predict the effectiveness of infliximab in chronic pulmonary sarcoidosis (116). Two studies have also analysed the usefulness of high-sensitivity CRP levels (31,117), reporting higher significant levels with respect to healthy controls, with a high specificity (91%) and lower sensitivity (53%).

5.2. Serum amyloid A

Serum amyloid A (SAA) is an acute-phase reactant related to high-density lipoprotein cholesterol. In sarcoidosis, SAA regulates granulomatous inflammation through Toll-like receptor-2 (118). Several studies have reported significantly higher serum SAA levels in patients with sarcoidosis in comparison with healthy control populations, with a very-high sensitivity rate (96%) and low specificity rates (37-52%) (31,119).

The results with respect to a potential clinical significance og high serum SAA levels are inconsistent, with no correlation with pulmonary involvement (48,120). Some studies reported a correlation with disease activity (50,121), and others showed no correlation with extrapulmonary involvement (50) or with disease progression (48).

5.3. ESR

Two studies have evaluated the usefulness of ESR levels, with higher values in around 40% of patients (17), and higher mean values with respect to healthy controls, but with no correlation with the clinical course of the disease (47).

6. Organ-specific serum biomarkers

6.1. Bronchial epithelium activation markers

Bronchial epithelium may contribute to the initiation and perpetuation of inflammatory processes. Bronchial epithelial cells are able to release a large number of mediators, not only cytokines and chemokines, but also growth factors and other molecules involved in innate immunity processes, which are able to regulate the recruitment, activation, and differentiation of immune cells. Serum levels of several epithelium-related molecules have been tested as potential biomarkers in patients with sarcoidosis.

Krebs von den Lungen 6 (KL-6) is a mucinous glycoprotein expressed on the surface membrane of alveolar and bronchiolar epithelial cells. Raised serum KL-6 levels are reported in a variety of pulmonary diseases such as radiation pneumonitis, drug induced pneumonitis, and interstitial lung disease (122–124). In patients with sarcoidosis, higher serum levels of KL-6 have been reported in comparison with healthy controls (77)

Several studies have demonstrated a close correlation between high KL-6 serum levels and pulmonary involvement, including a positive correlation with CT abnormalities (ground-glass, nodules, septal thickening and traction bronchiectasis) (125), radiological stages II/III (77,122), parenchymal infiltration (48), pulmonary gallium-67 scan uptake, BAL lymphocytosis (122), and progressive disease (48,77) (Table 3). In Japanese patients with uveitis, raised KL-6 levels may help in identifying sarcoidosis from other etiologies of uveitis (126,127), although no correlation with therapeutic response to corticosteroids was reported. KL-6 has demonstrated a weak predictive power to identify disease persistence or progression (48,77).

Raised serum levels of surfactant protein D (SP-D) have been reported in non-smoking patients with sarcoidosis in comparison with healthy controls (77), and in patients with uveitis related to sarcoidosis with respect to patients with uveitis of other etiologies (128). Other studies have reported significantly higher serum levels of a large number of markers in comparison with healthy controls, including ICAM-1 (129), VCAM-1 (130), EGF (75), VEGF and PDGF (131), alpha-defensin (132), cathelicidin (133), ficolin-3 (134), NGAL (135), CD163 (136), tryptase (137) and YKL-40 (138).

6.2. Cardiovascular markers

With respect to cardiac biomarkers, Date et al (139) reported a median plasma BNP levels significantly higher in patients with cardiac sarcoidosis than in those with pulmonary sarcoidosis, while Kiko et al (89) also reported BNP as a useful marker for detecting cardiac involvement in sarcoidosis and high-sensitivity cardiac troponin T (troponin I) as a predictor of fatal arrhythmia. Kandolin et al (140) measured troponin I in 62 patients with new-onset cardiac sarcoidosis (raised levels were found in 53% of patients), and found that left

ventricular ejection fraction was significantly reduced in those with raised troponin I levels, and that raised levels normalized after 4 weeks of glucocorticosteroid therapy; in addition, only 67% of patients with raised levels were free of cardiac events in a two-year follow-up in comparison with 93% of those with baseline normal levels. Baba et al (66) have reported that measurement of troponin I have a sensitivity and specificity of 87.5% and 75.0%, respectively, to detect active cardiac involvement confirmed by 18F-FDG PET findings. Finally, two studies have reported raised serum D-dimer levels in 20 (34.5%) out of 58 patients with sarcoidosis (141,142).

7. EXPERT COMMENTARY

7.1. Reliability and validity of diagnostic tests in sarcoidosis

There has been a long controversy about the limited reliability and validity of serum biomarkers as diagnostic tests for sarcoidosis (5). Reliability and validity are measures of diagnostic test performance, but they have separate and distinct meanings. Reliability (i.e., reproducibility or precision) refers to the capacity of a diagnostic test to give the same result on repeated measurements, and depends on the method of measurement and the variability in the specific disease on which the test is applied (143). In sarcoidosis, reliability of the most frequently tested serum biomarkers is poor, with a wide heterogeneity of techniques and cut-offs reported for these markers. The lack of a standardized international recommendations does not allow a homogeneous interpretation of those studies testing for a specific biomarker. In contrast to reliability, validity (i.e., accuracy) is the degree to which the data measure what they are intend to measure when compared with a confirmatory diagnostic test (often known as ‘‘gold standard’’) classifying subjects into a dichotomous category (diseased versus non-diseased) (143). In a diagnostic process, validity is described in terms of sensitivity (i.e., the rate of subjects with the disease who have a positive test) and specificity (i.e., the rate of those without the disease who have a negative test) (143). In sarcoidosis, the lack of an adequate “gold-standard” test complicates the analysis of any diagnostic test in terms of sensitivity and specificity. In addition, most studies measure sensitivity and specificity of a specific serum biomarker in sarcoidosis with respect to the values observed in healthy controls, an approach that although is methodologically correct, is not useful in daily practice in which biomarkers are mainly required for distinguishing between sarcoidosis and other similar diseases. Very few studies have included a control population with other similar diseases to sarcoidosis, not only pulmonary but also systemic diseases.

In the last 40 years, the usefulness of serum biomarkers in diagnosing sarcoidosis has been measured as the ability of a single biomarker to confirm the disease, a diagnostic approach

never applied in other complex systemic autoimmune diseases. Biomarkers should be considered a support for a suspected diagnosis of sarcoidosis, taking always into account the clinical context where we are searching for the diagnosis. Although the ideal diagnostic test should be both highly sensitive and highly specific, this is not usual in single markers testing complex diseases, as increasing sensitivity rate decreases specificity, and viceversa; this is the reason why these heterogeneous diseases are always diagnosed using a combination of various features (classification criteria); in addition, different clinical scenarios may require tests with different sensitivity and specificity rates (143). In the diagnostic workup of a patient with a suspected sarcoidosis, high sensitivity tests will be especially useful due to their highest ability to correctly classify the suspected individual as 'diseased', together with tests with high positive predictive values (PPV, percentage of patients with a positive test who actually have the disease) (144). In contrast, tests with high specificity and NPV will be useful mainly to discard sarcoidosis in some complex severe involvements in which a biopsy cannot be performed (i.e., cardiac and neurosarcoidosis) (**Figures 1 & 2**).

7.2. Multiple measurement of biomarkers

When raised levels of a specific biomarker for sarcoidosis (usually raised ACE levels) are accompanied by several other clues that physicians look for in diagnosing the disease, it may be a strong indication to consider sarcoidosis as a reasonable diagnostic option. But probably it could be more useful to test for a panel combining multiple markers than not testing a unique marker. There is a large variety of available markers from different cellular origin and with a differentiated balance between specificity and sensibility, and a simultaneous multiple measurement probably will cover better the diverse clinicopathological scenarios that may arise at diagnosis of sarcoidosis. Few studies have combined more than one serum biomarker. In 1987, Selroos and Klockars (111) measured serum concentrations of ACE and b2-microglobulin in patients with sarcoidosis in different clinical stages and found that b2-Microglobulin levels correlated better with early disease stages (granuloma formation) and ACE values with later etiopathogenic phases (granulomatous disease). In 2011, Mostard et al (70) measured ACE, sIL-2R and neopterin and found that sensitivity of combined serological biomarkers for the presence of inflammatory activity as detected by PET was 80% and specificity 100%. In a recent study in patients with uveitis, Groen-Hakan et al (28) tested 249 patients for detecting underlying sarcoidosis using ACE and sIL-2R serum levels, and found that the combined measurement of the two markers was not superior to the use of each marker individually in terms of validity; interestingly, sensitivity of chest X-Ray (56%) was quite similar to that of ACE (54%) and clearly lower than sIL-2R levels using a cut-off of 2500 pg/mL (92%).

Beirne et al (145) measured 30 circulating biomarkers in 20 patients with systemic sclerosis and 21 with sarcoidosis, and found raised levels of IL-1, -6, -8, TNF-RI, TNF-RII and growth factors EGF and HGF in both diseases, although only Th1 chemokines (IP-10, MIG, MIP-1α, MIP-1β and RANTES) were specifically raised in sarcoidosis. Loza et al (146) carried out a 92-analyte multiplex panel to assess the expression of serum proteins in 134 sarcoidosis patients compared with sera from 50 healthy controls, and found 29 markers significantly elevated in sarcoidosis, including chemokines, neutrophil-associated proteins, acute-phase proteins, and metabolism-associated proteins (CD40L, brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), CC-chemokine ligand 5/RANTES, myeloperoxidase, TNF-α levels, MIP-1β and ENA-78 (epithelial-derived neutrophil-activating protein 78). Those patients expressing the highest levels of TNF-α were those who had more severe disease and that following infliximab treatment, had the greatest improvement in pulmonary functional tests together with a reduction in serum levels of the inflammatory proteins MIP-1β and TNF-RII.

7.3. Clinical staging and therapeutic response

There is no current information about which or how many biomarkers are needed to characterize a specific clinical pattern, but some studies have evaluated certain molecules as early markers of progression toward chronic refractory disease, as predictors of disease relapse, or as markers of therapeutic response. The most solid association linked raised serum CTO and KL-6 levels with pulmonary involvement. With respect to the usefulness of serum biomarkers for predicting disease progression, the results were inconclusive for ACE and sIL-2R, since the number of studies reporting a significant correlation was similar to those that reported non-significant associations (Table 4); in contrast, most studies testing CTO (59,63) showed a good correlation with disease outcomes, including the prediction of relapses (63). With respect to therapeutic response, most studies showed the usefulness of measuring serum levels of ACE, chitotriosidase, sIL-2R and lysozyme (Table 4).

7.4. Phenotyping systemic disease

Another key message for daily practice is that the diagnostic validity of a specific biomarker may vary according to the clinical phenotype of sarcoidosis. Some studies have reported different validity rates of the same test in patients with a specific organ involved (ocular sarcoidosis) with respect to the values obtained in unselected populations of patients with sarcoidosis. In addition, some biomarkers seems to have a better ability in detecting sarcoidosis involving isolated organs (as has been reported for sIL-2R in pulmonary and cardiac sarcoidosis) (87,89), or are specifically increased in those patients with a systemic presentation

(three or more different organs involved) as has been reported for ACE, lysozyme and IL-12 (14,81). Even in patients with the same organ involved, different validity rates have been reported for the same biomarker among the different organ-specific types of involvement: in patients with uveitis, Niederer et al (25) have reported the highest sensitivity rate for ACE (82%) in patients presenting with intermediate/panuvetis, while the highest specificity rate (93%) was reported for those with posterior uveitis. Unfortunately, the association between raised levels of serum biomarkers and extrapulmonary or systemic sarcoidosis is unclear for the main markers (ACE, CTO, lysozyme and SAA), and only raised serum sIL-2R levels have been related to extrapulmonary involvement, not only for multiorgan disease but also for organ-specific involvements (skin, lymph nodes or spleen) (Table 4).

8. 5-YEAR VIEW

Sarcoidosis is a complex systemic disease with a silent, long-term pathological evolution, and a wide, heterogeneous clinical presentation. A large list of studies has tried to delineate clinical phenotypic subgroups that could predict the outcome of an individual patient and, therefore, to help the physician to decide specific diagnostic and therapeutic approaches. Considering the complexity and heterogeneity of sarcoidosis, "omics" and systems biology (147) may be future useful approaches to elucidate the biological mechanisms underlying the different disease phenotypes, and therefore, to identify more effective disease biomarkers (148). A recent review has analysed the application of these approaches to sarcoidosis research, including not only genome-wide association studies (GWASs), but also transcriptomic, proteomic, metabolomic and microbiomic studies (148). Several genetic studies have evaluated numerous gene expression signatures, while other studies have measured the whole blood transcriptome and the transcriptome of tissues. These studies have confirmed the predominant Th1 response in sarcoidosis and particularly the key role of interferon-γ (IFN-γ) and type I IFN-driven signaling pathways (149), including significant differences in enrichment of the interferon pathway (150), but have also identified new molecular mechanisms involving Tregs (reduced suppression activity), increased apoptosis or TLR-2 signaling inhibition pathways (151). The role of histopathological staining is also under investigation, and a recent study have reported that SAA staining of sarcoidosis granulomas has an overall specificity of 84% but with a low sensitivity (44%) (152).

Metabolic changes may also play a role in perpetuation of granulomatous inflammation in sarcoidosis. Geamanu et al (153) have used ^{1}H nuclear magnetic resonance (NMR)-based untargeted metabolomic analysis, and after the application of integrative pathway analyses, the authors identified deregulation of butanoate, ketone bodies, citric cycle, and

transmethylation metabolites, molecules that could be tested in further studies as potential biomarkers. With respect to microbiomic studies, a cross-sectional study compared the lung microbiota of 71 patients with sarcoidosis, and found that *Atopobium* spp and *Fusobacterium* spp were significantly more frequent in samples of patients with sarcoidosis in comparison with samples from healthy controls, with mycobacteria being found in only two of sarcoidosis samples (154). In contrast, a recent study using metagenomic sequencing have reported enrichment of microbes in sarcoidosis samples but with a limited concordance across sample types (155).

9. CONCLUSIONS

Diagnosis of sarcoidosis must be always approached as a multistep process based on a case-by-case integration of clinical, radiological, histological and serological data, none of which being pathognomonic in and of itself. Many different mediators, such as cytokines, chemokines, and other proteins with various functions, are involved in its complex pathogenesis and some have been proposed as potential biomarkers. This review has been centered on serological biomarkers, although there is a large number of studies that have evaluated other fluids (overwhelmingly the BAL fluid, because lungs are the most frequently involved organ). However, the analysis of biomarkers in serum would be preferable, because they are less invasive than BAL to obtain, and because nearly one third of patients with sarcoidosis do not have pulmonary involvement. Unfortunately, and in spite of the large number of studies published, the low level of evidence (most studies are retrospective and cross-sectional studies) together with a wide methodological heterogeneity (serum cut-off levels, control populations, inclusion criteria, definition of the main outcomes, etc.) resulted in inconsistent findings that do not allow to offering solid clinical recommendations, although previous reviews have reported some subjective qualitative scores (5,6)). As a summary, we found sIL-2R, CRP, SAA and chitotriosidase as the best markers to confirm sarcoidosis (highest sensitivity), while ACE, gammaglobulins and lysozyme may be more useful for discarding sarcoidosis in complex cases (highest specificity), taking into account that with the use of a higher cut-off we can increase the specificity and with a lower cut-off we can increase the sensitivity of a diagnostic test. About prognosis, sIL-2R and chitotriosidase are probably better prognostic markers in comparison with ACE [10,35], although head-to-head comparisons are limited (Table 5) (32,35,48,50,51,59). Other mediators, such as TNF-a and CCL18, could help to identify patients with an enhanced risk of developing pulmonary fibrosis or progressive disease [64,96]. However, proper validation in large cohorts of patients is required for most biomarkers [13]. The future scenario of the serological diagnostic approach of sarcoidosis will

be the use of multi-assays including biomarkers from different cellular sources, with a case-by-case interpretation of the different sensitivity and specificity values of each test according to the clinical presentation and phenotype of the patient.

KEY POINTS

- Reliability of the most frequently tested serum biomarkers is poor (wide heterogeneity of techniques, cut-offs, outcome definition).
- The diagnostic validity of a specific biomarker may vary according to the clinical phenotype.
- sIL-2R, CRP, SAA and chitotriosidase may be good markers to confirm sarcoidosis (high sensitivity)
- ACE, gammaglobulins and lysozyme may be more useful for discarding sarcoidosis in complex cases (high specificity)
- Biomarkers should be viewed as a support for a suspected diagnosis of sarcoidosis (not as a pathognomonic test)
- Most studies testing chitotriosidase showed a good correlation with disease outcomes and prediction of relapses
- Measuring serum levels of ACE, chitotriosidase, sIL-2R and lysozyme may be useful for evaluating therapeutic responses
- sIL-2R levels correlate with extrapulmonary/systemic sarcoidosis.

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Figure legends

Figure 1. Sensitivity (SE), specificity (SP), positive predictive values (PPV) and negative predictive values (NPV) in studies testing different biomarkers in patients with unselected sarcoidosis.

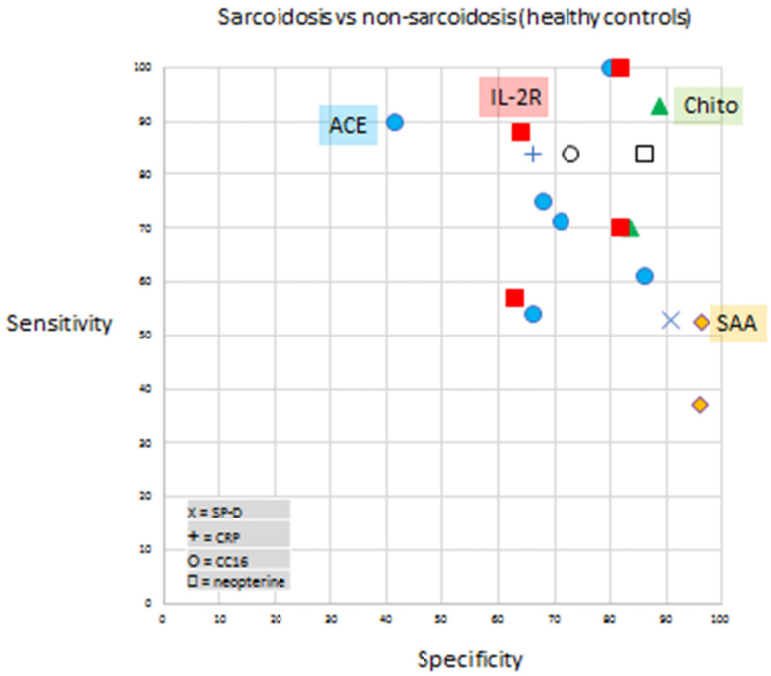

Figure 2. Sensitivity (SE), specificity (SP), positive predictive values (PPV) and negative predictive values (NPV) in studies testing different biomarkers in patients with ocular sarcoidosis.

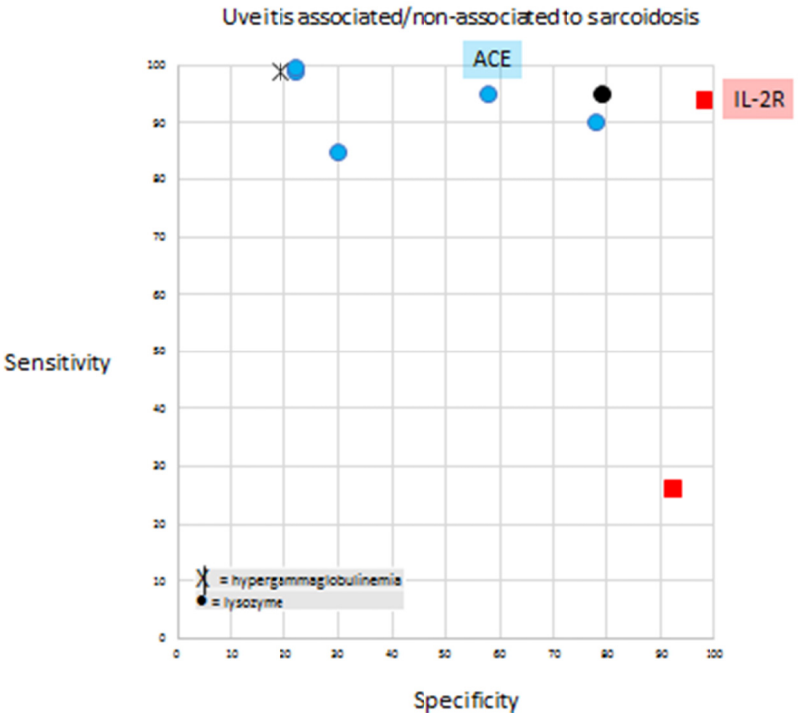

TABLE 1. Large studies (including more than 50 patients) testing serum ACE in patients with sarcoidosis.

Author (year)	Reference	Country	Clinical sarcoidosis phenotype	Patients with sarcoidosis (n)	Raised levels (n)	Frequency (%)	Cut-off
Loddenkemper et al (1998)	12	Germany	Unselected	715	443	61.96	NA
Doubkova et al (2015)	13	Czech	Unselected	306	124	40.52	68 U/L
Ungprasert et al (2016)	20	US	Unselected	251	104	41.43	ND
Gupta (2002)	19	India	Unselected	200	141	70.5	ND
Maña et al (1999)	21	Spain	Löfgren	186	71	50.35	ND
Kahkouee et al (2016)	22	Iran	Unselected	148	78	52.7	68 U/L
Gillman et al (2007)	23	Australia	Unselected	122	58	50.88	ND
Vorselaars et al (2015)	24	The Netherlands	Unselected	114	47	41.23	62 U/L
Niederer et al (2018)	25	UK	Ocular	110	85	77.27	52 U/L
Sejdic et al (2018)	26	Denmark	Unselected	101	48	47.52	52 U/L
Febvay et al (2015)	27	France	Ocular	83	50	60.24	62 U/L
Nguyen et al (2017)	14	Japan	Unselected	72	16	43.24	21 U/L
Kawaguchi et al (2007)	15	Japan	Ocular	67	35	58.33	ND
Thelier et al (2008)	16	France	Rheumatological	57	23	40.35	52 U/L
Leonhard et al (2016)	17	The Netherlands	Neurosarcoidosis	52	18	43.9	70 U/L
Khan et al (1998)	18	Pakistan	Unselected	51	44	86.27	52 U/L

TABLE 2. Sensitivity (SE), specificity (SP), positive predictive values (PPV) and negative predictive values (NPV) in studies testing serum ACE levels in patients with sarcoidosis.

Author (year)	Reference	Country	Sarcoidosis phenotype (patients tested)	Controls (patients tested)	Cut-off	SE	SP	PPV	NPV
Groen-Hakan et al (2017)	28	Netherlands	Uveitis associated with sarcoidosis (n=37)	Uveitis unassociated with sarcoidosis (n=212)	51 U/L 68 U/L	54 30	70 85	24 26	90 87
Gundlach et al (2016)	29	US	Uveitis associated with sarcoidosis (n=41)	Uveitis unassociated with sarcoidosis (n=220)	82 U/L	22	99	90	87
Kawaguchi et al (2007)	15	Japan	Uveitis associated with sarcoidosis (n=60)	Uveitis unassociated with sarcoidosis (n=86)	Not detailed	58	95	90	77
Niederer et al (2018)	25	UK	Uveitis associated with sarcoidosis (n=110)	Uveitis unassociated with sarcoidosis (n=925)	52 U/L	77	88	43	97
Bons et al (2007)	30	Netherlands	Unselected (n=35)	Healthy (n=35)	16.5 U/L	71	71	nd	nd
Ungprasert et al (2016)	20	US	Unselected (n=251)	Healthy (n=3016)	Nd	41	90	25	95

Rothkrantz-Kos et al (2003)	31	Netherlands	Untreated nonsmokers (n=73)	Healthy (n=282)	21 U/L	68	75	54	84
Khan AH,	18	Pakistan	Unselected (n=51)	TBC/other lung conditions (n=62)	52 IU/L 100 IU/L	86 37	61 93	65 83	84 64
Popevic et al (2016)	32	Serbia	Active (n=230)	Inactive (n=199)	32 U/L	66	54	nd	nd

ND: not determined

TABLE 3. Other serum biomarkers tested in at least two different case-control studies: number of studies with significant results in comparison with control

Serum biomarker	Cellular source	Number of studies	Patients tested (n)	Number of studies with significant differences (n, %)	Control populations
Chitotriosidase	M-M	5	509	5 (100%)	Healthy controls, other pulmonary diseases
Lysozyme	M-M	2	148	1 (50%)	Ocular diseases, healthy controls
CXCL10	M-M	5	237	5 (100%)	Healthy controls
CXCL9	M-M	3	101	2 (67%)	Healthy controls
IL-10	M-M	3	141	1 (33%)	Other cardiac diseases, healthy controls, other diseases
IL-12 p40	M-M	2	105	2 (100%)	Asthma, healthy controls
IL-18	M-M	3	160	3 (100%)	Healthy controls
TNF-alpha	M-M, T cells	2	133	2 (100%)	Healthy controls
sIL-2R	T cells	4	179	3 (75%)	Healthy controls
BAFF	B cells	3	158	3 (100%)	Healthy controls
Lymphopenia	B cells	4	200	4 (100%)	Uveitis, myelitis, healthy controls
CRP	Liver	6	491	5 (80%)	Healthy controls, other pulmonary diseases
SAA	Liver	3	168	3 (100%)	Healthy controls
KL-6	Epithelium	3	139	3 (100%)	Uveitis, healthy controls
CC16	Epithelium	2	196	2 (100%)	Healthy controls
SP-D	Epithelium	2	160	2 (100%)	Healthy controls
ICAM-1	Endothelium	2	46	1 (50%)	Healthy controls
GM-CSF	Endothelium	2	155	0 (0%)	Miscellaneous population

M-M: monocyte-macrophage system

Table 4. Summary of the results obtained for the main serum biomarkers in sarcoidosis: correlation with pulmonary and extrapulmonary involvement, therapeutic response and outcomes (references)

		Pulmonary involvement	Extrapulmonary involvement	Therapeutic response	Outcomes
ACE levels	Correlation	Parenchymal involvement (14) Stages I/II vs 0 (38,51)	BHL predictor (14) Stage I vs II/III (33) ↓ in Lofgren (35) Normal in EN (34) Extrathoracic manifestations (34) > 3 involved organs (14) Severe or systemic cardiac involvement (89)	↓ after Cs (36-39,41) ↓ after CQ (41) ↓ after IFX (42,43)	Progressive/chronic disease (22,39,92) Active disease (32,33,44)
	No correlation	Parenchymal involvement (48) Radiological stages (32,87) PFT (47)	Extrapulmonary involvement (32,38,50) Number of organs involved (32)	ND	Progressive/chronic disease (32,35,46-48,51,59) Active/acute disease (49,50) Disease duration (32)
CTO levels	Correlation	Stages I/II/III vs 0 (32) Stage III (59) PFT (63)	Extrapulmonary involvement (60)	↓ after Cs (59,60) ↓ after adding ID (59)	Active disease (32,60) Chronic disease (59) Clinical/radiological/PFT worsening (63) Predicting relapses (60) Clinical outcome status (32)

					Disease duration (32)
	No correlation	ND	Extrapulmonary involvement (32) Number of organs involved (32)	ND	Chronic disease (32)
Lysozyme levels	Correlation	Parenchymal involvement (14,34,48)	Extrathoracic involvement (34)	↓ after Cs (37,39)	ND
	No correlation	ND	> 3 involved organs (14) BHL (14)	ND	Progressive disease (48)
sIL-2R levels	Correlation	Stage II vs I (14) Stage I/II vs III (87) Parenchymal involvement (14,48)	BHL (14) EN (87) > 3 involved organs (14) Extrapulmonary disease (42,87) Spleen activity (88) Systemic cardiac sarcoidosis (89)	GC requirement (90) ↓ after Cs (91) ↓ after IFX (42,43)	Progressive disease (35,48,86) Need prolonged therapy (51) Active/acute disease (51,90)
	No correlation	PFT (47,87) Radiological stages (51)	ND	GC response (87)	Progressive/chronic disease (47,87,93) Active disease (50)
SAA levels	Correlation	ND	ND	ND	Clinical outcome status (120) Active disease (50,121)
	No correlation	PFT (120) Parenchymal involvement (48)	Extrapulmonary disease (50)	ND	Progressive disease (48)

KL-6 levels	Correlation	Parenchymal involvement (48) Stages II/III vs I (77) Stages II/III vs 0/I (122) PFT (77) Ground-glass opacity, nodules, interlobular septal thickening, traction bronchiectasis, architectural distortion and bronchial wall thickening (125)	ND	ND	Progressive disease (48,77) Active disease (122)
	No correlation	ND	BHL (125)	ND	ND

PFT: pulmonary functional tests; BHL: bilateral hilar lymphadenopathies; Cs: corticosteroids; IFX: infliximab; CQ: chloroquine; EN: erythema nodosum; ND: no data

Table 5. Summary of the results obtained in the main studies including a head-to-head comparison between several serum biomarkers in sarcoidosis (YES = positive correlation, NO = lack of correlation, nd = not analysed)

OUTCOME	AUTHOR (year)	Reference	SERUM BIOMARKERS						
			ACE	rs-IL2	CTO	SAA	Neopterin	Lysozyme	KL-6
Activity	Gungor et al (2015)	50	NO	NO	nd	YES	nd	nd	nd
	Popevic et al (2016)	32	YES	nd	YES	nd	nd	nd	nd
Extrapulmonary disease	Gungor et al (2015)	50	NO	YES	nd	NO	nd	nd	nd
	Popevic et al (2016)	32	NO	nd	NO	nd	nd	nd	nd
Progression	Prasse et al (2008)	51	NO	YES	nd	nd	YES	nd	nd
	Miyoshi et al (2010)	48	NO	YES	nd	NO	nd	YES	YES*
	Ziegenhagen et al (2003)	35	NO	YES	nd	nd	YES	nd	nd
	Bargagli et al (2013)	59	NO	nd	YES	nd	nd	nd	nd

*Statistically-significant in multivariate analysis