Chapter 9

Laboratory Investigations and Immunological Testing in Sarcoidosis

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Additional information is available at the end of the chapter

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1. Introduction

Sarcoidosis is a systemic granulomatous disease of unknown aetiology, which can affect virtually any organ and is thus characterised by a variable clinical presentation and course. The disease is generally considered to be a T helper-1 (T_H1) type of reaction, although T_H2 and T_H17 features have also been identified. Approximately 90% of patients demonstrate disease involvement of the lungs and thoracic lymph nodes and although sarcoidosis is usually subacute and self-limiting, progressive inflammation can lead to pulmonary fibrosis and death. Despite these features, there is currently no definitive single laboratory investigation used to identify sarcoidosis, indicating the need for improved understanding of the immunopathogenesis and identification of disease-specific biomarkers. Currently, sarcoidosis is generally a diagnosis of exclusion that is best confirmed by clinical and radiological findings and tissue biopsies revealing non-caseating granulomas in the absence of known granulomagenic agents. Laboratory testing is nonetheless beneficial in further supporting a diagnosis of sarcoidosis and assessing disease severity.

In this chapter, we focus on the laboratory and immunological testing used in sarcoidosis, including biomarkers that have been proposed as measures of the immunological response, as well as cellular markers present in blood and bronchoalveolar lavage. Comparisons will also be made with older immunological investigations including the Kveim-Siltzbach test and recent evidence of potential sarcoid antigens. Novel methods of sampling disease biomarkers, including the technique of exhaled breath analysis will be explored. Immunological testing and measurement of various biomarkers in body tissues has been a useful research tool in understanding sarcoid pathophysiology. There may be a useful role for some of these labora-

tory investigations as future clinical tools, improving diagnostic sensitivity and identifying novel targets for treatment [1].

2. Serum chemistries and other initial investigations

The diagnosis of sarcoidosis is based on a compatible clinical and radiological picture, histological evidence of non-caseating granulomas and exclusion of other diseases which show a similar clinical or histological picture [2]. The clinical, radiological and histological features of sarcoidosis are discussed elsewhere in this book. The recommended initial clinical and laboratory investigations for a patient suspected of having sarcoidosis are listed in Table 1. The rationale for these investigations is to detect frequent manifestations of the disease, as well as identification of serious, although rare complications of sarcoidosis such as cardiac disease.

	Routine testing	Additional testing
Peripheral blood	Peripheral blood counts: white blood cells, red blood cells, platelets	Angiotensin-converting enzyme (ACE)
	Serum chemistry: calcium, creatinine, renal function, liver enzymes, CRP, ESR	Glucose
Radiological procedures	Chest radiography	High resolution CT scan
Pulmonary function studies	Spirometry	Lung volumes and diffusing capacity of carbon monoxide. Six-minute walk with oximetry
Other testing	Urinalysis	24-hour urinary calcium
	Electrocardiogram (ECG)	
	Routine ophthalmologic examination	
	Tuberculin skin test	IFN-γ release assays
	Other tests depending on clinical manifestations and suspicion of specific organ involvement	

Table 1. Recommended initial clinical and laboratory investigations of patients with suspected sarcoidosis. *Source*: reference [11].

Simple baseline blood tests are useful to identify the presence and severity of specific organ involvement, including hepatic or renal impairment. Peripheral blood lymphopenia is a common finding in patients with sarcoidosis [3], as the activated T cells accumulate at the sites of granulomatous inflammation [4]. This may contribute to the systemic immunological abnormalities observed in sarcoidosis, with exaggerated local immune responses, but suppressed delayed-type hypersensitivity (DTH) skin tests and peripheral blood immune responses [5-7]. Other haematological abnormalities such as neutropenia or auto-immune

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haemolytic anaemia and/or thrombocytopenia appear very infrequently [8]. Approximately 10-20% of all patients with sarcoidosis have elevated serum aminotransferase and alkaline phosphatase levels [2, 9]. A cholestatic picture of hepatic impairment from granulomatous cholangitis can also occur with a syndrome of pruritis and jaundice. Hepatic failure or portal hypertension can also develop, although liver involvement is usually clinically silent [10].

Although renal disease is uncommon, increased serum creatinine and urea levels can reflect renal impairment related to chronic hypercalcemia, hypercalciuria, nephrolithiasis, nephrocalcinosis or granulomatous interstitial nephritis [12-14]. Hypercalciuria is observed in over 40% of patients with sarcoidosis and hypercalcemia in 5-10% of cases and occurs mainly in males over 40 years of age [8, 10]. Hypercalcemia and hypercalciuria in sarcoidosis are attributed to increased levels of serum 1,25-dihydroxyvitamin D_3 (also known as calcitriol), which increases serum calcium levels via increased intestinal calcium absorption and osteoclastic bone resorption. The kidney is normally the only organ that can hydroxylate vitamin D_3 to its biologically active form of 1,25-dihydroxyvitamin D_3. Sarcoid macrophages have also been shown to posses the enzyme 25-hydroxyvitamin D_3-1α-hydroxylase, which converts 25-hydroxyvitamin D_3 to its active form, which is produced in excess in sarcoid granulomas [12, 15, 16] and is not inhibited by normal negative feedback from hypercalcemia [15]. Alveolar macrophages have been shown to be the source of excess calcitriol in sarcoidosis, through elevated mRNA expression of the 25-hydroxyvitamin D_3-1α-hydroxylase gene [17]. It has been found that even anephric patients with sarcoidosis can develop hypercalcemia, through this alternative extrarenal source of the hydroxylase enzyme to create calcitriol [18]. Hypercalciuria is common in sarcoidosis and results from an increased calcium load filtered at the glomerulus, along with suppression of parathyroid hormone secretion by calcitriol, which diminishes renal tubular calcium reabsorption [12]. In an evaluation of 736 newly diagnosed sarcoidosis patients in the United States, 3.7% of all patients had abnormalities with calcium metabolism, with hypercalcaemia being more common in Caucasians than African Americans [9]. Therefore monitoring of serum calcium, as well as 24-hour urinary excretion of calcium should be measured in all patients with sarcoidosis [12]. As chronic hypercalcemia is a common and treatable cause of renal failure in sarcoidosis, it is important not to miss this complication. Hypercalcemia and increased calcitriol have also been described in infectious granulomatous disorders [19-22], again resulting from abnormal extrarenal metabolism of vitamin D_3.

The erythrocyte sedimentation rate (ESR) and the acute phase reactant C-reactive protein (CRP) have been used as nonspecific markers of inflammation in a wide variety of diseases. They are simple initial investigations for assessing the severity of systemic inflammation. In sarcoidosis very high levels of ESR and CRP have been observed in some patients with active disease [23]. The ESR level is more likely to be increased in patients with arthritis [24] and in those with erythema nodosum [25] than for other manifestations of the disease. CRP has been found to be associated with fatigue in sarcoidosis [26]. Levels of CRP are generally lower in patients with sarcoidosis compared with tuberculosis [27] and CRP measurement is less sensitive and specific for sarcoidosis compared to ACE [28].

3. Lymphocytic aspects, cytokines and chemokines in sarcoidosis

Sarcoidosis is characterised by an "immune paradox" of exaggerated T_H1 lymphocyte processes causing localised inflammation, although there is peripheral anergy to common antigens [29]. In patients with sarcoidosis total numbers of peripheral blood lymphocytes are normal or slightly reduced, but at disease sites there is a marked increase characterised by ratios of CD4+ to CD8+ T-cells ranging between 3.5:1 to 15:1 in about 50% of cases, compared to normal ratios of 2:1 [1, 30]. Peripheral anergy in sarcoidosis as displayed by suppression of delayed-type hypersensitivity (DTH) responses may be explained by expansion of a subgroup of $CD25^{bright}$ $FOXP3^+$ regulatory T-cells (Treg) in active sarcoidosis [6, 29]. The initial stimulus which induces local inflammation arises when an unknown insoluble antigen is presented on MHC class II molecules to CD4+ T_H1 lymphocytes. This leads to exaggerated activation and clonal proliferation of these lymphocytes, which produce increased amounts of interleukin-2 (IL-2), a local growth, survival and differentiation factor for T-lymphocytes [31]. These lymphocytes also release interferon-γ, together with cytokines and chemokines produced by mononuclear phagocytes (namely TNF-α, IL-12, IL-18, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP1α)) into the local milieu [1, 31), which leads to activation of blood monocytes that form non-caseating granulomas. The likely outcomes following granuloma formation are either resolution or fibrosis, which may be dependent on predominance of T_H1 or T_H2 T cell responses respectively.

T_H1 cytokines including IFN-γ promote granulomatous inflammation and inhibit fibrosis development, with Bronchoalveolar lavage (BAL) fluid IFN-γ levels being inversely related to progression to pulmonary fibrosis and are higher than BAL IFN-γ levels in healthy controls [1]. T_H2 type cytokines (e.g. IL-4, IL-5, IL-10, IL-13) and macrophage derived factors including fibronectin, platelet-derived growth factor, insulin-like growth factor-1 (IGF-1) and transforming growth factor-β_1 (TGF-β_1) promote fibroblast proliferation leading to either healing or progressive fibrosis [10]. The IL-17 producing T_H17 cells, considered developmentally distinct from T_H1 and T_H2 cells, have also recently been implicated in the pathogenesis of sarcoidosis. T_H17 cells are associated with autoimmune disease processes, granuloma formation and have a role in host defence against extracellular pathogens [32]. Recent findings from flow cytometry indicate that there are increased IL-17^+ and IL-$23R^+$ peripheral blood and BAL CD4+ T-cells from patients with active sarcoidosis compared to those with inactive disease or healthy controls and increased IL-17 and IL-23R expression in lung and lymph node specimens [33]. This was also confirmed with an increased presence of IL-$17A^+$, IL-$17A^+$ IFN-γ^+ and IL-$17A^+$ IL-4^+ memory T-cells in peripheral blood and BAL of patients with sarcoidosis and increased IL-22^+ cells in granuloma containing biopsies [34]. Gene profiling studies using sarcoid skin biopsies have also showed upregulated T_H1 and T_H17 gene expression along with increased IL-23 and IL-23R expression in patients with sarcoidosis compared with healthy volunteers [35]. Other groups found conflicting results using enzyme-linked immunospot (ELISPOT) assays [36], as well as finding reduced IL-17A gene expression in BAL CD4+ T-cells in patients with Löfgren's syndrome compared to controls [37]. These data indicate that the T_H17 subset may have a systemic role in active non-Löfgren's disease and may be involved in disease progression [1].

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Some of the principal cytokines and chemokines involved in sarcoidosis are summarised in Table 3. Sarcoidosis is also characterised by a polyclonal hypergammaglobulinaemia and circulating immune complexes, which is observed in 20-80% of cases. This may result from non-specific B-cell activation by activated T-helper lymphocytes in lymphoid organs [38].

Monocytes/macrophages	Lymphocytes
ACE	IL-2, IFN-γ
Lysozyme	TNF-α, TNF-β
Neopterin	TGF-β
Chitotriosidase	IL-6, IL-10, IL-12, IL-17
TGF-β	CCL-5
TNF-α, IL1-1β, IL-12, IL-18	GM-CSF
CCL2, CCL3, CCL4, CCL10, CCL18	
CXCL10	
GM-CSF	

Table 2. Summary of key cytokines, chemokines and factors expressed by activated lymphocytes and macrophages in the pathogenesis of sarcoidosis, which have be measured in biological samples. *Abbreviations*: CCL: C-C motif ligand; CXCL: C-X-C motif ligand; GM-CSF: granulocyte macrophage colony stimulating factor.

3.1. Lymphocyte markers: Soluble IL-2 receptor (sIL-2R)

Lung T-cells from patients with pulmonary sarcoidosis express both early and late activation cell surface markers, with IL-2R (CD25) being one of the most widely studied. The soluble form of the IL-2 receptor (sIL-2R) is a T-cell receptor for IL-2, which is used to monitor graft rejection after solid organ transplantation and can be elevated in a number of conditions including infection and autoimmune disease [39]. Its concentrations are elevated and easily detectable in the serum and BAL of patients with sarcoidosis and arises as a result of increased numbers and enhanced activation of macrophages and T-cells from granulomatous inflammation [40, 41]. In some studies, elevated serum sIL-2R falls during therapy or with spontaneous remission [42, 43]. Recent studies indicate that sIL-2R may have prognostic value as a marker of disease activity as the levels are significantly higher in patients with active sarcoidosis compared with inactive disease, correlating with BAL CD4+ T-cell numbers [41, 44]. Patients with extrapulmonary sarcoidosis excluding Löfgren's syndrome have also demonstrated greater serum sIL-2R levels compared to those with isolated pulmonary involvement [41], with sIL-2R appearing to be an independent marker for worse disease. sIL-2R has been compared with serum CRP, serum amyloid A and ACE activity indicating that only sIL-2R was predictive of sarcoidosis severity and could be used for patient follow-up [23].

3.2. β_2-microglobulin

β_2-microglobulin is a low molecular weight protein and a marker of lymphocyte activation. It has been described in a variety of infectious, inflammatory and neoplastic diseases and is used to monitor patients with lymphoma. Various studies have identified that approximately 25% of patients with sarcoidosis have elevated serum β_2-microglobulin concentrations [30, 45, 46]. Initial findings in sarcoidosis patients indicated that the levels were elevated at the time of diagnosis, rose during relapse and fell with corticosteroid therapy [47]. In a study of 107 patients with sarcoidosis, β_2-microglobulin levels were found to correlate with granuloma formation in the initial phases, whilst ACE activity reflected later phases. It was also noted that in patients with acute sarcoidosis and erythema nodosum, β_2-microglobulin was elevated and ACE was usually normal [46]. Another study of 132 sarcoidosis patients did not find an association between β_2-microglobulin and ACE [45], indicating that lymphocyte and macrophage activation are not always concurrently present. This is one limitation of this marker in that it only assesses lymphocyte activation, compared to sIL-2R which reflects both macrophage and lymphocyte activation. β_2-microglobulin concentrations have also been measured in the cerebrospinal fluid (CSF) and were found to be elevated in 68% of patients with neurosarcoidosis, although it was not elevated in patients who did not have neurological involvement [48]. As it has low specificity and sensitivity, serum β_2-microglobulin has limited use in clinical practice.

4. Immunological studies of alveolitis: BAL and induced sputum

BAL fluid analysis is a useful investigation for the diagnosis of pulmonary sarcoidosis by detecting a lymphocytosis with elevated ratios of CD4+/CD8+ cells, typically >3.5:1, in the absence of other causes [1]. BAL lymphocytosis with elevated CD4/CD8 ratios, normal percentages of eosinophils and neutrophils and the absence of plasma cells suggest a diagnosis of sarcoidosis. Cellular analysis of T-lymphocyte subsets and cytokine levels from BAL fluid and peripheral blood using flow cytometry have been compared and can provide useful diagnostic information on sarcoid alveolitis. Costabel et al. reported on the clinical utility of BAL CD4/CD8 ratios in the diagnosis of sarcoidosis. Ratios greater than 3.5 have a sensitivity of 53%, specificity of 94%, positive predictive value of 76% and a negative predictive value of 85% for sarcoidosis, and with higher ratios the specificity nearly reaches 100% [49]. For individual cases, CD4/CD8 ratios may not always be useful as some patients may have either decreased, normal or increased ratios, or in rare cases may present with a CD8 alveolitis, such as in sarcoid patients with HIV-1 infection [50].

Ex vivo studies in patients with sarcoidosis with flow cytometry identified greater activation of non-stimulated BAL CD4+ and CD8+ T cells when compared with peripheral blood lymphocytes [51], demonstrating compartmentalisation of the immune response. A large number of BAL lymphocytes from patients with active sarcoidosis express cell surface activation markers including CD26, CD54, CD69, CD95 and HLA-DR [51, 52]. CD4+/HLA-DR$^+$ T-cells spontaneously release IL-2. Some investigators identified the possibility of using the

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number of CD4+/HLA-DR$^+$ cells for evaluating the activation state of the IL-2 system and defining different phases of sarcoidosis, as numbers decrease in inactive disease [53]. It is also interesting to note studies with BAL fluid from sarcoidosis patients who have the HLA-DRB1*0301-positive genotype predominantly express the Vα2.3 (AV2S3+) T-cell receptor. The increase in AV2S3+ CD4+ T-cells may be very significant during acute disease in these patients and constitute more than 30% of BAL T-cells, as well as expressing cell surface activation markers including CD26, CD28, CD69 and HLA-DR [54], indicating acute clonal expansion and proliferation in response to inciting antigen(s) [55].

Intracellular cytokine expression has been compared in activated BAL and peripheral blood lymphocytes using non-specific lymphocyte mitogens in patients with sarcoidosis and healthy controls. Some studies suggest compartmentalised shifts in the T_H1/T_H2 cytokine balance modulate granulomatous lung inflammation and its evolution towards disease resolution or development of pulmonary fibrosis [1]. BAL T-cells from patients with pulmonary sarcoidosis show a dominant T_H1 cytokine expression, with elevated mRNA and protein levels of IFN-γ and IL-2, as well as TNF-α but not IL-4 [52, 56-59]. Additionally, BAL alveolar macrophages have been shown to be important regulators of the T_H1 response by producing IL-12 and IL-18, which stimulate IFN-γ production and differentiation of naïve T-cells into a T_H1 phenotype [60]. Following stimulation, significantly more BAL CD4+ cells express T_H1 receptors CXCR3, CCR5, IL-12R and IL-18R, but fewer T_H2 chemokine receptors (CXCR4, CCR4) when compared with paired peripheral blood CD4+ T-cells [61]. A recent study of 52 sarcoidosis patients and 21 healthy controls identified that circulating levels of the T_H1 chemokine IFN-inducible protein (IP-10/CXCL10) and the T_H2 chemokine CCL17 were both elevated in the serum of patients compared to controls [62]. They additionally found that there was significantly greater IP-10 production by BAL cells in patients with active sarcoidosis compared to controls but no difference in BAL CCL17 levels. Interestingly, increased numbers of CD4+ CD25^{bright} FOXP3$^+$ Treg cells have been identified in the peripheral blood and BAL fluid of patients with active sarcoidosis. These cells exhibit powerful anti-proliferative ability but are unable to completely down-regulate production of pro-inflammatory cytokines including IFN-γ and TNF-α, thus allowing granuloma formation [6, 29]. Further investigations are needed to evaluate the $T_H1/T_H2/T_H17$ network in sarcoidosis, during different disease stages and the regulatory mechanisms which may be involved.

A CD4+ IFN-γ^+ T-cell alveolitis with elevated ratios of CD4+/CD8+ T-cells has also been confirmed in patients with active pulmonary disease using induced sputum, a relatively less-invasive technique compared with BAL [63]. A strong correlation has been confirmed between T-cell subsets in BAL fluid and induced sputum in patients with sarcoidosis, although the proportion of alveolar macrophages was significantly lower in induced sputum [64]. Increased levels of regulatory CD4+ CD25^{bright} CD127^{low} T-cells have also been confirmed in induced sputum of patients with active pulmonary sarcoidosis [65]. This indicates that induced sputum may be a less invasive yet useful method of investigating the immunology of pulmonary disorders.

5. Exclusion of granulomatous diseases mimicking sarcoidosis

There has been an increase in the armamentarium of specific immunological and microbiological tests to identify granulomatous disorders which would have previously been mistakenly labelled as sarcoidosis. Table 3 lists common granulomatous conditions, some of which need to be considered in the differential diagnosis of a patient with suspected sarcoidosis.

Some investigations which can be used depending on the clinical context to identify other causes of granulomatous inflammation include: the beryllium lymphocyte proliferation test, which can be performed on peripheral blood or BAL mononuclear cells to test for chronic beryllium disease [66], tests for anti-neutrophil cytoplasmic antibodies for Wegener's granulomatosis and related vasculitides, anti-mitochondrial antibodies for primary biliary cirrhosis and serological and culture methods for infectious diseases. Investigations of special interest are discussed in detail below.

5.1. Tuberculin skin test, delayed-type hypersensitivity and interferon-γ release assays

Although patients with sarcoidosis exhibit an exaggerated T_H1 immune response at sites of disease, they commonly have depressed peripheral blood responses to common antigens [5, 67], are unresponsive to vaccinations [7, 68] and demonstrate suppression of DTH to tuberculin. Impaired DTH is a clinical feature of sarcoidosis, with skin anergy demonstrated to recall antigens and polyclonal mitogens including mumps virus, *Trichophyton*, *Candida*, streptokinase/streptodornase, dinitrochlorobenzene [5, 69-71] but not phytohaemagglutinin (PHA) [72]. Similarly, patients exhibit cutaneous anergy to the tuberculin skin test, which is considered as part of the diagnostic criteria for sarcoidosis [73]. It has also been determined that in populations with a high incidence of tuberculosis, the presence of tuberculin skin test anergy is less reliable in making a diagnosis of sarcoidosis, compared with the use of interferon-γ release assays (IGRA). In these populations and in patients with immune deficiencies, IGRA is more accurate in unmasking an actual case of latent tuberculosis infection, in patients who were labelled as having "sarcoidosis" [74, 75]. IGRAs have a higher sensitivity and specificity for detecting *Mycobacterium tuberculosis* (MTB) than the conventional tuberculin skin test, as they utilise antigens specific for MTB complex [76, 77]. The recent QuantiFERON-TB Gold IGRA is based on the principle that T-cells from a whole blood sample of a patient previously exposed to specific MTB complex antigens, including Culture Filtrate Protein-10 (CFP-10), Early Secretory Antigenic Target-6 (ESAT-6) and purified protein-derivative (PPD) will produce IFN-γ, which is measured using enzyme-linked immunosorbent assay (ELISA). These proteins are absent from BCG strains and most non-tuberculous mycobacteria, hence providing specific testing for *M. tuberculosis*. Screening for prior tuberculosis infection, with a detailed history, tuberculin skin testing or use of IGRAs is also required prior to starting anti-TNF therapy, as these drugs are associated with serious infection risk from reactivation of latent tuberculosis [78].

Disease associations	Characterisation
Infectious	Mycobacteria: tuberculosis, leprosy, Bacillus Calmette-Guérin (BCG) and atypical mycobacteria
	Propionibacterium acnes, P. granulosum; Borrelia burgdorferi; Yersinia spp.;Brucella spp., Cat-Scratch disease (*Bartonella henselae*)
	Protozoal: Toxoplasmosis, Leishmaniasis
	Spirochaetes: *Treponema pallidum* (secondary or tertiary syphilis), *T. carateum, T. pertunue* (yaws)
	Invasive fungal infections: histoplasmosis, sporotrichosis, aspergillosis, cryptococcosis, blastomycosis, coccidioidomycosis
	Pseudomycoses: actinomycoses, nocardiosis, botryomycosis
	Herpes simplex virus, Epstein-Barr virus, cytomegalovirus
	Helminth infections: Schistosomiasis, *Ascaris lumbricoides*
	Demodicidosis (*Demodex* species)
	Other sexually transmitted: Chancre (*Haemophilus ducreyi*); donovanosis (*Calymmatobacterium granulomatis*); lymphogranuloma venereum
	Whipple's disease (*Tropheryma whipplei*)
Inflammatory/unknown	Sarcoidosis
cause	Crohn's disease
	Granulomatous vasculitis: Wegener's, Churg-Strauss disease, bronchocentric granulomatosis, polyarteritis nodosa
	Primary biliary cirrhosis, hepatic granulomatosis
	Giant cell arteritis
	Granuloma annulare and actinic granuloma
	Granulomatous rosacea, Granulomatous cheilitis
	Necrobiosis lipoidica, Necrobiosis xanthogranuloma
	Langerhans cell histiocytosis (histiocytosis X)
	Granulomatous lesions of unknown significance (GLUS) syndromes
Identifiable inflammatory aetiology	Hypersensitivity pneumonitis- (e.g. farmer's lung, bird fancier's lung, hot tub lung, metal workers lung). Foreign body granulomas: beryllium, aluminium, titanium, zirconium, talc, paraffin, pine tree pollen, clay, interferon-α injections, tattoos
Neoplastic	Granulomatous mycosis fungoides
	Lymphomas with histiocytic infiltration (Lennert's disease)
Other causes	Blau's syndrome, chalazion, chronic granulomatous disease of childhood

Table 3. Some causes of granulomatous inflammation, table modified from references [79, 80].

IFN-γ production in response to PPD stimulation of BAL lymphocytes has been shown to distinguish *M. tuberculosis* infection from sarcoidosis in a patient with sarcoid-associated optic neuropathy [77]. The QuantiFERON TB Gold has also been investigated in a cohort of 90 Japanese patients with sarcoidosis and was found to be positive in 3 patients (3.3%), which is similar to the false-positive rate in healthy non-sarcoidosis subjects. In these 3 patients, their specimens were negative for *M. tuberculosis* by acid fast staining, culture and PCR evaluation of tissues and none developed tuberculosis infection at 1-year follow-up [81]. A recent study compared the release of IFN-γ by BAL mononuclear cells and PBMC following *ex vivo* stimulation with whole PPD, ESAT-6 and CFP-10 from German patients with sarcoidosis, tuberculosis and healthy controls. They similarly found that BAL and PBMC IFN-γ release was comparable amongst patients with sarcoidosis and controls, but less compared to patients with tuberculosis [82]. Hence IGRAs such as the QuantiFERON TB Gold are specific for tuberculosis infection and results are negative in patients with sarcoidosis.

Recent investigations have indicated that certain undegradable *M. tuberculosis* antigens (which are not present in tuberculosis-specific IGRAs) may be potential pathogenic antigens in sarcoidosis. Investigations utilising IFN-γ ELISPOT and flow cytometry indicated greater PBMC and BAL T_H1 responses to recombinant *M. tuberculosis* catalase-peroxidase (mKatG) [83] and mKatG peptides in patients with sarcoidosis compared to healthy controls, but no difference with PPD-positive (PPD+) control subjects, which profiles a possible pathogenic antigen in sarcoidosis [67, 84-88]. A greater frequency of peripheral blood T_H1 responses have also been shown in patients with sarcoidosis compared with healthy PPD- controls following stimulation with mycobacterial heat shock proteins [86, 89, 90] and *M. tuberculosis* peptides from ESAT-6 [67, 84-88], mycolyl-transferase Antigen 85A [86, 91] and superoxide dismutase A [86, 92]. Cellular immune responses against mycobacterial antigens were detected in cells from patients with sarcoidosis that did not react to *Trypanosoma brucei* lysates [88] or the neoantigen keyhole limpet hemocyanin [84], as well as Cytomegalovirus cell lysate and Cytomegalovirus, Epstein-Barr virus and Influenza (CEF) peptides [67]. Recent findings with flow cytometry have also shown significantly greater BAL CD4+ and CD8+ IFN-γ+ immune responses to *M. tuberculosis* ESAT-6 and *Propionibacterium acnes* proteins in sarcoidosis patients compared with healthy controls. This study also utilised matrix-assisted laser desorption ionisation mass spectrometry (MALDI-IMS) to localise ESAT-6 and *P. acnes* signals within sarcoidosis and control specimens. The authors identified localised signals consistent with ESAT-6 in sarcoid granulomas, although there was no specific localisation of *P. acnes* in sarcoid tissues [93]. This demonstrates specific mycobacterial antigen specificity inducing the immune response in some patients with sarcoidosis.

5.2. Histopathological testing and Polymerase-Chain Reaction (PCR)

In sarcoidosis tissue micro-organisms are not detected through conventional staining techniques or cultures of non-caseating granulomas. Important differential diagnoses, including infectious diseases must be excluded with histopathological testing using special stains for acid-fast bacilli, fungi and microbial cultures. This is especially important if the patient has a fever or when granulomas exhibit focal necrosis. Granulomas can also be found in regional

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lymph nodes of carcinomas or in primary tumours such as breast carcinoma and seminoma. However, with immunohistochemical techniques, granulomas associated with neoplastic processes are generally B-cell positive, whilst in sarcoidosis they are B-cell negative [94].

With the use of special stains or culture methods some investigators have also been able to identify micro-organisms in sarcoid tissues, most commonly those resembling mycobacteria [95]. Bacilli-like structures have also been observed using immunofluorescence techniques [96]. Schaumann bodies, are a type of inclusion body found in sarcoidal giant cells, which consist of small calcifications of calcium carbonate, iron and oxidised lipid with a lamellar morphology. They are identified in up to 88% of cases of sarcoidosis and arise from lysosomes [97]. They have interestingly been identified as sites of mycobacterial degradation by demonstrating the localisation of lysosomal components and mycobacterial antigens in immunohistologically stained sarcoidosis tissues [98]. Other investigators identified bacterial structures in skin and lymph node biopsies [99], as well as blood, bronchial washings, ocular anterior chamber fluid and cerebrospinal fluid from patients with sarcoidosis [100-104]. These organisms were identified as 'L-form' cell-wall deficient bacteria, which can occur during the life-cycle of mycobacteria or in response to inhospitable conditions [95, 103, 105]. However, in a larger multicentre study with 197 sarcoidosis cases and 150 controls an equal frequency of cell-wall deficient forms were observed in blood specimens [101]. Sarcoidosis can also be histologically similar to lesions in atypical mycobacterial infections, including *Mycobacterium avium-intracellulare* complex (MAC), *Mycobacterium marinum* and following BCG vaccination [95, 104]. Sarcoidosis is also an important differential diagnosis of *M. marinum* infection, where the acid-fast bacilli are detected in 22% of active cases and use of polymerase chain reaction (PCR) is more useful for diagnosis [106, 107].

In an attempt to improve the diagnostic sensitivity of traditional culture techniques, many investigators have used DNA amplification techniques to search for mycobacterial or propionibacterial infection in sarcoidosis. Investigations have used PCR and nested PCR techniques to identify mycobacterial and propionibacterial DNA or RNA in sarcoid tissue specimens, including fresh tissues, paraffin-embedded tissues, granulomas, lymph nodes, lung and BAL sediments and archival biopsy specimens. Several reports emerged indicating the presence of mycobacterial DNA in some sarcoid tissues using DNA primers for *M. tuberculosis* complex organisms [108-113], which could also suggest cell wall deficient mycobacterial infection. The results have been inconsistent, however, as other groups did not find fluorescent in situ hybridisation or PCR evidence of mycobacterial DNA or RNA in sarcoid tissues [114-116]. A recent meta-analysis of 31 such studies identified that 231 out of 874 (26.4%) sarcoidosis biopsy specimens had evidence of mycobacterial DNA, which is 9- to 19-fold higher than control tissue samples, supporting an association between mycobacterial infection and sarcoidosis [117]. However, it is important to note that these results are not reproducible in all sarcoidosis patients and that treatment of sarcoidosis with corticosteroids does not show reactivation of tuberculosis- indicating the lack of a direct role of mycobacterial infection in sarcoidosis [118]. In a patient with negative microscopy, culture and PCR for tuberculosis; in the presence of compatible clinical features and histology, a diagnosis of sarcoidosis can be made with confidence.

Propionibacterium acnes has also been isolated from sarcoid lesions [119] which has suggested a role for this commensal organism in sarcoidosis. Using PCR to amplify segments of the 16S rRNA of *P. acnes* or *P. granulosum*, several authors reported isolation of propionibacterial DNA from sarcoid tissues [120-123], with a DNA signal intensity greater than surrounding non-granulomatous tissue. These initial studies were followed by a cooperative study from Japanese and European investigators that confirmed the presence of *P. acnes* and *P. granulosum* DNA in all but two of 108 sarcoidosis specimens obtained from both Japanese and European biopsies [113]. However, *P. acnes* DNA was also reported in 57% of control tissues including from healthy controls, suggesting that it is a common commensal organism in peripheral lung tissues and mediastinal lymph nodes [124].

5.3. Exclusion of other infectious agents

A history of previous possible environmental exposure, or travel to endemic areas is important to exclude infectious granulomatous diseases. Apart from culture and microscopy of specimens with special stains for fungi and acid-fast bacilli, other investigations can be performed to exclude infection. These depend on the clinical context and may include serologic analyses. Some specialised investigations may be used including identification of the histoplasmosis urinary antigen and skin tests for fungi and protozoa (e.g. the Leishmanin test) [125]. In patients with leprosy the *ex vivo* lymphocyte proliferation test in response to *M. leprae* as well as the Mitsuda type of lepromin skin test [126] have been shown useful to differentiate leprosy from cutaneous sarcoidosis [127]. Tissue must also be analysed for presence of metals, and foreign bodies need to be excluded on microscopy.

5.4. Beryllium lymphocyte proliferation testing

The granulomas formed as a result of chronic beryllium exposure closely resembles that of sarcoidosis, such that some investigators have suggested berylliosis defines a sarcoidosis subset [128]. It is possible that in genetically susceptible individuals, distinct antigens can precipitate sarcoidosis or sarcoidosis-like diseases [129]. A history of occupational or environmental exposure to beryllium is important when considering a diagnosis of sarcoidosis, in patients who have been exposed to the metal dust or fumes. The beryllium lymphocyte proliferation test has been used in the diagnosis of chronic beryllium disease to distinguish it from sarcoidosis and other granulomatous diseases [128]. This laboratory investigation involves the addition of beryllium salts to a sample of peripheral blood or BAL fluid, which can lead to mononuclear cell proliferation, only in patients with berylliosis [66]. Beryllium sulphate stimulation of BAL from patients with chronic beryllium disease induced greater T_H1 immune responses, with markedly elevated numbers of CD4+ INF-γ and IL-2 secreting beryllium-specific lymphocytes, as well as *ex vivo* lymphocyte proliferation compared to healthy controls [130, 131], making it a useful investigation to distinguish berylliosis from sarcoidosis. Patients with chronic beryllium disease can also have preserved skin test-reactivity to common recall antigens such as candida, tetanus and mycobacteria, as well as hyperresponsive DTH to beryllium with the beryllium patch test, unlike sarcoidosis, where patients demonstrate cutaneous anergy [132]. Interestingly, patients with severe chronic

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beryllium disease may have lymphopenia, calcium metabolism abnormalities, elevation of serum ACE and elevated CD4+ T-cells at sites of inflammation- similar to sarcoidosis, but have negative Kveim test reactions [132].

6. The Kveim-Siltzbach test

Before the use of fibre-optic bronchoscopy and BAL, the Kveim-Siltzbach test was used as the diagnostic test for sarcoidosis, although now it remains of historical importance only. It was performed by intradermal injection of Kveim-reagent, a validated suspension of allogeneic human sarcoid tissue, typically sarcoid spleen or lymph nodes homogenised in phosphate-buffered saline, pasteurised, resuspended with 0.25% phenol, while later preparations were irradiated [133, 134]. The resultant papule at the injection site was biopsied three to six weeks later and presence of non-caseating granulomas indicated sarcoidosis [135, 136]. Kveim reactions can also be induced from similar preparations made from sarcoidosis BAL cells or peripheral blood monocytes, suggesting systemic dissemination of the inciting agent by mononuclear phagocytes [38, 137, 138]. Ansgar Kveim was the first to report in the 1940s that biopsy of these papules demonstrated the presence of epithelioid granulomas that were histologically identical to granulomas observed in sarcoid tissues [135]. In 1967 Siltzbach demonstrated that >80% of sarcoid patients worldwide reacted positively to a single batch of Kveim reagent, with <1% false positive rate of non-specific reactions in control subjects, indicating the possibility of a common antigen in the aetiology of sarcoidosis [139]. The test was very useful in distinguishing sarcoidosis from other granulomatous diseases [140]. A medical centre that performed >10,000 Kveim-Siltzbach tests over fifty years identified a true positive rate of >50% and false negative rate of nearly zero [10]. This test is now rarely used as no commercially available preparation of the reagent exists, with the additional problem that each new Kveim-Siltzbach preparation requires validation *in vivo* [136]. Furthermore, use of human tissue extracts for clinical purposes presents constraints, including risks of transmitting infections such as Creutzfeldt-Jakob disease even after heating, phenol and irradiation [141].

Because of the four week delay in response and need for biopsies, attempts were made to develop a rapid *in-vitro* Kveim-Siltzbach test [142, 143]. These have been based on examining morphology of lymphoblastic transformation or macrophage activation in response to Kveim reagent, which was identified but most results were controversial and negative [144-146]. A study investigating stimulation of BAL and blood lymphocytes from sarcoidosis patients using Kveim antigen did not show any significant increase in lymphocyte responses to Kveim antigen as measured by lymphocyte DNA synthesis [147]. Hence no comprehensive *in vitro* Kveim-Siltzbach test has been developed that could be used for diagnostic purposes. Nevertheless, Kveim reagent or sarcoid tissues can theoretically be utilised as a lymphocyte stimulus *ex vivo*. Improvements to *in vitro* Kveim tests could include purer validated preparations [148], addition of macrophages for enhanced antigen presentation and reactivity, avoidance of sarcoid sera known to inhibit lymphocyte function and advanced immunological techniques [149].

It was later identified that the granulomagenic factor was an insoluble undegradable particulate, devoid of a consistently identifiable infectious agent by electron microscopy that aggregated in phagolysosomes of antigen presenting cells [38]. The Kveim reaction is characterised by an influx of mainly CD4+ T cells which express a restricted variable-β (Vβ) region of the T-cell receptor (TCR), indicating oligoclonal expansion in response to a limited number of antigens [150]. This may also indicate that the aetiological agent may be present in Kveim reagent as Vα and Vβ specific TCR oligoclonality has also been identified in T-cells from sites of sarcoid inflammation [150, 151]. Based on hypotheses that pathogenic antigens in sarcoidosis have similar physicochemical properties as Kveim reagent (including poor solubility in neutral detergent and resistance to acidity and protease digestion) [152], this led to a limited proteomics approach to determine potential antigens with these characteristics in sarcoidosis tissue homogenates [153]. Using matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectroscopy and protein immunoblotting, mycobacterial catalase-peroxidase (KatG) protein was identified in 55% of samples that was also a target of circulating IgG in 48% of sarcoidosis subjects [153]. This suggested that this remnant mycobacterial protein is one target of the adaptive immune response driving granulomatous inflammation in at least a subset of sarcoidosis tissues. Subsequently, investigations with IFN-γ ELISPOT and flow cytometry following intracellular staining for IFN-γ indicated greater peripheral blood mononuclear cell (PBMC) and BAL T_H1 responses to recombinant *Mycobacterium tuberculosis* KatG [83] and KatG peptides in patients with sarcoidosis compared to healthy controls. However, there was no difference when compared with PBMC from PPD+ control subjects, which profiles a pathogenic antigen in some patients with sarcoidosis [84,86-88]. We also determined that stimulation with pooled peptides from *M. tuberculosis* Early-Secretory Antigen Target-6 (ESAT-6) and KatG induced greater numbers of IFN-γ producing T-cells and elevated IL-2, IL-6 and TNF-α in sarcoidosis compared to PPD- healthy control subjects [67]. Since these mycobacterial antigens do not induce immune responses in all patients with sarcoidosis, newer, more specific approaches are thus needed to identify other potential antigens in Kveim reagent.

7. Important markers of granulomatous inflammation

7.1. Angiotensin Converting Enzyme (ACE)

Most clinicians are familiar with ACE, as it is the glycoprotein enzyme responsible for converting angiotensin I to angiotensin II, for which ACE inhibitors are used to treat hypertension and congestive heart failure. ACE is typically measured using a functional assay which measures ACE activity rather than ACE concentrations [154]. As the test is a functional biological assay, the presence of ACE inhibitors in the patient's serum can affect measurements [155]. ACE activity levels also tend to be higher in younger subjects [156].

ACE is a ubiquitous enzyme secreted by monocytes and macrophages, as well as pulmonary endothelial cells into the bloodstream where it exerts its actions. Serum ACE was first reported by Lieberman in 1975 as being elevated in patients with untreated active sarcoido-

sis [157, 158]. Sarcoid granulomas produce ACE, with the source being epithelioid and giant cells from the macrophage line [159]. Serum ACE activity is elevated in ~60% of patients with sarcoidosis [10, 160, 161], although values can vary depending on the time of diagnosis, extent of disease, acute or chronic disease and radiological stage and corticosteroid treatment. It is a useful diagnostic and prognostic tool, but normal levels do not exclude sarcoidosis and false positives are not uncommon. Serum ACE may also be elevated in a variety of other granulomatous and non-granulomatous diseases such as pulmonary silicosis, asbestosis [162], chronic beryllium disease [163], histoplasmosis [164], miliary tuberculosis, leprosy [165, 166], diabetes mellitus [167], hyperthyroidism [168] and Gaucher's disease [169]. Hence serum ACE activity in sarcoidosis is a marker of granuloma formation but with limited sensitivity and specificity. It must be interpreted with other markers of sarcoidosis, along with clinical and radiological features, although serial values may be helpful in disease monitoring. One confounding factor is that ACE activity in biological fluids is also affected by insertion/deletion (I/D) polymorphisms of the ACE gene and the Angiotensin II receptor 1 (AT2R1) gene [170]. Patients can be classified into three groups based on the ACE gene polymorphisms, including II, ID and DD. Homozygous carriers of the deletion mutation (DD) or the insertion (II) are associated with the highest and lowest ACE levels respectively, which can lead to underestimation or overestimation [171]. Patient genotyping for ACE I/D polymorphisms may improve assessment of ACE activity, however, there is a need for genotype-corrected reference levels.

ACE activity is also measurable and elevated in BAL fluid of patients with sarcoidosis [172] and is considered to provide better prognostic information than serum ACE. ACE has also been measured in urine and cerebrospinal fluid (CSF) and other biological fluids. Elevated CSF ACE has been identified as a useful marker in patients with suspected neurosarcoidosis, with values ≥8nmol/mL/min having a specificity of 94% and sensitivity of 55% in one study [173].

7.2. Lysozyme

Lysozyme is another monocyte-macrophage derived enzyme that may be considered a potential marker of macrophage activity. It is normally found in the granules of monocytes, macrophages, and neutrophils, where it may be released and is readily detectable in tears, saliva, airway secretions and CSF. Elevated serum lysozyme has been found in patients with active sarcoidosis, in ~30% of patients at clinical onset [30, 174, 175] and had been identified before the ACE test became available [176]. In sarcoidosis and in the Kveim reaction, immunohistochemical studies have identified the source of lysozyme as macrophages and epithelioid giant cells involved in granuloma formation [177]. Several authors have compared serum lysozyme with ACE and have identified a positive correlation between them, as both tests are positive in the majority of patients with acute disease [178, 179]. Lysozyme may be used to aid in the diagnosis of sarcoidosis and monitor disease course. However, the use of serum lysozyme is limited in clinical practice by its low sensitivity and specificity compared to other biomarkers, as it is also elevated in several other diseases [30].

7.3. Neopterin

Neopterin is a metabolite of guanosine triphosphate released by activated macrophages in response to IFN-γ [30, 180] and is elevated in serum and urine of patients with sarcoidosis [181, 182]. Other groups have detected elevated neopterin in patients with active disease [40, 183, 184] and have noted that levels fall with disease resolution [185, 186]. Interestingly, comparing neopterin to other serum and BAL markers demonstrated that elevated neopterin and sIL-2R were present in patients who were more likely to have progressive disease requiring long-term treatment with corticosteroids [40, 44].

7.4. Chitotriosidase

Chitotriosidase is a member of a group of enzymes involved in the breakdown of chitins (polymers of *N*-acetylglucosamine- commonly found in cell walls of fungi and exoskeletal elements of some animals and arthropods) [187]. The role of chitotriosidase in the pathogenesis of sarcoidosis is not clearly understood. It is believed that chronic over-expression, along with CCL18 over-expression (a chemokine involved in fibrotic remodelling of diffuse lung diseases) may induce pro-fibrotic T_H2 cytokines and fibronectin, predisposing to development of fibrosis [188]. In situ hybridisation has confirmed that alveolar macrophages from BAL fluid of patients with sarcoidosis are the primary source of this mediator [188, 189]. Serum chitotriosidase has been identified to be elevated in the serum of >90% of patients with sarcoidosis [190]. Significantly greater chitotriosidase activity was also demonstrated in the BAL and serum of sarcoidosis patients than in controls and levels generally increase with disease progression. BAL chitotriosidase activity also correlated with sarcoidosis radiological stages, serum ACE activity and radiological CT findings of fibrotic lung involvement [191]. Levels also decrease significantly with therapeutic interventions indicating that it may be a suitable marker of disease severity and granulomatous inflammation in sarcoidosis [187] and that it may have potential for identifying patients at risk of developing chronic fibrotic disease [191].

7.5. Other markers

Serum amyloid A is an amyloid precursor protein related to the high-density lipoprotein and is an innate receptor ligand with some physicochemical properties seen in the Kveim reagent [152]. It has been found to regulate granulomatous inflammation through Toll-like receptor-2 in experimental models of mKatG induced granulomatous lung inflammation and using alveolar macrophages from sarcoid patients [192]. Serum amyloid A is also an acute phase protein that is released together with C-reactive protein by the liver under systemic IL-1 and IL-6 stimulation and is hence also regarded as a nonspecific inflammatory marker of sarcoidosis, although it is less sensitive and specific than other markers such as sIL-2R [23].

Adenosine deaminase is an enzyme for purine metabolism that is important for differentiation of T lymphocytes. It has been studied in sarcoidosis and tuberculosis and is elevated in BAL fluid and serum in some cases of sarcoidosis [193]. Some studies have found serum adenosine deaminase to be useful, finding elevated levels in active sarcoidosis compared to inactive

disease and healthy controls [194], whilst others found conflicting results indicating no relationship with disease activity [179].

Endothelin-1 is a vasoactive bronchoconstrictive peptide identified in pulmonary fibroproliferative processes and has been assessed in the serum, BAL, urine and lung tissues in sarcoidosis [195-197]. Elevated endothelin-1 levels have been associated with the clinical course of sarcoidosis and the degree of lymphocytic alveolitis and number of BAL macrophages [196, 197]. It is hypothesised that endothelin-1 may be involved in the development of pulmonary hypertension and fibrosis, although further studies are needed to confirm this in sarcoidosis.

Other markers studied in sarcoidosis include neutrophils and neutrophil-associated markers, which have been noted to be associated with chronic disease in sarcoidosis with progressive fibrosis and diffuse fibrotic lung disease. Important neutrophil markers studied in sarcoidosis include elastase and collagenase. Elastase appears to have a limited role in monitoring sarcoidosis although is elevated in those with chronic Stage III radiological disease [198]. Collagenase is elevated in the serum of some patients with sarcoidosis and elevated BAL collagenase has also been measured in conjunction with elevated fibronectin and is associated with more progressive and fibrotic disease [199].

8. A proteomics approach

Proteomics is emerging as a useful method of simultaneously analysing large numbers of proteins, including protein structure and functions in biological samples, as well as identifying profiles characteristic of disease processes. This approach has the potential to make new discoveries as the findings are usually independent of any earlier specific protein biomarkers. In sarcoidosis various proteomic techniques have been used for profiling protein patterns in BAL fluid and serum. Two-dimensional electrophoresis and mass spectrometry identified a total of 85 proteins in BAL fluid, of which 38 were newly identified in BAL from patients with sarcoidosis and idiopathic pulmonary fibrosis [200]. Proteins identified included locally secreted, plasma derived, proteolytic or cell damage products that have pro-inflammatory, anti-inflammatory and anti-protease activity. Compared with systemic sclerosis and idiopathic pulmonary fibrosis, BAL from patients with sarcoidosis also had greater acute phase proteins including ceruloplasmin, haptoglobin β, β_2-microglobulin and α_1-antichymotrypsin [59]. Other techniques have included applying narrow range pH gradients, which also identified new groups of proteins in the BAL and serum of patients with sarcoidosis, many of which are proteins involved in inflammatory and oxidative stress processes [201, 202]. This same group also applied difference gel electrophoresis proteomics to the analysis of BAL from patients at risk of developing chronic sarcoidosis (with a HLA-DRB1*15 genotype) compared with patients with chronic beryllium disease and controls. The investigators identified differing protein patterns between the three groups including increased peroxiredoxin 5, heat shock protein 70, complement C3, annexin A2 and transthyretin in sarcoidosis patients compared to the control group [203]. Other novel approaches have utilised surface-enhanced laser desorption ionization-time of flight-mass spectrometry (SELDI-TOF-MS) and have found different

disease-related proteins and protein patterns in serum; identifying the α_2 chain of haptoglobin as a potential serologic marker [204]. This was also performed in BAL fluid of patients with sarcoidosis identifying proteins that may related to clinical course, including α_1-antitrypsin, protocadherin-2 precursor and albumin [205]. Proteomic analysis has provided a large-scale of novel data identifying protein biomarkers in sarcoidosis, which are different to those of healthy controls and patients with other interstitial lung diseases. However, much still needs to be done in identifying their role in the pathogenesis and validating the clinical utility of these markers in patients with sarcoidosis through further large-scale studies.

9. Exhaled breath analysis

Approximately 90% of patients with sarcoidosis have pulmonary disease involvement. It has hence been proposed that exhaled biomarkers from the lungs of patients with sarcoidosis could potentially be used to provide novel insights into the immunopathogenesis of the disease as well as for diagnosis and disease monitoring [1]. Initial studies investigating exhaled breath in sarcoidosis identified increased exhaled nitric oxide (FENO) in patients with sarcoidosis compared to healthy controls, which then reduced significantly after 6 weeks of treatment with corticosteroids [206]. Increased exhaled nitric oxide possibly reflects disease activity, and is associated with increased T_H1 immune responses. This may arise through up-regulation of nitric oxide synthase, which is induced (iNOS) by increased IFN-γ and TNF-α in active disease [206]. Other investigators however, found conflicting results, identifying that FENO from 59 patients with sarcoidosis did not differ significantly from 44 healthy controls and were not related to the extent of individual CT scan abnormalities or pulmonary function impairment [207]. Similar results have been obtained in a recent study examining multiple flow rates measurement of exhaled nitric oxide in patients with sarcoidosis and healthy controls, as well as patients treated with corticosteroids [208], which indicated that exhaled nitric oxide measurement did not appear to be a clinically useful method of monitoring disease progression in sarcoidosis. One study recently investigated the use of exhaled carbon monoxide (CO) in sarcoidosis, an oxidative stress biomarker for alveolar macrophage heme oxygenase activity. The authors identified a significantly elevated exhaled CO in patients with sarcoidosis compared to healthy controls and patients with miscellaneous interstitial lung disease [209]. However, the prognostic value of this measurement remains undefined and may be affected by cigarette smoke.

Exhaled breath condensate (EBC) analysis is a simple method of sampling airway lining fluids that has been shown to be useful for analysing exhaled breath markers [210-212] and is less invasive compared with induced sputum and BAL. Total protein levels are much higher in BAL than EBC [213], but nevertheless, the presence of a few biomarkers has been demonstrated in the EBC of patients with sarcoidosis. Levels of inflammatory markers e.g. TNF-α, Insulin-like growth factor-1 (IGF-1), and plasminogen activator inhibitor-1 (PAI-1) have been shown to be comparable and closely correlated in EBC and BAL samples, however, EBC IL-6 concentration was significantly lower when compared with that in BAL fluid in patients with sarcoidosis [214]. This study concluded that besides IL-6, EBC reflects cytokine production in

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the lung as effectively as BAL and may allow for a simplified sampling method. Hepatocyte growth factor (HGF) is produced by fibroblasts, causing strong epithelial proliferative responses and has been found to be elevated in EBC of patients with pneumonia [215]. However, levels of HGF are comparable in both EBC and BAL fluid of patients with sarcoidosis and healthy controls, suggesting it is not useful as an EBC biomarker in sarcoidosis [216]. Transforming growth factor-β_1 (TGF-β_1) has also recently been identified in the EBC of patients with sarcoidosis, along with vascular endothelial growth factor (VEGF), PAI-1, TNF-α and IL-8 but not urokinase-type plasminogen activator (uPA) [217]. TGF-β is implicated in fibrosis by inducing extracellular matrix synthesis and has been detected in *ex vivo* BAL cell cultures from patients with sarcoidosis [30], suggesting it may be a useful sarcoid EBC marker. Markers of granulomatous inflammation identified in our laboratory at greater levels in the EBC of patients with sarcoidosis compared to healthy controls include neopterin and TGF-β_1 (H. Ahmadzai, D. Wakefield, P.S. Thomas; unpublished observations), which could potentially be measured as airway markers of sarcoid activity.

Recent investigations have identified exhaled eicosanoids including 8-isoprostane and cysteinyl leukotrienes as being elevated in the EBC and BAL fluid of sarcoidosis patients [218]. A correlation has been identified between the levels of 8-isoprostane and leukotriene B_4 in BAL fluid and EBC of patients with sarcoidosis [219]. EBC levels of 8-isoprostane also positively correlate with the percentage of eosinophils in BAL and negatively with neutrophils [220, 221]. 8-isoprostane levels are increased in active sarcoidosis compared to healthy subjects, which may serve as a marker of disease severity and indicate increased oxidation [220]. Increased markers of oxidative stress including elevated hydrogen peroxide levels have also been demonstrated in EBC and BAL of sarcoidosis patients, as well as end-products of lipid peroxidation [222]. Growth factors, reactive oxygen species and products of oxidative stress in BAL and exhaled breath cannot yet be considered specific prognostic markers in sarcoidosis. Further research is needed into their potential clinical applications for disease monitoring.

10. Novel and experimental testing and conclusions

Laboratory and immunological testing has provided significant advances in the understanding of sarcoid immunopathogenesis and has allowed for easier diagnosis. Important recent findings have included the investigation of the immunology of the disease, through the $T_H1/T_H2/T_H17$ paradigm, which could provide further new insights into immunopathogenesis and potential treatments. Novel methods of identifying peripheral blood T-cell activation, such as with an in vitro Kveim reaction, could identify causative antigenic peptides. Large-scale studies validating initial proteomics findings from BAL and serum need to be conducted to identify the different clinical phenotypes of sarcoidosis and recognise patients at risk of chronic disease and pulmonary fibrosis. Simple, less invasive investigations such as exhaled breath analysis also need to be improved for clinical use. There is also potential for advanced immunological investigations including multiplex protein and gene expression technology to further investigate this intriguing disease. Although various biomarkers have been identified

and proposed for diagnosis and monitoring, there is still no sufficiently specific or sensitive disease marker for clinical practice and much is yet to be understood.

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