BACTERIAL DNA ARE FOUND IN LYMPH NODES OF ALL CHRONICALLY SYMPTOMATIC SARCOIDOSIS PATIENTS

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ARTICLE SUMMARY:

ARTICLE FOCUS:

- Sarcoidosis is a common yet incurable, chronic granulomatous disease of unknown etiology treated with non-specific anti-inflammatory and/or immunosuppressive drugs.
- Persistently symptomatic patients worsen despite treatment with a disabling, potentially fatal clinical course.
- We propose that sarcoidosis results from a chronic granulomatous infection from a treatable pathogen in certain susceptible individuals, much like granulomatous ileitis (Crohn's disease) which shows an encouraging response to multi-drug antimicrobial therapy.

KEY MESSAGES:

- This case-control, retrospective study correlated clinical outcomes with the presence of detectable bacterial DNA in sarcoidosis lymph nodes versus control lymph nodes.
- The entire group of sarcoidosis patients had significantly more detectable bacterial DNA than control patient lymph nodes.
- However, **all** persistently symptomatic patients have detectable bacterial DNA in their lymph nodes suggesting they have more aggressive sarcoidoisis that

potentially might benefit from antimicrobial treatment directed against a presumed chronic granulomatous infection.

STRENGTHS AND LIMITATIONS:

- Although a number of prior studies have demonstrated the consistent presence of bacterial DNA (mostly atypical mycobacteria and *Propionibacterium acnes*) in sarcoidosis tissue, the current study is the first to correlate clinical outcomes with the presence of detectable bacterial DNA, suggesting the most promising candidates for treatment.
- Nevertheless, the molecular approach to bacterial detection has distinct limitations including possible false-positive results secondary to contaminated PCR reagents, the paraffin imbedding process, or post-embedding handling and processing of the paraffin block.
- Additionally, the number of lymph nodes positive for bacterial DNA may be significantly underestimated because of the tendency of the formalin-fixation and paraffin embedding process to breakdown prokaryotic DNA.

ABSTRACT

INTRODUCTION: Sarcoidosis is an incurable, chronic granulomatous disease primarily involving the lungs and lymph nodes of unknown etiology, treated with non-specific anti-inflammatory/immunosuppressive drugs. Persistently symptomatic patients worsen with a disabling, potentially fatal clinical course. To determine a possible infectious cause, we correlated in a case control study clinical information with the presence of bacterial DNA in sarcoidosis mediastinal lymph nodes compared to control lymph nodes resected during cancer surgery.

METHODS: We retrospectively studied formalin-fixed, paraffin-embedded, mediastinal lymph nodes from 30 sarcoidosis patients and 30 control lung cancer patients. Nucleic acids were extracted from nodes, were evaluated by rRNA PCR for bacterial 16S rDNA, and the result was sequenced and compared to a bacterial sequence library. Clinical information was correlated.

RESULTS: 11/30 (36.7%) of lymph nodes from sarcoidosis patients had detectable bacterial DNA, significantly more than control patient lymph nodes (2/30, 6.7%), $p = 0.00516$. At presentation, 19/30 (63.3%) sarcoidosis patients were symptomatic including all patients with detectable bacterial DNA. Radiographically, there were 18 Stage I and 12 Stage II patients. All Stage II patients were symptomatic and 75% had PCR-detectable bacteria. After a mean follow-up of 52.8 $\pm$ 32.8 months, **all** patients with PCR-detectable bacteria were persistently symptomatic requiring treatment.

DISCUSSION: 36.6% of sarcoidosis patients had detectable bacteria DNA on presentation, **all** were quite symptomatic, and most were radiographically-advanced stage II patients. These findings suggest bacterial DNA-positive, symptomatic patients have more aggressive

sarcoidoisis that persists long term, and might benefit from antimicrobial treatment directed against this presumed chronic granulomatous infection.

INTRODUCTION

Sarcoidosis is a multisystem, granulomatous disease whose etiology is obscure and controversial. Nevertheless, the condition is relatively common with a significantly higher age-adjusted annual incidence in African-Americans (35.5 cases per 100,000) versus Caucasian-Americans (10.9 cases per 100,000). An estimated one million people in the U. S. have this disease. Based on the current U.S. population of 315,556,000, there will be approximately 38,605 new cases of sarcoidosis this year and just over 1000 (2.6%) will die of the illness.[1,2]

The fundamental pathologic abnormality in the disease is the formation of non-caseating epitheliod granulomas, which usually confine poorly soluble foreign material that simply cannot be removed by a single phagocytic cell. The key feature in sarcoidosis is activated CD4+ T cells which differentiate into type 1 helper T cells (Th1), secreting interleukin-2 and interferon-γ, augmenting macrophage TNF-α, and amplifying the local cellular immune response.[3,4] This granulomatous inflammation interferes with local tissue homeostasis leading to organ impairment.

Since sarcoidosis primarily involves the lungs, eyes and skin, attention has focused on airborne environmental antigens that might trigger this presumed hypersensitivity response with its T cell-mediated cellular immune response.[3] Similar granulomatous responses can be seen from a variety of infectious agents including mycobacteria, parasites (schistosoma), and fungi (coccidiomycosis). Early studies reported associations with non-infective agents including beryllium, zirconium, aluminum, wood-burning stoves, tree pollen, clay soil, talc, insecticides, inorganic particles, and moldy environments, but none of these theoretical causes has endured.[3,5,6] There is also a several-fold increased incidence of sarcoidosis occurring in siblings

and parents, as well as a consistent strong association with specific gene products such as class I and class II HLA antigens, which may add to the familial connection.

Although no infectious agent has been cultured directly from sarcoidosis tissues, clinical and immunologic characteristics of the disorder provide the strongest support for a microbial etiology, at least in some patients.[5-7] To explore a possible infectious cause in patients seen at the Moffitt Cancer Center, we correlated the clinical presentation and long-term follow-up of sarcoidosis patients with the presence of bacterial DNA in archived, surgically-resected mediastinal lymph nodes. Results from sarcoidosis nodes were compared to control lymph nodes resected at the time of lung surgery in node-negative, Stage I non-small cell lung cancer patients.

METHODS

Regulatory Oversight

Tissue and clinical data in this case-control study was obtained after approval by the Moffitt Cancer Center Scientific Review Committee Protocol MCC #16131 and the University of South Florida IRB Protocol #108656.

Study Design

By searching the Moffitt Cancer Center surgical pathology database between January 1, 2000 and April 1, 2010, we retrospectively identified 30 randomly-chosen patients who were diagnosed with sarcoidosis based on the typical radiographic and clinical presentation, and the histologic finding of non-caseating epitheliod granulomata in lymph nodes obtained sterilely only by mediastinoscopy, to avoid possible microorganism contamination by endoscopic biopsies. Special stains for microorganisms were negative on the specimens. For inclusion in

this group, the following criteria were used to make the diagnosis of sarcoidosis: 1) chest radiograph and chest CT findings of symmetrical mediastinal and hilar adenopathy with or without reticulonodular infiltrates in the lung fields (see Figure 1); 2) when performed, PET scans demonstrated glucose avidity in the enlarged lymph nodes (see Figure 2); 3) asymptomatic presentation or typical symptoms of dyspnea, cough, chest tightness/pain, night sweats, fevers, fatigue, malaise, skin rash or weight loss; 4) lymph nodes showing histologic features of confluent, non-caseating granulomata; and 5) any known microorganism causes of granulomata were excluded by history or culture. All histopathologic specimens were reviewed by one of us (P.S.) to reconfirm the diagnosis made originally by departmental pathologists at Moffitt.

To insure sterile collection, all control specimens were taken from lymph nodes removed at open thoracotomy by one of us (L.R.) in 30 (1:1 match with cases) randomly-chosen patients with Stage IA non-small lung cancer. Lymph nodes were selected only from patients with small peripheral tumors, no obstructive atalectasis, and no evidence of active infection. One of us (P.S) reviewed all control lymph node histology to verify there were no metastases, acute inflammation, or granulomata.

Clinical Data

Clinical data on the patient demographics, initial presenting symptoms and objective findings were extracted from the electronic medical record on all sarcoidosis patients by one of us (L.R.). Patients were staged using the modified Scadding radiographic staging system: stage 0 normal chest x-ray; stage 1 hilar and mediastinal adenopathy alone; stage 2 adenopathy and pulmonary infiltrates; stage 3 pulmonary infiltrates alone; and stage 4 pulmonary fibrosis.[4] Long-term follow-up clinical status and subsequent treatment regimens for all sarcoidosis patients were

obtained from the electronic medical record and telephone calls placed to the patients or their immediate family.

DNA Extraction

De-identified, formalin-fixed, paraffin-embedded blocks of lymph nodes from sarcoidosis and control patients were sent to the Departments of Laboratory Medicine and Microbiology at the University of Washington (Seattle, WA), where investigators were blinded as to the identity of the specimens. The DNA extraction from paraffin-embedded blocks was performed after paraffin was removed by incubation in xylene.[8]

PCR Analysis for 16S ribosomal DNA, Heat Shock Protein 65(hsp65), RNA polymerase subunit (rpoB)

The 16S gene fragment was amplified as previously described.[8] The hsp65 gene was amplified using TB11 and TB12 primers, and the RNA polymerase subunit gene (rpoB) was amplified using MF and MR primers.[9] The amplified products were then sequenced using the Big Dye Sequencing kit (Applied Biosystems, Foster City, CA) using the vendor's recommended protocol. The sequences of two strands were assembled into doubled-stranded contig using Sequencher software (Gene Codes, Ann Arbor, MI). The final sequences were used to search the National Center for Biotechnology Information (National Institutes of Health) database using BLAST (Basic Local Alignment Search Tool) to identify the amplified DNA.

Quantitative Variables

The primary variable to be compared between the sarcoidosis and controls patients is the number of patients in each group with bacterial DNA found in lymph nodes. The N-1 Two Proportion test for comparing independent proportions for small sample sizes is used to compare the results between the two groups.[10] All numerical data is expressed as the mean ± standard deviation.

RESULTS

Demographic and clinical characteristics of the 30 sarcoidosis study patients are found in Table 1.

Demographics

Patient ages are: mean 52.5 ± 12.3 years (median 53 years, range 30-75 years). The male:female ratio is 14:16. The ethnicity: Caucasian 73.3% (22), African-American 16.7% (5), and Hispanic 10% (3). Most patients were overweight: mean BMI 31.4 ± 6.9, median 28.5, range 18.8-47.3.

Clinical Presentation

At the time of initial presentation, 19 of 30 patients (63.3%) were symptomatic, usually with multiple symptoms. Of these 19 patients, the duration of symptoms before diagnosis was a mean 22.1 ± 30.0 months (median 12 months, range 1-120 months). The most common symptoms were night sweats 9 (30.0%); dyspnea 8 (26.7%); chest pain 7 (23.3%); chest tightness 5 (16.7); fevers 3 (10.0%); fatigue 3 (10%); skin rash 2 (6.7%); and stomach ulcer 2 (6.7%). Other symptoms present in at least one patient include: dyspepsia, dysphagia, diarrhea,

Table 1. Sarcoidosis Patient Results

No.	Age/ Sex	Race	X-ray Stage	Pack-Year Smoking	Prior Cancer	Chemo	PCR Result on Lymph Nodes	Initial Symptoms (year)	Follow-up (mo.)	Current Status (Long-Term)
1	**41/F**	**AA**	**I**	**7**	**Uterine**	**No**	***Mycobacteria chelonae***	**Yes (2009)**	**36**	**Sympt., alive**
2	**63/F**	**C**	**II**	**20**	**Breast**	**Yes**	***Mycobacteria chelonae***	**Yes (2008)**	**12**	**Sympt., deceased from COPD 2009**
3	**64/F**	**C**	**II**	**None**	**None**	**No**	***Mycobacteria mucogenicum***	**Yes (2008)**	**41**	**Sympt., alive**
4	43/M	C	I	None	Melanoma	No	Negative	No (2009)	31	Asympt., alive
5	61/F	C	I	None	Synovial cell sarcoma	Yes	Negative	No (2009)	23	Asympt.; new endomet. ca, alive
6	**39/M**	**C**	**II**	**5**	**None**	**No**	***Proprionobacterium acnes***	**Yes (2008)**	**35**	**Sympt., alive**
7	**58/M**	**C**	**I**	**3**	**Tonsil cancer**	**Yes**	***Corynebacterium propinquum***	**Yes (2006)**	**65**	**Sympt.; tonsil ca. relapse, alive**
8	**54/M**	**C**	**II**	**None**	**None**	**No**	***Proprionobacterium acnes***	**Yes (2006)**	**60**	**Sympt., alive**
9	52/F	AA	I	6	None	No	Negative	Yes (2007)	58	**Sympt., alive**
10	53/F	C	I	5	Melanoma	Yes	Negative	No (2007)	53	Asympt., alive
11	**34/M**	**AA**	**II**	**None**	**None**	**No**	***Proprionobacterium acnes***	**Yes (2007)**	**53**	**Sympt., alive**
12	40/M	C	I	Cigars	None	No	Negative	Yes (2007)	36	**Sympt., alive**
13	49/M	AA	II	None	None	No	***Duganella zoogloeoides***	Yes (2010)	Lost	Unknown, alive
14	**57/F**	**AA**	**II**	**5**	**Breast**	**Yes**	***Proprionobacterium acnes***	**Yes (2000)**	**136**	**Sympt; breast ca. relapse; polymyalgia rheumatica; Hashimoto's thyroditis, alive**
15	67/M	C	I	None	None	No	Negative	No (2001)	Lost	Unknown, alive
16	30/M	H	I	None	None	No	Negative	No (2001)	Lost	Unknown, alive
17	75/F	C	I	None	Ovarian	Yes	Negative	No (2002)	36	Asymptomatic, deceased from ca., 2005
18	67/F	C	I	50	Liposarcoma	No	Negative	No (2003)	106	Sympt., alive
19	60/F	C	I	None	Nerve sheath tumor	No	Negative	No (2003)	60	Asympt., deceased unknown cause 2008
20	41/F	C	I	None	None	No	Negative	No (2005)	78	Asympt., alive
21	50/F	C	I	None	None	No	Negative	Yes (2005)	73	Asympt., alive
22	**48/F**	**C**	**I**	**None**	**Breast**	**Yes**	***Proprionobacterium acnes***	**Yes (2003)**	**24**	**Sympt.; deceased from ca. 2005**
23	41/F	C	II	None	Adrenal ca; melanoma	Yes	Negative	Yes (2006)	4	Deceased from ca. 2007
24	35/M	H	I	2.5	None	No	Negative	Yes (2006)	24	Asympt., alive
25	32/M	C	I	1.0	None	No	Negative	No (2006)	Lost	Unknown, alive
26	**37/M**	**C**	**II**	**15**	**Hodgkin's disease**	**Yes**	***Proprionobacterium acnes***	**Yes (2007)**	**66**	**Sympt., alive**
27	51/F	AA	II	None	None	No	Negative	No (2007)	64	Sympt; (mildly), alive
28	56/F	C	II	None	Colon ca.	Yes	Negative	No (2008)	Lost	Unknown, alive
29	33/M	C	I	5	Melanoma	No	Negative	No (2009)	38	Asympt., alive
30	52/F	H	II	1	Uterine	No	Negative	Yes (2002)	48	Asympt., alive

Table 1 abbreviations: AA = African-American; Asympt. = asymptomatic; C = Caucasian; Ca. = cancer; Chemo = chemotherapy; COPD = chronic obstructive pulmonary disease; H = Hispanic; Sympt. = symptomatic. All patients positive on PCR for microorganism DNA in lymph nodes are shown in bold type.

constipation, kidney stones, joint and muscle pains, orthopnea, nose and mouth skin lesions, intermittent bronchospasm, malaise, and weight loss.

Co-morbidities: asthma 2 (6.7%); coronary artery disease 2 (6.7%); diabetes mellitus 4 (13.3%); hypertension 5 (16.6), and one each of gout, hypothyroidism, eczema, fibromyalgia, and Crohn's disease. Malignancies were extremely common with over half (53.3%) having a current or prior tumor. The malignancies prior to or at the time of presentation are: breast 3, melanoma 3, uterine 2, sarcomas 3, tonsil 1, ovary 1, adrenal 1, colon 1, and Hodgkin's lymphoma 1.

Radiographic Studies

Chest CT was performed on all 30 patients and all had symmetrical mediastinal and hilar adenopathy. Four of 30 (13.3%) had obvious abdominal adenopathy. Lung nodules were present in 12 patients (40.0%) and were radiographic Stage II sarcoidosis. The other 18 patients (60.0%) had Stage I disease. All 12 Stage II patients were symptomatic. PET/CT scans were done in 25 of 30 sarcoidosis patients. All demonstrated glucose avidity in the enlarged mediastinal and hilar nodes (see Figure 2 for typical example), and glucose avidity was seen in the abnormal abdominal nodes in the 4 patients with radiographic adenopathy below the diaphragm.

Laboratory Results

Twelve of 30 patients had lymph node tissue sent at the time of mediastinoscopy for aerobic, fungal and mycobacterial cultures. All cultures showed no growth after six weeks incubation.

Bacterial DNA Detected by PCR

Eleven of 30 lymph nodes (36.7%) in sarcoidosis patients had bacterial DNA present by PCR. Only 2 of 30 (6.7%) control patients were found to have bacterial DNA in their lymph nodes. The microorganisms present in each group are shown in Table 2. There are significantly more sarcoidosis patient lymph nodes positive for microorganism DNA than control lymph nodes: 11/30 versus 2/30, $p = 0.00516$ (2-tailed p-value).

All sarcoidosis patients with detectable bacterial DNA in lymph nodes (36.7%) were symptomatic at presentation. Additionally, 73% (8/11) of bacterial DNA-positive sarcoidosis patients were both symptomatic at presentation and had radiographic Stage II disease.

Long-Term Follow-up

Long-term follow-up was complete in 25 of 30 (83.3%) of sarcoidosis patients, for a mean follow-up of 50.4 $\pm$ 28.2 months (median 48 months, range 4 -132 months). Five of these patients are deceased: 3 from cancers, 1 from chronic obstructive pulmonary disease, and one from unknown causes. The other five patients lost to direct follow-up are still living based on information obtained from the Social Security Death Index.[11]

Of the 10 sarcoidosis patients with bacterial DNA found in their lymph nodes in whom long-term follow-up was available, **all** were symptomatic at follow-up a mean 52.8 $\pm$ 32.4 months (median 47 months, range 12-136 months). The one additional bacterial DNA-positive patient was lost to follow-up.

Table 2. Bacterial DNA detected by PCR

Sarcoidosis (11/30)	Control (2/30)
Propionibacterium acnes: 7	*Mycobacterium avium*: 1
Mycobacterium	*Propionibacterium acnes:* 1
chelonae: 2	
mucogenicum: 1	
Duganella zooloeoides: 1	
Corynebacterium propinquum: 1	

DISCUSSION

The objectives of this case-control study were two-fold: a) evaluate sterilely-resected lymph nodes in documented sarcoidosis patients for the presence of bacterial DNA by molecular methods and b) correlate the results with clinical characteristics of the patients.

Bacterial DNA

As expected, our molecular testing using PCR demonstrated that over one–third of sarcoidosis patients (36.7%) had evidence of bacterial DNA in the nodes, indicating either past or current involvement with these microorganisms. This percentage of bacterial DNA-positive specimens falls in the range found in numerous prior published studies from the last two decades (using various methodologies), which range from 26%-80% positive (Table 3[5,7,12-15]). Furthermore, atypical mycobacteria and *Proprionibacterium acnes* represented almost all DNA identified, also consistent with the findings of the multiple prior studies (Table 3). Additionally identified were one skin and mucous membrane organism (*Corynebacterium propinquum*), and one aerobic Gram negative bacillus (*Duganella zoogloeoides*) that is usually found in aqueous environments. Interestingly, the latter patient (no. 13) with *Duganella zoogloeoides* was an asbestos technician originally from tropical Haiti. As a disclaimer, just the finding of DNA from a microorganism in lymph nodes does not tell us whether the viable organism is present nor whether it caused the granulomatous reaction.

Similar to prior published studies summarized in Table 3, significantly less (only 2 of 30 or 6.7%, $p = 0.00516$) of control lymph nodes resected at the time of lung cancer surgery showed evidence of bacterial DNA (*Mycobacterium avium intracellulare* and *Propionibacterium acnes*). This difference strongly suggests that the demonstration of bacterial DNA in sarcoidosis lymph

Table 3. Selected studies of DNA of infectious agents found in sarcoidosis tissues

Author/Year	Sarcoid Tissue (No. patients)	Technique	Organisms (%)	Control No. (% organisms)
Li, 1999[11]	Skin (20)	PCR (restriction enzyme pattern)	*Mycobacteria* (2 *tuberculosis*, 14 other *mycobacteria*. 80% total positive)	20 Normals (0% organisms)
Du Bois, 2003[7] (Review of pre-1999 studies)	Lymph nodes (12 studies with 295 patients)	PCR (various methods)	*Mycobacterium* sp. (34%)	Various
Eishi, 2002[12] (5 center study)	Lymph nodes (108)	Quantitative real-time PCR	*P. acnes* (72%) *P. granulosum* (55%) *M. tuberculosis* (4%) *E. coli* (2%)	86 Normals (29% *P. acnes*, 12% *P. granulosum*, 2% *M. tuberculosis*)
Drake, 2002[13]	Lymph nodes (25)	Nested PCR	*Mycobacterium* sp. (60%)	25 Normals (0%)
Gupta, 2007[5] (metanalysis)	Various (31 studies with 874 patients	PCR (various methods)	*Mycobacterium* sp. (26%)	745 Controls (3%)
Ichikama, 2008[14]	Bronchoalveolar lavage (42)	Quantitative PCR	*Propionibacterium* sp. (3X higher genome levels vs. controls)	30 Controls (low levels same genome)

nodes is a real finding in our study (and in over 35 prior published studies) and they are not just processing contaminants, therefore pointing to microorganisms as potential contributors to the genesis of this disease. In addition, *Propionibacterium acnes* DNA was found in only 1 of 30 (3.3%) control lymph nodes in our study, in stark contrast to Ishige and associates in Japan who reported this microorganism is an ubiquitous pulmonary lymph node commensal found in 8 of 11 (72.7%) non-sarcoid patients in their study.[16] Such a very high positive result in their study is likely due to either geographical/ethic/racial differences or potential contamination in processing.

Clinical Characteristics

Perhaps the most intriguing findings came from correlation of the PCR findings with the clinical information. All patients with lymph nodes containing bacteria DNA on presentation were also highly symptomatic and 75% of them had the poorer-prognosis radiographic stage II findings. Moreover, after a median 4 years follow-up, all bacterial DNA-positive patients were *still* highly symptomatic. This striking correlation strongly suggests that demonstration of bacterial DNA by PCR in lymph nodes on initial presentation is an adverse prognostic factor and makes it unlikely that these patients will have a spontaneous remission.

Indeed, if infection with one of these microorganisms triggers an exuberant granulomatous immune response, the 50-80% of patients who usually have a spontaneous remission[1] likely clear the offending organism and the immune reaction subsides. We postulate that those patients who have persisting symptomatic disease, likely continue to harbor the microorganism which perpetuates the vigorous, destructive immune response, and as well as permit the microorganism to travel elsewhere to other organs to create distant granulomatous inflammation.

Limitations of Study

Many pathogenic microorganisms such as *Tropheryma whippelii* (Whipple's disease) or coronaviruses (severe acute respiratory syndrome) cannot be grown directly in culture or they are very slow growing or fastidious such as *Mycobacterium leprae* (leprosy).[5] In these instances, detection and identification rely on molecular mechanisms such PCR used in this study. Nevertheless, the molecular approach has distinct limitations including possible false-positive results secondary to contaminated PCR reagents, the paraffin imbedding process, or post-embedding handling and processing of the paraffin block. However in our study, thirty control lymph nodes were processed in an identical manner and bacterial DNA was detected in only 2/30 (6.6%), significantly less than the sarcoidosis nodes (36.7%, $p = 0.00516$), suggesting that contamination is unlikely to account for the findings.

Additionally, the number of lymph nodes positive for bacterial DNA may be significantly underestimated because of the tendency of the formalin-fixation and paraffin embedding process to breakdown prokaryotic DNA. Also, over time other investigators have found degradation of the prokaryotic bacteria DNA (especially mycobacteria) with aging of the paraffin-embedded specimens.[14] Of note, the only 3 sarcoidosis lymph nodes positive for mycobacteria in our study were less than 3 years old when evaluated by PCR. Had we used fresh lymph node tissue like Drake and associates[14] who found 60% PCR positive for mycobacteria species, there may have been a much higher rate of positive bacterial DNA results (particularly mycobacteria) in our study.

Implications of the Study

Sarcoidosis is a granulomatous disease primarily involving the lungs, lymph nodes and other organs that appears to be the result of an exuberant T cell and macrophage immunologic response to the continued presentation of a poorly degradable antigen. Numerous non-infective agents have been implicated based on epidemiologic basis but none have stood up to scrutiny.[1,3,5] The focus over the last two decades has been on infective agents that might trigger sarcoidosis, with the strongest suspects found in the mycobacteria family and the common commensal *Propionibacterium acnes.* And like classical tuberculosis where up to 90% of people infected with *Mycobacterium tuberculosis* remain in remission without treatment,[17] sarcoidosis also has a 65-80% spontaneous remission rate without treatment.[1] One may speculate that similar to tuberculosis, the immune system, after its initial response to a triggering microorganism, is successful in eradicating the agent and the immune response subsides. Then in the 20% or so with persistent and progressive sarcoidosis, the organism remains viable and perpetuates the destructive immune response.

Symptomatic sarcoidosis is usually treated with various anti-inflammatory and immunosuppressive agents such as corticosteroids, methotrexate and TNF-inhibitors (biologics).[18] The similarities in immunologic abnormalities and treatment to another debilitating granulomatous disease, Crohn's disease, are striking.[19] Granulomatous ileitis (Crohn's) has been suspected by many investigators to be the result of a chronic infection with the obligate intracellular microorganism *Mycobacterium avian* subspecies *paratuberculosis* (MAP), that is known to cause a granulomatous ileitis in cattle and other ruminants called Johne's disease.[20] Although the classical treatment of Crohn's disease has been with immunosuppressive agents just like sarcoidosis, many recent studies suggest a much more effective treatment with less side

effects may be a triple antibiotic regimen geared toward the putative triggering agent MAP.[21-23] In fact, many in the field suspect that this intracellular organism (MAP) that resides in the macrophage impairs the normal autophagy that would usually eradicate the organism.[21] Agents that enhance autophagy such as 16α-bromoepiandersterone,[24,25] currently in human trials, may prove effective along with antibiotics in Crohn's disease.[21]

Can some antibacterial/anti-mycobacterial regimen such as that used in Crohn's disease alter the natural history of sarcoidosis in chronically symptomatic patients? Sixty years ago a number of small trials using classical anti-tuberculous drugs (isoniazide, streptomycin, or cortisone) were published with discouraging results.[26] However, atypical mycobacteria (rather than *M. tuberculosis*) that are more likely to be one of the etiologic agents in sarcoidosis, are almost all resistant to the standard anti-tuberculosis agents such as isoniazid.[27-32] And if other organisms such as *Proprionibacterium acnes* or perhaps cell-wall deficient (L-forms) bacteria trigger and perpetuate sarcoidosis in some individuals, then the standard anti-tuberculous drugs would also be ineffective.

The tetracycline derivatives (minocycline and doxycycline), as well as the anti-malarial drug chloroquine have been shown to be quite effective in treating cutaneous sarcoidosis.[33] Minocycline can produce complete responses in up two-thirds of cases, although it is debated whether this is an anti-microbial effect or an immunomodulating effect.

Conclusions

Over the last three decades or more, numerous studies have examined every aspect of sarcoidosis including its dysfunctional immune response. The primary therapy is immune

suppression in various forms but this treats only symptoms and does not seem to alter the natural history of the disease.[4,18] Dozens of studies (Table 3) have repeatedly demonstrated evidence of microorgnisms in 30-80% of sarcoidosis tissues, mostly various mycobacteria and *Proprionibacterium acnes,* and more of these molecular studies is not likely warranted.

Perhaps we should follow the lead of the Crohn's disease gastroenterologists[21,22] and proceed with a therapeutic clinical trial using a regimen of multiple antibiotics in persistently-symptomatic, advanced stage sarcoidosis patients. Indeed, if there is a persistent, viable microorganism infection causing the continuing or progressive debilitating symptoms and organ failure, antibiotics might favorably impact the course of this disease.

FIGURE LEGENDS

Figure 1. Contrast-enhanced computed chest tomography at 2 different axial levels showing typical symmetrical hilar and mediastinal adenopathy.

Figure 2. PET/CT (coronal view) of symmetrical hypermetabolic mediastinal and hilar lymph nodes (arrows).

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Dr. Robinson: Contributed to the conception, hypotheses delineation and design of the study; data acquisition, analysis and interpretation; and writing and revision of the article prior to submission.

Dr. Smith: Contributed to the conception, hypothesis delineation, and design of the study; data acquisition, analysis and interpretation; and revision of the article prior to publication.

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ABBREVIATIONS

BMI = body mass index

CD4+ T cells = cluster of differentiation 4 thymic lymphocyte cells

CT = computed tomography

DNA = deoxyribonucleic acid

MAP = *Mycobacterium avium* subspecies *paratuberculosis*

PCR = polymerase chain reaction

PET = positron emission tomography

RNA = ribonucleic acid

rRNA PCR = ribosomal ribonucleic acid PCR

16S rDNA= 16 subunit of ribosomal DNA

TNF-α = tumor necrosis factor-alpha

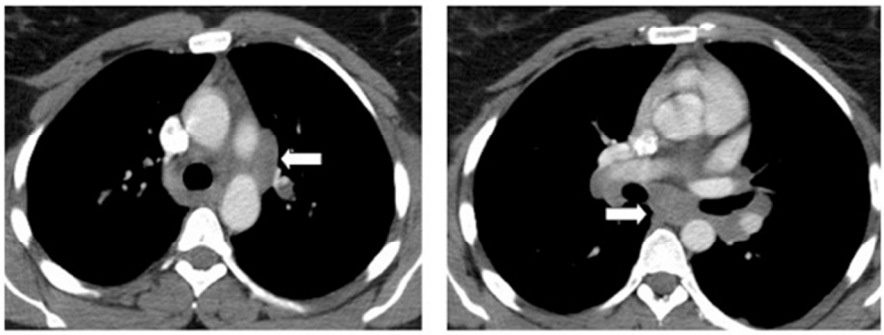

Fig. 1. Contrast-enhanced computed chest tomography at 2 different axial levels showing typical symmetrical hilar and mediastinal adenopathy

Contrast-enhanced computed chest tomography at 2 different axial levels showing typical symmetrical hilar and mediastinal adenopathy

225x169mm (72 x 72 DPI)

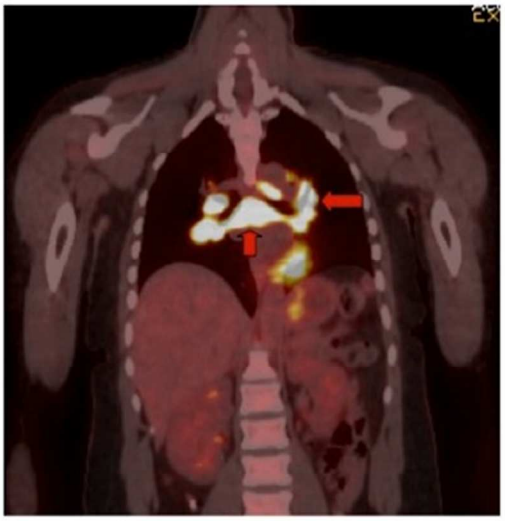

Fig 2. PET/CT (coronal view) of symmetrical hypermetabolic mediastinal and hilar lymph nodes

PET/CT (coronal view) of symmetrical hypermetabolic mediastinal and hilar lymph nodes (arrows)

225x169mm (72 x 72 DPI)

STROBE 2007 (v4) checklist of items to be included in reports of observational studies in epidemiology*

Checklist for cohort, case-control, and cross-sectional studies (combined)

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract Page 1, 3, 4	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	
Introduction			
Background/rationale **Pages 5-6**	2	Explain the scientific background and rationale for the investigation being reported	
Objectives **Pages 5-6**	3	State specific objectives, including any pre-specified hypotheses	
Methods			
Study design Page6	4	Present key elements of study design early in the paper	
Setting Pages 6-7	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	
Participants Pages 7-8	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables Pages 6-7	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
Data sources/ measurement Pages 6-7	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	
Bias	9	Describe any efforts to address potential sources of bias	
Study size Not applicable in this case control observational trial	10	Explain how the study size was arrived at	
Quantitative variables Pages 6-7	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods Pages 6, 7, 9	12	(*a*) Describe all statistical methods, including those used to control for confounding	
		(*b*) Describe any methods used to examine subgroups and interactions	

		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	
Results			
Participants Table 1	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data Table 1	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data Tables 1 and 2	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results Tables 1 and 2	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses Not applicable	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion Discussion section			
Key results	18	Summarise key results with reference to study objectives	
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability **Conclusion section**	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding Title page	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

MOLECULAR ANALYSIS OF SARCOIDOSIS LYMPH NODES FOR MICROORGANISMS: A CASE-CONTROL STUDY WITH CLINICAL CORRELATES

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Title: "MOLECULAR ANALYSIS OF SARCOIDOSIS LYMPH NODES FOR MICROORGANISMS: A CASE-CONTROL STUDY WITH CLINICAL CORRELATES"

Running Head: Sarcoidosis and Bacteria

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ABSTRACT

INTRODUCTION: Sarcoidosis is an incurable, chronic granulomatous disease primarily involving the lungs and lymph nodes of unknown etiology, treated with non-specific anti-inflammatory/immunosuppressive drugs. Persistently symptomatic patients worsen with a disabling, potentially fatal clinical course. To determine a possible infectious cause, we correlated in a case control study clinical information with the presence of bacterial DNA in sarcoidosis mediastinal lymph nodes compared to control lymph nodes resected during cancer surgery.

METHODS: We retrospectively studied formalin-fixed, paraffin-embedded, mediastinal lymph nodes from 30 sarcoidosis patients and 30 control lung cancer patients. Nucleic acids were extracted from nodes, were evaluated by rRNA PCR for bacterial 16S rDNA, and the result was sequenced and compared to a bacterial sequence library. Clinical information was correlated.

RESULTS: 11/30 (36.7%) of lymph nodes from sarcoidosis patients had detectable bacterial DNA, significantly more than control patient lymph nodes (2/30, 6.7%), $p = 0.00516$. At presentation, 19/30 (63.3%) sarcoidosis patients were symptomatic including all patients with detectable bacterial DNA. Radiographically, there were 18 Stage I and 12 Stage II patients. All Stage II patients were symptomatic and 75% had PCR-detectable bacteria. After a mean follow-up of 52.8 $\pm$ 32.8 months, **all** patients with PCR-detectable bacteria in this series were persistently symptomatic requiring treatment.

DISCUSSION: 36.6% of sarcoidosis patients had detectable bacteria DNA on presentation, **all of these patients** were quite symptomatic, and most were radiographically-advanced stage II.. These findings suggest bacterial DNA-positive, symptomatic patients have more aggressive

sarcoidoisis that persists long term, and might benefit from antimicrobial treatment directed against this presumed chronic granulomatous infection.

ARTICLE SUMMARY:

ARTICLE FOCUS:

- Sarcoidosis is a common yet incurable, chronic granulomatous disease of unknown etiology treated with non-specific anti-inflammatory and/or immunosuppressive drugs.
- Persistently symptomatic patients worsen despite treatment with a disabling, potentially fatal clinical course.
- We propose that sarcoidosis results from a chronic granulomatous infection from a treatable pathogen in certain susceptible individuals, much like granulomatous ileitis (Crohn's disease) which shows an encouraging response to multi-drug antimicrobial therapy.

KEY MESSAGES:

- This case-control, retrospective study correlated clinical outcomes with the presence of detectable bacterial DNA in sarcoidosis lymph nodes versus control lymph nodes.
- The entire group of sarcoidosis patients had significantly more detectable bacterial DNA than control patient lymph nodes.

- However, **all** persistently symptomatic patients have detectable bacterial DNA in their lymph nodes suggesting they have more aggressive sarcoidosis that potentially might benefit from antimicrobial treatment directed against a presumed chronic granulomatous infection.

STRENGTHS AND LIMITATIONS:

- Although a number of prior studies have demonstrated the consistent presence of bacterial DNA (mostly atypical mycobacteria and *Propionibacterium acnes*) in sarcoidosis tissue, the current study is the first to correlate clinical outcomes with the presence of detectable bacterial DNA, suggesting the most promising candidates for treatment.
- Nevertheless, the molecular approach to bacterial detection has distinct limitations including possible false-positive results secondary to contaminated PCR reagents, the paraffin imbedding process, or post-embedding handling and processing of the paraffin block. Also the mere physical presence of the bacterial DNA in the lymph nodes does not prove that the disease is caused by the microorganism.
- Additionally, the number of lymph nodes positive for bacterial DNA may be significantly underestimated because of the tendency of the formalin-fixation and paraffin embedding process to breakdown prokaryotic DNA.

INTRODUCTION

Sarcoidosis is a multisystem, granulomatous disease whose etiology is obscure and controversial. Nevertheless, the condition is relatively common with a significantly higher age-adjusted annual incidence in African-Americans (35.5 cases per 100,000) versus Caucasian-Americans (10.9 cases per 100,000). An estimated one million people in the U. S. have this disease. Based on the current U.S. population of 315,556,000, there will be approximately 38,605 new cases of sarcoidosis this year and just over 1000 (2.6%) will die of the illness.[1,2]

The fundamental pathologic abnormality in the disease is the formation of non-caseating epitheliod granulomas, which usually confine poorly soluble foreign material that simply cannot be removed by a single phagocytic cell. The key feature in sarcoidosis is activated CD4+ T cells which differentiate into type 1 helper T cells (Th1), secreting interleukin-2 and interferon-γ, augmenting macrophage TNF-α, and amplifying the local cellular immune response.[3,4] This granulomatous inflammation interferes with local tissue homeostasis leading to organ impairment.

Since sarcoidosis primarily involves the lungs, eyes and skin, attention has focused on airborne environmental antigens that might trigger this presumed hypersensitivity response with its T cell-mediated cellular immune response.[3] Similar granulomatous responses can be seen from a variety of infectious agents including mycobacteria, parasites (schistosoma), and fungi (coccidiomycosis). Early studies reported associations with non-infective agents including beryllium, zirconium, aluminum, wood-burning stoves, tree pollen, clay soil, talc, insecticides, inorganic particles, and moldy environments, but none of these theoretical causes has endured.[3,5,6] There is also a several-fold increased incidence of sarcoidosis occurring in siblings

and parents, as well as a consistent strong association with specific gene products such as class I and class II HLA antigens, which may add to the familial connection.

Although no infectious agent has been cultured directly from sarcoidosis tissues, clinical and immunologic characteristics of the disorder provide the strongest support for a microbial etiology, at least in some patients.[5-7] To explore a possible infectious cause in patients seen at the Moffitt Cancer Center, we correlated the clinical presentation and long-term follow-up of sarcoidosis patients with the presence of bacterial DNA in archived, surgically-resected mediastinal lymph nodes. Results from sarcoidosis nodes were compared to control lymph nodes resected at the time of lung surgery in node-negative, Stage I non-small cell lung cancer patients.

METHODS

Regulatory Oversight

Tissue and clinical data in this case-control study was obtained after approval by the Moffitt Cancer Center Scientific Review Committee Protocol MCC #16131 and the University of South Florida IRB Protocol #108656.

Study Design

By searching the Moffitt Cancer Center surgical pathology database between January 1, 2000 and April 1, 2010, we retrospectively identified 30 randomly-chosen patients who were diagnosed with sarcoidosis based on the typical radiographic and clinical presentation, and the histologic finding of non-caseating epitheliod granulomata in lymph nodes obtained sterilely only by mediastinoscopy, to avoid possible microorganism contamination by endoscopic biopsies. Special stains for microorganisms were negative on the specimens. For inclusion in

this group, the following criteria were used to make the diagnosis of sarcoidosis: 1) chest radiograph and chest CT findings of symmetrical mediastinal and hilar adenopathy with or without reticulonodular infiltrates in the lung fields (see Figure 1); 2) when performed, PET scans demonstrated glucose avidity in the enlarged lymph nodes (see Figure 2); 3) asymptomatic presentation or typical symptoms of dyspnea, cough, chest tightness/pain, night sweats, fevers, fatigue, malaise, skin rash or weight loss; 4) lymph nodes showing histologic features of confluent, non-caseating granulomata; and 5) any known microorganism causes of granulomata were excluded by history or culture. All histopathologic specimens were reviewed by one of us (P.S.) to reconfirm the diagnosis made originally by departmental pathologists at Moffitt.

To insure sterile collection, all control specimens were taken from lymph nodes removed at open thoracotomy by one of us (L.R.) in 30 (1:1 match with cases) randomly-chosen patients with Stage IA non-small lung cancer. Lymph nodes were selected only from patients with small peripheral tumors, no obstructive atalectasis, and no evidence of active infection. One of us (P.S) reviewed all control lymph node histology to verify there were no metastases, acute inflammation, or granulomata.

Clinical Data

Clinical data on the patient demographics, initial presenting symptoms and objective findings were extracted from the electronic medical record on all sarcoidosis patients by one of us (L.R.). Patients were staged using the modified Scadding radiographic staging system: stage 0 normal chest x-ray; stage 1 hilar and mediastinal adenopathy alone; stage 2 adenopathy and pulmonary infiltrates; stage 3 pulmonary infiltrates alone; and stage 4 pulmonary fibrosis.[4] Long-term follow-up clinical status and subsequent treatment regimens for all sarcoidosis patients were

obtained from the electronic medical record and telephone calls placed to the patients or their immediate family.

DNA Extraction

De-identified, formalin-fixed, paraffin-embedded blocks of lymph nodes from sarcoidosis and control patients were sent to the Departments of Laboratory Medicine and Microbiology at the University of Washington (Seattle, WA), where investigators were blinded as to the identity of the specimens. The DNA extraction from paraffin-embedded blocks was performed after paraffin was removed by incubation in xylene using the Roche HighPure PCR template purification kit (Roche Diagnostics GmbH, Mannheim, Germany.[8] Several negative patient samples (unrelated to the present study) were included in each batch to rule out contamination. Inhibition was ruled out by checking the ability of exogenously added target DNA to be amplified in the same PCR mix that contained DNA extracted from clinical specimen. *Mycobacterium tuberculosis* and *Bartonella henselae* have been detected multiple times in the past using these PCR assays. 16S primers used were broad range for all bacteria. hsp65 and rpoB were broad range for Mycobacteria spp only. 16S PCR detected non-Mycobacterium species DNA such as *Propionibacterium acnes*. Mycobacterium sp. DNA were detected by hsp65 and/or rpoB primers. Primers used for amplification were also used for amplicon sequencing. The PCR amplicon was directly sequenced; no cloning was performed. Mixed infection was not detected in this set of specimens. For alignment, BLASTN was used. Identification was based on exact match on all cases. No sequence that could not be linked to a microbe was detected.

P. acnes was detected by 16S primers and *M. avium* was detected by hsp65 primers. *Mycobacterium avium* was detected and identified by sequence analysis.

PCR Analysis for 16S ribosomal DNA, Heat Shock Protein 65(hsp65), RNA polymerase subunit (rpoB)

The 16S gene fragment was amplified as previously described.[8] The hsp65 gene was amplified using TB11 and TB12 primers, and the RNA polymerase subunit gene (rpoB) was amplified using MF and MR primers.[9] The amplified products were then sequenced using the Big Dye Sequencing kit (Applied Biosystems, Foster City, CA) using the vendor's recommended protocol. The sequences of two strands were assembled into doubled-stranded contig using Sequencher software (Gene Codes, Ann Arbor, MI). The final sequences were used to search the National Center for Biotechnology Information (National Institutes of Health) database using BLAST (Basic Local Alignment Search Tool) to identify the amplified DNA.

Quantitative Variables

The primary variable to be compared between the sarcoidosis and controls patients is the number of patients in each group with bacterial DNA found in lymph nodes. The N-1 Two Proportion test for comparing independent proportions for small sample sizes is used to compare the results between the two groups.[10] Additionally, odds ratios with 95% confidence intervals were calculated.[11] All numerical data is expressed as the mean $\pm$ standard deviation.

RESULTS

Demographic and clinical characteristics of the 30 sarcoidosis study patients are found in Table 1.

Demographics

Patient ages are: mean 52.5 ± 12.3 years (median 53 years, range 30-75 years). The male:female ratio is 14:16. The ethnicity: Caucasian 73.3% (22), African-American 16.7% (5), and Hispanic 10% (3). Most patients were overweight: mean BMI 31.4 ± 6.9, median 28.5, range 18.8-47.3.

Clinical Presentation

At the time of initial presentation, 19 of 30 patients (63.3%) were symptomatic, usually with multiple symptoms. Of these 19 patients, the duration of symptoms before diagnosis was a mean 22.1 ± 30.0 months (median 12 months, range 1-120 months). The most common symptoms were night sweats 9 (30.0%); dyspnea 8 (26.7%); chest pain 7 (23.3%); chest tightness 5 (16.7); fevers 3 (10.0%); fatigue 3 (10%); skin rash 2 (6.7%); and stomach ulcer 2 (6.7%). Other symptoms present in at least one patient include: dyspepsia, dysphagia, diarrhea,

Table 1. Sarcoidosis Patient Results

No.	Age/ Sex	Race	X-ray Stage	Pack-Year Smoking	Prior Cancer	Chemo	PCR Result on Lymph Nodes	Initial Symptoms (year)	Follow-up (mo.)	Current Status (Long-Term)
1	**41/F**	**AA**	**I**	**7**	**Uterine**	**No**	***Mycobacteria chelonae***	**Yes (2009)**	**36**	**Sympt., alive**
2	**63/F**	**C**	**II**	**20**	**Breast**	**Yes**	***Mycobacteria chelonae***	**Yes (2008)**	**12**	**Sympt., deceased from COPD 2009**
3	**64/F**	**C**	**II**	**None**	**None**	**No**	***Mycobacteria mucogenicum***	**Yes (2008)**	**41**	**Sympt., alive**
4	43/M	C	I	None	Melanoma	No	Negative	No (2009)	31	Asympt., alive
5	61/F	C	I	None	Synovial cell sarcoma	Yes	Negative	No (2009)	23	Asympt.; new endomet. ca, alive
6	**39/M**	**C**	**II**	**5**	**None**	**No**	***Proprionibacterium acnes***	**Yes (2008)**	**35**	**Sympt., alive**
7	**58/M**	**C**	**I**	**3**	**Tonsil cancer**	**Yes**	***Corynebacterium propinquum***	**Yes (2006)**	**65**	**Sympt.; tonsil ca. relapse, alive**
8	**54/M**	**C**	**II**	**None**	**None**	**No**	***Proprionibacterium acnes***	**Yes (2006)**	**60**	**Sympt., alive**
9	52/F	AA	I	6	None	No	Negative	Yes (2007)	58	Sympt., alive
10	53/F	C	I	5	Melanoma	Yes	Negative	No (2007)	53	Asympt., alive
11	**34/M**	**AA**	**II**	**None**	**None**	**No**	***Proprionibacterium acnes***	**Yes (2007)**	**53**	**Sympt., alive**
12	40/M	C	I	Cigars	None	No	Negative	Yes (2007)	36	Sympt., alive
13	49/M	AA	II	None	None	No	***Duganella zoogloeoides***	Yes (2010)	Lost	Unknown, alive
14	**57/F**	**AA**	**II**	**5**	**Breast**	**Yes**	***Proprionibacterium acnes***	**Yes (2000)**	**136**	**Sympt; breast ca. relapse; polymyalgia rheumatica; Hashimoto's thyroditis, alive**
15	67/M	C	I	None	None	No	Negative	No (2001)	Lost	Unknown, alive
16	30/M	H	I	None	None	No	Negative	No (2001)	Lost	Unknown, alive
17	75/F	C	I	None	Ovarian	Yes	Negative	No (2002)	36	Asymptomatic, deceased from ca., 2005
18	67/F	C	I	50	Liposarcoma	No	Negative	No (2003)	106	Sympt., alive
19	60/F	C	I	None	Nerve sheath tumor	No	Negative	No (2003)	60	Asympt., deceased unknown cause 2008
20	41/F	C	I	None	None	No	Negative	No (2005)	78	Asympt., alive
21	50/F	C	I	None	None	No	Negative	Yes (2005)	73	Asympt., alive
22	**48/F**	**C**	**I**	**None**	**Breast**	**Yes**	***Proprionibacterium acnes***	**Yes (2003)**	**24**	**Sympt.; deceased from ca. 2005**
23	41/F	C	II	None	Adrenal ca; melanoma	Yes	Negative	Yes (2006)	4	Deceased from ca. 2007
24	35/M	H	I	2.5	None	No	Negative	Yes (2006)	24	Asympt., alive
25	32/M	C	I	1.0	None	No	Negative	No (2006)	Lost	Unknown, alive
26	**37/M**	**C**	**II**	**15**	**Hodgkin's disease**	**Yes**	***Proprionibacterium acnes***	**Yes (2007)**	**66**	**Sympt., alive**
27	51/F	AA	II	None	None	No	Negative	No (2007)	64	Sympt; (mildly), alive
28	56/F	C	II	None	Colon ca.	Yes	Negative	No (2008)	Lost	Unknown, alive
29	33/M	C	I	5	Melanoma	No	Negative	No (2009)	38	Asympt., alive
30	52/F	H	II	1	Uterine	No	Negative	Yes (2002)	48	Asympt., alive

Table 1 abbreviations: AA = African-American; Asympt. = asymptomatic; C = Caucasian; Ca. = cancer; Chemo = chemotherapy; COPD = chronic obstructive pulmonary disease; H = Hispanic; Sympt. = symptomatic. All patients positive on PCR for microorganism DNA in lymph nodes are shown in bold type.

constipation, kidney stones, joint and muscle pains, orthopnea, nose and mouth skin lesions, intermittent bronchospasm, malaise, and weight loss.

Co-morbidities: asthma 2 (6.7%); coronary artery disease 2 (6.7%); diabetes mellitus 4 (13.3%); hypertension 5 (16.6), and one each of gout, hypothyroidism, eczema, fibromyalgia, and Crohn's disease. Malignancies were extremely common with over half (53.3%) having a current or prior tumor, a finding noted previously by others.[12] The malignancies prior to or at the time of presentation are: breast 3, melanoma 3, uterine 2, sarcomas 3, tonsil 1, ovary 1, adrenal 1, colon 1, and Hodgkin's lymphoma 1.

Radiographic Studies

Chest CT was performed on all 30 patients and all had symmetrical mediastinal and hilar adenopathy. Four of 30 (13.3%) had obvious abdominal adenopathy. Lung nodules were present in 12 patients (40.0%) and were radiographic Stage II sarcoidosis. The other 18 patients (60.0%) had Stage I disease. All 12 Stage II patients were symptomatic. PET/CT scans were done in 25 of 30 sarcoidosis patients. All demonstrated glucose avidity in the enlarged mediastinal and hilar nodes (see Figure 2 for typical example), and glucose avidity was seen in the abnormal abdominal nodes in the 4 patients with radiographic adenopathy below the diaphragm.

Laboratory Results

Twelve of 30 patients had lymph node tissue sent at the time of mediastinoscopy for aerobic, fungal and mycobacterial cultures. All cultures showed no growth after six weeks incubation.

Bacterial DNA Detected by PCR

Eleven of 30 lymph nodes (36.7%) in sarcoidosis patients had bacterial DNA present by PCR. Only 2 of 30 (6.7%) control patients were found to have bacterial DNA in their lymph nodes. The microorganisms present in each group are shown in Table 2. There are significantly more sarcoidosis patient lymph nodes positive for microorganism DNA than control lymph nodes: 11/30 versus 2/30, $p = 0.00516$ (2-tailed p-value); the odds ratio is 8.1053 with 95% confidence intervals 1.6115-40.7675, $p = 0.0111$.

All sarcoidosis patients with detectable bacterial DNA in lymph nodes (36.7%) were symptomatic at presentation. Additionally, 73% (8/11) of bacterial DNA-positive sarcoidosis patients were both symptomatic at presentation and had radiographic Stage II disease.

Long-Term Follow-up

Long-term follow-up was complete in 25 of 30 (83.3%) of sarcoidosis patients, for a mean follow-up of 50.4 $\pm$ 28.2 months (median 48 months, range 4 -132 months). Five of these patients are deceased: 3 from cancers, 1 from chronic obstructive pulmonary disease, and one from unknown causes. The other five patients lost to direct follow-up are still living based on information obtained from the Social Security Death Index.[13]

Of the 10 sarcoidosis patients with bacterial DNA found in their lymph nodes in this series in whom long-term follow-up was available, **all** were symptomatic at follow-up a mean 52.8 $\pm$ 32.4 months (median 47 months, range 12-136 months). The one additional bacterial DNA-positive patient was lost to follow-up.

Table 2. Bacterial DNA detected by PCR

Sarcoidosis (11/30)	Control (2/30)
***Propionibacterium acnes*: 7**	***Mycobacterium avium*: 1**
Mycobacterium	***Propionibacterium acnes:* 1**
***chelonae*: 2**	
***mucogenicum*: 1**	
***Duganella zooloeoides*: 1**	
***Corynebacterium propinquum*: 1**	

DISCUSSION

The objectives of this case-control study were two-fold: a) evaluate sterilely-resected lymph nodes in documented sarcoidosis patients for the presence of bacterial DNA by molecular methods and b) correlate the results with clinical characteristics of the patients.

Bacterial DNA

As expected, our molecular testing using PCR demonstrated that over one–third of sarcoidosis patients (36.7%) had evidence of bacterial DNA in the nodes, indicating either past or current involvement with these microorganisms. This percentage of bacterial DNA-positive specimens falls in the range found in numerous prior published studies from the last two decades (using various methodologies), which range from 26%-80% positive (Table 3[5,7,14-17]). Furthermore, atypical mycobacteria and *Proprionibacterium acnes* represented almost all DNA identified, also consistent with the findings of the multiple prior studies (Table 3). Additionally identified were one skin and mucous membrane organism (*Corynebacterium propinquum*), and one aerobic Gram negative bacillus (*Duganella zoogloeoides*) that is usually found in aqueous environments. Interestingly, the latter patient (no. 13) with *Duganella zoogloeoides* was an asbestos technician originally from tropical Haiti. As a disclaimer, just the finding of DNA from a microorganism in lymph nodes does not tell us whether the viable organism is present or whether it caused the granulomatous reaction.

Similar to prior published studies summarized in Table 3, significantly less (only 2 of 30 or 6.7%, $p = 0.00516$) of control lymph nodes resected at the time of lung cancer surgery showed

evidence of bacterial DNA (*Mycobacterium avium* and *Propionibacterium acnes*). This difference strongly suggests that the demonstration of bacterial DNA in sarcoidosis lymph

Table 3. Selected studies of DNA of infectious agents found in sarcoidosis tissues

Author/Year	Sarcoid Tissue (No. patients)	Technique	Organisms (%)	Control No. (% organisms)
Li, 1999[11]	Skin (20)	PCR (restriction enzyme pattern)	*Mycobacteria* (2 *tuberculosis*, 14 other *mycobacteria*. 80% total positive)	20 Normals (0% organisms)
Du Bois, 2003[7] (Review of pre-1999 studies)	Lymph nodes (12 studies with 295 patients)	PCR (various methods)	*Mycobacterium* sp. (34%)	Various
Eishi, 2002[12] (5 center study)	Lymph nodes (108)	Quantitative real-time PCR	*P. acnes* (72%) *P. granulosum* (55%) *M. tuberculosis* (4%) *E. coli* (2%)	86 Normals (29% *P. acnes*, 12% *P. granulosum*, 2% *M. tuberculosis*)
Drake, 2002[13]	Lymph nodes (25)	Nested PCR	*Mycobacterium* sp. (60%)	25 Normals (0%)
Gupta, 2007[5] (metanalysis)	Various (31 studies with 874 patients	PCR (various methods)	*Mycobacterium* sp. (26%)	745 Controls (3%)
Ichikama, 2008[14]	Bronchoalveolar lavage (42)	Quantitative PCR	*Propionibacterium* sp. (3X higher genome levels vs. controls)	30 Controls (low levels same genome)

nodes is a real finding in our study (and in over 35 prior published studies) and they are not just processing contaminants, therefore pointing to microorganisms as potential contributors to the genesis of this disease. In addition, *Propionibacterium acnes* DNA was found in only 1 of 30 (3.3%) control lymph nodes in our study, in stark contrast to Ishige and associates in Japan who reported this microorganism is an ubiquitous pulmonary lymph node commensal found in 8 of 11 (72.7%) non-sarcoid patients in their study.[18] Such a very high positive result in their study is likely due to either geographical/ethic/racial differences or potential contamination in processing.

Clinical Characteristics

Perhaps the most intriguing findings came from correlation of the PCR findings with the clinical information. All patients with lymph nodes containing bacteria DNA on presentation were also highly symptomatic and 75% of them had the poorer-prognosis radiographic stage II findings. Moreover, after a median 4 years follow-up, **all** bacterial DNA-positive patients were *still* highly symptomatic. This striking correlation strongly suggests that demonstration of bacterial DNA by PCR in lymph nodes on initial presentation is an adverse prognostic factor and makes it unlikely that these patients will have a spontaneous remission.

Indeed, if infection with one of these microorganisms triggers an exuberant granulomatous immune response, the 50-80% of patients who usually have a spontaneous remission[1] likely clear the offending organism and the immune reaction subsides. We postulate that those patients who have persisting symptomatic disease, likely continue to harbor the microorganism which perpetuates the vigorous, destructive immune response, and as well as permit the microorganism to travel elsewhere to other organs to create distant granulomatous inflammation.

Limitations of Study

Many pathogenic microorganisms such as *Mycobacterium leprae* (leprosy) or coronaviruses (severe acute respiratory syndrome) cannot be grown directly in culture or they are very slow growing or difficult to culture such as*Tropheryma whippelii* (Whipple's disease) .[5,19] In these instances, detection and identification rely on molecular mechanisms such PCR used in this study. Nevertheless, the molecular approach has distinct limitations including possible false-positive results secondary to contaminated PCR reagents, the paraffin imbedding process, or post-embedding handling and processing of the paraffin block. However in our study, thirty control lymph nodes were processed in an identical manner and bacterial DNA was detected in only 2/30 (6.6%), significantly less than the sarcoidosis nodes (36.7%, $p = 0.00516$), suggesting that contamination is unlikely to account for the findings.

Another obvious limitation in interpreting the results of this and other prior molecular studies relates to colonization versus causation. Just the finding of microbial DNA in the nodes does not prove that the organism is actively involved in the pathogenesis of the disease. The microorganism may just be a commensal or theoretically it might even be attracted to the area of granulomatous inflammation. Nevertheless, the marked difference in the percentage of microbial DNA-positive nodes in sarcoidosis versus control patients is certainly suggestive of disease causation by the microorganisms.

Additionally, the number of lymph nodes positive for bacterial DNA may be significantly underestimated because of the tendency of the formalin-fixation and paraffin embedding process to breakdown prokaryotic DNA. Over time other investigators have found degradation of the prokaryotic bacteria DNA (especially mycobacteria) with aging of the paraffin-embedded

specimens.[16] Of note, the only 3 sarcoidosis lymph nodes positive for mycobacteria in our study were less than 3 years old when evaluated by PCR. Had we used fresh lymph node tissue like Drake and associates[16] who found 60% PCR positive for mycobacteria species, there may have been a much higher rate of positive bacterial DNA results (particularly mycobacteria) in our study.

Implications of the Study

Sarcoidosis is a granulomatous disease primarily involving the lungs, lymph nodes and other organs that appears to be the result of an exuberant T cell and macrophage immunologic response to the continued presentation of a poorly degradable antigen. Numerous non-infective agents have been implicated based on epidemiologic basis but none have stood up to scrutiny.[1,3,5] The focus over the last two decades has been on infective agents that might trigger sarcoidosis, with the strongest suspects found in the mycobacteria family and the common commensal *Propionibacterium acnes.* And like classical tuberculosis where up to 90% of people infected with *Mycobacterium tuberculosis* remain in remission without treatment,[20] sarcoidosis also has a 65-80% spontaneous remission rate without treatment.[1] One may speculate that similar to tuberculosis, the immune system, after its initial response to a triggering microorganism, is successful in eradicating the agent and the immune response subsides. Then in the 20% or so with persistent and progressive sarcoidosis, the organism remains viable and perpetuates the destructive immune response.

Symptomatic sarcoidosis is usually treated with various anti-inflammatory and immunosuppressive agents such as corticosteroids, methotrexate and TNF-inhibitors

(biologics).[21] The similarities in immunologic abnormalities and treatment to another debilitating granulomatous disease, Crohn's disease, are striking.[22] Granulomatous ileitis (Crohn's) has been suspected by many investigators to be the result of a chronic infection with the obligate intracellular microorganism *Mycobacterium avium* subspecies *paratuberculosis* (MAP), that is known to cause a granulomatous ileitis in cattle and other ruminants called Johne's disease.[23] Although the classical treatment of Crohn's disease has been with immunosuppressive agents just like with sarcoidosis, many recent studies suggest a much more effective treatment with less side effects may be a triple antibiotic regimen geared toward the putative triggering agent MAP.[24-26] In fact, many in the field suspect that this intracellular organism (MAP) that resides in the macrophage impairs the normal autophagy that would usually eradicate the organism.[24] Agents that enhance autophagy such as 16α-bromoepiandersterone,[27,28] currently in human trials, may prove effective along with antibiotics in Crohn's disease.[24]

Can some antibacterial/anti-mycobacterial regimen such as that used in Crohn's disease alter the natural history of sarcoidosis in chronically symptomatic patients? Sixty years ago a number of small trials using classical anti-tuberculous drugs (isoniazide, streptomycin, or cortisone) were published with discouraging results.[29] However, atypical mycobacteria (rather than *M. tuberculosis*) that are more likely to be one of the etiologic agents in sarcoidosis, are almost all resistant to the standard anti-tuberculosis agents such as isoniazid.[30-35] And if other organisms such as *Proprionibacterium acnes* or perhaps cell-wall deficient (L-forms) bacteria trigger and perpetuate sarcoidosis in some individuals, then the standard anti-tuberculous drugs would also be ineffective.

The tetracycline derivatives (minocycline and doxycycline), as well as the anti-malarial drug chloroquine have been shown to be quite effective in treating cutaneous sarcoidosis.[36]

Minocycline can produce complete responses in up two-thirds of cases, although it is debated whether this is an anti-microbial effect or an immunomodulating effect.[37]

Attention has recently turned to randomized sarcoidosis treatment trials with various anti-microbial agents. W. P. Drake and associates just published positive results of the first randomized trial (NCT01074554) of an anti-microbial regimen (directed at atypical mycobacteria) in the United States using oral levofloxacin, ethambutol, azithromycin and rifampin (CLEAR) to treat 30 patients with cutaneous sarcoidosis, with quite significant reductions in cutaneous lesion size.[38] In 2012, D. Gupta and associates in their comprehensive review of sarcoidosis and its similarities to tuberculosis presents a convincing case for anti-tuberculous treatment of sarcoidosis.[39] D. Gupta is also the principal investigator in an ongoing clinical trial in India using more standard anti-tuberculous therapy "Rifampicin and Isoniazid Along With Prednisolone Compared to Prednisolone Alone in Treatment of Sarcoidosis: a Pilot Randomized Controlled Trial" (ClinicalTrials.gov Identifier: NCT01245036).[40] The results of this trial in India with its high burden of tuberculosis will be available next year, though the drug regimen used may not be as effective in countries with a low tuberculosis burden. If indeed sarcoidosis arises from an abnormal immunologic response to a microorganism(s), the patient's geographical location may dictate which microorganism is involved and what anti-microbial regimen will be most effective.

Conclusions

Over the last three decades or more, numerous studies have examined every aspect of sarcoidosis including its dysfunctional immune response. The primary therapy is immune suppression in various forms but this treats only symptoms and does not seem to alter the natural

history of the disease.[4,21] Dozens of studies (Table 3) have repeatedly demonstrated evidence of microorgnisms in 30-80% of sarcoidosis tissues, mostly various mycobacteria and *Proprionibacterium acnes,* and more of these molecular studies is not likely warranted.

Perhaps we should follow the lead of the Crohn's disease gastroenterologists[24,25] and proceed with a therapeutic clinical trial using a regimen of multiple antibiotics in persistently-symptomatic, advanced stage sarcoidosis patients. Indeed, if there is a persistent, viable microorganism infection causing the continuing or progressive debilitating symptoms and organ failure, antibiotics might favorably impact the course of this disease.

FIGURE LEGENDS

Figure 1. Contrast-enhanced computed chest tomography at 2 different axial levels showing typical symmetrical hilar and mediastinal adenopathy.

Figure 2. PET/CT (coronal view) of symmetrical hypermetabolic mediastinal and hilar lymph nodes (arrows).

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Dr. Robinson: Contributed to the conception, hypotheses delineation and design of the study; data acquisition, analysis and interpretation; and writing and revision of the article prior to submission.

Dr. Smith: Contributed to the conception, hypothesis delineation, and design of the study; data acquisition, analysis and interpretation; and revision of the article prior to publication.

Dr. SenGupta: Contributed to the data acquisition, analysis and interpretation; and revision of the article prior to publication.

Ms. Prentice: Contributed to the data acquisition, analysis and interpretation.

Dr. Sandin: Contributed to the conception, hypothesis delineation, and design of the study; and revision of the article prior to publication.

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Title: "~~BACTERIAL DNA ARE FOUND IN LYMPH NODES OF ALL CHRONICALLY SYMPTOMATIC SARCOIDOSIS PATIENTS~~MOLECULAR ANALYSIS OF SARCOIDOSIS LYMPH NODES FOR MICROORGANISMS: A CASE-CONTROL STUDY WITH CLINICAL CORRELATES"

Running Head: Sarcoidosis and Bacteria

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ARTICLE SUMMARY:

ARTICLE FOCUS:

- Sarcoidosis is a common yet incurable, chronic granulomatous disease of unknown etiology treated with non-specific anti-inflammatory and/or immunosuppressive drugs.
- Persistently symptomatic patients worsen despite treatment with a disabling, potentially fatal clinical course.
- We propose that sarcoidosis results from a chronic granulomatous infection from a treatable pathogen in certain susceptible individuals, much like granulomatous ileitis (Crohn's disease) which shows an encouraging response to multi-drug antimicrobial therapy.

KEY MESSAGES:

- This case-control, retrospective study correlated clinical outcomes with the presence of detectable bacterial DNA in sarcoidosis lymph nodes versus control lymph nodes.
- The entire group of sarcoidosis patients had significantly more detectable bacterial DNA than control patient lymph nodes.

- However, **all** persistently symptomatic patients have detectable bacterial DNA in their lymph nodes suggesting they have more aggressive sarcoidoisis that potentially might benefit from antimicrobial treatment directed against a presumed chronic granulomatous infection.

STRENGTHS AND LIMITATIONS:

- Although a number of prior studies have demonstrated the consistent presence of bacterial DNA (mostly atypical mycobacteria and *Propionibacterium acnes*) in sarcoidosis tissue, the current study is the first to correlate clinical outcomes with the presence of detectable bacterial DNA, suggesting the most promising candidates for treatment.
- Nevertheless, the molecular approach to bacterial detection has distinct limitations including possible false-positive results secondary to contaminated PCR reagents, the paraffin imbedding process, or post-embedding handling and processing of the paraffin block. Also the mere physical presence of the bacterial DNA in the lymph nodes does not prove that the disease is caused by the microorganism.
- Additionally, the number of lymph nodes positive for bacterial DNA may be significantly underestimated because of the tendency of the formalin-fixation and paraffin embedding process to breakdown prokaryotic DNA.

ABSTRACT

INTRODUCTION: Sarcoidosis is an incurable, chronic granulomatous disease primarily involving the lungs and lymph nodes of unknown etiology, treated with non-specific anti-inflammatory/immunosuppressive drugs. Persistently symptomatic patients worsen with a disabling, potentially fatal clinical course. To determine a possible infectious cause, we correlated in a case control study clinical information with the presence of bacterial DNA in sarcoidosis mediastinal lymph nodes compared to control lymph nodes resected during cancer surgery.

METHODS: We retrospectively studied formalin-fixed, paraffin-embedded, mediastinal lymph nodes from 30 sarcoidosis patients and 30 control lung cancer patients. Nucleic acids were extracted from nodes, were evaluated by rRNA PCR for bacterial 16S rDNA, and the result was sequenced and compared to a bacterial sequence library. Clinical information was correlated.

RESULTS: 11/30 (36.7%) of lymph nodes from sarcoidosis patients had detectable bacterial DNA, significantly more than control patient lymph nodes (2/30, 6.7%), $p = 0.00516$. At presentation, 19/30 (63.3%) sarcoidosis patients were symptomatic including all patients with detectable bacterial DNA. Radiographically, there were 18 Stage I and 12 Stage II patients. All Stage II patients were symptomatic and 75% had PCR-detectable bacteria. After a mean follow-up of 52.8 ± 32.8 months, **all** patients with PCR-detectable bacteria in this series were persistently symptomatic requiring treatment.

DISCUSSION: 36.6% of sarcoidosis patients had detectable bacteria DNA on presentation, **all of these patients** were quite symptomatic, and most were radiographically-advanced stage II. ~~patients~~. These findings suggest bacterial DNA-positive, symptomatic patients have more

aggressive sarcoidoisis that persists long term, and might benefit from antimicrobial treatment directed against this presumed chronic granulomatous infection.

INTRODUCTION

Sarcoidosis is a multisystem, granulomatous disease whose etiology is obscure and controversial. Nevertheless, the condition is relatively common with a significantly higher age-adjusted annual incidence in African-Americans (35.5 cases per 100,000) versus Caucasian-Americans (10.9 cases per 100,000). An estimated one million people in the U. S. have this disease. Based on the current U.S. population of 315,556,000, there will be approximately 38,605 new cases of sarcoidosis this year and just over 1000 (2.6%) will die of the illness.[1,2]

The fundamental pathologic abnormality in the disease is the formation of non-caseating epitheliod granulomas, which usually confine poorly soluble foreign material that simply cannot be removed by a single phagocytic cell. The key feature in sarcoidosis is activated CD4+ T cells which differentiate into type 1 helper T cells (Th1), secreting interleukin-2 and interferon-γ, augmenting macrophage TNF-α, and amplifying the local cellular immune response.[3,4] This granulomatous inflammation interferes with local tissue homeostasis leading to organ impairment.

Since sarcoidosis primarily involves the lungs, eyes and skin, attention has focused on airborne environmental antigens that might trigger this presumed hypersensitivity response with its T cell-mediated cellular immune response.[3] Similar granulomatous responses can be seen from a variety of infectious agents including mycobacteria, parasites (schistosoma), and fungi (coccidiomycosis). Early studies reported associations with non-infective agents including beryllium, zirconium, aluminum, wood-burning stoves, tree pollen, clay soil, talc, insecticides, inorganic particles, and moldy environments, but none of these theoretical causes has endured.[3,5,6] There is also a several-fold increased incidence of sarcoidosis occurring in siblings

and parents, as well as a consistent strong association with specific gene products such as class I and class II HLA antigens, which may add to the familial connection.

Although no infectious agent has been cultured directly from sarcoidosis tissues, clinical and immunologic characteristics of the disorder provide the strongest support for a microbial etiology, at least in some patients.[5-7] To explore a possible infectious cause in patients seen at the Moffitt Cancer Center, we correlated the clinical presentation and long-term follow-up of sarcoidosis patients with the presence of bacterial DNA in archived, surgically-resected mediastinal lymph nodes. Results from sarcoidosis nodes were compared to control lymph nodes resected at the time of lung surgery in node-negative, Stage I non-small cell lung cancer patients.

METHODS

Regulatory Oversight

Tissue and clinical data in this case-control study was obtained after approval by the Moffitt Cancer Center Scientific Review Committee Protocol MCC #16131 and the University of South Florida IRB Protocol #108656.

Study Design

By searching the Moffitt Cancer Center surgical pathology database between January 1, 2000 and April 1, 2010, we retrospectively identified 30 randomly-chosen patients who were diagnosed with sarcoidosis based on the typical radiographic and clinical presentation, and the histologic finding of non-caseating epitheliod granulomata in lymph nodes obtained sterilely only by mediastinoscopy, to avoid possible microorganism contamination by endoscopic biopsies. Special stains for microorganisms were negative on the specimens. For inclusion in

this group, the following criteria were used to make the diagnosis of sarcoidosis: 1) chest radiograph and chest CT findings of symmetrical mediastinal and hilar adenopathy with or without reticulonodular infiltrates in the lung fields (see Figure 1); 2) when performed, PET scans demonstrated glucose avidity in the enlarged lymph nodes (see Figure 2); 3) asymptomatic presentation or typical symptoms of dyspnea, cough, chest tightness/pain, night sweats, fevers, fatigue, malaise, skin rash or weight loss; 4) lymph nodes showing histologic features of confluent, non-caseating granulomata; and 5) any known microorganism causes of granulomata were excluded by history or culture. All histopathologic specimens were reviewed by one of us (P.S.) to reconfirm the diagnosis made originally by departmental pathologists at Moffitt.

To insure sterile collection, all control specimens were taken from lymph nodes removed at open thoracotomy by one of us (L.R.) in 30 (1:1 match with cases) randomly-chosen patients with Stage IA non-small lung cancer. Lymph nodes were selected only from patients with small peripheral tumors, no obstructive atalectasis, and no evidence of active infection. One of us (P.S) reviewed all control lymph node histology to verify there were no metastases, acute inflammation, or granulomata.

Clinical Data

Clinical data on the patient demographics, initial presenting symptoms and objective findings were extracted from the electronic medical record on all sarcoidosis patients by one of us (L.R.). Patients were staged using the modified Scadding radiographic staging system: stage 0 normal chest x-ray; stage 1 hilar and mediastinal adenopathy alone; stage 2 adenopathy and pulmonary infiltrates; stage 3 pulmonary infiltrates alone; and stage 4 pulmonary fibrosis.[4] Long-term follow-up clinical status and subsequent treatment regimens for all sarcoidosis patients were

obtained from the electronic medical record and telephone calls placed to the patients or their immediate family.

DNA Extraction

De-identified, formalin-fixed, paraffin-embedded blocks of lymph nodes from sarcoidosis and control patients were sent to the Departments of Laboratory Medicine and Microbiology at the University of Washington (Seattle, WA), where investigators were blinded as to the identity of the specimens. The DNA extraction from paraffin-embedded blocks was performed after paraffin was removed by incubation in xylene using the Roche HighPure PCR template purification kit (Roche Diagnostics GmbH, Mannheim, Germany.[8] Several negative patient samples (unrelated to the present study) were included in each batch to rule out contamination. Inhibition was ruled out by checking the ability of exogenously added target DNA to be amplified in the same PCR mix that contained DNA extracted from clinical specimen. *Mycobacterium tuberculosis* and *Bartonella henselae* have been detected multiple times in the past using these PCR assays. 16S primers used were broad range for all bacteria. hsp65 and rpoB were broad range for Mycobacteria spp only. 16S PCR detected non-Mycobacterium species DNA such as *Propionibacterium acnes*. Mycobacterium sp. DNA were detected by hsp65 and/or rpoB primers. Primers used for amplification were also used for amplicon sequencing. The PCR amplicon was directly sequenced; no cloning was performed. Mixed infection was not detected in this set of specimens. For alignment, BLASTN was used. Identification was based on exact match on all cases. No sequence that could not be linked to a microbe was detected.

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P. acnes was detected by 16S primers and *M. avium* was detected by hsp65 primers. *Mycobacterium avium* was detected and identified by sequence analysis.[8]

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PCR Analysis for 16S ribosomal DNA, Heat Shock Protein 65(hsp65), RNA polymerase subunit (rpoB)

The 16S gene fragment was amplified as previously described.[8] The hsp65 gene was amplified using TB11 and TB12 primers, and the RNA polymerase subunit gene (rpoB) was amplified using MF and MR primers.[9] The amplified products were then sequenced using the Big Dye Sequencing kit (Applied Biosystems, Foster City, CA) using the vendor's recommended protocol. The sequences of two strands were assembled into doubled-stranded contig using Sequencher software (Gene Codes, Ann Arbor, MI). The final sequences were used to search the National Center for Biotechnology Information (National Institutes of Health) database using BLAST (Basic Local Alignment Search Tool) to identify the amplified DNA.

Quantitative Variables

The primary variable to be compared between the sarcoidosis and controls patients is the number of patients in each group with bacterial DNA found in lymph nodes. The N-1 Two Proportion test for comparing independent proportions for small sample sizes is used to compare the results between the two groups.[10] Additionally, odds ratios with 95% confidence intervals were calculated.[11] All numerical data is expressed as the mean ± standard deviation.

RESULTS

Demographic and clinical characteristics of the 30 sarcoidosis study patients are found in Table 1.

Demographics

Patient ages are: mean 52.5 ± 12.3 years (median 53 years, range 30-75 years). The male:female ratio is 14:16. The ethnicity: Caucasian 73.3% (22), African-American 16.7% (5), and Hispanic 10% (3). Most patients were overweight: mean BMI 31.4 ± 6.9, median 28.5, range 18.8-47.3.

Clinical Presentation

At the time of initial presentation, 19 of 30 patients (63.3%) were symptomatic, usually with multiple symptoms. Of these 19 patients, the duration of symptoms before diagnosis was a mean 22.1 ± 30.0 months (median 12 months, range 1-120 months). The most common symptoms were night sweats 9 (30.0%); dyspnea 8 (26.7%); chest pain 7 (23.3%); chest tightness 5 (16.7); fevers 3 (10.0%); fatigue 3 (10%); skin rash 2 (6.7%); and stomach ulcer 2 (6.7%). Other symptoms present in at least one patient include: dyspepsia, dysphagia, diarrhea,

Table 1. Sarcoidosis Patient Results

No.	Age/ Sex	Race	X-ray Stage	Pack-Year Smoking	Prior Cancer	Chemo	PCR Result on Lymph Nodes	Initial Symptoms (year)	Follow-up (mo.)	Current Status (Long-Term)
1	**41/F**	**AA**	**I**	**7**	**Uterine**	**No**	***Mycobacteria chelonae***	**Yes (2009)**	**36**	**Sympt., alive**
2	**63/F**	**C**	**II**	**20**	**Breast**	**Yes**	***Mycobacteria chelonae***	**Yes (2008)**	**12**	**Sympt., deceased from COPD 2009**
3	**64/F**	**C**	**II**	**None**	**None**	**No**	***Mycobacteria mucogenicum***	**Yes (2008)**	**41**	**Sympt., alive**
4	43/M	C	I	None	Melanoma	No	Negative	No (2009)	31	Asympt., alive
5	61/F	C	I	None	Synovial cell sarcoma	Yes	Negative	No (2009)	23	Asympt.; new endomet. ca, alive
6	**39/M**	**C**	**II**	**5**	**None**	**No**	***Propionibacterium acnes***	**Yes (2008)**	**35**	**Sympt., alive**
7	**58/M**	**C**	**I**	**3**	**Tonsil cancer**	**Yes**	***Corynebacterium propinquum***	**Yes (2006)**	**65**	**Sympt.; tonsil ca. relapse, alive**
8	**54/M**	**C**	**II**	**None**	**None**	**No**	***Propionibacterium acnes***	**Yes (2006)**	**60**	**Sympt., alive**
9	52/F	AA	I	6	None	No	Negative	Yes (2007)	58	Sympt., alive
10	53/F	C	I	5	Melanoma	Yes	Negative	No (2007)	53	Asympt., alive
11	**34/M**	**AA**	**II**	**None**	**None**	**No**	***Propionibacterium acnes***	**Yes (2007)**	**53**	**Sympt., alive**
12	40/M	C	I	Cigars	None	No	Negative	Yes (2007)	36	Sympt., alive
13	49/M	AA	II	None	None	No	***Duganella zoogloeoides***	Yes (2010)	Lost	Unknown, alive
14	**57/F**	**AA**	**II**	**5**	**Breast**	**Yes**	***Propionibacterium acnes***	**Yes (2000)**	**136**	**Sympt; breast ca. relapse; polymyalgia rheumatica; Hashimoto's thyroditis, alive**
15	67/M	C	I	None	None	No	Negative	No (2001)	Lost	Unknown, alive
16	30/M	H	I	None	None	No	Negative	No (2001)	Lost	Unknown, alive
17	75/F	C	I	None	Ovarian	Yes	Negative	No (2002)	36	Asymptomatic, deceased from ca., 2005
18	67/F	C	I	50	Liposarcoma	No	Negative	No (2003)	106	Sympt., alive
19	60/F	C	I	None	Nerve sheath tumor	No	Negative	No (2003)	60	Asympt., deceased unknown cause 2008
20	41/F	C	I	None	None	No	Negative	No (2005)	78	Asympt., alive
21	50/F	C	I	None	None	No	Negative	Yes (2005)	73	Asympt., alive
22	**48/F**	**C**	**I**	**None**	**Breast**	**Yes**	***Propionibacterium acnes***	**Yes (2003)**	**24**	**Sympt.; deceased from ca. 2005**
23	41/F	C	II	None	Adrenal ca; melanoma	Yes	Negative	Yes (2006)	4	Deceased from ca. 2007
24	35/M	H	I	2.5	None	No	Negative	Yes (2006)	24	Asympt., alive
25	32/M	C	I	1.0	None	No	Negative	No (2006)	Lost	Unknown, alive
26	**37/M**	**C**	**II**	**15**	**Hodgkin's disease**	**Yes**	***Propionibacterium acnes***	**Yes (2007)**	**66**	**Sympt., alive**
27	51/F	AA	II	None	None	No	Negative	No (2007)	64	Sympt; (mildly), alive
28	56/F	C	II	None	Colon ca.	Yes	Negative	No (2008)	Lost	Unknown, alive
29	33/M	C	I	5	Melanoma	No	Negative	No (2009)	38	Asympt., alive
30	52/F	H	II	1	Uterine	No	Negative	Yes (2002)	48	Asympt., alive

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Table 1 abbreviations: AA = African-American; Asympt. = asymptomatic; C = Caucasian; Ca. = cancer; Chemo = chemotherapy; COPD = chronic obstructive pulmonary disease; H = Hispanic; Sympt. = symptomatic. All patients positive on PCR for microorganism DNA in lymph nodes are shown in bold type.

constipation, kidney stones, joint and muscle pains, orthopnea, nose and mouth skin lesions, intermittent bronchospasm, malaise, and weight loss.

Co-morbidities: asthma 2 (6.7%); coronary artery disease 2 (6.7%); diabetes mellitus 4 (13.3%); hypertension 5 (16.6), and one each of gout, hypothyroidism, eczema, fibromyalgia, and Crohn's disease. Malignancies were extremely common with over half (53.3%) having a current or prior tumor, a finding noted previously by others.[12] The malignancies prior to or at the time of presentation are: breast 3, melanoma 3, uterine 2, sarcomas 3, tonsil 1, ovary 1, adrenal 1, colon 1, and Hodgkin's lymphoma 1.

Radiographic Studies

Chest CT was performed on all 30 patients and all had symmetrical mediastinal and hilar adenopathy. Four of 30 (13.3%) had obvious abdominal adenopathy. Lung nodules were present in 12 patients (40.0%) and were radiographic Stage II sarcoidosis. The other 18 patients (60.0%) had Stage I disease. All 12 Stage II patients were symptomatic. PET/CT scans were done in 25 of 30 sarcoidosis patients. All demonstrated glucose avidity in the enlarged mediastinal and hilar nodes (see Figure 2 for typical example), and glucose avidity was seen in the abnormal abdominal nodes in the 4 patients with radiographic adenopathy below the diaphragm.

Laboratory Results

Twelve of 30 patients had lymph node tissue sent at the time of mediastinoscopy for aerobic, fungal and mycobacterial cultures. All cultures showed no growth after six weeks incubation.

Bacterial DNA Detected by PCR

Eleven of 30 lymph nodes (36.7%) in sarcoidosis patients had bacterial DNA present by PCR. Only 2 of 30 (6.7%) control patients were found to have bacterial DNA in their lymph nodes. The microorganisms present in each group are shown in Table 2. There are significantly more sarcoidosis patient lymph nodes positive for microorganism DNA than control lymph nodes: 11/30 versus 2/30, $p = 0.00516$ (2-tailed p-value); the odds ratio is 8.1053 with 95% confidence intervals 1.6115-40.7675, $p = 0.0111$.

All sarcoidosis patients with detectable bacterial DNA in lymph nodes (36.7%) were symptomatic at presentation. Additionally, 73% (8/11) of bacterial DNA-positive sarcoidosis patients were both symptomatic at presentation and had radiographic Stage II disease.

Long-Term Follow-up

Long-term follow-up was complete in 25 of 30 (83.3%) of sarcoidosis patients, for a mean follow-up of 50.4 ± 28.2 months (median 48 months, range 4 -132 months). Five of these patients are deceased: 3 from cancers, 1 from chronic obstructive pulmonary disease, and one from unknown causes. The other five patients lost to direct follow-up are still living based on information obtained from the Social Security Death Index.[13]

Of the 10 sarcoidosis patients with bacterial DNA found in their lymph nodes in this series in whom long-term follow-up was available, **all** were symptomatic at follow-up a mean 52.8 ± 32.4 months (median 47 months, range 12-136 months). The one additional bacterial DNA-positive patient was lost to follow-up.

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Table 2. Bacterial DNA detected by PCR

Sarcoidosis (11/30)	Control (2/30)
***Propionibacterium acnes*: 7**	***Mycobacterium avium*: 1**
Mycobacterium	***Propionibacterium acnes:* 1**
***chelonae*: 2**	
***mucogenicum*: 1**	
***Duganella zooloeoides*: 1**	
***Corynebacterium propinquum*: 1**	

DISCUSSION

The objectives of this case-control study were two-fold: a) evaluate sterilely-resected lymph nodes in documented sarcoidosis patients for the presence of bacterial DNA by molecular methods and b) correlate the results with clinical characteristics of the patients.

Bacterial DNA

As expected, our molecular testing using PCR demonstrated that over one–third of sarcoidosis patients (36.7%) had evidence of bacterial DNA in the nodes, indicating either past or current involvement with these microorganisms. This percentage of bacterial DNA-positive specimens falls in the range found in numerous prior published studies from the last two decades (using various methodologies), which range from 26%-80% positive (Table 3[5,7,14-17]). Furthermore, atypical mycobacteria and *Proprionibacterium acnes* represented almost all DNA identified, also consistent with the findings of the multiple prior studies (Table 3). Additionally identified were one skin and mucous membrane organism (*Corynebacterium propinquum*), and one aerobic Gram negative bacillus (*Duganella zoogloeoides*) that is usually found in aqueous environments. Interestingly, the latter patient (no. 13) with *Duganella zoogloeoides* was an asbestos technician originally from tropical Haiti. As a disclaimer, just the finding of DNA from a microorganism in lymph nodes does not tell us whether the viable organism is present ~~n~~or whether it caused the granulomatous reaction.

Similar to prior published studies summarized in Table 3, significantly less (only 2 of 30 or 6.7%, $p = 0.00516$) of control lymph nodes resected at the time of lung cancer surgery showed

evidence of bacterial DNA (*Mycobacterium avium* ~~*intracellulare*~~ and *Propionibacterium acnes*).

This difference strongly suggests that the demonstration of bacterial DNA in sarcoidosis lymph

Table 3. Selected studies of DNA of infectious agents found in sarcoidosis tissues

Author/Year	Sarcoid Tissue (No. patients)	Technique	Organisms (%)	Control No. (% organisms)
Li, 1999[11]	Skin (20)	PCR (restriction enzyme pattern)	*Mycobacteria* (2 *tuberculosis*, 14 other *mycobacteria*. 80% total positive)	20 Normals (0% organisms)
Du Bois, 2003[7] (Review of pre-1999 studies)	Lymph nodes (12 studies with 295 patients)	PCR (various methods)	*Mycobacterium* sp. (34%)	Various
Eishi, 2002[12] (5 center study)	Lymph nodes (108)	Quantitative real-time PCR	*P. acnes* (72%) *P. granulosum* (55%) *M. tuberculosis* (4%) *E. coli* (2%)	86 Normals (29% *P. acnes*, 12% *P. granulosum*, 2% *M. tuberculosis*)
Drake, 2002[13]	Lymph nodes (25)	Nested PCR	*Mycobacterium* sp. (60%)	25 Normals (0%)
Gupta, 2007[5] (metanalysis)	Various (31 studies with 874 patients	PCR (various methods)	*Mycobacterium* sp. (26%)	745 Controls (3%)
Ichikama, 2008[14]	Bronchoalveolar lavage (42)	Quantitative PCR	*Propionibacterium* sp. (3X higher genome levels vs. controls)	30 Controls (low levels same genome)

nodes is a real finding in our study (and in over 35 prior published studies) and they are not just processing contaminants, therefore pointing to microorganisms as potential contributors to the genesis of this disease. In addition, *Propionibacterium acnes* DNA was found in only 1 of 30 (3.3%) control lymph nodes in our study, in stark contrast to Ishige and associates in Japan who reported this microorganism is an ubiquitous pulmonary lymph node commensal found in 8 of 11 (72.7%) non-sarcoid patients in their study.[18] Such a very high positive result in their study is likely due to either geographical/ethic/racial differences or potential contamination in processing.

Clinical Characteristics

Perhaps the most intriguing findings came from correlation of the PCR findings with the clinical information. All patients with lymph nodes containing bacteria DNA on presentation were also highly symptomatic and 75% of them had the poorer-prognosis radiographic stage II findings. Moreover, after a median 4 years follow-up, **all** bacterial DNA-positive patients were *still* highly symptomatic. This striking correlation strongly suggests that demonstration of bacterial DNA by PCR in lymph nodes on initial presentation is an adverse prognostic factor and makes it unlikely that these patients will have a spontaneous remission.

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Indeed, if infection with one of these microorganisms triggers an exuberant granulomatous immune response, the 50-80% of patients who usually have a spontaneous remission[1] likely clear the offending organism and the immune reaction subsides. We postulate that those patients who have persisting symptomatic disease, likely continue to harbor the microorganism which perpetuates the vigorous, destructive immune response, and as well as permit the microorganism to travel elsewhere to other organs to create distant granulomatous inflammation.

Limitations of Study

Many pathogenic microorganisms such as *Mycobacterium leprae* (leprosy)~~*Tropheryma whippelii* (Whipple's disease)~~ or coronaviruses (severe acute respiratory syndrome) cannot be grown directly in culture or they are very slow growing ~~or fastidious~~ or difficult to culture such as-*Tropheryma whippelii* (Whipple's disease) ~~*Mycobacterium leprae* (leprosy)~~.[5,19] In these instances, detection and identification rely on molecular mechanisms such PCR used in this study. Nevertheless, the molecular approach has distinct limitations including possible false-positive results secondary to contaminated PCR reagents, the paraffin imbedding process, or post-embedding handling and processing of the paraffin block. However in our study, thirty control lymph nodes were processed in an identical manner and bacterial DNA was detected in only 2/30 (6.6%), significantly less than the sarcoidosis nodes (36.7%, $p = 0.00516$), suggesting that contamination is unlikely to account for the findings.

Another obvious limitation in interpreting the results of this and other prior molecular studies relates to colonization versus causation. Just the finding of microbial DNA in the nodes does not prove that the organism is actively involved in the pathogenesis of the disease. The microorganism may just be a commensal or theoretically it might even be attracted to the area of granulomatous inflammation. Nevertheless, the marked difference in the percentage of microbial DNA-positive nodes in sarcoidosis versus control patients is certainly suggestive of disease causation by the microorganisms.

Additionally, the number of lymph nodes positive for bacterial DNA may be significantly underestimated because of the tendency of the formalin-fixation and paraffin embedding process to breakdown prokaryotic DNA. ~~Also, over~~Over time other investigators have found degradation

of the prokaryotic bacteria DNA (especially mycobacteria) with aging of the paraffin-embedded specimens.[16] Of note, the only 3 sarcoidosis lymph nodes positive for mycobacteria in our study were less than 3 years old when evaluated by PCR. Had we used fresh lymph node tissue like Drake and associates[16] who found 60% PCR positive for mycobacteria species, there may have been a much higher rate of positive bacterial DNA results (particularly mycobacteria) in our study.

Implications of the Study

Sarcoidosis is a granulomatous disease primarily involving the lungs, lymph nodes and other organs that appears to be the result of an exuberant T cell and macrophage immunologic response to the continued presentation of a poorly degradable antigen. Numerous non-infective agents have been implicated based on epidemiologic basis but none have stood up to scrutiny.[1,3,5] The focus over the last two decades has been on infective agents that might trigger sarcoidosis, with the strongest suspects found in the mycobacteria family and the common commensal *Propionibacterium acnes.* And like classical tuberculosis where up to 90% of people infected with *Mycobacterium tuberculosis* remain in remission without treatment,[20] sarcoidosis also has a 65-80% spontaneous remission rate without treatment.[1] One may speculate that similar to tuberculosis, the immune system, after its initial response to a triggering microorganism, is successful in eradicating the agent and the immune response subsides. Then in the 20% or so with persistent and progressive sarcoidosis, the organism remains viable and perpetuates the destructive immune response.

Symptomatic sarcoidosis is usually treated with various anti-inflammatory and immunosuppressive agents such as corticosteroids, methotrexate and TNF-inhibitors (biologics).[21] The similarities in immunologic abnormalities and treatment to another debilitating granulomatous disease, Crohn's disease, are striking.[22] Granulomatous ileitis (Crohn's) has been suspected by many investigators to be the result of a chronic infection with the obligate intracellular microorganism *Mycobacterium* ~~*avian*~~ *avium* subspecies *paratuberculosis* (MAP), that is known to cause a granulomatous ileitis in cattle and other ruminants called Johne's disease.[23] Although the classical treatment of Crohn's disease has been with immunosuppressive agents just like with sarcoidosis, many recent studies suggest a much more effective treatment with less side effects may be a triple antibiotic regimen geared toward the putative triggering agent MAP.[24-26] In fact, many in the field suspect that this intracellular organism (MAP) that resides in the macrophage impairs the normal autophagy that would usually eradicate the organism.[24] Agents that enhance autophagy such as 16α-bromoepiandersterone,[27,28] currently in human trials, may prove effective along with antibiotics in Crohn's disease.[24]

Can some antibacterial/anti-mycobacterial regimen such as that used in Crohn's disease alter the natural history of sarcoidosis in chronically symptomatic patients? Sixty years ago a number of small trials using classical anti-tuberculous drugs (isoniazide, streptomycin, or cortisone) were published with discouraging results.[29] However, atypical mycobacteria (rather than *M. tuberculosis*) that are more likely to be one of the etiologic agents in sarcoidosis, are almost all resistant to the standard anti-tuberculosis agents such as isoniazid.[30-35] And if other organisms such as *Proprionibacterium acnes* or perhaps cell-wall deficient (L-forms) bacteria trigger and perpetuate sarcoidosis in some individuals, then the standard anti-tuberculous drugs would also be ineffective.

The tetracycline derivatives (minocycline and doxycycline), as well as the anti-malarial drug chloroquine have been shown to be quite effective in treating cutaneous sarcoidosis.[36] Minocycline can produce complete responses in up two-thirds of cases, although it is debated whether this is an anti-microbial effect or an immunomodulating effect.[37]

Attention has recently turned to randomized sarcoidosis treatment trials with various anti-microbial agents. W. P. Drake and associates just published positive results of the first randomized trial (NCT01074554) of an anti-microbial regimen (directed at atypical mycobacteria) in the United States using oral levofloxacin, ethambutol, azithromycin and rifampin (CLEAR) to treat 30 patients with cutaneous sarcoidosis, with quite significant reductions in cutaneous lesion size.[38] In 2012, D. Gupta and associates in their comprehensive review of sarcoidosis and its similarities to tuberculosis presents a convincing case for anti-tuberculous treatment of sarcoidosis.[39] D. Gupta is also the principal investigator in an ongoing clinical trial in India using more standard anti-tuberculous therapy "Rifampicin and Isoniazid Along With Prednisolone Compared to Prednisolone Alone in Treatment of Sarcoidosis: a Pilot Randomized Controlled Trial" (ClinicalTrials.gov Identifier: NCT01245036).[40] The results of this trial in India with its high burden of tuberculosis will be available next year, though the drug regimen used may not be as effective in countries with a low tuberculosis burden. If indeed sarcoidosis arises from an abnormal immunologic response to a microorganism(s), the patient's geographical location may dictate which microorganism is involved and what anti-microbial regimen will be most effective.

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~~Minocycline can produce complete responses in up two thirds of cases, although it is debated whether this is an anti-microbial effect or an immunomodulating effect.~~

Conclusions

Over the last three decades or more, numerous studies have examined every aspect of sarcoidosis including its dysfunctional immune response. The primary therapy is immune suppression in various forms but this treats only symptoms and does not seem to alter the natural history of the disease.[4,21] Dozens of studies (Table 3) have repeatedly demonstrated evidence of microorgnisms in 30-80% of sarcoidosis tissues, mostly various mycobacteria and *Proprionibacterium acnes,* and more of these molecular studies is not likely warranted.

Perhaps we should follow the lead of the Crohn's disease gastroenterologists[24,25] and proceed with a therapeutic clinical trial using a regimen of multiple antibiotics in persistently-symptomatic, advanced stage sarcoidosis patients. Indeed, if there is a persistent, viable microorganism infection causing the continuing or progressive debilitating symptoms and organ failure, antibiotics might favorably impact the course of this disease.

FIGURE LEGENDS

Figure 1. Contrast-enhanced computed chest tomography at 2 different axial levels showing typical symmetrical hilar and mediastinal adenopathy.

Figure 2. PET/CT (coronal view) of symmetrical hypermetabolic mediastinal and hilar lymph nodes (arrows).

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Dr. Robinson: Contributed to the conception, hypotheses delineation and design of the study; data acquisition, analysis and interpretation; and writing and revision of the article prior to submission.

Dr. Smith: Contributed to the conception, hypothesis delineation, and design of the study; data acquisition, analysis and interpretation; and revision of the article prior to publication.

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Ms. Prentice: Contributed to the data acquisition, analysis and interpretation.

Dr. Sandin: Contributed to the conception, hypothesis delineation, and design of the study; and revision of the article prior to publication.

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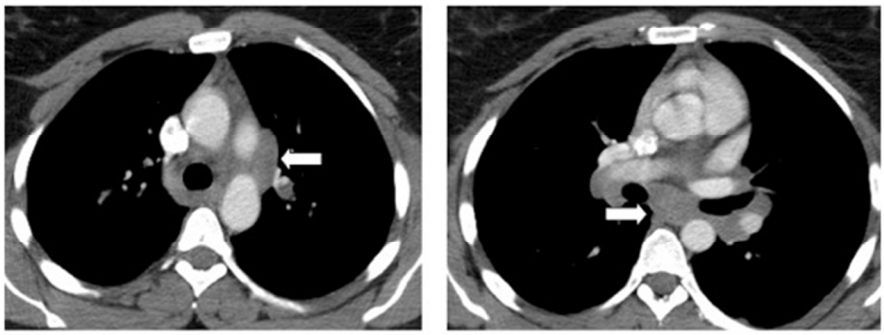

Fig. 1. Contrast-enhanced computed chest tomography at 2 different axial levels showing typical symmetrical hilar and mediastinal adenopathy

Contrast-enhanced computed chest tomography at 2 different axial levels showing typical symmetrical hilar and mediastinal adenopathy

225x169mm (72 x 72 DPI)

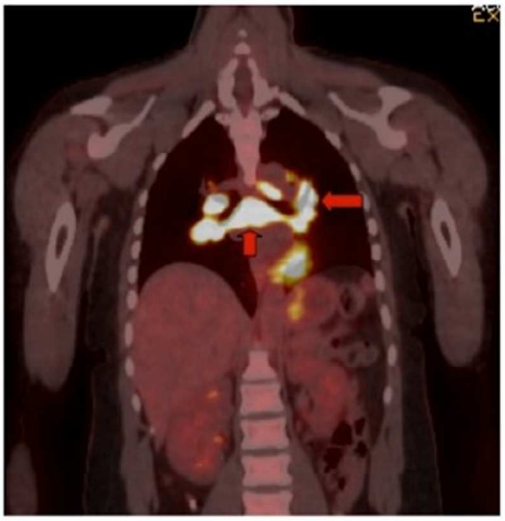

Fig 2. PET/CT (coronal view) of symmetrical hypermetabolic mediastinal and hilar lymph nodes

PET/CT (coronal view) of symmetrical hypermetabolic mediastinal and hilar lymph nodes (arrows)

225x169mm (72 x 72 DPI)

STROBE 2007 (v4) checklist of items to be included in reports of observational studies in epidemiology*

Checklist for cohort, case-control, and cross-sectional studies (combined)

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract Page 1, 3, 4	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	
Introduction			
Background/rationale **Pages 5-6**	2	Explain the scientific background and rationale for the investigation being reported	
Objectives **Pages 5-6**	3	State specific objectives, including any pre-specified hypotheses	
Methods			
Study design Page6	4	Present key elements of study design early in the paper	
Setting Pages 6-7	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	
Participants Pages 7-8	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables Pages 6-7	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
Data sources/ measurement Pages 6-7	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	
Bias	9	Describe any efforts to address potential sources of bias	
Study size Not applicable in this case control observational trial	10	Explain how the study size was arrived at	
Quantitative variables Pages 6-7	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods Pages 6, 7, 9	12	(*a*) Describe all statistical methods, including those used to control for confounding	
		(*b*) Describe any methods used to examine subgroups and interactions	

		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	
Results			
Participants Table 1	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data Table 1	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data Tables 1 and 2	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results Tables 1 and 2	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses Not applicable	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion Discussion section			
Key results	18	Summarise key results with reference to study objectives	
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability **Conclusion section**	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding Title page	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.