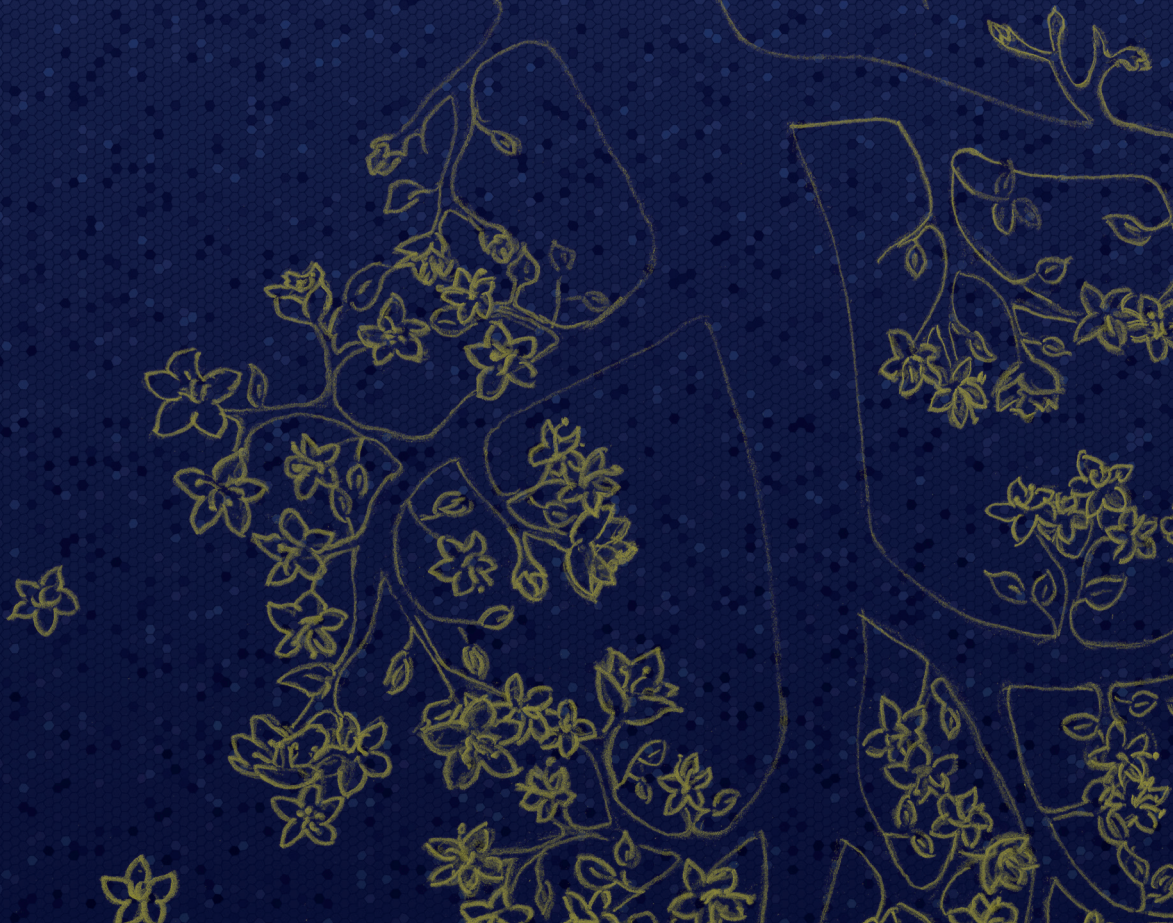


Identification of trigger-related phenotypes in sarcoidosis

Els Beijer



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Identification of trigger-related phenotypes in sarcoidosis

Identificatie van trigger-gerelateerde fenotypes in sarcoïdose
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 15 juni 2021 des middags te 2.15 uur

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Never give up

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Chapter 1

Introduction and outline of the thesis

Introduction

Sarcoidosis is a systemic disease which is characterized by the formation of non-caseating granulomas (1). Virtually any part of the body can be involved, but intrathoracic lymph nodes, the lungs, skin and eyes are most commonly involved (2). Granulomas are compact organized structures of mature macrophages accompanied by other immune cells including lymphocytes and plasma cells. They are thought to be formed in response to inability of phagocytes to destroy certain particles. Granulomas are not specific for sarcoidosis since infectious agents, foreign bodies, many inflammatory and autoimmune conditions coincide with granuloma formation (3). Therefore, a diagnosis of sarcoidosis can only be made when other causes of granuloma formation are excluded (4). Sarcoidosis mainly effects middle-aged individuals, with the highest incidence among men between 30 and 50 years of age and women between 40 and 60 years of age (5). The incidence and prevalence of sarcoidosis vary between different regions of the world (6). In the Netherlands the incidence and prevalence of sarcoidosis are estimated to be 10-20 and 40 per 100.000 respectively (7).

Etiology of sarcoidosis

The etiology of sarcoidosis is not clear, but both the innate and adaptive immune system are suggested to be involved in its disease pathogenesis (8). A more detailed description of cellular players of our innate and adaptive immune system possible involved in sarcoidosis pathogenesis can be found in **chapter 2**. Sarcoidosis is thought to develop due to the interplay between genetic susceptibility, the immune system and exposure to particular etiologic agents. Agents described in relation to sarcoidosis pathogenesis are bacteria (9), inorganic agents (10,11) and auto-antigens (12,13). Those possible “triggers” are reviewed in **chapter 2**. The suggestion that inorganic agents are related to sarcoidosis pathogenesis originates from the observation that certain inorganic agents such as metals or silica are able to induce granulomas (14). The most extensively studied metal in the context of granulomatous disease is probably beryllium. Chronic beryllium disease (CBD), a granulomatous disease related to beryllium exposure, can be histopathological and clinical indistinguishable from sarcoidosis (15). Activation of CD4⁺ T-cells by beryllium in genetic susceptible individuals will lead to beryllium sensitization (BeS). BeS can be measured in blood by a lymphocyte proliferation test (LPT), since peripheral blood mononuclear cells (PBMCs) of beryllium sensitized patients show a proliferative response when stimulated *in vitro* with beryllium (16). The relation between other metals and silica and sarcoidosis have mainly been described in case reports (17,18) or epidemiological studies (19). Examples of clinical observations are described in detail in **chapter 3**. Sensitization to metals other than beryllium and silica have, however, not been examined in cohorts of sarcoidosis patients and controls. This issue is further addressed in **chapter 4**.

Clinical presentation and phenotypes of sarcoidosis

As mentioned before, the presentation of sarcoidosis can be very heterogeneous and virtually any organ can be affected. The lungs and hilar and mediastinal lymph nodes are the most frequently involved (in about 90% of cases), followed by involvement of the skin, eyes and liver (2). Moreover, the course of the disease is unpredictable, meaning that the disease can spontaneously resolve, while a chronic disease course can also develop (20).

The best known phenotype in sarcoidosis is Löfgren's syndrome, an acute, mainly self-limiting presentation of sarcoidosis. The clinical presentation of Löfgren's syndrome is accompanied by arthritis, erythema nodosum and bilateral hilar lymphadenopathy (21). Some studies already tried to identify other phenotypic subgroups among sarcoidosis patients, for instance by clustering involved organs and disease symptoms in relation to gender, geographic origin and occupational categories or by relating disease course to sex or composition of bronchoalveolar lavage (BAL) fluid (22–24). Since clinical characteristics including organ involvement, Scadding stage and disease course have never been studied in relation to certain etiologic agents in sarcoidosis, in **chapter 5** we tried to identify new clinical phenotypes by relating sensitization to organic and inorganic agents to clinical characteristics. An association between sensitization to mycobacterial antigens, one of the identified phenotypes in chapter 5, was further evaluated in **chapter 6**.

The pathological hallmark of sarcoidosis: Non-caseating granulomas

As mentioned above, the hallmark of sarcoidosis are non-caseating granulomas (25). The diagnosis of sarcoidosis is made based on exclusion of other causes of granulomas since granulomas are observed also in particular infectious diseases, summarized in (4). Moreover, as previously mentioned, several inorganic agents have been found to cause a granulomatous reaction (14). Among sarcoidosis patients, such a granulomatous reaction in response to inorganic agents is observed in tattoos in some cases, probable induced by ink pigments (26,27). Figure 1 shows an example of non-caseating lymph node granulomas including inorganic particles.

Since tuberculosis and sarcoidosis share radiological, clinical and histological similarities (28), mycobacteria have been extensively studied in relation to sarcoidosis pathogenesis (29). Also other bacteria, including *Propionibacterium acnes* (*P. acnes*), currently known as *Cutibacterium acnes* (*C. acnes*), are able to induce granuloma formation (30,31) and are

related to sarcoidosis disease pathogenesis mainly by Japanese studies (32). A relation between *P. acnes* and sarcoidosis is difficult to prove, since this bacterium is a commensal of the human skin (33). Some studies have focused on cellular and humoral immune responses to *P. acnes* in sarcoidosis (34,35). However *P. acnes* sensitization to and antibodies against *P. acnes* can also be observed in healthy individuals (36–38). Furthermore, sensitization may be difficult to demonstrate in sarcoidosis patients due to T-cell anergy and exhaustion (39). Another way of examining a relation between *P. acnes* and sarcoidosis is to study its presence in granulomas of patients, which we describe in **chapter 7**. Although *P. acnes* have previously been related to sarcoidosis pathogenesis, granulomas observed in other interstitial lung diseases (ILDs) have not been studied with regard to presence of *P. acnes*. In **chapter 8** we examined whether presence of *P. acnes* is limited to granulomas of sarcoidosis patients or that this bacterium can be identified in granulomas of other diseases as well.

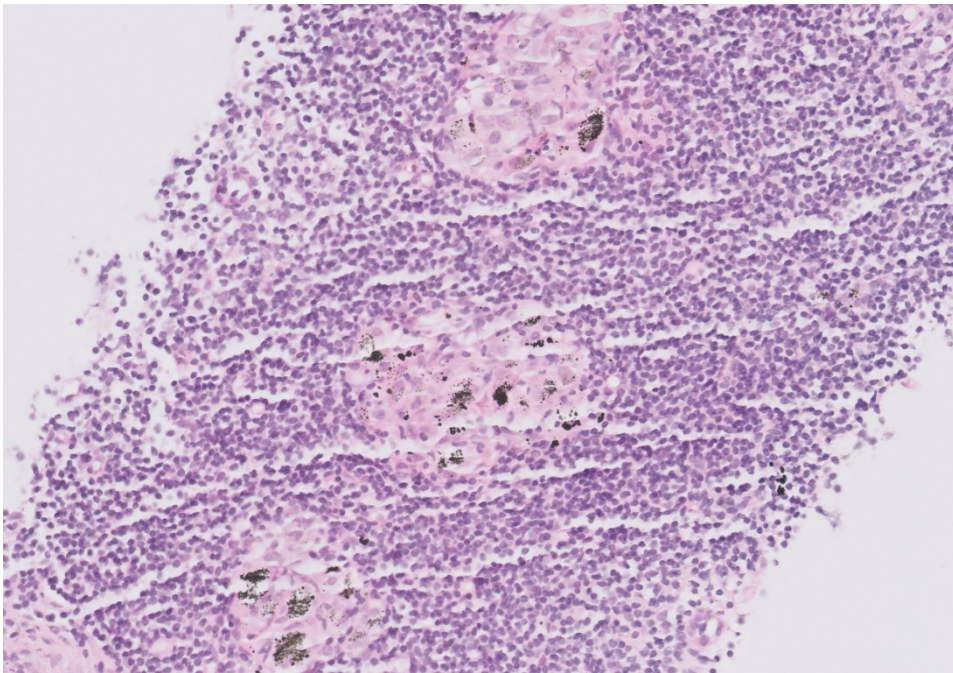


Figure 1. Axillary lymph node of a sarcoidosis patient with large tattoos on both arms, showing ink particles within non-caseating granulomas.

Serum levels of Serum amyloid A (SAA), an acute phase protein (40), have been observed in increased amounts in inflammatory diseases including rheumatoid arthritis, Crohn's disease

sarcoidosis. The pattern of SAA in granulomas of sarcoidosis was shown to differ from granulomas of Crohn's disease and CBD patients (12). For this reason we investigated in **chapter 9** whether SAA levels were higher in serum from patients with granulomatous diseases including sarcoidosis, hypersensitivity pneumonitis and (eosinophilic) granulomatosis with polyangiitis than in controls or patients without granulomatous disease. Furthermore, since the SAA pattern differed between CBD and sarcoidosis patients, we examined whether SAA serum levels differed among sarcoidosis patients sensitized to bacterial antigens, inorganic antigens and self-antigens.

Aim of this thesis

Sarcoidosis has a very heterogeneous presentation and multiple agents have been related to granuloma formation. It is therefore plausible that sarcoidosis is not a single homogeneous disease but rather a collection of several granulomatous diseases induced by different granuloma stimulating triggers in genetically susceptible individuals. As described above, several possible triggers have already been related to sarcoidosis pathogenesis. However, studies combining multiple possible triggers in the same cohort of patients are lacking. Furthermore, relations between possible triggers and clinical data have not been described before but may lead to the identification of trigger-related phenotypes. This may be clinically relevant as it has the potential to improve the prediction of disease prognosis and a more personalized based treatment regime could be explored in subcategories of patients. For example, in sarcoidosis patients with a possible bacterial antigen as cause of the disease the use of antibiotics could be further explored. In patients with an inorganic cause of the disease the focus in clinical management should incorporate the avoidance of further exposure. This thesis therefore aimed to simultaneously study organic as well as inorganic agents in sarcoidosis in order to define trigger-related phenotypes which may allow a more personalized based clinical management in the future.

Scope and outline of this thesis

Chapter 2 provides an overview of current knowledge on the etiology and immunopathogenesis of sarcoidosis.

Chapter 3 describes three clinical cases in which sarcoidosis possibly developed in response to aluminum, beryllium and silica exposure.

In **Chapter 4** metal and silica exposure and sensitization was examined in a Dutch sarcoidosis and control cohort.

Chapter 5 describes peripheral blood responses to organic and inorganic antigens in the same cohort of sarcoidosis patients. Antigen sensitization was linked to clinical characteristics for identification of trigger-related phenotypes.

In **Chapter 6** the association between a latent tuberculosis infection (LTBI) and cardiac involvement in sarcoidosis, one of the trigger-related-phenotypes identified in chapter 5, is further evaluated.

In **Chapter 7** the presence of *P. acnes* in granulomas of Dutch sarcoidosis patients was demonstrated with the use of immunohistochemistry. Results of the staining were studied in relation to disease course.

Chapter 8 examines tissue and granulomas of GPA, EGPA and HP patients for presence of *P. acnes*.

Chapter 9 studies SAA levels in serum of sarcoidosis, HP, (E)GPA and IPF patients.

Chapter 10 gives a summary and discusses the main findings of this thesis.

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Chapter 2

Etiology and Immunopathogenesis of Sarcoidosis: Novel Insights

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Semin Respir Crit Care Med. 2017 Aug;38(4):404-416

Abstract

Sarcoidosis is a disorder of unknown etiology. It is a systemic disease, frequently involving the lungs, skin, eyes and lymph nodes. It is characterized by formation of noncaseating granulomas at the site(s) of disease. Sarcoidosis has a complex disease pathogenesis, with involvement of both the innate and adaptive immune system. Several innate immune system receptors including NLRs and TLRs appear to be involved in the development of sarcoidosis as well as cellular players like DCs and macrophages. Furthermore, lymphocytes from the adaptive immune system including Th1, Th17, Tregs and B-cells are likely to play a role in the sarcoidosis disease pathogenesis as well. Possibly, genetic susceptibility and exposure to particular etiologic agents including mycobacterial and propionibacterial antigens, metals and silica can cause sarcoidosis. Besides exogenous triggers, also self-compounds such as SAA and vimentin have been found to play a role in the development of sarcoidosis. It is likely that sarcoidosis does not have one single cause but rather is the result of the interplay between different etiologic agents and the immune system in predisposed individuals.

Key words: Sarcoidosis, pathogenesis, immune system, Mycobacteria, *P. acnes*, metals, silica, vimentin, SAA

Sarcoidosis is a disorder of unknown etiology. It is characterized by formation of noncaseating granuloma mostly affecting the lungs, skin, eyes and lymph nodes. In the last decades, different organic as well as inorganic triggers have been related to sarcoidosis pathogenesis. First, it is suggested that sarcoidosis is the result of an infection, based on overlap found between infectious granulomatous diseases such as tuberculosis and leprosy (1). Secondly, inorganic compounds such as metals may have a role in the onset of sarcoidosis as well (2–5). Furthermore the disease is also considered to be an auto-immune disease due to a possible immune response towards self-antigens (6). The current review describes the role of the innate as well as the adaptive immune system in sarcoidosis disease pathogenesis and the current knowledge on possible etiologic agents.

Innate Immune System

The innate immune system is an evolutionary-conserved system that provides first-line protection against pathogens. Microorganisms contain a wide range of invariable and highly conserved molecular patterns that differentiate them from eukaryotic cells of the host. These exogenous molecular patterns are called pathogen-associated molecular patterns (PAMPs). Furthermore, endogenous proteins released during tissue injury, such as heat shock protein (HSP), hyaluronan peptide and uric acid crystal can also be recognized by the innate immune system and are known as danger-associated molecular patterns (DAMPs) (7). DAMPs and PAMPs are recognized by the immune system via pattern recognition receptors (PRRs), including Toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD) like receptors (NLRs), RIG-I like receptors (RLRs), absent in melanoma 2 (AIM-2) like receptors and the receptor for advanced glycation end products (RAGE) (8). Upon activation of these receptors an innate immune response is initiated.

NLRs and TLRs

Studies have investigated the contribution of several of those receptors in the onset of sarcoidosis. Mutations in the NOD2 gene are associated with auto inflammatory diseases, Blau syndrome and early onset sarcoidosis (9). *Propionibacterium acnes*, a possible causative agent of sarcoidosis, was shown to activate NF- κ B via NOD1 and NOD2. Two allelic NOD1 polymorphisms, 796G-haplotype and 796A-haplotype, have been identified in Japanese sarcoidosis patients. The frequency of the latter haplotype was significantly higher among sarcoidosis patients than among the healthy controls. Functional experiments have shown that this polymorphism leads to reduced NOD1 expression and, consequently, to a decreased NF- κ B mediated signal transduction in response to *P. acnes*. The altered capacity to recognize PAMPs may lead to increased sarcoidosis susceptibility in the Japanese population (10). Besides NLRs, multiple studies have focused on the contribution of TLRs to

sarcoidosis. When stimulated with TLR2 and TLR4 ligands, bronchoalveolar lavage (BAL) cells obtained from sarcoidosis patients showed increased cytokine responses to TLR2/1 ligand 19-kDa lipoprotein of *Mycobacterium tuberculosis*. In addition, in an animal model of sarcoidosis, granuloma formation in response to heat-killed *P. acnes* was attenuated in TLR2 knock out mice compared to the wild-type C57BL/6 ones (11). In one study, it was suggested that sarcoidosis patients with the TLR-2 promotor polymorphism -16934AA have a higher risk of developing a chronic disease course due to a higher production of tumor necrosis factor-alpha (TNF-alpha) (12). The same group identified that the absence of a common haplotype in the TLR10-TLR1-TLR6 gene cluster, all co receptors of TLR2 located in a gene cluster on chromosome 4, increased the risk of developing a chronic disease course as well (13). Also TLR9 has been the topic of investigations as it is involved in every response against microorganisms, and both *Mycobacterium tuberculosis* and *P. acnes* are potential causative agents of sarcoidosis. A TLR9 polymorphism was found to be more common in chronic sarcoidosis patients than in the control group (14), however these results could not be replicated (15). In addition to the receptors itself, different regulation of TLR and NOD like receptor signaling have been found in sarcoidosis. When ligands bound to TLRs and NLRs, MAPK cascades are activated which leads to phosphorylation of three principal kinases: P38, JNKs and ERKs. Activated P38 is involved in the production of many cytokines including TNF- α and interleukin-1 (Il-1) which are important factors that contribute to Th1 commitment. MAPKs are inactivated by dephosphorylation through MAPK phosphatases (MKPs). Interestingly, p38 phosphorylation was sustained in BAL cells from sarcoidosis patients stimulated with NOD-1 and TLR4 agonist. Compared to healthy controls, there was no significant induction of MKP-1 in sarcoidosis BAL cells. This finding points to a yet to explore in more detail regulatory defect in sarcoidosis and may explain the sustained production of Th1 cytokines observed in this disease (16).

Neutrophil Granulocytes

Neutrophil granulocytes are a type of leukocyte that are the first to move through the endothelium towards the inflammatory site. For this reason they are important contributors in the host defense against invading microorganisms (17). Neutrophils express several receptors that contribute to phagocytosis. Furthermore, they form phagolysosomes containing several proteolytic and antibacterial enzymes capable of microbial killing (18). Although neutrophils are not typically seen within granulomas in sarcoidosis tissues, neutrophils have been found in higher numbers in BAL of sarcoidosis patients compared to healthy controls (19) and the neutrophil percentage in BAL fluid of sarcoidosis patients may very well be related to disease prognosis. A Chinese study found that sarcoidosis patients with higher neutrophil amount in the BAL had more reticular lung changes and lower

diffusing capacity for carbon monoxide (DLCO) values (20) and Tutor-Ureta and co-workers observed less neutrophils in BAL from radiological stage I sarcoidosis patients compared to stage II and III patients. Löfgren's patients had a significantly lower amount of neutrophils in BAL than non-Löfgren's patients and indeed, patients with a poor disease outcome had a significantly higher amount of neutrophils than patients with a favorable disease course (21). Others have investigated the neutrophil / lymphocyte ratio (NLR) as a marker of disease prognosis in sarcoidosis. Ocal and colleagues observed an increasing NLR with increasing radiological stages in pulmonary sarcoidosis patients (22). Also Dirican *et al.* found such a relation, by showing that more patients in higher radiological stages had an increased NLR (19). How neutrophil increase leads to a worse disease prognosis is not completely understood, but it may be related to their capacity to secrete matrix proteases that cause collagen destruction and remodeling and, ultimately, promote formation of fibrosis (21) and the development of bronchiectasis and fibrocystic changes.

Dendritic Cells

Dendritic cells (DCs) are antigen presenting cells and act as messengers between the innate and adaptive immune system (23). In peripheral blood from sarcoidosis patients a decreased number of DCs was observed (24,25). In contrast, in BAL fluid from pulmonary sarcoidosis patients an increased number of DCs can be found compared to healthy controls (26). Probably, peripheral blood DCs are attracted to and matured DCs have been found to accumulate near the granulomas (25). Moreover, in *in vitro* co-culture experiments with CD4⁺T-cells, DCs were able to produce more TNF- α when obtained from sarcoidosis patients compared to healthy controls (26). TNF is involved in DC maturation and migration, as well as granuloma formation (26), and TNF inhibitors have successfully been used in the treatment of sarcoidosis, which suggests that DCs are important in the sarcoidosis pathogenesis (27).

Macrophages

Sarcoidosis is characterized by granuloma formation, a dynamic process in which monocytes are recruited from the circulation and accumulate near already recruited macrophages. Material that is either insoluble or too bulky for phagocytoses may lead to the persistence of the granulomas. Macrophages are the major contributors of the granuloma, suggesting that these cells contribute to the sarcoidosis pathology (28). Kjellin *et al.* profiled the membrane-associated proteome of alveolar macrophages from sarcoidosis patients and healthy controls to identify proteins and associated signaling pathways possibly involved in sarcoidosis development. They found eighty proteins that were different in abundance between patients and controls. Interestingly, Fc γ receptor

mediated phagocytosis and clathrin mediated endocytosis signaling pathways were upregulated. These are important pathways in pathogen degradation and antigen presentation to memory T-cells (29). The phagocytosis, degradation and clearance of antigens are dependent on several factors including functions of the receptor for Fc fragment for IgG and complement receptors. Significantly higher expression of surface Fc γ receptors CD16, CD32 and CD64 and complement receptors CD35 on peripheral blood mononuclear cells (PBMCs) of sarcoidosis patients were found in a study by Dubaniewicz and co-workers (30). Once macrophages are activated via PRRs a strong inflammatory response is initiated, not only due to production of cytokines but activated macrophages can be effective antigen presenting cells (APCs), as well thereby serving as the bridge between the innate and adaptive immune response (31).

Adaptive Immune System

In contrast to the innate immune system, the adaptive immune system is highly specific, and includes APCs, T-cells and antibody producing B-cells. Each T-cell expresses a specific T-cell receptor on its surface, capable of recognizing processed antigens presented in major histocompatibility complex (MHC) molecules on antigen presenting cells. In response to recognized non self-antigens, T-cells will proliferate and produce cytokines, that mediates microbial killing (32). The cytokines that will be produced by the T-cell depends on which effector T-cell type is differentiated from the Th0-lymphocyte. This differentiation is already set at the first contact with the antigen and is dependent on the cytokines present in the surrounding of the T-cell. Il-4 produced by mast cells causes differentiation into T-helper-2 cells, Il-12 produced by macrophages and dendritic cells and interferon-gamma (IFN- γ) produced by Natural killer (NK) cells leads to differentiation into T-helper-1 cells, while Il-1 and Il-23 produced by macrophages and dendritic cells cause differentiation into T-helper-17 cells. Thus, the kind of response induced by the innate immune system already determines the type of T-cell differentiation in the adaptive immune response (33).

Th1-cells

Sarcoidosis is considered to be a T-helper-1 (Th1) - cell mediated disease. Due to an ongoing immune stimulation, T-cells accumulate around granulomas (34). So far it is not known why the hyperactive Th1-cell response occurs in sarcoidosis. It might be that the immune system of a predisposed host reacts to specific antigens without a normally occurring negative feedback signal, leading to an enhanced and ongoing immune response (35). Sarcoidosis patients generally have more Th1 cytokine production, including Il-2 and IFN- γ , than healthy controls (36). Also Il-12 and Il-18 are important cytokines in the Th1 immune response, since they stimulate IFN- γ production. In addition, as mentioned above, Il-12 stimulates the

differentiation from Th0-cells into Th1-cells. Importantly, IL-12 (37) and IL-18 (38) were found to be increased in BAL fluid from sarcoidosis patients compared to healthy controls. Furthermore, these cytokines were able to induce higher IFN- γ production by cells present in sarcoid BAL from sarcoidosis patients (38). Th1-cells present in sarcoidosis lung patients express the chemokine receptor CXCR-3. Expression of this receptor on CD4 T-cells is controlled by T-bet, a Th1 transcription factor, along with typical cytokines such as IFN- γ . It was found that T-bet expression levels are higher in sarcoidosis BAL cells compared to healthy controls cells, positively correlated with IFN- γ and CXCR-3 expression levels in sarcoidosis patients (39). In Scandinavian sarcoidosis patients it was found that HLA-DRB1*0301 positive patients had lower Th1 IFN- γ expression in peripheral blood Th-cells and a tendency towards lower IFN- γ expression in BALF Th1 cells. In this population, expression of HLA-DRB1*0301 is associated with more acute onset of the disease and a better prognosis (40). This could indicate that HLA-DRB1*0301 positive patients have a less pronounced Th1 response that may result in better disease prognosis, considering the role of IFN- γ in driving granulomatous inflammation (41).

PD-1

Programmed cell death protein 1 (PD-1) is a receptor expressed on T-cells and inhibits T-cell effector functions, by blocking cytokine production, T-cell proliferation and impairing T-cell survival. For this reason, this receptor is important in auto-immunity and self-tolerance, and it is regarded as an immune checkpoint (42,43). The dysregulation of cellular immunity could play a role in sarcoidosis through T-cell mediated responses to so far unknown antigens (44). It has been found that PD-1 pathway blockade restores T-cell function in chronic beryllium disease (CBD) (45). Interestingly, this has also been found in sarcoidosis. Sarcoidosis CD4+ T-cells have increased PD-1 expression and reduced proliferation capacity, which was restored upon blockage of the PD-1 pathway (46,47). In addition, patients with resolving disease were found to have reduced PD-1 expressing CD4+ T-cells and normal proliferative capacity compared to the patients with progressive disease (48). P13K and Akt are part of the signaling pathway that is critical for cell proliferation. Signaling through PD-1 disturbs the phosphorylation of P13K, which has a negative impact on cell proliferation. Besides increased PD-1 expression on sarcoidosis T-cells, also reduced expression of the P13/Akt pathway was found. Those expression levels were restored to levels found in healthy controls when the PD-1 pathway was blocked. In response to foreign antigens T-cells undergo proliferation and differentiation to facilitate antigen clearance, loss of T-cell proliferation might therefore lead to antigen persistence. Impaired T-cell proliferation possibly leads to disease progression in some sarcoidosis cases (47) as sarcoidosis might be caused by an intracellular infection in which the cellular immune response is the most

important defense mechanism. Those studies indicate that blocking PD-1 pathways could be a new therapeutic option in sarcoidosis patients. However, given the lack of microbiologic or pathologic evidence that sarcoidosis is associated with an active infection, PD-1 blockade may be counterproductive if not toxic to sarcoidosis patients if as in the example of chronic beryllium disease, PD-1 is upregulated to control the adaptive immune response to persistent tissue antigens that the patient is unable to clear (45). Furthermore, there are several cases described where blockage of PD-1 led to the occurrence of sarcoidosis symptoms. A refractory stage IV Hodgkin lymphoma patient treated with pembrolizumab, a human antibody targeting PD-1, developed a flare of her sarcoidosis (49). In addition, a lung adenocarcinoma patient with a combined treatment of ipilimumab (a CTLA-4 inhibitor) and nivolumab (a PD-1 inhibitor), developed sarcoidosis as an immune related adverse event (44). Finally, two patients diagnosed with metastatic melanoma treated with nivolumab alone developed a sarcoid like reaction (50,51). Remarkably, the same phenomenon has been observed in patients treated with TNF- α inhibitors. The use of TNF-alpha blockers is a well-established and effective treatment in patients with severe and refractory sarcoidosis (52). However new onset sarcoidosis cases are observed in rheumatoid arthritis patients and a psoriatic arthritis patient receiving TNF- α inhibitors (53,54). Those cases point to the complex immunopathogenesis in sarcoidosis.

Th17-cells

Th17-cells produce the pro-inflammatory cytokine IL-17, which is involved in the immune response towards extracellular and intracellular pathogens, including *Listeria monocytogenes*, *Salmonella enterica* as well as *Mycobacterium tuberculosis* (55). For this reason, this cell type has been related to sarcoidosis and it was found to induce granuloma formation (56). IL-17 expressing cells were found in granuloma containing lung biopsies of sarcoidosis patients. The IL-17 positive cells were mainly present in and surrounding the granulomas (57). The number of CD4+ IL-17 producing cells was higher in both blood and BAL of sarcoidosis patients with active disease compared to patients with inactive disease (56). As mentioned before, mycobacterial antigens are suggested to play a role in the etiology of sarcoidosis. Richmond and colleagues report mycobacterial specific Th17 responses in blood and BALF of sarcoidosis patients, which were not observed in healthy controls (58). Ostadkarampour and colleagues found that when BAL cells from either Lofgren or non-Lofgren patients were stimulated with the candidate mycobacterial pathogenic antigen mKatG, Th1 cells (defined by release of INF- γ) were more than twice as prevalent than Th17 cells (defined by release of IL17) suggesting Th1 responses may dominate sarcoidosis specific immunity at sites of disease when directly compared (59). Next to Th1 cells, also Th17 cells produce IFN- γ . This subtype of IFN- γ producing Th17-cells

are known as Th17.1-cells (60). The fraction of IFN- γ producing Th17-cells was found to be two times higher in sarcoidosis BALF than in controls. In addition, the percentage of Th17.1-cells correlated positively with the radiological score for staging sarcoidosis (61). Ramstein *et al.* suggest that IFN- γ producing cells may have been misclassified as Th1-cells in the past. IFN- γ and IL-17A cytokine measurement has mostly been used to define Th1 and Th17-cells respectively, IFN- γ producing Th17-cells would have been missed in this way (60). Lexberg and co-workers (62) have shown that Th17-cells can be transformed to Th17.1-cells following incubation with the Th1 cytokines IL-12 and IFN- γ . In conclusion, IFN- γ producing CD4 T-helper-cells are observed in higher numbers in sarcoidosis. Th1 as well as Th17-cells are capable of IFN- γ production and Th1 cytokines have been shown to skew Th17 cells into Th17.1 producing IFN- γ cells. Therefore, sarcoidosis can be considered as an Th1 as well as Th17 mediated disease, but fundamentally these studies confirm that sarcoidosis is an IFN- γ (and likely T-bet) driven disorder.

Regulatory T-cells

Regulatory T-cells (Tregs) are essential for maintenance of immune tolerance and prevent development of auto-immune diseases. Immune homeostasis is established by production of immune suppressive cytokines such as IL-10 and transforming growth factor-beta (TGF β) (63,64). As sarcoidosis has many elements resembling a deregulated immune response, impaired function of Tregs may be a possible contribution factor to the response seen in sarcoidosis. Indeed, an impaired suppressive function of peripheral Tregs from sarcoidosis patients has been observed in several studies (48,65,66). Broos *et al.* found increased levels of peripheral Tregs in patients developing chronic disease compared to healthy controls. However, Treg mediated suppression of pro-inflammatory cytokine production and proliferation of Th-cells was significantly impaired in the sarcoidosis patients. Furthermore, Tregs obtained from pulmonary sarcoidosis patients suffered from impaired survival and increased apoptosis (67). Moreover, restoration of Treg function was associated with disease remission (48). Tafllin and colleagues have suggested that Tregs may be able to prevent sarcoidosis development, but cannot reverse the granuloma process. This was supported by the observation that [1] healthy Tregs prevent granuloma formation *in vitro* whereas sarcoidosis Tregs do not, and [2] Tregs are found to accumulate and proliferate within sarcoidosis granulomas, but are clearly not able to reduce their growth (68).

B-cells

Next to innate and cellular immune factors in sarcoidosis, also the humoral immune response is thought to contribute to the sarcoidosis disease process. Sarcoidosis patients with active disease were found to have a higher frequency of naive B-cells and IL-10

producing regulatory B-cells in blood than healthy controls (69) and B-cells were found within and near granulomas. Differences in immunoglobulin producing capacities of B-cell blasts were not found between sarcoidosis patients and healthy controls (70). B-cell activating factor from the TNF family (BAFF) plays a role in the survival and maturation of B-cells. Increased BAFF levels have been found in sarcoidosis patients with active disease (68,71). It is suggested that BAFF overexpression can lead to severe auto-immune disease by rescuing self-reactive B-cells from deletion (71). A study among chronic sarcoidosis patients showed indeed disturbed B-cell homeostasis (72). There are some case reports describing the successful treatment of sarcoidosis with rituximab, an antibody against CD20 (73). CD20 is a non-glycosylated phosphoprotein present on all B-cells, except on plasma cells (74). The fact that rituximab is an effective treatment in some sarcoidosis patients (73,75–77) points towards the possible involvement of B-cells in the pathology of the disease.

Etiologic agents

Although sarcoidosis is a systemic disease, the intrathoracic lymph nodes and lungs are the most frequent involved organs. This has led to the hypothesis that the etiologic agent of this disease must be airborne (78). Both exogenous organic (79) and inorganic components (80–82) can be involved in sarcoidosis, of which the exposure to the inorganic components is often related to particular professions (5). More recent data suggest that also self-antigens can be at least involved in the sarcoidosis process (6).

Mycobacteria

In clinical, radiological as well as immunological features, there is similarity between sarcoidosis and tuberculosis (78), leading to the suggestion of mycobacteria as etiologic agent in sarcoidosis (83). Several studies have described a role of mycobacteria in the involvement of sarcoidosis. Gupta and colleagues wrote a meta-analysis of 31 studies published between 1980 and 2006 that have used molecular techniques to determine the histological as well as the cellular presence of mycobacteria in sarcoidosis. The odds ratio to identify mycobacterial DNA in sarcoidosis samples versus control samples was calculated to be 9.67 and it was estimated that a mycobacterial signature was present in granulomatous tissue of almost 30% of sarcoidosis patients (84). A recently published meta-analysis by Fang *et al.* showed a significantly higher frequency of immune responses towards mycobacteria among sarcoidosis patients than controls (85). The presence of an immune response against antigens at least indicates past exposure that has resulted in induction of immunological memory, which can also be accessed *in vitro* in the absence of an active infection. For this reason, immune assays are a powerful tool to study involvement

of particular antigens in sarcoidosis (86). To identify specific antigens involved in the granuloma formation in sarcoidosis, Song *et al.* used a proteomic approach and identified protein aggregates containing mycobacterial catalase-peroxidase (mKatG) in tissue biopsies of a subset of sarcoidosis patients. KatG is found in most mycobacterial species and is an important virulence factor that provides protection from peroxide, which leads to the survival of the organism in macrophages. Mycobacterial organisms may trigger sarcoidosis in a subset of patients, in which mKatG possibly serves as a target antigen (87). Chen *et al.* investigated the T-cell response against mKatG in a cohort of Swedish as well as American sarcoidosis patients. In this cohort of 150 patients, 50% had an increased IFN- γ response against mKatG compared to no responses in the group of healthy volunteers (88). Studies by Oswald-Richter *et al.* and Drake *et al.* demonstrated significantly increased IFN- γ responses towards mKatG and ESAT-6 in BAL and PBMCs respectively, from sarcoidosis patients compared to controls (89,90). Those results were confirmed in a latter study of Oswald-Richter and colleagues, where 70 % (n=31) BAL samples of sarcoidosis patients produced a CD4+ T-cell response to at least one of the mycobacterial epitopes of KatG, ESAT-6, Ag85A, sodA or Heat shock protein (HSP). ESAT-6 was the most frequently recognized antigen by sarcoidosis patients in this study (91). Although immune responses to HSP were not different in the above mentioned study, Dubaiewicz *et al.* did show significant differences between sarcoidosis patients and controls in TNF- α and IL-6 responses upon *in vitro* stimulation using mycobacteria hsp (92). Next to Oswald-Richter and colleagues, also Carlisle *et al.* observed significantly more sarcoidosis patients responding to the virulence factor sodA compared to PPD- controls (93). An overview of different mycobacterial antigens studied in sarcoidosis is given in Table 1.

If mycobacteria play a role in disease pathogenesis a key question is why some patients develop tuberculosis while others develop sarcoidosis. One could speculate that there is a different immune reaction in patients against the same pathogen. Interestingly, such a phenomenon has already been observed in patients infected with *Mycobacterium leprae* (94). In leprosy there is a type I as well as a type II reaction based on variety in strength of the cellular immune response. The type I reaction, also called the reversal reaction, might result from an exacerbated Th1 reaction in response to increased release of mycobacterial antigens (95). The type II reaction, also called erythema nodosum leprosum, is a more immunocomplex-mediated disease and involves Th1 as well as Th2 cells (96). Different immune reactions to the same pathogen lead to different clinical manifestations and outcomes in patients with leprosy (97).

Table 1. Overview of mycobacterial antigens recognized by the immune system of sarcoidosis patients

Mycobacterial antigen	Studies showing immune response
AG85A	Oswald-Richter <i>et al.</i> , 2010 (91)
ESAT-6	Carlisle <i>et al.</i> , 2007 (93) Drake <i>et al.</i> , 2007 (90) Oswald-Richter <i>et al.</i> , 2009 (89) Oswald-Richter <i>et al.</i> , 2010 (91)
HSP	Dubaniewicz <i>et al.</i> , 2007 (92)
KatG	Carlisle <i>et al.</i> , 2007 (93) Chen <i>et al.</i> , 2008 (88) Drake <i>et al.</i> , 2007 (90) Oswald-Richter <i>et al.</i> , 2009 (89) Oswald-Richter <i>et al.</i> , 2010 (91)
sodA	Carlisle <i>et al.</i> , 2007 (93) Oswald-Richter <i>et al.</i> , 2010 (91)

AG85A = Antigen 85A, ESAT-6 = early secreted antigenic target of 6 kDa, HSP = Heat shock protein, KatG = catalase-peroxidase, soda = superoxide dismutase A.

Based on possible involvement of mycobacteria in sarcoidosis pathogenesis, Drake *et al.* investigated the use of levofloxacin, ethambutol, azithromycin, and rifampin (CLEAR) therapy in patients with pulmonary and cutaneous sarcoidosis. In the first pilot study of 15 sarcoidosis patients with pulmonary sarcoidosis, a significant increase of forced vital capacity (FVC) was seen after treatment with the CLEAR regimen. Interestingly, the highest increase of FVC was seen in patients with a positive IFN- γ response to ESAT-6 (98). The second trial was a RCT of CLEAR versus placebo in sarcoidosis patients with cutaneous localization. A significant decrease in lesion diameter was seen in the CLEAR group compared to the placebo group after 8 weeks of treatment (99). In both studies, the authors acknowledged the lack of evidence for an active mycobacterial infection in either the lung or skin, but rather suggested this antibiotic combination had significant anti-inflammatory effects, suppressing NF κ B and related pathways. In summary, histological and cellular presence of mycobacteria in sarcoidosis lesions, immune response towards mycobacterial antigens and beneficial effects of anti-mycobacterial therapy in sarcoidosis patients suggest an etiological link between mycobacteria and sarcoidosis. However, regarding the role of mycobacteria, there is a lack of consensus on whether an active replicating mycobacterial infection is the cause of chronic sarcoidosis or an ongoing immune response to persistent mycobacterial antigens in the absence of an active infection. The challenge for those who

propose chronic sarcoidosis is caused by an active infection is to explain the lack of clinical, microbiologic and pathologic evidence for an active infection in sarcoidosis patients throughout their course, despite treatment with corticosteroid, immunosuppressive or anti-TNF therapies for many years. There is no credible evidence that directly supports the concept of an active replicating infection despite this hypothesis being formulated after the initial description of the disease over 100 years ago.

Propionibacteria

Mycobacteria and propionibacteria have several features in common. The most important shared feature in the context of sarcoidosis is that both bacteria are intracellular pathogens, which persist in macrophages (100). *Propionibacterium acnes* (*P. acnes*) has been cultured from lymph nodes of sarcoidosis patients, and DNA of this bacterium was found in BAL from sarcoidosis patients as well (101–103), though widespread clinical experience suggests this is a rare finding during routine clinical biopsies or BAL procedures. In a study by Zhou et al. ribosomal RNA (rRNA) of *P. acnes* and *P. granulosum* was measured by RT-PCR in lymph node tissue from Chinese sarcoidosis patients, which is considered proof for the presence of viable bacteria. They found that the proportion of samples positive for *P. acnes* and *P. granulosum* rRNA was significantly higher in sarcoidosis patients compared with tuberculosis patients or controls with other diseases. In addition, the copy number of rRNA from both the bacteria was also higher in sarcoidosis patients compared to the two control groups. These results indicate that Propionibacteria are associated with the development of sarcoidosis (104). Ebe et al. screened a genomic DNA expression library of *P. acnes* with sera of sarcoidosis patients. Using this methodology, they identified RP35, the c-terminal region of the trigger factor of *P. acnes* as a potential antigen involved in the sarcoidosis disease process. They demonstrated that RP35 caused proliferation of the mononuclear cells in 18% of the sarcoidosis patients investigated compared to none in healthy controls (105). Minami et al. investigated whether RP35 was able to induce granulomas in a mouse model. They found a strong immune response and pulmonary granuloma forming in mice sensitized with RP35 in CFA (106). Interestingly, administration of antibiotics before and during the experiment prevented granuloma formation in this experimental model of sarcoidosis. In a study using immunoblotting, Yorozu et al. identified *P. acnes* catalase-peroxidase (KAT) as a one of the immunogenic antigens in sarcoidosis. The identified *P. acnes* KAT antigen showed increased peripheral blood mononuclear cell (PBMC) IFN- γ responses in sarcoidosis patients compared to patients with other interstitial lung diseases and healthy controls (107). Schupp et al. also demonstrated the link between sarcoidosis and *P. acnes* in Caucasian sarcoidosis patients. In this study BAL cells were exposed to heat-

killed *P. acnes* which resulted in significantly higher production of IFN- γ and granulocyte macrophage-colony stimulating factor (GM-CSF) in case of sarcoidosis patients. Furthermore, a higher concentration of *P. acnes*-IgGs antibodies was found in BALF from sarcoidosis patients as well (100). The above mentioned studies suggest different antigens of *P. acnes* as possible causative agents in some sarcoidosis patients. A key question related to *P. acnes* is why some patients develop sarcoidosis as a reaction to this human commensal. In an excellent review by Eishi, it is hypothesized that *P. acnes*, just as mycobacteria, can be present as a latent infection in the form of Hamazaki-Wesenberg bodies. Intracellular proliferation of latent *P. acnes* in macrophages triggers granuloma formation in patients with a hypersensitivity to this bacterium (Figure 1) (108).

A case report successfully using clarithromycin in the treatment of a sarcoidosis patient further strengthens the link between *P. acnes* and sarcoidosis pathogenesis (109), though, in contrast, it is the widespread clinical experience that antimicrobial therapy including antibiotics effective against *P. acnes*, is not effective in the vast majority of sarcoidosis patients.

In conclusion, culturing of *P. acnes* from sarcoid lymph nodes in rare cases, the presence of *P. acnes* rRNA in lymph nodes of sarcoidosis patients and immunological responses towards *P. acnes* antigens suggest that propionibacteria are associated with the development of sarcoidosis. As for a mycobacterial link to sarcoidosis, there is a lack of consensus as to whether an active, replicating *P. acnes* infection is central to chronic sarcoidosis. In experimental models of allergic diseases using immunization with self-antigens such as thyroglobulin (thyroiditis) or myelin basic protein (encephalomyelitis) autoimmune inflammatory lesions could only be induced in the organs from which these self-antigens originated (110,111). In theory, when sarcoidosis is seen as an autoimmune disease, one could speculate that in the absence of an active infection *P. acnes* serves as a self-antigen in some patients where this bacterium is a commensal of the lungs.

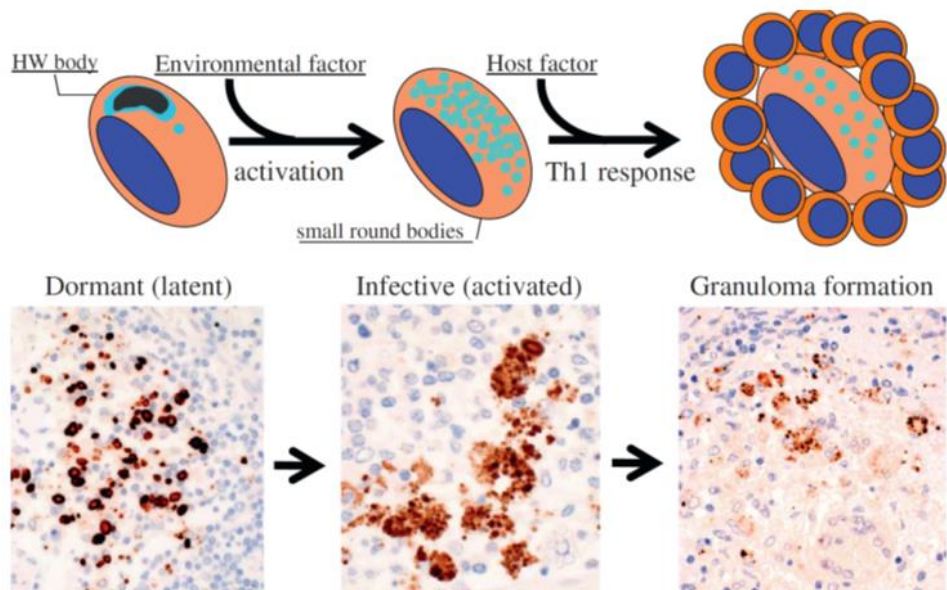


Figure 1. Dormant and cell-wall deficient forms of *P.acnes* may be present as Hamazaki-Wesenberg bodies and can be activated under certain conditions, which lead to proliferation of the bacteria in cells at latent infection sites. Infective proliferating forms of the bacteria may be present in macrophages as small round bodies. When macrophages release those small round bodies they can infect other cells and organs via the lymph and blood stream. To prevent this spread, granulomas are formed as a host defense mechanism in patients having a hypersensitive immune response towards this bacterium. (Adapted with permission from Eishi Y. Etiologic link between sarcoidosis and *Propionibacterium acnes* . Respir Investig. 2013 Jun;51(2):56–68. ¹⁰⁸)

Metals

The inhalation of metal dust has been related to lung diseases for several years and has been proposed as a cause of sarcoidosis. Chronic beryllium disease (CBD) and sarcoidosis can present with the same clinical findings in the lung. The only way to distinguish between CBD and isolated pulmonary sarcoidosis is by an occupational history that shows beryllium exposure, in combination with a positive *in vitro* T-cell proliferation test in which beryllium is included (112). Many investigators do not believe CBD or other metals should be included under the disease classification of sarcoidosis (but rather “sarcoidosis –like”) because of the clinical differences in organ involvement with only rare cases of metal induced granulomatous pneumonitis having extra-pulmonary involvement. Chronic beryllium disease can be misdiagnosed by sarcoidosis when exposure is not examined properly and a beryllium proliferation test is not performed. In a well-designed cohort of 536 sarcoidosis

patients, Müller-Quernheim and colleagues identified 34 patients who were exposed to beryllium and had a positive result in the beryllium lymphocyte proliferation test. Therefore, almost 6% of this cohort could be diagnosed as CBD instead of sarcoidosis (113). Genetic susceptibility towards CBD is found in patients expressing HLA-DPB1 alleles that are characterized by the presence of a glutamic acid at position 69 of the beta chain (114–116). Also other metals like aluminum and zirconium have been linked to sarcoidosis onset in different case reports (4,117–120). Furthermore, a study in Switzerland found an association between the regional frequency of sarcoidosis and the distribution of different sub-branches of the metal industry (121). How metals activate lymphocytes via T-cell receptors to induce an immune reaction is still not completely clear. A possible mechanism is that non-proteinaceous compounds like metals can function as haptens (122). Haptens are molecules not immunogenic themselves but only when bound to a carrier protein (123). Another mechanism has been recently identified upon detailed analysis of T-cell proliferation process mediated by beryllium. The T-cell receptor does not interact with beryllium directly. Rather, the Be ion non-covalently interacts with the peptide binding pocket of HLA-DP, thereby altering the position of peptides that are presented by HLA-DP. Under non-infectious condition, a self-peptide will be present in the peptide binding pocket of HLA-DP, against which T-cells are trained not to respond. Due to the presence of beryllium in the pocket, the self-peptide gets displaced and is seen as foreign by HLA-DP specific CD4 T-cells (124,125). Peptides of the plexin A protein may be the potential source of the displaced self-peptide (126). Plexins are proteins involved in cell movement and response (127). Expression of plexin A was found in BAL and lung tissue from CBD patients (126), strongly supporting the role of plexin A as possible endogenous antigen in this disease.

Silica

Silicon (Si) is one of the most abundant elements on the earth and the combination of two oxygen atoms is well known as silicon oxide or silica (SiO_2) (128). Silica is abundantly present in nature and several industrial products like glass, ceramics and cement contain silica (129). Silica exposure is known to cause a fibrotic and sometimes progressive lung disease called silicosis (130) however, a relation with rheumatoid arthritis, systemic lupus erythematosus as well as sarcoidosis pathogenesis has been suggested (131). For example, sarcoidosis was found to be more common among an Iceland's cohort exposed to crystalline silica in the form of cristobalite, resulting in an increased odds ratio in case of exposure (81). Furthermore, a few case reports have described occupational as well as non-occupational silica exposure as possible causes of sarcoidosis (132–135). In a pilot study by Fireman *et al.* a teacher and a dental technician diagnosed with sarcoidosis showed a positive lymphocyte proliferation response towards silica and metals, determined by the MELISA test. This is a

memory lymphocyte immuno-stimulation assay used to identify sensitization towards metals and silica (136). To induce granulomatous inflammation, silica must be able to react with the immune system. This process is proposed as follows: silica particles are inhaled and reach the alveolar space. Here it is covered by proteins and surfactants and reacts with endogenous antioxidants (129). By binding to proteins, silica possibly cause antibody formation in a hapten like manner. Alveolar macrophages will ingest silica particles and try to clear it from the lungs. Modified silica particles could end up in MHC I or II, which leads to a cellular immune response. When the clearance process is not successful, macrophages are activated and will release chemotactic factors, pro-inflammatory- and pro-fibrotic mediators. In addition, macrophages will be physically damaged by the silica particles which eventually results in cell death, and activation of DAMPs (129). This might eventually result in auto-immune disease or sarcoidosis. Silica-containing macrophages together with surrounding lymphocytes will release macrophage attracting chemokines which lead to the attraction of other macrophages which eventually could lead to granuloma formation (137). As in CBD, the lack of extra pulmonary or multiorgan involvement makes inclusion of this exposure as a cause of sarcoidosis uncertain to many investigators.

SAA

Serum amyloid A (SAA) is produced by the liver during the acute phase reaction (APR). The acute phase reaction serves to eliminate foreign pathogens. In inflammatory conditions, macrophages are a major source of SAA. Elevated SAA levels have been found in several inflammatory diseases including rheumatoid arthritis, diabetes type II and Crohn's disease (138). Significantly higher SAA levels in serum and BAL fluid were observed in sarcoidosis as well (139–142). SAA levels are elevated during infection and inflammation, and this APR protein can activate cells via several mechanisms and receptors including TLR2, TLR4 and RAGE (138). Chen *et al.* have studied the role of SAA in the regulation of granulomatous inflammation in sarcoidosis. SAA expression was found in granulomas of sarcoidosis patients. This SAA expression in granulomas seemed to be sarcoidosis specific, because SAA expression was both quantitatively as well as qualitatively different in other granulomatous disorders including chronic beryllium disease, Wegener granulomatosis, Crohn's disease and tuberculosis. The authors observed *in vitro* that SAA can signal via TLR2, since SAA mediated activation of NF- κ B signaling was inhibited by antibody mediated blockage of TLR2 in a human cell line. Furthermore, it was found that SAA induced higher TNF, IL-10 and IL-18 cytokine production in BAL cells from sarcoidosis patients compared to healthy control cells. This coincided with higher TLR2 expression on BAL macrophages from these sarcoidosis patients. When TLR2 was blocked, cytokine production was inhibited. To summarize, the results suggest that SAA is involved in the sarcoidosis disease pathogenesis in a TLR2

dependent way (142). Chen and colleagues proposed a model of the contribution of SAA in sarcoidosis (143,144) (Figure 2).

In a (myco)bacterial infection, SAA is produced as part of acute phase response. Due to genetic and environmental factors, a Th1 dominated response is induced towards microbial antigens. The intensity of the immune response results in overproduction of SAA, resulting in misfolding or aggregation of SAA. These SAA aggregates form complexes with other matrix proteins, leading to granuloma formation and entrapment of microbial antigens or autoantigens. Being a TLR2 ligand, SAA functions as a feed forward loop to conserve granuloma formation. Granulomas are only resolved when both SAA as well as the other (microbial) antigens are cleared (143). Therefore, SAA serum levels may be helpful in order to determine sarcoidosis activity. In a study by Gungor *et al.* disease activity was determined by clinical findings, pulmonary function tests and radiological findings. They observed that SAA serum levels were significantly higher in sarcoidosis patients with active disease (140), which was confirmed by Salazar *et al.* (145).

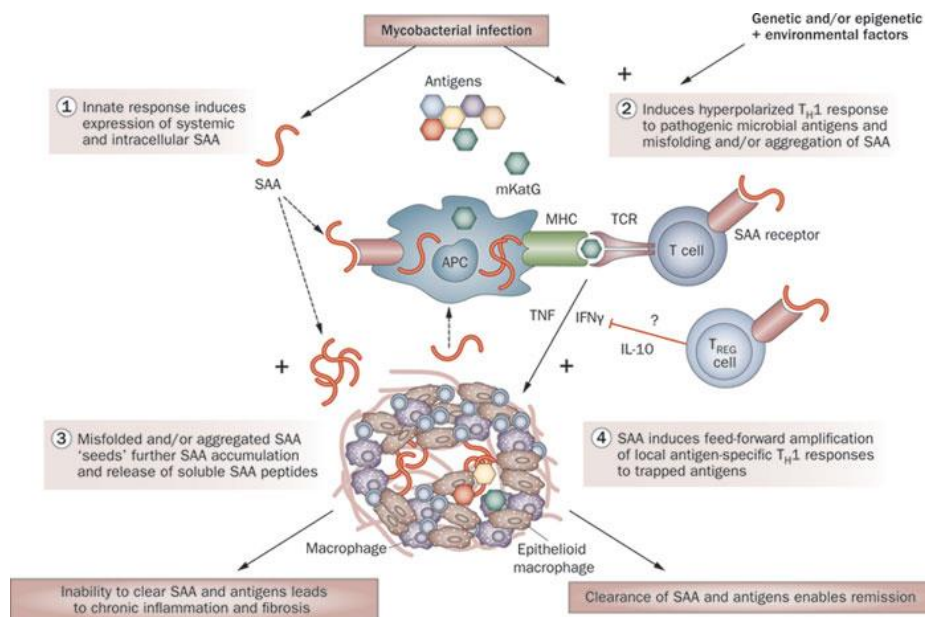


Figure 2. Hypothesized contribution of SAA in the pathogenesis of sarcoidosis. SAA released from granulomas reacts with receptors including TLR2, which leads to the feed-forward stimulation of macrophages and Th1 cells. In combination with uncleared microbial antigens, granulomas will persist. (With permission from Chen ES, Moller DR. Sarcoidosis--scientific progress and clinical challenges . Nat Rev. 2011 Jul 12;7(8):457–67 (144))

Vimentin

Vimentin is a filament protein present in the cell cytoskeleton. Mesenchymal cells, including endothelial cells, express vimentin. It is also expressed in cells that experience physiological and pathological changes, such as wound healing and metastasis. In addition, vimentin might be involved in the host defense to clear microbial infections (146). By immunostaining, asteroid bodies in granulomas of sarcoidosis patients were found to contain vimentin (147). Wahlström and colleagues identified peptides extracted from HLA-DRB1*0301 molecules on BAL cells to be derived from vimentin, along with β -actin, lysyl-tRNA synthase, ATP synthase and kininogen (148). In continuation on this study, antigenic capacity of those peptides was investigated by ELISPOT tests. Significantly higher IFN- γ ELISPOT responses against vimentin were observed in the HLA-DRB1*0301 positive group compared to the HLA-DRB1*0301 negative group. In 4 recovered HLA-DRB1*0301 subjects, no strong responses against vimentin were seen (149). Furthermore, Grunewald and colleagues identified highly clonal T-cells expressing V α 2.3/V β 22 receptors in the lungs of HLA-DRB1*03 positive patients. The expression of specific V α and V β genes suggest clonal expansion due to abundant availability of cognate peptide – MHC class II combination. Using in silico modeling, they made very plausible that the combination of vimentin peptide bound to HLA matches with the T-cell receptor (150). Recently, Eberhardt *et al.* identified vimentin as a Kveim specific protein. Stimulation with vimentin could induce a specific pro-inflammatory cytokine response only in patients with sarcoidosis (151). These data further strengthen the role of vimentin as a possible autoantigen in sarcoidosis pathogenesis. Given the lack of immune responses to vimentin in all patients, it is likely other autoantigens may be involved in disease pathogenesis. A hypothesis that combines a central role for SAA driving persistent Th1 immunity to tissue autoantigens such as vimentin is consistent with the known clinical biology of chronic sarcoidosis.

Conclusion

Sarcoidosis is a complex disease and seems an interplay between genetic susceptibility, cellular function of both innate as well as adaptive immunity, and several exogenous and endogenous triggers. Studies addressing both the innate and adaptive immune response can result in successfully targeting specific mediators such as been demonstrated by blocking TNF-alpha. Elucidating possible triggers in sarcoidosis can help in development of a more personalized-based treatment regime such as use of anti-mycobacterial or anti-propionibacterial drugs. Increasing knowledge of exposure to metals or silica helps to identify sarcoidosis patients which could benefit more from exposure prevention rather than increasing the amount of immune suppression. Further studies are therefore eagerly awaited to definitely confirm the link between possible triggers and sarcoidosis etiology.

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Chapter 3

Clinical observations: is metal or silica associated sarcoidosis a new trigger-related phenotype?

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Introduction

Sarcoidosis is a systemic disorder of unknown etiology, although initiating inorganic triggers such as metals or silica have previously been suggested to play a role (1–4). Interestingly, some epidemiological studies have even demonstrated an elevated risk of sarcoidosis among occupationally silica-exposed individuals (5,6) and patients with chronic beryllium disease (CBD) have been found among cohorts of sarcoidosis patients (7). In this chapter, 3 patients with a biopsy proven diagnosis of sarcoidosis are described in which exposure to metals or silica may have contributed in the development of the disease.

Case 1: Beryllium

The clinical presentation of CBD and sarcoidosis can be identical (8), and the only way to distinguish between both diseases is to assess beryllium sensitization using a lymphocyte proliferation test (LPT) on beryllium (9). An LPT examines the presence of sensitized lymphocytes or memory lymphocytes, for a specific antigen. If memory/sensitized T-cells are present, proliferation of these cells will be observed after stimulation of these cells *in vitro* by the particular antigen. The degree of proliferation is expressed as stimulation index (SI), where an SI between 2 and 3 is defined as a weak positive response and an SI > 3 as a positive response (10).

A 53 year old man was referred to our hospital because of persistent complaints of cough and dyspnea. He first experienced dyspnea while working as a smelter in a foundry about 25 years ago. In this period, fever and dyspnea particular presented during the evening. He was diagnosed with metal fume fever and therefore changed his job. He improved, although the complaints did not completely disappear over the years. At presentation in our ILD clinic, his dyspnea had progressed over the last couple of months. Upon physical examination the patient had a normal breathing rate with an oxygen saturation of 92% at room air. During auscultation of the lung, dorsobasal inspiratory crackles were heard and digital clubbing was seen. His pulmonary function demonstrated a restrictive pattern with a significant reduced diffusion capacity of 33% of predicted. A High-resolution computed tomography (HRCT) scan of the thorax showed bilateral hilar and mediastinal lymphadenopathy with extensive peribronchovascular located ground glass densities present at both lungs. Furthermore, small areas of air trapping and fibrotic abnormalities were seen at both lungs without the presence of honeycombing (Figure 1).

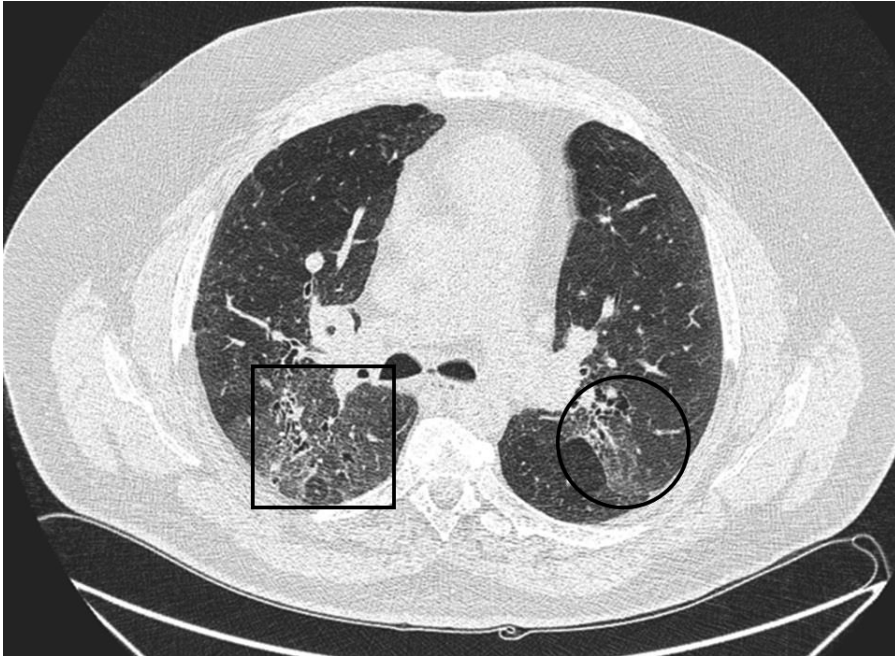


Figure 1. HRCT scan of the thorax which shows ground glass areas in the left lung (circle) and areas of fibrosis (square) in the right lung.

In order to make a diagnosis, an Endoscopic Ultra Sound (EUS) guided puncture of the subcarinal lymph node was performed revealing non-caseating granulomas. Culture for bacteria such as mycobacteria was negative and a diagnosis of pulmonary sarcoidosis was made. However, due to his occupational history, presence of pulmonary fibrosis and clubbing, it was decided to perform an LPT on aluminum, beryllium and zirconium. A positive LPT for beryllium, with an SI of 24.5, was observed indicating beryllium sensitization. Based on exposure to metals, presence of non-caseating granulomas and sensitization for beryllium, the diagnosis was changed into CBD according to the current guideline (9). Due to the disease progression of this patient, treatment with methotrexate and later with infliximab was initiated, both with only a marginal effect. Although the patient already quitted his occupational activities in the foundry, he was advised to avoid further exposure to beryllium, for example in hobby related activities.

Case 2: Aluminum

A 31 old man without relevant medical history, was referred to our hospital because of complaints of dyspnea. He had been working for 12 years as a logistic employee in the dock of a steel factory, with direct exposure to lignite, coal and iron ores.

During physical examination the patient had a normal breathing rate with an oxygen saturation of 96% at room air. On auscultation, inspiratory crackles were heard in the basal fields of both lungs and presence of digital clubbing was observed. Pulmonary function tests showed a minimal restricted pattern and a moderate to severe impairment of diffusion capacity (52% of predicted) An HRCT scan of the thorax showed ground glass densities with mosaic attenuation in both lungs. Lymphadenopathy or fibrosis were not observed (Figure 2).

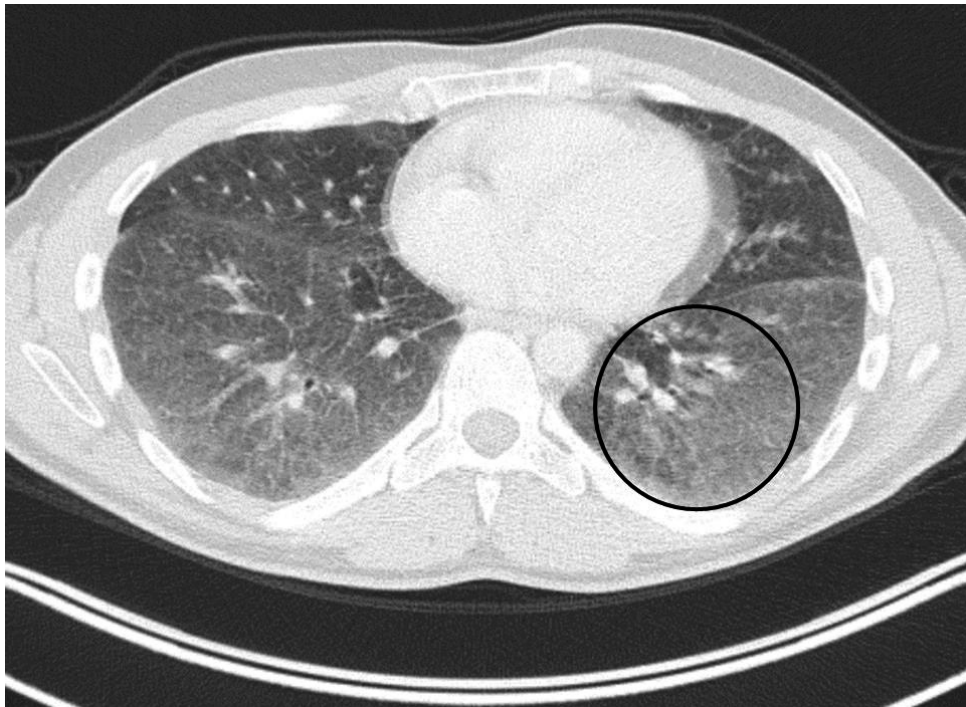


Figure 2. HRCT scan of the thorax. An extensive reticulonodular interstitial pattern was observed with ground glass areas and mosaic attenuation (circle) at both lungs.

The differential diagnosis included pneumoconiosis or an atypical presentation of sarcoidosis. To make a definite diagnosis, an open lung biopsy was performed revealing mature non-caseating granulomas, suggesting a diagnosis of sarcoidosis instead of pneumoconiosis. Besides granulomas, however, presence of birefringent material was

found in the lung biopsy. An Energy-dispersive X-ray spectroscopy analysis (EDXA) to determine the origin of the birefringent material failed due to technical reasons. Based on his exposure to metals, the extensive ground glass on HRCT and findings on physical examination suggesting pulmonary fibrosis, an LPT was performed on aluminum, beryllium and zirconium. A weakly positive response for aluminum (SI 2.7) was found. For this reason, the patient was advised to avoid further metal exposure. Exposure avoidance combined with prednisone treatment resulted in the decrease of dyspnea complaints as well as an improvement in the patient's radiological picture.

Case 3: Silica

Silicosis is caused by the inhalation of crystalline silicon dioxide, or silica, and is one of the most important occupational diseases worldwide (11). A diagnosis of silicosis is made based on exposure and typical radiological findings, according to the ILO's International Classification of Radiographs of Pneumoconiosis (12). In addition, competing diagnoses such as miliary tuberculosis or sarcoidosis should be excluded, as radiologic patterns of these diseases can be similar (11).

A 49-year-old male who had worked as a plasterer for 30 years presented with complaints of fatigue and progressive dyspnea, and was diagnosed as having silicosis. The patient was referred to our hospital because of deterioration, and to confirm the diagnosis. Occupational history was taken by an occupational hygienist and did indeed show exposure to construction dust including both crystalline and amorphous silica. HRCT showed mediastinal and bilateral hilar lymphadenopathy, subpleural nodules, consolidations, bronchiectasis and fibrotic lesions (Figure 3).

The radiology findings did not allow a diagnostic distinction to be made between silicosis and sarcoidosis. Hence, an open lung biopsy was performed, which showed inflammatory infiltration in the pleura, non-necrotizing granulomas including multinucleated giant cells with hyalinization, and birefringent material in the lung parenchyma (Figure 4).

Since yeast, mycobacterial and fungus cultures were negative and malignancy was excluded as well, the biopsy fitted within a diagnosis of sarcoidosis. In view of the presence of birefringent material in the tissue, most likely deposited as a result of the patient's masonry and plasterwork, an EDXA was performed. This revealed aluminum, silicon and titanium, elements that are all present in cement. An LPT was used to test whether the patient showed immunoreactivity to any of these compounds. Since beryllium is known to be capable of inducing a granulomatous reaction (9), the LPT was performed for beryllium as well. The stimulation indexes for the metals tested were all below 2.0. Interestingly, a positive stimulation index of 3.2 was found for silica, indicating sensitization.

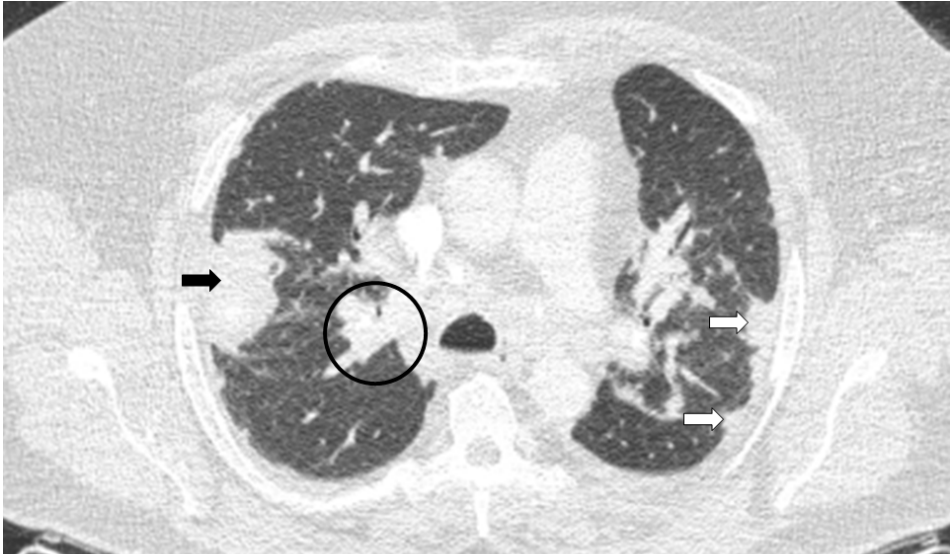


Figure 3. HRCT scan of the thorax showed bilateral hilar and mediastinal lymphadenopathy (circle) with subpleural nodules (white arrows) and consolidations (black arrow).

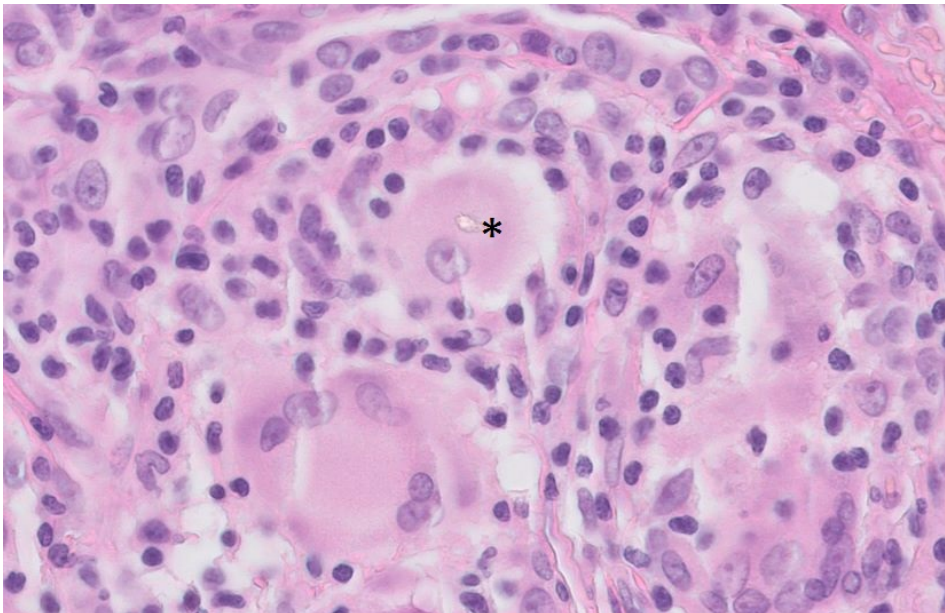


Figure 4. Open lung biopsy tissue demonstrating non-necrotizing granulomas including birefringent material, as indicated by the asterisk.

While being treated with a combination of prednisone and azathioprine, the patient deteriorated further, with increased dyspnea and pulmonary consolidations and worsening of the pulmonary function tests. In view of the final diagnosis of sarcoidosis instead of silicosis, treatment with infliximab was started. After 7 months of treatment with this agent, the patient's pulmonary function test had improved and pulmonary consolidations on HRCT as well as the inflammatory activity measured by ^{18}F -fluorodeoxyglucose by positron emission tomography/computed tomography (^{18}F -FDG PET/CT) had decreased (Figure 5). Forced Vital Capacity (FVC) rose from 1.97 L to 2.35 L (44 and 53 percent of predicted, respectively) and the diffusing capacity of the lung for carbon monoxide (DLCO) rose from 5.46 mmol/(min*kPa) (54 percent of predicted) to 6.26 mmol/(min*kPa) (62 percent of predicted). carbon monoxide (DLCO) rose from 5.46 mmol/(min*kPa) (54 percent of predicted) to 6.26 mmol/(min*kPa) (62 percent of predicted).

Discussion

The patients described in this chapter highlight an interesting clinical problem in the differential diagnosis of pneumoconiosis versus sarcoidosis. The appearance of non-caseating granulomas in the biopsies of these patients pointed towards a diagnosis of sarcoidosis and not pneumoconiosis, where more sclerotic nodules are found (13). However, demonstration of sensitization to metals or silica in these patients makes it difficult to apply the criterion "per exclusionem" for diagnosis of sarcoidosis in the last two of these patients. In the first patient, the diagnosis of sarcoidosis could be changed to CBD according the current guideline (9). The patient had exposure to beryllium, presence of noncaseating granulomas and proven immunological sensitization for beryllium. It is well known that the non-necrotizing granulomas found in CBD are identical to the granulomas found in sarcoidosis, so one could speculate that CBD can be seen as a form of beryllium-induced sarcoidosis (14,15). A current guideline for diagnosing aluminum or silica induced granulomatous pulmonary disease is lacking. Keeping in mind the above mentioned criteria to diagnose CBD, and applying them to aluminum and silica, a diagnosis of sarcoidosis associated with aluminum or silica exposure seems reasonable in our opinion for the last 2 patients. In the future, we could perhaps use the diagnosis of "chronic aluminum disease (CAD)" or "chronic silica disease (CSD)" when we demonstrate immunological sensitization against the above mentioned antigens in patients with biopsy proven sarcoidosis.

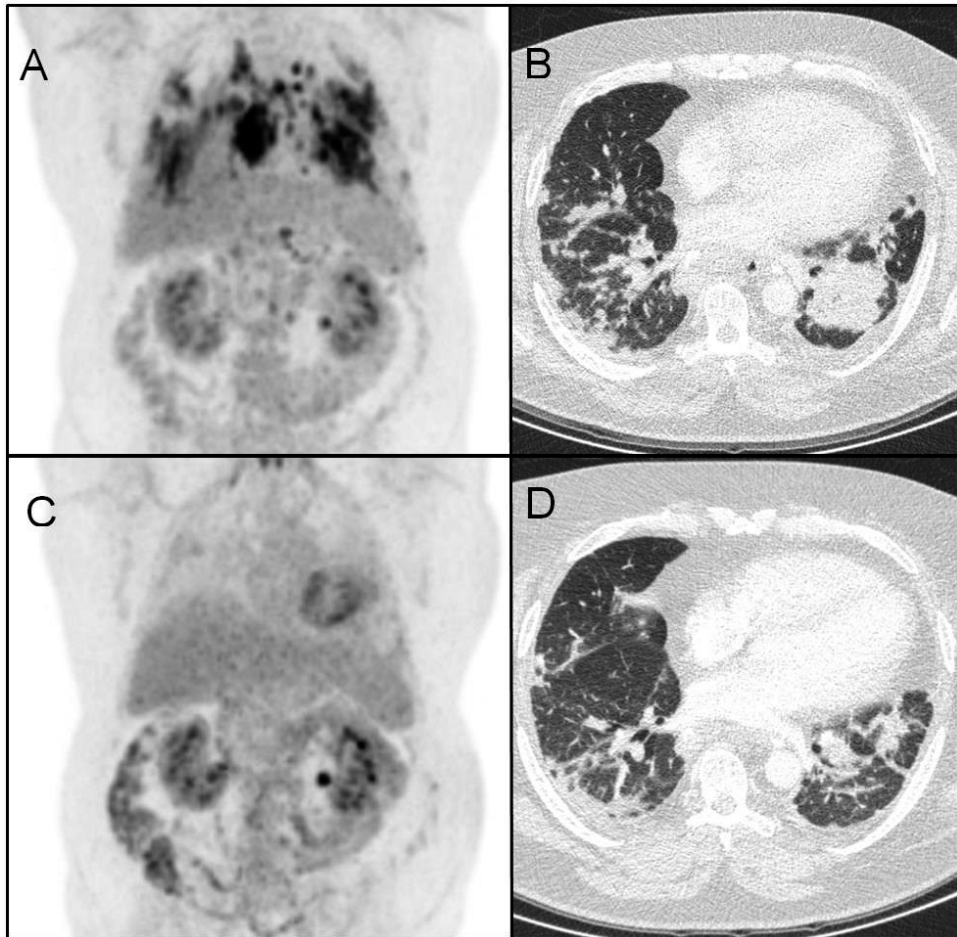


Figure 5. PET and HRCT scans before and after 7 months of infliximab therapy. Upper part: PET (A) and HRCT scan (B) 2 months prior to the start of infliximab. At this time, the patient had been on azathioprine therapy for 8 months, and prednisone therapy had been stopped 14 months ago. Lower part: PET (C) and HRCT scans (D) after 7 months of infliximab therapy, showing decreased consolidations.

The clinical, radiological as well as histological presentation of these 3 patients can provide some clinical guidance. Indications of pulmonary crackles on auscultation (case 1 and 2) are not typical findings in sarcoidosis patients, and might point to the development of pulmonary fibrosis. Furthermore, also diffuse ground glass abnormalities were observed in patient 1 and 2, which are not common in the presentation of sarcoidosis either. Last, birefringent material in biopsies are neither frequently seen in sarcoidosis patients.

Therefore, when above mentioned features are observed in a patient suspected for sarcoidosis, the clinician should assess the occupational history in more detail and assess the possible presence of an inorganic inducing agent. This could be done by the performance of an EDXA in case of presence of birefringent material in biopsy. However, beryllium has proven to be too small to be detected by EDXA. For this reason, patients with CBD will be missed when only the biopsy is analyzed for presence of inorganic agents while an LPT is not performed. It could therefore be important to carefully examine occupational and exposure history of patients suspected for sarcoidosis and to perform an LPT.

In conclusion, it can be difficult to distinguish between pneumoconiosis and sarcoidosis based on only exposure, radiological or histological patterns. Sensitization to metals and silica could be involved in an underlying immunological mechanism resulting in sarcoidosis, explaining why people with metal or silica exposure have an elevated risk of developing sarcoidosis (5,16). Future studies in larger patients groups should be initiated to address the possible causality of metal and silica exposure and the development of sarcoidosis, and clarify the underlying immunological mechanisms in more detail.

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Chapter 4

**Immunoreactivity to metal and silica
associates with sarcoidosis in Dutch
patients**

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Abstract

Involvement of metals or silica in the pathogenesis of sarcoidosis has been suggested by several case reports and specific epidemiological studies. However, the combination of occupational exposure and an immunological reaction has not been studied before in a group of sarcoidosis patients and non-sarcoidosis controls.

In 256 sarcoidosis patients and 73 control patients with obstructive sleep apnea, exposure to metal and silica was assessed using a questionnaire consisting of a complete occupational history subsequently linked to job-exposure matrices. Next, immunoreactivity to aluminum, beryllium, zirconium and silica was determined in 33 sarcoidosis and 19 control patients using a lymphocyte proliferation test.

In sarcoidosis, 83 out 256 patients (32.4%) had occupational exposure to metals or silica, compared to 24.7% in the control group ($P = 0.21$). A significantly higher percentage of the sarcoidosis patients tested showed immunoreactivity to metals or silica compared to the control group (21.2% and 0% respectively, $P = 0.039$).

Immunoreactivity to silica and metals only found in sarcoidosis patients, supporting the hypothesis that these antigens may be involved in the pathogenesis of a distinct subgroup of sarcoidosis patients. This indicates that when searching for causative agents in sarcoidosis patients, besides beryllium, zirconium, aluminum and silica also deserve clinical investigation.

Keywords: Sarcoidosis, aluminum, beryllium, zirconium, metals, silica

Introduction

Sarcoidosis is a systemic disease characterized by the formation of noncaseating granuloma, mostly affecting the lungs, skin, eyes and lymph nodes (1). The etiology of sarcoidosis remains unclear to date and possible etiologic factors such as metals or silica are not included in the current work-up of sarcoidosis. When beryllium exposure is clinically suspected in patients with sarcoidosis, chronic beryllium disease (CBD) can be excluded by testing for beryllium immunoreactivity using a Lymphocyte Proliferation Test (LPT). This is important since CBD can be histopathological and clinical indistinguishable from sarcoidosis (2). Also other antigens such as aluminum, zirconium and silica are suggested to play a role in the pathogenesis of sarcoidosis (3–7). At present, however, a causal relationship between these antigens and sarcoidosis has not been established. First because studies using a control group are lacking and second because exposure is difficult to assess. Identification of possible etiologic antigens in sarcoidosis is important since it may lead to a more personalized treatment regime. Retrospective studies on CBD showed that termination of beryllium exposure could lead to disease reversal (8,9). Avoiding exposure might therefore be the first step in the treatment of sarcoidosis patients in whom metal or silica might be involved in the disease pathogenesis.

Although involvement of metals and silica in sarcoidosis pathogenesis has been suggested by several case reports and epidemiological studies, it has not been systematically studied in a larger group of sarcoidosis patients. The aim of this study was to characterize both occupational exposure and immunoreactivity to metals and silica in a group of sarcoidosis patients and controls. Since sarcoidosis is a very heterogeneous disease, we hypothesize that metals or silica are involved in the pathogenesis of the disease in only a subgroup of patients. Therefore, we have investigated whether occupational history linked to a job exposure matrix (JEM) can be an appropriate tool to identify which sarcoidosis patients have to be further tested for metal and silica immunoreactivity.

Material and methods

Study subjects

Sarcoidosis patients visiting the Interstitial Lung Disease (ILD) outpatient clinic of St. Antonius Hospital (Nieuwegein, The Netherlands) received an invitation letter to participate in the study. The diagnosis of sarcoidosis had been established according to the criteria of the American Thoracic Society/European Respiratory Society (10). The characteristics of the sarcoidosis patients, including age at diagnosis and inclusion, Scadding stage at diagnosis and inclusion, organ involvement and the use of immunosuppressive medication, were collected from medical records. As a control group, patients with obstructive sleep apnea (OSA) visiting the Pulmonary outpatient clinic of the St. Antonius Hospital were asked to

participate. Patients diagnosed with OSA were used as controls based on the fact that there is no relationship between environmental triggers and development of OSA (11). This study was approved by the Medical research Ethics Committees United (MEC-U) of the St. Antonius Hospital (R14.023) and written informed consent was obtained from all participants.

Assessment of metal and silica exposure

Sarcoidosis and control patients received an online questionnaire asking for a complete occupational history. The patients' job histories were coded using the International Standard Classification of Occupations 1968 (ISCO-68) (12). This classification system consists of seven major groups, which are subdivided into several minor groups. These minor groups are further divided into unit groups, and eventually into single jobs. Every single job has a unique 5-digit code. This allows jobs to be organized into clearly defined sets according to the tasks and duties undertaken in the job. The complete job history, until the diagnosis of sarcoidosis, was taken into account. Occupational exposure to silica, and the metals nickel and chromium was assigned through the DOM-JEM developed by experts in the Netherlands for application in general population studies (13). Occupational exposure to metals in general was assigned through the ALOHA + JEM, developed by the same experts (14). Because this JEM needs jobs coded into ISCO-88 (15), initial ISCO-68 job codes were recoded into ISCO-88 codes.

Lymphocyte proliferation tests to determine metal and silica immunoreactivity

Patient selection

A random selection of 45 sarcoidosis patients with occupational exposure to silica or metals were asked to donate a blood sample for an LPT on beryllium, aluminum, zirconium and silica. From the group of sarcoidosis patients without exposure, 10 patients who had an appointment in the near future were asked for the LPT as well. Those patients were selected in such way that age and sex did not differ from the exposed sarcoidosis patients invited for the LPT. The first 20 control patients included were also asked to donate a blood sample.

MELISA®

Blood was drawn in citrate tubes and sent within 24 hours to Prohealth Medical, Nederweert, The Netherlands, where the memory lymphocyte immunostimulation assay (MELISA®) LPT was performed. MELISA® is a clinically validated blood test that detects type-IV allergy (sensitization) to multiple metals (16,17). Furthermore, it can also be used to determine sensitization for silica (18). Lymphocytes were isolated from citrated blood, and 1×10^6 cells were incubated for 5 days in HEPES-buffered media containing at least two

consecutive dilutions of an antigen: aluminum nitrate, beryllium sulfate, silicon dioxide or zirconium dioxide. After cultivating for 5 days, proliferation was measured by incorporation of H^3 -labelled thymidine. Radioactivity was measured with a scintillation counter (Microbeta, Perkin Elmer). Stimulation indexes (SI) were calculated by dividing the antigen specific counts per minute by the mean counts per minute of the basal controls. SI of ≥ 3.0 were considered as a positive test results, based on the MELISA[®] guidelines (16).

Beryllium LPT

Patients with an SI of ≥ 3.0 for beryllium were invited to donate a second blood sample, which was sent to the department of Laboratory Medicine, University Hospitals Leuven, Belgium, where a second beryllium LPT was performed according to the protocol described in the ATS official statement on diagnosis and management of beryllium sensitivity and chronic beryllium disease (19). Briefly, $1.8 \cdot 10^5$ PBMCs/200 μ L were incubated for 6 days in medium (RPMI-1640 GlutaMax supplemented with 5% autologous plasma and penicillin/streptomycin) containing concentrations of 10^{-4} M till 10^{-10} M $BeSO_4$. Cell proliferation was measured by incorporation of H^3 -labelled thymidine. An SI of ≥ 3.0 was considered positive. The highest SI measured for each sample was used in the analysis. For calculating the correlation between the SI for beryllium obtained by MELISA[®] and obtained in Leuven, one additional patient, who had already performed both test for clinical evaluation, was included.

Statistical analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at St. Antonius hospital. Data were analyzed using IBM SPSS statistics version 24. An unpaired T-test was used to compare numerical data between the sarcoidosis and control group. Non-parametric tests were used for non-normally distributed data (Mann-Whitney U test). Categorical data were compared between the sarcoidosis and control group using the Chi-squared test. If expected cell frequencies were below 5, Fisher's exact test was used for categorical data up to two categories. Odds ratios were estimated for sarcoidosis and occupational metal and silica exposure. Correlation between the MELISA[®] LPT and validated beryllium LPT was determined with Spearman's rho correlation. P-values <0.05 were considered significant.

Results

Patient characteristics

A total of 590 patients were invited to participate in the study, of whom 314 responded and were willing to fill out the questionnaire. Finally, 256 sarcoidosis patients completed the occupational history and were included in the study. Seventy-three patients with OSA were included to serve as a control group. Demographics of all sarcoidosis and control patients are presented in Table 1. No difference in age at inclusion, sex or ethnicity was observed between the sarcoidosis and control patients. Only smoking status was significantly different between the sarcoidosis and control patients, with a higher proportion of never smokers in the sarcoidosis group.

Table 1. Demographics of sarcoidosis and control patients

	Controls (%) (n=73)	Sarcoidosis (%) (n=256)	P*
Male / Female	64.4 / 35.6	63.7 / 36.3	0.91
Age at diagnosis (Y)	N/A	43.57±10.63	-
Age at inclusion (Y)	55.42±14.37	54.50±10.83	0.61
Ethnicity (W/B/O)	93.2 / 2.7 / 4.1	93.4 / 3.5 / 3.1	0.88
Smoking status ^a (Never/former or current)	34.2 / 65.8	57.7 / 42.3	<0.001
Scadding stage at inclusion (0/I/II/III/IV/ unknown)	N/A	30.1 / 9.8 / 18.4 / 12.9 / 26.2 / 2.7	
Extra pulmonary involvement	N/A	46.5	

Age at diagnosis and age at inclusion are shown as means ± std. deviation. Scadding stages were determined by chest X-ray. If Scadding stages at the time of inclusion were not available, Scadding stage was determined from the last obtained chest X-ray before the inclusion date. 0 = Normal chest radiograph; I = Bilateral hilar lymphadenopathy (BHL); II = BHL with pulmonary infiltrates; III = pulmonary infiltrates without BHL; IV = fibrosis. B: Black; O: Other; W: White

^a Smoking status was not available from 8 sarcoidosis patients. *Statistical differences for sex, ethnicity and smoking status were assessed using Pearson Chi-Square, statistical differences for age were calculated using an unpaired T-test.

No differences in occupational history and exposure between sarcoidosis patients and controls

The distribution of sarcoidosis patients and controls over the major occupational groups is shown in Table 2. Almost all occupations classified by the JEMs as involving exposure to metals or silica, were jobs in major group 7/8/9 (Production and Related Workers, Transport Operators and laborers). Hence we compared the distribution of sarcoidosis and controls patients also over the minor groups belonging to major group 7/8/9 (Table 2). No significant differences between sarcoidosis patients and controls were observed for any of these major or minor group. Occupational metal or silica exposure was assigned to 83 sarcoidosis patients (32.4%) and 18 control patients (24.7%) ($P = 0.21$) (OR = 1.47; 95% CI 0.81 to 2.97, Table 3). No significant differences were observed in occupational metal or silica exposure between the sarcoidosis and control group (Table 4).

Immunoreactivity to metals and silica was only observed in sarcoidosis patients

Forty-five patients occupationally exposed to metals or silica were invited for the LPT, of whom 37 were willing to donate a blood sample. Unfortunately, LPTs on 11 samples could not be performed due to an insufficient number of lymphocytes. Ten sarcoidosis patients who had no exposure based on the JEMs were invited for the LPT as well. Of those 10 samples, 3 patients also had an insufficient number of lymphocytes. Of the 14 patients in whom the lymphocyte count was insufficient, 8 were on immunosuppressive medication and 6 were not. In total, an LPT was performed on samples of 33 sarcoidosis patients, of whom 26 had JEM assigned exposure and 7 had no JEM assigned exposure to either metals or silica. Of the 20 blood samples from control patients, 1 sample could not be analyzed for technical reasons. Three of those 19 control patients had JEM assigned exposure to either metals or silica. No significant differences regarding ethnicity, age at time of the LPT, sex or smoking were observed between the sarcoidosis and control patients of whom an LPT was performed. We found that 45.5% of the sarcoidosis patients, compared to none of the controls, were on immunosuppressive drugs at the time of the LPT ($P < 0.01$) (Table 5).

Table 2. Percentage of patients within ISCO-68 major groups and in minor groups of major group 7/8/9.

Major occupational group	Sarcoidosis (%) (n=256)	Controls (%) (n=73)
0/1. Professional, Technical and Related Workers	34.8	30.1
2. Administrative and Managerial Workers.	11.7	11.0
3. Clerical and Related Workers	23.0	27.4
4. Sales Workers	9.8	15.1
5. Service Workers	13.3	16.4
6. Agricultural, Animal Husbandry and Forestry Workers, Fishermen and Hunters	3.5	2.7
7/8/9. Production and Related Workers, Transport Operators and Laborers	41.4	42.5
Minor occupational groups of major group 7/8/9		
70. Production Supervisors and General Foremen	3.5	4.1
71. Miners, Quarrymen, Well Drillers and Related Workers	0.4	0.0
72. Metal Processers	2.7	0.0
73. Wood Preparation Workers and Paper Makers	0.8	0.0
77. Food and Beverage Processers	3.1	4.1
79. Tailors, Dressmakers, Sewers, Upholsterers and Related Workers	1.2	1.4
81. Cabinetmakers and Related Woodworkers	0.4	0
83. Blacksmiths, Toolmakers and Machine-Tool Operators	3.1	6.8
84. Machinery Fitters, Machine Assemblers and Precision Instrument Makers (except Electrical)	9.4	8.2
85. Electrical Fitters and Related Electrical and Electronics Workers	10.9	9.6
87. Plumbers, Welders, Sheet Metal and Structural Metal Preparers and Erectors	7.4	2.7

88. Jewelry and Precious Metal Workers	0.8	0.0
90. Rubber and Plastics Product Makers	0.4	0.0
91. Paper and Paperboard Products Makers	0.0	1.4
92. Printers and Related Workers	0.4	0.0
93. Painters	1.6	1.4
94. Production and Related Workers Not Elsewhere Classified	1.6	0.0
95. Bricklayers, Carpenters and Other Construction Workers	4.7	8.2
97. Material-Handling and Related Equipment Operators, Dockers and Freight Handlers	3.5	4.1
98. Transport Equipment Operators	6.3	8.2
99. laborers Not Elsewhere Classified	0.8	1.4

Percentages of sarcoidosis patients and controls are based on all jobs performed during working life until the diagnosis of sarcoidosis was made (sarcoidosis group) or until the moment of study enrolment (control group). If participants had more than one job within the same group, only one was counted. Minor occupation groups without sarcoidosis patients and controls are not shown.

Table 3. Estimated odds ratios for sarcoidosis and JEM assigned exposure

Exposure	Controls (%) (n=73)	Sarcoidosis (%) (n=256)	OR (95% CI)	OR (95% CI) Adjusted*
Silica and/or metals	24.7	32.4	1.47 (0.81, 2.65)	1.62 (0.88, 2.97)
Silica	5.5	7.4	1.38 (0.46, 4.20)	1.52 (0.49, 4.70)
Metals	23.3	29.3	1.37 (0.75, 2.50)	1.48 (0.80, 2.76)

*adjusted for smoking

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Table 4. JEM assigned exposure of sarcoidosis patients and controls

JEM Exposure	Controls (n=73)	Sarcoidosis (n=256)	P*
Silica	4 (5.5)	19 (7.4)	0.57
Metals	17 (23.3)	75 (29.3)	0.31
Chromium	6 (8.2)	35 (13.7)	0.21
Nickel	6 (8.2)	31 (12.1)	0.35
Silica and/or metal	18 (24.7)	83 (32.4)	0.21

Values are shown as absolute numbers with percentages in brackets.

* Statistical differences were accessed using Pearson Chi-Square

Table 5. Demographics of the sarcoidosis and control patients who had an LPT performed

	Controls (%) (n=19)	Sarcoidosis (%) (n=33)	P*
Male / Female	68.4 / 31.6	90.9 / 9.1	0.06
Age at inclusion (Y)	60.53±14.21	54.36±11.18	0.09
Ethnicity (W/B/O)	94.7 / 0 / 5.3	97.0 / 3.0 / 0	0.31
Smoking status ^a (Never/Former or current)	47.4 / 52.6	62.5 / 37.5	0.29
Using immunosuppressive medication	0	45.5	<0.01

Age at inclusion is shown as means ± std. deviation.. B: Black; O: Other; W: White.

^aSmoking status was not available from 1 sarcoidosis patient *Statistical differences for sex was accessed using Fisher's Exact Test, P-values of ethnicity, smoking status and use of medication were calculated using Pearson Chi-Square and statistical differences for age were calculated using an unpaired T-test.

Immunoreactivity was only observed within the sarcoidosis group. Seven (21.2%) sarcoidosis patients had a positive LPT for at least one of the antigens tested, compared to none of the controls (Figure 1, P = 0.039). One sarcoidosis patient had a positive LPT for multiple antigens, namely zirconium and beryllium. In total, 3 sarcoidosis patients had an SI of ≥ 3.0 for beryllium and were invited for a second beryllium LPT using the ATS protocol. The highest SI values obtained by MELISA® for those three patients and the additional clinical patient were 3.96, 4.94, 9.05 and 24.48. The highest SI values of the second beryllium LPT following the ATS protocol for these patients were 1.27, 3.42, 5.93 and 379,

respectively. When the results of those 4 patients were combined, a strong correlation of 1.00 ($P < 0.01$) was found between the beryllium SI of the MELISA® and LPT using the ATS protocol.

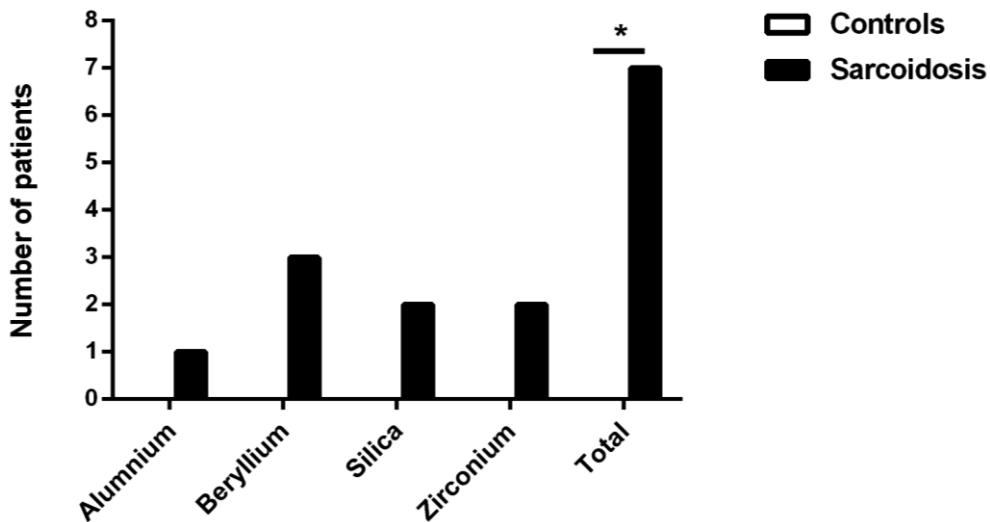


Figure 1. Number of sarcoidosis patients and controls with a positive LPT to the antigens tested. None of the control patients had a positive LPT for aluminum, silica, zirconium or beryllium. The difference between sarcoidosis patients and controls with a positive LPT to any of the antigens was significant ($P = 0.039$). An LPT was considered positive when the stimulation index was ≥ 3.0 .

Occupational exposure does not predict immunoreactivity

Occupational exposure as assigned by JEMs was compared with LPT results. Four of the 26 sarcoidosis patients (15.4%) with metal or silica exposure showed immunoreactivity to at least one of the antigens tested. Among the 7 sarcoidosis patients without JEM assigned exposure, 3 (42.9%) had a positive LPT for the antigens tested (Table 6). The difference in percentage positive LPTs between patients with and without JEM assigned exposure was not significant ($P = 0.145$).

Table 6. JEM assigned exposure of LPT positive sarcoidosis and control patients

Sarcoidosis or control	LPT positive for	SI	JEM assigned exposure
Sarcoidosis	Al	13.62	-
Sarcoidosis	Be	4.94	-
Sarcoidosis	Be, Zir	9.05, 3.51	-
Sarcoidosis	Be	3.96	Silica
Sarcoidosis	Si	3.20	Silica
Sarcoidosis	Si	6.90	Metals
Sarcoidosis	Zir	3.01	Metals

Al: aluminum; Be: Beryllium; Si: Silica; Zir: zirconium; SI: Stimulation index

Clinical phenotypes of LPT-positive sarcoidosis and LPT-negative sarcoidosis patients

Clinical characteristics of LPT-positive sarcoidosis patients and LPT-negative sarcoidosis patients are shown in Table 7. We also determined the clinical outcome status (COS), and divided it into three groups: resolved and minimal disease (COS 1,2,3,4), persistent disease without therapy (COS 5,6) and persistent disease with need for therapy (COS 7,8,9). No differences between the two groups were observed for any of the clinical characteristics or COS.

Discussion

This study is to our knowledge the first to combine both exposure analysis and lymphocyte proliferations tests for multiple inorganic antigens in patients with sarcoidosis and controls. We found immunoreactivity to metals and silica only in patients with sarcoidosis. Although a large group of patients with sarcoidosis was included, no differences in occupational exposure to metals and silica with the OSA control group was observed. Previous studies addressing such questions were either large epidemiological studies without immunological data, or smaller studies and case reports describing only one specific antigen (3,7,20,21).

Table 7. Clinical features of LPT-positive and LPT-negative sarcoidosis patients

	LPT-negative (%) (n=26)	LPT-positive (%) (n=7)	P*
Male / Female	92.3 / 7.7	85.7/ 14.3	0.52
Age at diagnosis (Y)	43.46±11.61	41.40±7.97	0.66
Time after diagnosis (Y)	13.65±12.39	9.21±8.21	0.36
Medication	46.2	42.9	1.00
Scadding stage at diagnosis (0/I/II/III/IV/unknown)	11.5/26.9/42.3/3.8/0/ 15.4	0/42.9/28.6/0/14.3/ 14.3	0.36
Scadding stage at inclusion (0/I/II/III/IV/unknown)	38.5/7.7/11.5/11.5/30.8 /0	28.6/14.3/14.3/0/28.6 /14.3	0.42
Extra pulmonary involvement	57.7	57.1	1.00
COS	n= 21	n= 5	
Resolved & minimal disease	4.8	40	0.09
Persistent disease without therapy	33.3	20	1.00
Persistent disease with need for therapy	61.9	40	0.62

Age at diagnosis and time after diagnosis are shown as means ± std. deviation. COS: Clinical outcome status. COS was defined 5 years after diagnosis. Information to determine COS was missing for 7 patients (5 LPT-negative and 2 LPT positive patients).

* Statistical differences for sex, medication, COS groups, extra pulmonary involvement, was accessed using Fisher's Exact Test, P-values for scadding stages were calculated using Pearson Chi-Square. Statistical differences for age at diagnosis were calculated using an unpaired T-test and statistical differences for time after diagnosis was calculated using Mann-Whitney U test.

The fact that only patients within the sarcoidosis group demonstrated immunoreactivity for different antigens supports the concept of metals and silica involvement in the disease pathogenesis of a distinct subgroup of sarcoidosis patients. Different epidemiological studies have demonstrated a higher risk of sarcoidosis among persons occupationally exposed to silica (4,22). Immunoreactivity to silica in some sarcoidosis patients, suggests an underlying immunological mechanism that could explain these epidemiological observations. Regarding the role of zirconium and aluminum in sarcoidosis pathogenesis,

no other studies than case-reports exist (3,7,23). Müller-Quernheim and colleagues (20) established that in a well-defined cohort of biopsy-proven sarcoidosis patients, over 6% should be diagnosed with CBD based on the diagnostic criteria for this disease, which includes presence of non-caseating granulomas in lung or lymph node biopsies, beryllium exposure and sensitization (19). In our cohort, 3 out of 256 sarcoidosis patients (1.2%) had a positive LPT for beryllium defined by MELISA[®], of which two also had elevated SI values on the beryllium LPT using the ATS guideline protocol (19). These results demonstrate that also in the Netherlands, patients with CBD can be identified within a group of biopsy proven sarcoidosis patients. This is a clinically relevant finding since termination of exposure to beryllium may be the first step in the treatment of CBD to prevent progression of the disease in these patients. In light of diagnostic criteria for CBD, it is tempting to speculate that patients with a positive LPT to zirconium, aluminum or silica, have a disease etiology clinically comparable to that of CBD. In CBD, genetic susceptibility has been demonstrated, since a Glu at position 69 in the HLA-DPB1 gene is found in approximately 90% of patients compared to about 40% of healthy controls. The presence of this genetic variant has proven to be an important contributing factor to the development of CBD (24,25). Since immunoreactivity, but not occupational exposure, differed between patients and controls, genetic variants may very well be involved in the ability to respond to the metals and silica addressed in this study.

Another important conclusion from our study is that our screening method, including the questionnaire linked to the JEMs, appears unable to predict immunoreactivity. Almost half of our patients with a positive LPT were classified by JEMs as unexposed. The JEMs are designed to be very specific (i.e. high probability of exposure within a certain job) and will, by definition, not capture individuals experiencing coincidental high risk exposure while carrying out otherwise low-risk jobs (26). It is important to state that even very low occasional exposures can be relevant, as suggested by the work of Infante *et al.* (27), who described that bystander beryllium exposure can lead to CBD. Perhaps a simple occupational history is not detailed enough, and additional (job-specific) questionnaires will be required to identify occasional/recreational exposures that can be sufficient to initiate sarcoidosis or a sarcoid-like disease. However, the addition of more detailed methods to establish exposure will also lead to less specificity, i.e. there will be a higher number of exposure assigned patients who may not show immunoreactivity. Furthermore, since even low non-occupational exposures (28) can be of relevance in light of immunoreactivity, it will be difficult to distinguish between meaningful and negligible exposures. Therefore, testing all sarcoidosis patients for immunoreactivity to metals and silica in future studies, irrespective of exposure, will probably be more convenient to address this issue.

A limitation of the study is that the group for whom an LPT has been performed was quite small. The LPT could not be performed on blood samples of some sarcoidosis patients, due to insufficient lymphocyte counts. Lymphocytopenia is a common observation in sarcoidosis patients (29), not related to immunosuppressive medication (30). As a consequence, less LPTs were performed than anticipated. LPT subgroups proved to be too small to detect differences in clinical phenotype between the LPT-positive and LPT-negative sarcoidosis patients. Further studies are needed to indicate whether metal and silica immunoreactive patients have a distinct disease phenotype or particular genetic variants comparable to the Glu at position 69 in HLA-DPB1 in CBD. For beryllium, it has already been discovered that the Be^{2+} cation induces a conformational change in the HLA-DP2/peptide complex. Immunoreactivity develops when T lymphocytes regard the displaced peptide as foreign (non-self) and become activated(31). Possibly, other metals, including aluminum and zirconium, may induce similar conformational changes in the MHCII-peptide binding complex. Therefore, future studies are warranted as well to clarify these underlying immunological mechanisms leading to aluminum, silica and zirconium immunoreactivity.

Another study limitation may be the sensitivity of the assays performed. For beryllium LPTs, about 1% false positive and 30% false negative results are observed. The specificity of this assay is quite high (0.969) although the sensitivity is determined to be lower (0.686) (32). Data on sensitivity and specificity regarding aluminum, zirconium and silica LPTs are lacking but probably comparable. The MELISA[®] test is using a higher number of lymphocytes per test suggesting that the sensitivity could be slightly higher than the classical LPT (17). Due to the low sensitivity observed for the beryllium LPT, it is possible that the number of sarcoidosis patients with metal/silica immunoreactivity we observed is an underestimation, and the real number of patients with a positive LPT may actually be higher.

In conclusion, immunoreactivity to silica and metals found in sarcoidosis patients only, supports the hypothesis that these antigens may be involved in the disease pathogenesis of a distinct subgroup of sarcoidosis patients. Since job history with assigned exposure did not differ between sarcoidosis patients and controls, our data indicate that even very low incidence or occasional exposures could be relevant in sarcoidosis patients. Future studies may consider testing all sarcoidosis patients for immunoreactivity without screening them on exposure beforehand. Finally, the data presented in this paper suggests that besides beryllium, also zirconium, aluminum and silica can be considered as possible antigens in this heterogeneous disease. Future studies should however be initiated to clarify underlying immunological pathways and mechanisms in order to establish a more definitive role for these antigens in sarcoidosis.

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Chapter 5

Simultaneous testing of immunological sensitization to multiple antigens in sarcoidosis reveals an association with inorganic antigens specifically related to a fibrotic phenotype

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Abstract

Organic and inorganic antigens were studied simultaneously in the same cohort of sarcoidosis patients, to investigate whether correlations between clinical characteristics and immunological sensitization could reveal new phenotypes.

Sensitization to antigens of mycobacteria, *P. acnes* catalase and vimentin was investigated in 201 sarcoidosis and 51 obstructive sleep apnea patients, serving as control group. Sensitization to aluminum, beryllium, silica and zirconium was also studied in 105 of the sarcoidosis patients and in 24 of the controls.

A significantly higher percentage of sarcoidosis patients (27.6%) than controls (4.2%) had an immunological response to metals or silica ($P = 0.014$). A higher percentage of these sarcoidosis patients showed fibrosis on chest X-ray 5 years after the diagnosis (69.2% vs. 30.3%, $P = 0.016$). No significant differences in mycobacterial or vimentin elispot assay results were observed between sarcoidosis and control patients. A significantly lower percentage of sarcoidosis patients (3.5%) than control patients (15.7%) had a positive elispot for *P. acnes* catalase ($P = 0.003$). However, sarcoidosis patients sensitized to *P. acnes* catalase were more likely to have skin involvement, while sarcoidosis patients sensitized to mycobacterial antigens were more likely to have cardiac involvement.

Our study suggests a more prominent role for inorganic triggers in sarcoidosis pathogenesis than previously thought. Immunological sensitization to inorganic antigens was associated with development of fibrotic sarcoidosis. No association was found between sensitization to bacterial antigens or vimentin and sarcoidosis in Dutch patients. However, our data does suggest that trigger-related phenotypes can exist in the heterogeneous population of sarcoidosis patients.

Key words: Sarcoidosis, triggers, metals, silica, *P. acnes*

Introduction

Sarcoidosis is a systemic disease characterized by formation of noncaseating granulomas, mostly affecting the lungs, skin, eyes and lymph nodes (1). The course of the disease is unpredictable, meaning that the disease can spontaneously resolve, while a chronic disease course can also develop (2). Currently, no curative treatment exists for sarcoidosis patients. Patients with symptomatic organ involvement or at risk of permanent damage are treated with immunosuppressive drugs. This kind of treatment is associated with several side effects, and approximately 10% of patients still develop progressive disease while receiving immunosuppressives (2).

Considerable research effort has focused on identification of triggers of sarcoidosis, including antigens of mycobacteria (3,4), *Propionibacterium acnes* (*P. acnes*) (5,6), vimentin (7,8), metals and silica (9,10). Although all these antigens have shown potential, results demonstrate that sarcoidosis probably has multiple triggers. Importantly, none of the above organic and inorganic antigens have been studied simultaneously in one group of sarcoidosis patients. Identification of triggers in sarcoidosis will allow personalized treatment options, which may include anti-bacterial drugs. In addition, increased knowledge about sensitization to metals and silica can lead to identification of sarcoidosis patients for whom exposure prevention may be more beneficial than increasing immune suppression.

The aim of this study was to examine possible triggers of sarcoidosis in Dutch patients. Correlations between clinical characteristics and immunological response to microbial antigens or metals and silica may result in identification of new clinical phenotypes. Identification of these subgroups can lead the way to clinical trials in the future, to explore more accurate treatment options.

Materials and methods

Study subjects

Biopsy proven sarcoidosis patients or Löfgren syndrome patients seen for the first time at the ILD outpatient clinic of the St Antonius Hospital (Nieuwegein, The Netherlands) from 1 May 2016, were asked to participate, until at least 200 patients had been included. The diagnosis of sarcoidosis had been established according to the criteria of the American Thoracic Society/European Respiratory Society (11). Patients with obstructive sleep apnea (OSA) are also seen at the pulmonary outpatient clinic, and this disorder has not been associated with the antigens tested in this study (12). OSA patients were therefore included as a control group. The study was approved by the Medical research Ethics Committees United (MEC-U) of the St Antonius Hospital (R14.023), and written informed consent was obtained from all participants.

IFN- γ elispot

A blood sample was drawn from all participants. Peripheral blood mononuclear cells (PBMCs) were collected and stored at -150°C until time of analysis. IFN- γ elispot assays were performed following the manufacturer's instructions (Mabtech, Nacka Strand, Sweden). Briefly, 2×10^5 PBMCs were seeded per well, together with 33.33 $\mu\text{g}/\text{mL}$ ESAT-6 peptides, 33.33 $\mu\text{g}/\text{mL}$ MKatG peptides, 10 $\mu\text{g}/\text{mL}$ MKatG protein, 10 $\mu\text{g}/\text{mL}$ *P. acnes* catalase or 20 $\mu\text{g}/\text{mL}$ vimentin, in duplicate. Responses were calculated by subtracting the number of spot-forming-cells (SFCs) of the negative control wells from that of the antigen specific wells. Following Drake *et al.* (13), positive responses were defined as at least 50 SFC per 10^6 PBMCs, at least 3 times higher than the negative control well. Details of PBMC isolation, the antigens used and elispot are provided in the supplement.

Clinical tuberculosis IGRAs

Medical records of sarcoidosis patients were searched for data of interferon gamma release assays (IGRAs) (TB ELISpot or QuantiFERON tests). When data of those tests were not available, medical records were searched for results of tuberculin skin tests (TSTs).

Cytokine measurements

Supernatant of elispot plates was collected and stored at -80°C until time of analysis. Concentrations of TNF- α , IL-2, IL-17A and IL-1 β were measured using Human Magnetic Luminex Assays (Biotechne, Minneapolis, USA) following the manufacturer's instructions. Values beyond the range of the standard curve were given the lowest or highest value the standard curve reached. Baseline cytokine concentrations were subtracted from cytokine concentrations of the antigen stimulated-wells. Negative values were set to zero.

Lymphocyte proliferation tests to determine metal and silica sensitization

All sarcoidosis and control patients were asked to provide a second blood sample for lymphocyte proliferation tests (LPTs) on beryllium, aluminum, zirconium and silica. Blood was drawn in citrate tubes and sent within 24 hours to Prohealth Medical, Nederweert, The Netherlands, where the memory lymphocyte immunostimulation assay (MELISA[®]) LPT was performed, as described in the supplementary file. Following MELISA[®] guidelines, a stimulation index (SI) > 2.0 was considered a weakly positive test result and an SI > 3.0 a positive test result.

HLA-DRB1 genotyping

Presence of HLA-DRB1*0301 and HLA-DRB1*1501 alleles was determined for sarcoidosis patients of whom residual DNA was available. Tag single nucleotide polymorphisms (SNPs) rs2040410A and rs3135388A were used to capture HLA-DRB1*0301 and HLA-DRB1*1501 respectively, using the methods described by Karakaya *et al.* (14).

Identification of trigger-related phenotypes

Scadding stage at diagnosis and follow-up, organ involvement and the use of immunosuppressive medication were collected from medical records. In addition, the clinical outcome status (COS) (15) was determined 2 and 5 years after diagnosis and classified into two groups: group 1: resolved, minimal or persistent disease without treatment (COS 1-6) and group 2: persistent disease with need for treatment (COS 7-9). Clinical characteristics and COS were related to elispot and LPT results to explore trigger-related phenotypes.

Statistical analysis

Study data was collected and managed using Research Electronic Data Capture (REDCap) tools hosted at St. Antonius Hospital (16). Data was analyzed using IBM SPSS statistics version 24. An unpaired T-test was used to compare numerical data. Non-parametric tests were used for non-normally distributed data (Mann-Whitney U test). Categorical data were compared using the Chi-squared test. If expected cell frequencies were below 5, Fisher's exact test was used for categorical data up to two categories. P-values <0.05 were considered significant.

Results

Patient characteristics

A total of 205 sarcoidosis patients and 51 control patients were included in the study (Table 1). Control patients were older at inclusion and a higher proportion of control patients were ever smokers compared to the sarcoidosis patients.

Table 1. Demographics of sarcoidosis and control patients

	Sarcoidosis (n=205)	Controls (n=51)	P-value*
Sex (Male / Female) (%)	53.2 / 46.8	62.7 / 37.3	0.219
Age at diagnosis (Y)	43.74 ± 12.48	NA	
Age at inclusion (Y)	48.93 ± 12.21	55.45 ± 13.71	0.001
Ethnicity (W/NW) (%)	89.2 / 10.8	92.2 / 7.8	0.535
Smoking status (Never/ former or current) (%)	51.2 / 48.8	29.4 / 70.6	0.005
Scadding stage at inclusion (0/I/II/III/IV) (%)	19.6 / 29.4 / 28.4 / 5.9 / 16.7	NA	
Extra pulmonary involvement (%)	59.5	NA	

Age at diagnosis and age at inclusion are expressed as mean ± std. deviation.

W: White, NW: Non White; 0 = Normal chest radiograph; I = Bilateral hilar lymphadenopathy (BHL); II = BHL with pulmonary infiltrates; III = pulmonary infiltrates without BHL; IV = fibrosis.

* A P-value regarding sex, ethnicity and smoking status was calculated using Pearson Chi-square test; an independent samples T-test was used to calculate a P-value for differences regarding age at inclusion.

Elispot results

An elispot assay was performed on PBMCs of 201 sarcoidosis patients and 51 controls. Ninety-one sarcoidosis patients (45.3%) were using immunosuppressive medication at the time of blood collection. No differences in number of spots upon stimulation with anti-CD3 antibody were observed between sarcoidosis patients with and without immunosuppressive medication, while more controls had a low response (Supplementary Figures 1 and 2). Only 1 control and 2 sarcoidosis patients had a positive elispot for ESAT-6, and neither controls nor patients had a positive elispot for MKatG peptide, although one sarcoidosis patient had a positive elispot for MKatG protein. Eight controls (15.7%) compared to 7 sarcoidosis patients (3.5%) had a positive elispot for *P. acnes* catalase (P = 0.003). A vimentin-positive elispot was found for 9 controls (17.6%) and 18 sarcoidosis patients (9.0%)(Figure 1).

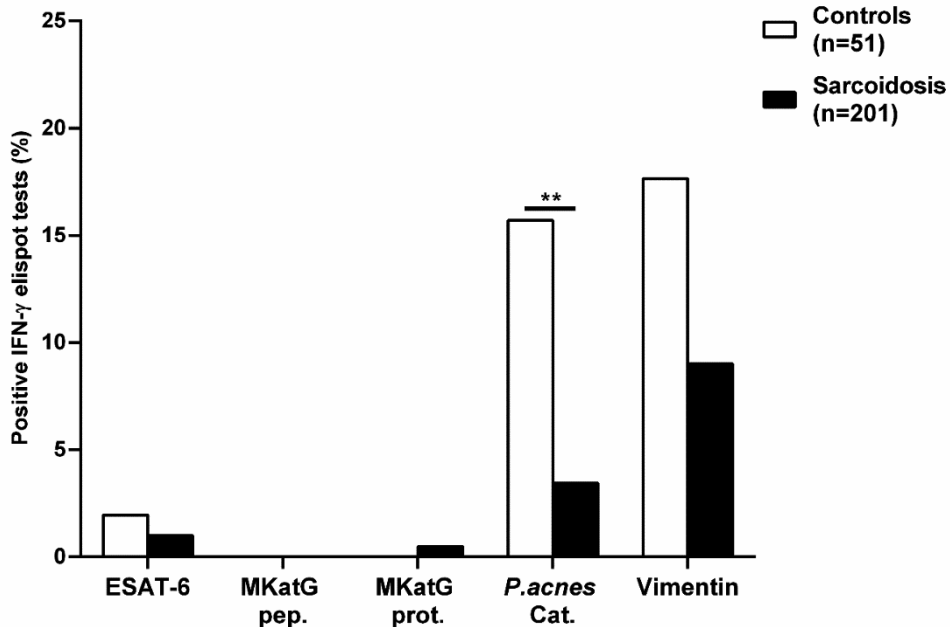


Figure 1. Percentage positive IFN- γ elispot tests of sarcoidosis and control patients.

A positive response was defined as at least 50 spot-forming cells per million cells after subtraction of the negative control, being at least three times higher than the negative control. A significantly higher percentage of the control patients ($P = 0.003$) had a positive elispot for *P. acnes* catalase.

ESAT-6 = peptides 14 and 15 of early secretory antigenic target produced by *Mycobacterium tuberculosis*; MKatG pep.= peptide 31 of *Mycobacterium tuberculosis* catalase-peroxidase; MKatG prot. = whole *Mycobacterium tuberculosis* catalase-peroxidase protein; *P. acnes* Cat. = *Propionibacterium acnes* catalase-peroxidase

To determine sensitization to mycobacterial antigens, results of our elispot and clinical data of diagnostic IGRAs or TSTs were combined. A total of five sarcoidosis patients had a positive test for mycobacterial antigens.

Antigen-specific cytokine measurements

Median baseline concentrations of IL-1 β , IL-17, IL-2 and TNF- α were significantly higher in sarcoidosis patients (Supplementary Table 1). Median concentrations of IL-1 β were significantly higher in sarcoidosis patients after PBMC stimulation with ESAT-6 (Supplementary Figure 3A, $P = 0.024$), *P. acnes* catalase (Supplementary Figure 3C, $P = 0.001$) and vimentin (Supplementary Figure 1D, $P = 0.012$).

Metal and silica LPT results

Of all patients, 24 controls and 105 sarcoidosis patients agreed to provide a second blood sample for an LPT. Fifty-nine sarcoidosis patients (56.2%) were using immunosuppressive medication at the time of LPT. No differences in the SI upon stimulation with pokeweed (used as positive control) were observed between sarcoidosis patients with and without immunosuppressive medication, or between sarcoidosis patients and controls (Supplementary Figure 4).

Five sarcoidosis and no control patients had a positive LPT. A significantly higher percentage of sarcoidosis patients (27.6 % compared to 4.17% controls, $P = 0.014$) had a weakly positive LPT (Figure 2 and Table 2). Four sarcoidosis patients had a weakly positive LPT for two antigens (Supplementary Table 2). The single weakly positive control patient had a weakly positive LPT for all the antigens tested. Occupations at time of diagnosis of the 29 sensitized sarcoidosis patients and 1 sensitized control patient are provided in Supplementary Table 5.

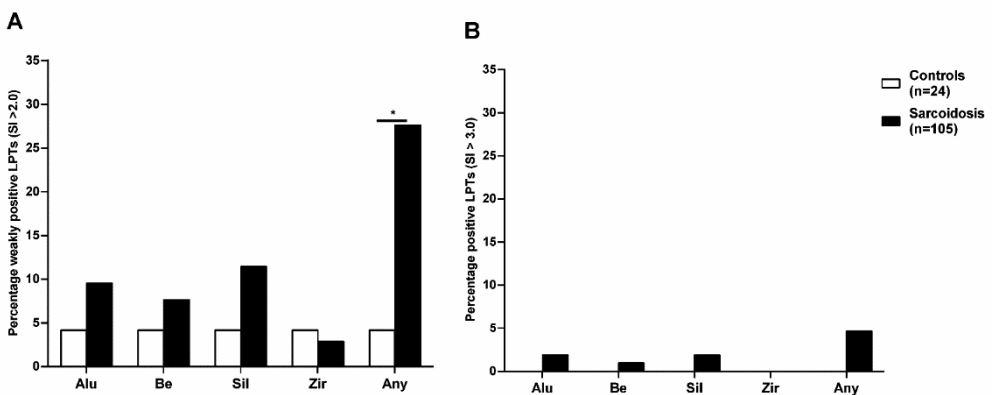


Figure 2. Percentage positive and weakly positive lymphocyte proliferation tests of sarcoidosis and control patients.

A: Percentages of sarcoidosis patients and controls with a weakly positive LPT. A stimulation index > 2.0 was defined as a weakly positive result. A significantly higher percentage of sarcoidosis patients had a weakly positive LPT ($P = 0.014$). Four sarcoidosis patients had a weakly positive LPT for two antigens. The only weakly positive control patient had a weakly positive LPT for all the antigens tested. B: Percentages of sarcoidosis patients and controls with positive LPT, which was defined as a stimulation index > 3.0

Alu: aluminum, Be: beryllium, Sil: silica, Zir: zirconium, LPT: lymphocyte proliferation test, SI: stimulation index.

Table 2. Percentage positive and weakly positive lymphocyte proliferation tests of sarcoidosis and control patients

	Controls n=24 (%)	Sarcoidosis n=105 (%)	P*
SI > 2.0			
Alu	1 (4.17)	10 (9.52)	0.688
Be	1 (4.17)	8 (7.62)	1.000
Sil	1 (4.17)	12 (11.43)	0.460
Zir	1 (4.17)	3 (2.86)	0.566
Any	1 (4.17)	29 (27.62)	0.014
SI > 3.0			
Alu	0	2 (1.90)	1.000
Be	0	1 (0.95)	1.000
Sil	0	2 (1.90)	1.000
Zir	0	0	NA
Any	0	5 (4.67)	0.583

A stimulation index > 2.0 was defined as a weakly positive result. A positive LPT was defined as a stimulation index > 3.0

Alu: aluminum, BE: beryllium, Sil: silica, Zir: zirconium, LPT: lymphocyte proliferation test, SI: stimulation index.

* A P-value of any positive LPT SI > 2.0 was calculated using Pearson Chi-square test; the other P-values were calculated using Fisher's exact test. The two silica positive patients were also included in a previous study (19).

Trigger-related phenotypes

Bacterial antigens

One sarcoidosis patient with a positive mycobacterial IGRA also had a positive elispot for vimentin (Supplementary Table 3), and two sarcoidosis patients with a positive LPT also had a positive vimentin elispot (Supplementary Table 4). No other overlap between elispot tests or between elispot and LPT was observed. A significantly higher proportion of sarcoidosis patients with a positive response to mycobacterial antigens had cardiac involvement (3/5) compared to sarcoidosis patients without sensitization to mycobacterial antigens (34/196, $P = 0.044$). Although not statistically significant, patients with a positive elispot for *P. acnes* catalase showed a trend towards more skin involvement (3/7, compared to 27/194 *P. acnes* catalase negative patients, $P = 0.069$) (table 3). Furthermore, *P. acnes* catalase positive patients were significantly younger at diagnosis than sarcoidosis patients without a positive *P. acnes* catalase elispot (median age 27.94 compared to 42.76, $P = 0.002$).

Table 3. Organ involvement and elispot

	Mycobacteria			<i>P. acnes</i> catalase			Vimentin		
	Neg. n=196	Pos. n= 5	P	Neg. n=194	Pos. n=7	P	Neg. n=183	Pos. n=18	P
Extra pulm.	116 (59.2)	3 (60)	1.00	113 (58.2)	6 (85.7)	0.244	108 (59)	11 (61.1)	0.863
Skin	30 (15.3)	0	1.00	27 (13.9)	3 (42.9)	0.069	27 (14.8)	3 (16.7)	0.736
Eyes	24 (12.2)	0	1.00	23 (11.9)	1 (14.3)	0.595	21 (11.5)	3 (16.7)	0.457
Heart	34 (17.3)	3 (60)	0.044	36 (18.6)	1 (14.3)	1.00	33 (18)	4 (22.2)	0.749

Data is shown in absolute numbers with percentages in brackets. P-values were calculated using the Chi-squared test. If expected cell frequencies were below 5, Fisher's exact test was used.

Vimentin

A higher percentage of Löfgren's syndrome patients were positive for HLA-DRB1*0301 than non-Löfgren patients (50% versus 16.5%, $P = 0.006$). None of the vimentin-positive sarcoidosis patients were diagnosed with Löfgren's syndrome. No difference in presence of HLA-DRB1*0301 was observed between vimentin positive and vimentin negative patients (11.8% and 19.7%, respectively, $P = 0.75$). A significantly higher percentage of vimentin-positive patients were positive for HLA-DRB1*1501, than vimentin-negative patients (47.1% vs. 23.1%, $P = 0.041$) (Figure 3).

Inorganic antigens

No relation was observed between organ involvement and sensitization to metals or silica (data not shown), although a higher percentage of the LPT-positive sarcoidosis patients showed fibrosis on chest X-ray 5 years after the diagnosis, than LPT-negative sarcoidosis patients (69.2% vs. 30.3%, $P = 0.016$). This difference was not observed 2 years after the diagnosis of sarcoidosis (Table 4). No relations were observed between HLA types and LPT-positive sarcoidosis patients (Supplementary Figure 5).

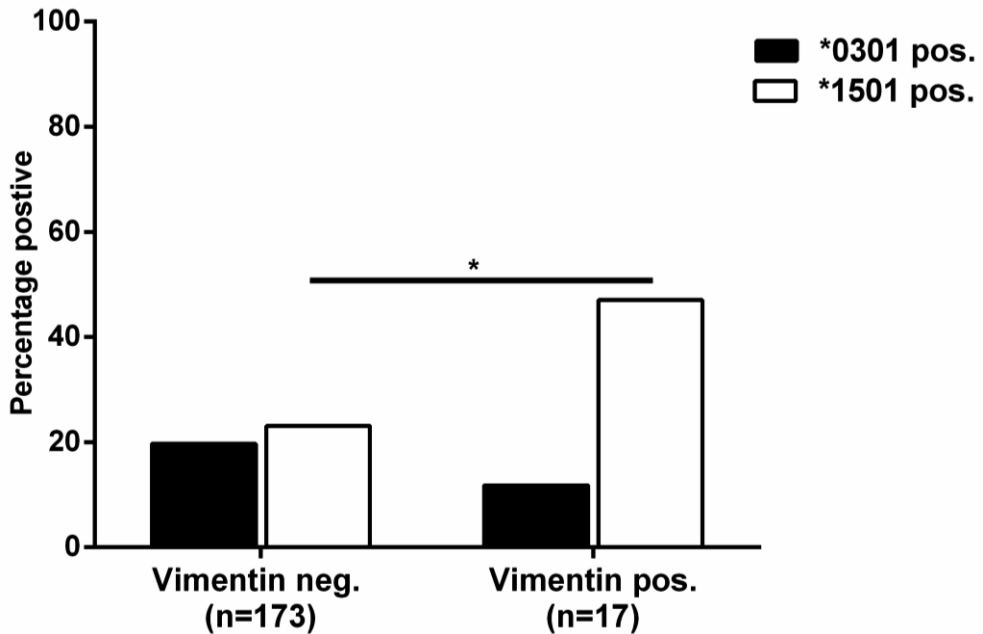


Figure 3. Percentage of vimentin positive patients positive for HLA-DRB1*0301 and HLA-DRB1*1501.

Discussion

To the best of our knowledge, this is the first study that simultaneously investigated organic and inorganic antigens in the pathogenesis of sarcoidosis within one cohort of patients. We found immunological sensitization, defined by an LPT SI above 3.0, to inorganic antigens in 5% of sarcoidosis patients. In addition to beryllium, we identified sarcoidosis patients sensitized to aluminum and silica. Even though only observed in a small percentage of patients, these findings imply clinical significance. It makes sense, in our opinion, to carefully re-assess work or home exposure in this subgroup of patients. In patients with chronic beryllium disease (CBD), improved lung function and radiographic findings can be observed when exposure is terminated or decreased (17,18). It seems plausible to assume that this will be the case for other inorganic triggers as well. In a previous paper we showed that occupational exposure to metal / silica determined by a job exposure matrix (JEM) is not predictive for a positive LPT (19). A possible explanation may be that the JEMs are designed to be very specific and will not capture individuals experiencing coincidental high risk exposure while carrying out low-risk jobs. However even very low occasional exposures can be relevant, as bystander beryllium exposure can lead to CBD (20,21).

Table 4. Fibrosis, COS and LPT results

2 years after diagnosis	Silica		Metals		Silica and/or metals		P
	Neg. n=81	Pos. n=10	Neg. n=73	Pos. n=18	Neg. n=65	Pos. n=26	
Fibrosis on chest X-ray ^a	12 (14.8)	1 (10)	9 (12.3)	4 (22.2)	8 (12.3)	5 (19.2)	0.51
Persistent disease with medication ^b	47 (52.8)	3 (27.3)	41 (50.6)	9 (47.4)	38 (52.8)	12 (42.9)	0.37
5 years after diagnosis	Silica		Metals		Silica and/or metals		P
	Neg. n=40	Pos. n=6	Neg. n=38	Pos. n=8	Neg. n=33	Pos. n=13	
Fibrosis on chest X-ray ^c	15 (37.5)	4 (66.7)	14 (36.8)	5 (62.5)	10 (30.3)	9 (69.2)	0.016
Persistent disease with medication ^d	25 (62.5)	4 (66.7)	26 (66.7)	3 (42.9)	22 (64.7)	7 (58.3)	0.74

Data is shown in absolute numbers with percentages in brackets. P-values were calculated using the Chi-squared test. If expected cell frequencies were below 5, Fisher's exact test was used. ^a Chest X-rays 2 years after diagnosis were not available from 14 out of 105 patients (11 of the 76 patients without any positive LPT test, 2 of the 12 silica-positive patients, 1 of the 19 metal-positive patients, and 3 of the 29 LPT-positive patients). ^b COS 2 years after diagnosis could not be determined for 5 out of 105 patients (4 of the 76 patients without any positive LPT, 1 of the 12 silica-positive patients and 1 of the 29 LPT-positive patients). ^c Chest X-rays 5 years after diagnosis were not available from 59 out of 105 patients (43 of the 76 patients without any positive LPT test, 6 of the 12 silica-positive patients, 11 of the 19 metal-positive patients, and 16 of the 29 LPT-positive patients). ^d COS 5 years after diagnosis could not be determined for 59 out of 105 patients (42 of the 76 patients without any positive LPT, 6 of the 12 silica-positive patients, 12 of the 19 metal-positive patients and 17 of the 29 LPT-positive patients).

Perhaps a simple occupational history is not detailed enough, and additional (job-specific) questionnaires will be required to identify occasional/recreational exposures that can be sufficient to initiate sarcoidosis in genetic predisposed individuals. Considering above mentioned explanations, in the current study, we decided to test all sarcoidosis patients for metal and silica sensitivity, without determining exposure beforehand.

Regarding the LPT, an SI between 2.0 and 3.0 is considered weakly positive, making interpretation difficult. A weakly positive test does not prove, but rather suggest sensitization. However, when comparing LPT results using an SI > 2.0, we found a remarkable difference between patients and controls, with 27.6% of sarcoidosis patients showing an immunological response compared to only 4.2% of controls. Clinical relevance is further supported by our finding that sensitization for inorganic triggers, suggested by an SI > 2.0, was associated with development of fibrotic sarcoidosis 5 years after diagnosis. An unresolved issue is why some sarcoidosis patients experience spontaneous remission while others develop chronic disease. It has been proposed that difference in antigen clearance could play a role (22). Patients in whom the antigen cannot be cleared are likely to develop chronic disease. Our results of a higher prevalence of fibrotic disease among the subgroup of sarcoidosis patients sensitized to inorganic antigens, fit this hypothesis very well.

Interpretation of our findings regarding *P. acnes* are quite challenging as well. *P. acnes* is a commensal of the human skin but can also be detected in lymph nodes or lung tissue of sarcoidosis as well as non-sarcoidosis patients (23). Unlike T-cells specific for self-antigens, commensal-specific T-cells do not undergo negative selection in the thymus and can be found in healthy individuals (24). Indeed, in our study, a *P. acnes* catalase specific T-cell response was present in approximately 15% of controls. However, the percentage of sarcoidosis patients with such a T-cell response was significantly lower. Our observations are not in line with previous publications which described an increased response to *P. acnes* in Japanese patients with sarcoidosis (25,26). Differences in ethnicity and genetic susceptibility might explain why an increased *P. acnes* immune response is observed in Japanese but not in Dutch sarcoidosis patients. It seems too early, however, to exclude a role for *P. acnes* in the pathogenesis of sarcoidosis in Dutch patients. First, the lower percentage of *P. acnes* T-cell responses in our sarcoidosis patients could reflect homing of these cells to the lungs. In inflammatory bowel disease it has been demonstrated that circulating microbiota-reactive CD4-positive T-cell frequencies are decreased, which might indeed reflect their selective recruitment to the inflamed gut (27). One may therefore argue that peripheral blood is not the right compartment to study *P. acnes* sensitization in order to clarify its role in sarcoidosis pathogenesis. Accordingly, in our opinion, future sarcoidosis research should focus either on testing sensitization for *P. acnes* in the alveolar compartment or on detection of *P. acnes* in biopsy material. Second, our results

demonstrate that sarcoidosis patients sensitized to *P. acnes* catalase are significantly younger at the time of diagnosis and are more likely to have skin involvement. While in theory it seems reasonable to relate *P. acnes* to cutaneous sarcoidosis, more research is needed to confirm this possible association. Finally, it could be that *P. acnes* is a mitogen rather than a specific trigger in sarcoidosis, explaining the relative absence of a specific T-cell response. It has been reported that *P. acnes* has a mitogenic effect on T-lymphocytes, and consequently promotes an inflammatory response, activates specific T-cell subsets and stimulates production of cytokines (28). This way, *P. acnes* may rather serve as an adjuvant, by providing an inflammatory environment during presentation of other antigens to antigen-specific immune cells (29).

Our results suggest that mycobacterial antigens do not play a significant role in sarcoidosis pathogenesis of Dutch patients, which is in line with the fact that the Netherlands is a country with a low tuberculosis incidence: about 5 per 100 000 (30). Other studies investigating the role of mycobacterial antigens in sarcoidosis have shown conflicting results. In cohorts of Danish and Japanese sarcoidosis patients no or a very low percentage of latent tuberculosis infection (LTBI) was found (0 and 3%, respectively (31,32)). However, in a study using US and Swedish patients with sarcoidosis, 50% of patients demonstrated T-cell reactivity against MKatG (33). The contradicting results regarding MKatG between this and our study may be explained by a different definition of a positive elispot result. Whereas we defined it as 50 SFCs per 10^6 PBMCs, being at least 3 times higher than background, Chen *et al.* used the 95% confidence interval of the control group as a cut-off, making comparison of results difficult. In our search for trigger-related phenotypes, a remarkable observation was that sarcoidosis patients sensitized to mycobacterial antigens were more likely to have cardiac involvement. Since only 5 sarcoidosis patients were sensitized for mycobacterial antigens, this association requires further investigation as cardiac involvement is a major cause of death in sarcoidosis patients.

We did not observe a significant difference in vimentin-positive elispots between sarcoidosis and control patients, which is in line with an Australian study that found no difference in IFN- γ producing cells after stimulation with pooled peptides of vimentin and lysyl tRNA synthetase (34). Based on a previous study reporting a higher number of IFN- γ producing cells after vimentin stimulation in DRB1*0301 positive sarcoidosis patients, (35) we also performed HLA-DRB1 genotyping. In contrast to DRB1*0301, we observed a significantly higher percentage of DRB1*1501 carriers among vimentin positive patients. This can be explained by findings of Wahlström *et al.*, who identified by peptide binding predictions, that apart from DRB1*0301, other HLA molecules are also possible binders for vimentin, including DRB1*1501 (35). Where we used the whole protein in elispot assays, Wahlström *et al.* used a vimentin peptide. The observed association with DRB1*1501

instead of DRB1*0301 might be caused by difference in vimentin epitopes. Unfortunately, no residual DNA was available from controls, so we were not able to investigate whether sensitization to vimentin in the control group was related to DRB1*1501 as well, or whether this was sarcoidosis-specific.

To study other cytokines as well, concentrations of TNF- α , IL-2, IL-17A and IL-1 β were measured in supernatant of elispot plates. Higher median IL-1 β concentrations were found in sarcoidosis patients. However, since higher concentrations were observed after stimulation with vimentin, mycobacterial and *P. acnes* antigens, this seems to be an unspecific inflammatory reaction rather than a specific T-cell response.

There are some limitations to our study. Presence of fibrosis was determined by chest X-ray. Only moderate agreement on Scadding stages is reported in literature (36). Furthermore, we are aware that HRCT is preferable to chest X-ray to determine fibrosis. However, follow-up data of HRCT was available only for a proportion of patients, which would have decreased the cohort size and so the power of the analysis. A second limitation was that only half of all patients agreed to provide a second blood sample required for an LPT. Hence, overlap between sensitization to organic and inorganic antigens could not be analyzed for the whole cohort. However, since only a few of the 105 patients demonstrated more than 1 potential trigger, it is unlikely that this would have been the case in the patients not analyzed for inorganic agents.

In conclusion, our study suggests a more prominent role for inorganic triggers such as metals and silica in sarcoidosis pathogenesis than previously thought. Immunological sensitization to inorganic antigens seems related to development of fibrotic sarcoidosis. Future studies including a higher number of patients and controls are warranted to clarify the meaning of a weak positive LPT in sarcoidosis and to definitely confirm the role of metal and silica sensitization in its pathogenesis. We did not find an association between sarcoidosis and an immunological response to bacterial antigens or vimentin. However, our data on LTBI and presence of cardiac sarcoidosis, and the possible relation between sensitization to *P. acnes* and cutaneous involvement, further suggest that trigger-related phenotypes can exist in the heterogeneous population of sarcoidosis patients.

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Supplement

Methods

PBMC isolation

A blood sample (4 x 9 mL) was drawn from all participants. Peripheral blood mononuclear cells (PBMCs) were collected by the Ficoll-Hypaque density gradient centrifugation method (GE Healthcare) within 24 hours. PBMCs were frozen in RPMI 1640 (Gibco) supplemented with glutamax (Thermo Fisher), 20% heat-inactivated fetal bovine serum (Gibco) and 20% dimethylsulfoxide (DMSO) and stored at -150° C until time of analysis. Full details can be found in the online supplement.

Antigens used for PBMC stimulation

Based on previous findings (1–4), the following antigens and bacteria were used in the enzyme-linked immunospot (elispot) assay:

Mycobacterium tuberculosis

Mycobacterial 6 kDa early secretory antigenic target (ESAT-6) peptide 14 and 15, sequences NNALQNLARTISEAG and NLARTISEAGQAMAS respectively (purity 90%), Mycobacterial catalase-peroxidase antigen (MKatG) peptide 31, peptide sequence WTNTPTKWDNSFLEI and the whole MKatG protein (purity 90%). Peptides and protein were produced at Genscript, Piscataway, USA.

Propionibacterium acnes

Propionibacterium acnes (*P.acnes*) catalase antigen was kindly provided by the research group of Y. Eishi from the department of Human Pathology, Tokyo Medical and Dental University Graduate school, Tokyo, Japan.

Vimentin

Human recombinant vimentin (GF181) was bought from Merck Chemicals B.V., Amsterdam, The Netherlands.

IFN- γ elispot

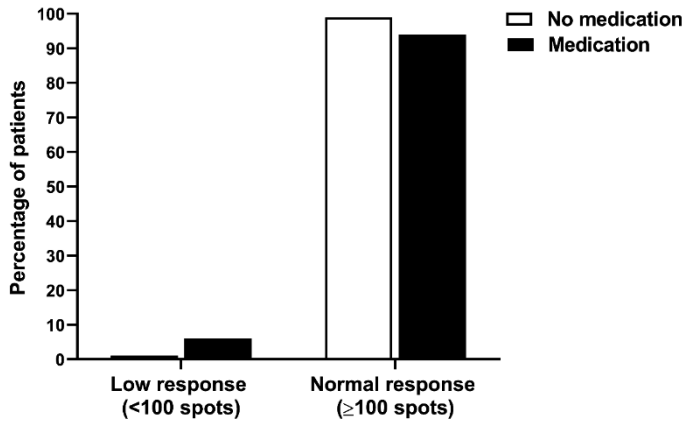
A blood sample (4 x 9 mL) was drawn from all participants. Peripheral blood mononuclear cells (PBMCs) were collected and stored at -150° C until time of analysis. IFN- γ elispot assays were performed following the manufactures' instructions (Mabtech, Nacka Strand, Sweden). Briefly, 2*10⁵ PBMCs were seeded per well, together with either 33.33 μ g/mL

ESAT-6 peptides, 33.33 µg/mL MKatG peptides, 10 µg/mL MKatG protein, 10 µg/mL *P.acnes* catalase or 20 µg/mL vimentin, in duplicate. As a positive control, PBMCs were seeded together with anti-CD3 mAb (1:50.000 diluted). PBMCs without stimulation served as a negative control. The plates were incubated for 18-22 hours (ESAT-6, MKatG, *P.acnes* catalase) or ~40 hours (vimentin) at 37 ° C in 5% CO₂. Hereafter, spots were developed following the manufactures protocol. Spots were counted using the AID EliSpot Reader System (Advanced Imaging Devices GmbH, Strassberg, Germany). Responses were calculated by subtracting the spot-forming cells (sfc) of the negative control wells from the antigen specific wells. Following methods of Drake et al. (1), positive responses were defined as at least 50 sfc per 10⁶ PBMCs, being at least 3 times higher than the negative control well.

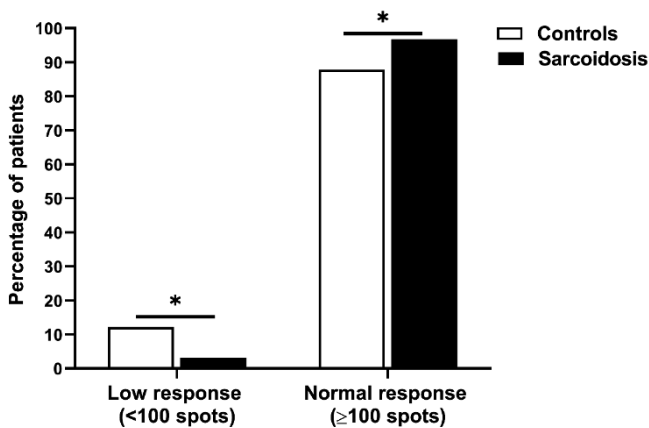
MELISA®

Blood was drawn in citrate tubes and sent within 24 hours to Prohealth Medical, Nederweert, The Netherlands, where the memory lymphocyte immunostimulation assay (MELISA®) LPT was performed. MELISA® is a clinically validated blood test that detects type-IV allergy (sensitization) to multiple metals (5,6). Furthermore, it can also be used to determine sensitization for silica (7). Lymphocytes were isolated from citrated blood, and 1*10⁶ cells were incubated for 5 days in HEPES-buffered media containing at least two consecutive dilutions of an antigen: aluminium nitrate, beryllium sulphate, silicon dioxide or zirconium dioxide. After cultivating for 5 days, proliferation was measured by incorporation of H³-labelled thymidine. Radioactivity was measured with a scintillation counter (Microbeta, Perkin Elmer). Stimulation indexes (SI) were calculated by dividing the antigen specific counts per minute by the mean counts per minute of the basal controls. Regarding the MELISA® guidelines, a stimulation index (SI) > 2.0 was considered a weakly positive test result and a SI > 3.0 a positive test result.

Results



Supplementary Figure 1. Elispot response after stimulation with the positive control (anti-CD3 mAb), compared between sarcoidosis patients with and without immunosuppressive medication at time of PBMC isolation for the elispot assay. No significant differences were observed between patients with and without

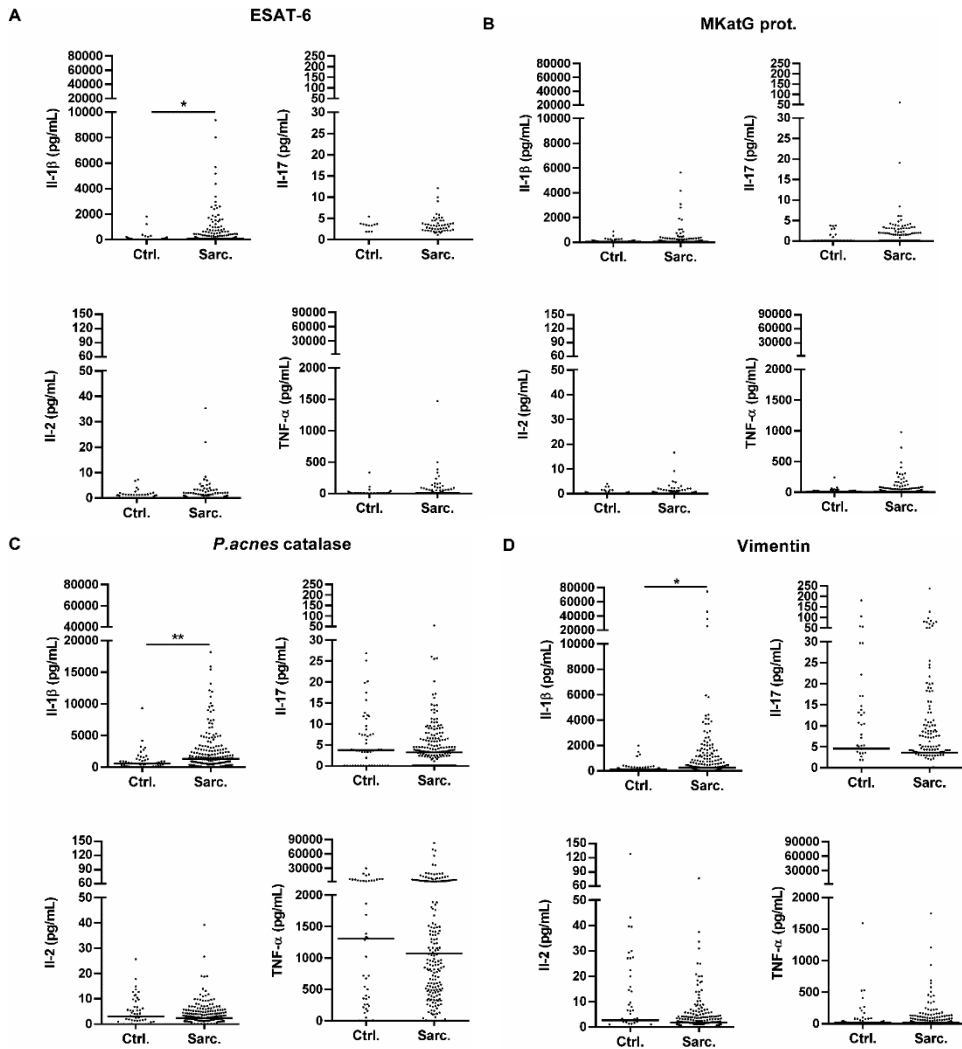


Supplementary Figure 2. Elispot response after stimulation with the positive control (anti-CD3 mAb), compared between sarcoidosis patients and controls. A significant higher percentage of controls had a low response (<100 spots) after stimulation with anti-CD3 mAb used as a positive control ($P = 0.020$).

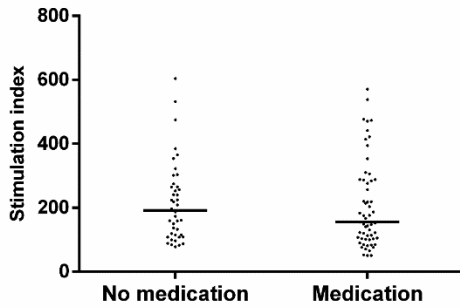
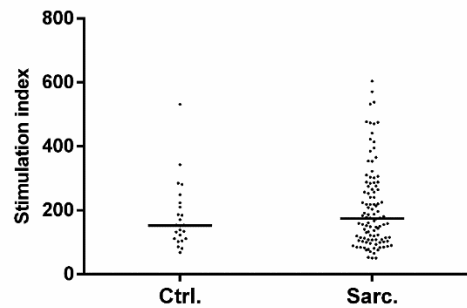
Supplementary Table 1. Median baseline cytokine levels

Cytokine baseline	Controls	Sarcoidosis	P*
Il-1β (pg/mL)	994.24 (IQR 1548.13)	2365.65 (IQR 3080.18)	<0.001
Il-17 (pg/mL)	3.55 (IQR 5.00)	6.16 (IQR 6.76)	<0.001
Il-2 (pg/ mL)	3.10 (IQR 4.15)	6.95 (IQR 5.46)	<0.001
TNF-α (Pg/mL)	133.80 (IQR 215.07)	322.60 (IQR 438.49)	<0.001

P values were calculated using a Mann-Whitney U test. IQR = interquartile range



Supplementary Figure 3. Median cytokine concentration in cell supernatant after subtraction of baseline cytokine levels. A: IL-1 β , IL-17, IL-2 and TNF- α concentrations in supernatant after PBMC stimulation with ESAT-6. B: IL-1 β , IL-17, IL-2 and TNF- α concentrations in supernatant after PBMC stimulation with the whole MKatG protein. C: IL-1 β , IL-17, IL-2 and TNF- α concentrations in supernatant after PBMC stimulation with *P.acnes* catalase. D: IL-1 β , IL-17, IL-2 and TNF- α concentrations in supernatant after PBMC stimulation with vimentin

A**B**

Supplementary Figure 4. Median stimulation index after lymphocyte stimulation with pokeweed, used as a positive control in de LPT

A. No difference was observed in median stimulation index between sarcoidosis patient with and without immunosuppressive medication at time of LPT (191.80 vs 155.40, $p = 0.344$).

B. No difference was observed in median stimulation index between sarcoidosis patients and controls (152.45 vs 173.40, $p = 0.492$).

Supplementary Table 2. Overlap in weakly positive LPT results of sarcoidosis patients

	Aluminum (n=10)	Beryllium (n=8)	Silica (n=12)	Zirconium (n=3)
Aluminum	10	1	1	1
Beryllium	1	8	1	0
Silica	1	1	12	0
Zirconium	1	0	0	3

Supplementary Table 3. Overlap in positive elispot results of sarcoidosis patients

	Myco. (n=5)	P.ac. cat. (n=7)	Vim (n=18)
Myco	5	0	1
P.ac KAT	0	7	0
Vim	1	0	18

Data is shown as absolute numbers. Myco = positive elispot or IGRA for mycobacterial antigens, P.ac. cat. = positive elispot for *P.acnes* catalase, Vim = positive elispot for vimentin.

Supplementary Table 4. Overlap in positive elispots and LPTs of sarcoidosis patients

	Myco. (n=2)	P.ac cat. (n=4)	Vim (n=6)
Sil (n=12)	0	0	2
Metal (n=17)	0	0	1
LPT (n=27)	0	0	2

Data is shown as absolute numbers. Since a LPT was only performed on 105 of the 205 sarcoidosis patients, not all elispot positive patient had a LPT performed. Myco = positive elispot or IGRA for mycobacterial antigens, P.ac. cat. = positive elispot for *P.acnes* catalase, Vim = positive elispot for vimentin. Sil = weakly positive LPT for silica, Metal = weakly positive LPT for aluminum, beryllium or zirconium LPT = a weakly positive lymphocyte proliferation test for any of the antigens tested (aluminum, beryllium zirconium or silica).

Supplementary Table 5. Occupations of the LPT positive and weakly positive sarcoidosis patients and control patient

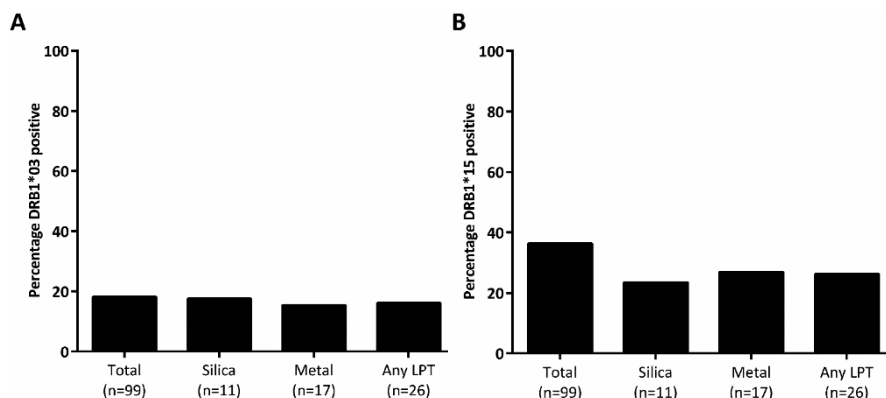
Patient	LPT weak positive (SI >2.0) for	LPT positive (SI >3.0) for	Occupation	Exposure to metal/silica* based on current occupation	Exposure to metal/silica based on all occupations
Sarc 15	Sil		Civil Engineer		
Sarc 18	Be	Be	Lorry and Van Driver (Long-Distance Transport)		Metal
Sarc 43	Be		Business Services Salesman		
Sarc 63	Sil		Office clerk		
Sarc 73	Be, Sil		Government Executive Official		
Sarc 74	Be		Cleaner		
Sarc 75	Zir		Office clerk		
Sarc 79	Zir		Social Worker		
Sarc 85	Alu		Tailor, Made-to-Measure Garments		
Sarc 100	Sil		Other Managers		
Sarc 104	Alu		Office clerk		
Sarc 113	Sil		Machinery Fitter-Assembler	Metal	Metal
Sarc 124	Sil	Sil	Plasterer, General.	Silica	Silica
Sarc 146	Alu		Office clerk		
Sarc 159	Sil		Roof Thatcher.		
Sarc 166	Sil		House builder	Silica	Silica
Sarc 167	Be		Supervisor and General Foreman (Construction Work)		
Sarc 170	Alu	Alu	Office clerk		
Sarc 171	Alu, Be		Office clerk		

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Sarc 177	Sil	Sil	Sheet-Metal Worker	Metal	Metal
Sarc 178	Be		Office clerk		
Sarc 186	Be		Other Primary Education Teachers		
Sarc 189	Sil		Excavating-Machine Operator	Silica	Silica
Sarc 193	Alu		Insurance Salesman		
Sarc 194	Sil		House builder	Silica	Silica
Sarc 199	Alu, Sil		Other Clerical Supervisors		
Sarc 207	Alu		General Manager		
Sarc 209	Alu, Zir		Social Worker		
Sarc 212	Alu	Alu	Retail Trade Salesman		
CTRL 38	Alu, Be, Zir, Sil		Concierge (Apartment House)		Silica, Metal

If patients had no occupation at time of their diagnosis, the most recent performed occupation before the diagnosis is shown. In the last column of the table metal and silica exposure based on all occupations performed before the diagnosis is shown instead of only exposure based on the current occupation.

* Exposure was determined by job exposure matrices (DOM-JEM and ALOHA JEM) coupled to jobs encode by ISCO-



Supplementary Figure 5. Percentage of sarcoidosis patients positive for HLA DRB1*03 (A) and *15 (B).

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Chapter 6

Latent tuberculosis infection associates with cardiac involvement in patients with sarcoidosis

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Sarcoidosis, Vasc Diffus Lung Dis. 2020;37(3).

Abstract

Sarcoidosis is a systemic disease characterized by formation of non-caseating granulomas. About 5% of patients have symptoms of cardiac sarcoidosis. Identification of cardiac involvement is important since it is a major cause of death. Mycobacterial antigens have been linked to sarcoidosis pathogenesis. Previous findings suggest that a latent tuberculosis infection (LTBI) might associate with development of cardiac involvement in patients with sarcoidosis. The aim of the present study was to further evaluate these findings in another cohort of cardiac sarcoidosis patients.

Interferon release assays (IGRAs) or tuberculin skin tests (TST) were analyzed in a cohort of cardiac sarcoidosis patients (n=103) and compared to non-cardiac sarcoidosis patients (n=153).

In the cohort of patients with cardiac sarcoidosis, 7 could be diagnoses with an LTBI (6.8%) compared to only one of the non-cardiac patients (0.7%), $p = 0.008$.

To conclude, we were able to show an association between an LTBI and cardiac involvement in patients with sarcoidosis. Future research is however required to unravel the mechanism involved in this association.

Keywords: Sarcoidosis, Cardiac sarcoidosis, Mycobacteria, Tuberculosis

Introduction

Sarcoidosis is a systemic disease characterized by the formation of noncaseating granulomas. Although intrathoracic lymph nodes and the lungs are the most common affected organs, any organ can be involved in this disease (1). About 5% of sarcoidosis patients have symptoms of cardiac sarcoidosis (CS), although the prevalence of CS is 20-30% in autopsy and imaging studies (2). Identification of cardiac involvement is important since it is a major cause of death in sarcoidosis patients (3,4).

The etiology of sarcoidosis is not clear do date. However, several antigens have been related to sarcoidosis, including inorganic agents (5,6), auto-antigens (7) and bacteria (8). The two most extensive studied bacteria in relation to sarcoidosis pathogenesis are *Propionibacterium acnes* (*P. acnes*) (9) and Mycobacteria (10). Studies regarding *P. acnes* and mycobacteria in relation to sarcoidosis mainly focused on cellular immune responses to antigens of *P. acnes* and mycobacteria (11,12) as well as detecting DNA or antigens in biopsy material of sarcoidosis patients and controls (13,14). Trigger related phenotypes, however, have not been described previously. In recent work from our group (15) sarcoidosis patients were tested for an immunological response towards several antigens related to sarcoidosis pathogenesis. In a total cohort of 201 patients with sarcoidosis, a latent tuberculosis infection (LTBI) was found in 5 patients (2.5%). Interestingly, when defining trigger-related phenotypes it was found that three of these LTBI patients had cardiac involvement of their sarcoidosis. The aim of the present study was to further evaluate these unexpected results. We used another cohort of CS patients to investigate whether an LTBI associates with cardiac involvement in sarcoidosis.

Methods

Study subjects

A cohort of CS patients registered in our hospital was retrospectively studied and included as CS group (approved by the local institutional review board (Z.19.004) of the St Antonius Hospital. Due to the use of clinical data, the need for informed consent was waived). A diagnosis of CS was made after advanced imaging with cardiac resonance imaging (CMR) and fluorodeoxyglucose positron emission tomography (FDG-PET). The likelihood of CS was assessed in a multidisciplinary team consisting of a pulmonologist, cardiologist, radiologist, nuclear specialist and nurse, predominantly based on the diagnostic criteria from the Heart Rhythm Society (HRS) consensus statement (16). Definite and probable CS were the gold standard for the diagnosis of CS.

Biopsy proven sarcoidosis patients or Löfgren syndrome patients without cardiac involvement, seen for the first time at the ILD outpatient clinic of the St Antonius hospital (Nieuwegein, The Netherlands) from May 1st 2016 till December 2017, were also

retrospectively studied and included in the non-cardiac sarcoidosis (non-CS) group (approved by the Medical research Ethics Committees United (MEC-U) (R14.023), all these patients signed informed consent). The diagnosis of sarcoidosis had been established according to the criteria of the American Thoracic Society/European Respiratory Society (17).

Diagnosis of LTBI

Following the diagnostic criteria for sarcoidosis to exclude other causes of granulomatous disease, an Interferon Gamma Release Assay (IGRA) or tuberculin skin test (TST) is performed in all sarcoidosis patients in the St. Antonius Hospital. Medical records of patients were searched for data of IGRAs (TB ELISpot or QuantiFERON tests). When data of those tests were not available, medical records were searched for results of tuberculin skin tests (TST). If IGRAs or TSTs were not performed in our hospital, available data of referring hospitals were retrieved. If data of IGRAs or TSTs were not available, patients were excluded from the study. Results of IGRAs or TSTs were compared between the CS group and the non-CS group. A diagnosis of LTBI was made when a cellular immune response against antigens of *Mycobacterium tuberculosis* (MTB) was present without bacterial, radiological or clinical signs of an active tuberculosis infection according to the current guidelines (18,19).

Statistics

Data were analyzed using IBM SPSS statistics version 24. An unpaired T-test was used to compare numerical data between the non-CS and CS group. Non-parametric tests were used for non-normally distributed data (Mann-Whitney U test). Categorical data were compared using the Chi-squared test. If expected cell frequencies were below 5, Fisher's exact test was used for categorical data up to two categories. Odds ratios were calculated using a binary logistic regression model.

Results

Retrospective data of IGRAs or TSTs was available from 153 non-CS patients and 103 CS patients, which were included in the study (Table 1). Establishment of the diagnosis of cardiac sarcoidosis is shown in Supplementary Table1. The CS group was significantly older at time of the IGRA or TST and consisted of more men. No difference in ethnicity was observed between the two study groups and also the percentage of patients that originated from another country than the Netherlands did not significantly differ between the non-CS and CS group either (3.9% vs 7.8%, $P = 0.189$, Supplementary Table 2).

Table 1. Demographics of study patients

	Non-CS (n=153)	CS (n=103)	P *
IGRA / TST	149 / 4	96 / 7	0.124
Age (mean ± SD)	47.23±12.64	51.39±10.87	0.005
Sex (male / female)	80 / 73	77 / 26	<0.001
Ethnicity (white / non-white)	135 / 18	92 / 11	0.788
Organ involvement:			
Lymph nodes (%)	146 (96.1)	99 (97.1)	0.744
Lungs (%)	114 (74.5)	78 (75.7)	0.825
Nervous system (%)	28 (18.3)	8 (7.8)	0.017
Skin (%)	28 (18.3)	4 (3.9)	0.001
Eyes (%)	23 (15.0)	8 (7.8)	0.081
Bone (%)	8 (5.2)	8 (7.8)	0.411
Liver (%)	7 (4.6)	7 (6.8)	0.443
Spleen (%)	5 (3.3)	13 (12.6)	0.004

Age is age at time of IGRA or TST. * P-values for sex, ethnicity, lung, eye, skin, nervous system, bone, liver and spleen involvement were calculated using Pearson Chi-Square. Statistical differences for IGRA/TST and lymph node involvement were accessed using Fisher's Exact Test. A P-value for age was calculated using an unpaired T-test.

IGRA: Interferon gamma release assay, TST: tuberculin skin test, CS: Cardiac sarcoidosis

In total, 7 CS patients were diagnosed with LTBI (6.8%) compared to only one patient of the non-CS group (0.7%), $P = 0.008$ (Figure 1). When looking at the two different assays, in the CS group 6 of 96 patients had a positive IGRA (6.3%) compared to 1 of 149 patients of the non-CS group (0.7%) ($P = 0.016$). A positive TST was found in 1 of 7 CS patients (14.3%) and in none of the 4 non-CS patients ($P = 1.000$). An increased OR of 11.08 (CI: 1.34; 91.49) was observed for CS and an LTBI and an increased odds ratio (OR) of 9.87 (CI: 1.17; 83.29) was observed for a positive IGRA and CS. Furthermore, when these ORs were adjusted for age, also significant increased ORs of respectively 10.17 (CI: 1.23; 84.50) and 9.06 (CI: 1.06; 77.13) were observed.

Besides cardiac involvement, we also compared other involved organs between the 8 sarcoidosis patients with an LTBI and the other sarcoidosis patients. Prevalence of other involved organs was not different between the patients with an LTBI and the remaining patients (Table 2).

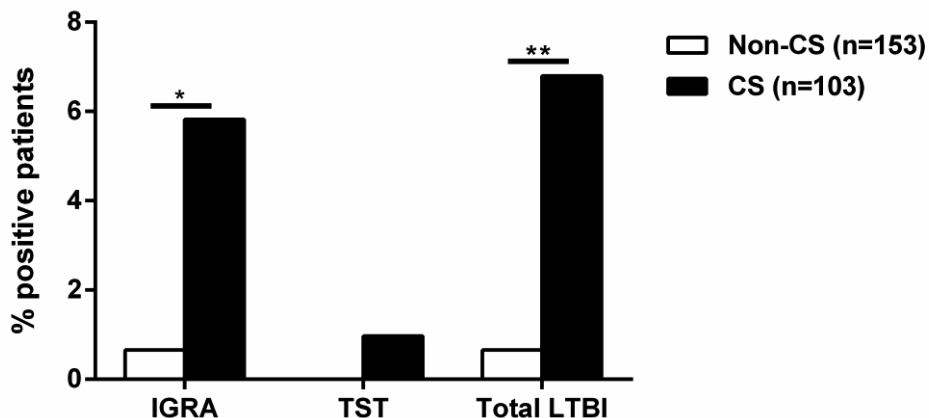


Figure 1. Percentage of patients with a positive IGRA or TST, to determine a latent tuberculosis infection. Significant more CS patients had a positive IGRA test compared to the non-CS group (6 versus 1, respectively) ($P = 0.016$). In total, significant more CS patients were diagnosed with a LTBI compared to non-CS patients (7 versus 1, respectively) ($P = 0.008$).

IGRA included a QuantiFERON test or TB elispot.

Non-CS: Non-cardiac sarcoidosis, CS: Cardiac sarcoidosis, IGRA: Interferon gamma release assay, TST: tuberculin skin test, LTBI: latent tuberculosis infection.

Table 2. Organ involvement of LTBI and no LTBI sarcoidosis patients

Involved organ:	No LTBI (n=248)	LTBI (n=8)	P-value*
Heart (%)	96 (38.7)	7 (87.5)	0.008
Lymph nodes (%)	237 (96.3)	8 (100)	1.000
Lungs (%)	185 (74.6)	7 (87.5)	0.684
Nervous system (%)	36 (14.5)	0 (0.0)	0.605
Skin (%)	32 (12.9)	0 (0.0)	0.601
Eyes (%)	30 (12.1)	1 (12.5)	1.000
Bone (%)	16 (6.5)	0 (0.0)	1.000
Liver (%)	13 (5.2)	1 (12.5)	0.366
Spleen (%)	18 (7.3)	0 (0.0)	1.000

* P-values were calculated using Fisher's Exact Test

LTBI: Latent tuberculosis infection

Discussion

In this study we were able to show that a latent tuberculosis infection is associated with cardiac involvement in patients already diagnosed with sarcoidosis, which is in line with our initial observation (15).

Although the finding that 6.8% of patients with CS were diagnosed with an LTBI seems low, this is an interesting observation since the Netherlands is a very low tuberculosis incidence country with a tuberculosis incidence of about 5 per 100.000 and an LTBI incidence of about 8 per 100.000 (20).

In previous papers, a possible link between LTBI and sarcoidosis has already been suggested (21). However, to the best of our knowledge, this is the first study to relate LTBI specifically to CS. It is known from literature that myocardial involvement in tuberculosis exists, but this is rare and has been occasionally described in case reports (22). Luk *et al.* described a case of CS who underwent heart transplantation, developing recurrent CS in the graft following a mycobacteria tuberculosis infection (23). Since data of IGRAs or TSTs before heart transplantation were not presented, it is unclear whether this patient was suffering from a reactivation of an LTBI already present before heart transplantation, or a newly acquired tuberculosis infection. In a recent study in mice it was demonstrated that after intranasal infection, *Mycobacterium avium* was able to disseminate into cardiac tissue (24). Interestingly, infection with *Mycobacterium avium* was able to induce intracardiac inflammatory gene expression and induce intracardiac tissue fibrosis. Although quite speculative, if other species of mycobacteria are also capable of inducing such damage to cardiac tissue, this could perhaps trigger a local granulomatous reaction in the heart we define as cardiac sarcoidosis. A possible explanation for our findings, may be molecular mimicry. Molecular mimicry has been described in T cell specific autoimmune diseases including multiple sclerosis and rheumatoid arthritis (RA) but also myocarditis (25,26). Chodisetti *et al.* identified several T cell epitopes that are similar to peptides of mycobacterial antigens. Those epitopes may act as molecular mimics and result in an autoimmune response during an infection with *M. tuberculosis* (27). Cross-reactive antibodies with heart tissue were observed in rheumatic carditis patients (28). Further studies should clarify whether such cross reactive antibodies or shared epitopes are also present for mycobacteria and cardiac tissue.

If the association between LTBI and CS can be confirmed in further studies, this could be of interest for clinical management. For instance, it could be relevant to screen sarcoidosis patients with an LTBI for CS, even though symptoms may not be present (yet). Identification of cardiac involvement seems important since it is a major cause of death. Furthermore, in current clinical practice, if a patient is diagnosed with an LTBI they can either choose follow-up without medication or start LTBI treatment using Isoniazid/Rifampicin (29). One could

speculate whether it would be beneficial for this group to start LTBI treatment instead of a wait-and-see policy directly after the diagnosis of LTBI is made.

A limitation of this retrospective study design is that the diagnosis of an LTBI was based on a positive result of different assay types (TST or IGRA). So, not all study patients had the same assay performed, although an IGRA was performed in over 95% of cases and results remained statistical significant when only LTBI's diagnosed by an IGRA were taken into account. A study regarding RA patients showed that results of IGRA tests did not correlate with use of corticosteroids, making these assays useful in RA patients (30), and likewise in sarcoidosis patients (31). Where results of IGRA are not affected by previous BCG vaccination, the TST is. However, no evidence for a previous BCG vaccination was found for the patient with an LTBI based on a positive TST. Moreover, there are several methods to search for an *Mycobacterium tuberculosis* (MTB) infection, which all have a different sensitivity and specificity. For instance, Masoud *et al.* showed that purified protein derivative antigens of MTB were present in tissue cells of 3 out of 10 sarcoidosis patients even when MTB DNA could not be detected (14). Based on our retrospective study design, we were not able to examine different methods to search for a MTB infection such as detection of mycobacterial antigens in tissue of patients. It would however be interesting to examine in future studies whether mycobacterial antigens are present in myocardial tissue of cardiac sarcoidosis patients diagnosed with an LTBI.

Another study limitation was that the non-CS and CS group were not similar regarding sex and age. Although tuberculosis is more common in the Netherlands among people with a higher age (32), the OR was still significantly increased after adjustment for age, suggesting that this difference between the groups have not induced a bias in our results. Although patients in the non-CS group did not have symptoms associated with CS, we cannot completely exclude the possibility that there might be some asymptomatic CS patients in this group, since a PET or MRI was not performed for the complete non-CS group.

To conclude, our data suggest that a latent tuberculosis infection associates with cardiac involvement in patients with sarcoidosis. Future research is required to unravel pathways involved in the association between a latent tuberculosis infection and cardiac involvement in sarcoidosis.

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Supplement

Supplementary Table 1. Establishment of the diagnosis of cardiac sarcoidosis

Total CS	103	
1.Histological diagnosis from myocardial tissue	3	
2a + 2b	Histological diagnosis of extra-cardiac sarcoidosis (n=78)	Consensus diagnosis extra-cardiac sarcoidosis based on expert opinion (n=21)
Steroid+/- immunosuppressant responsive cardiomyopathy or heart block	1	3
Unexplained reduced LVEF (<40%)	11	2
Unexplained sustained (spontaneous or induced) VT	9	4
Mobitz type II 2nd degree heart block or 3rd degree heart block	11	9
Patchy uptake on dedicated cardiac PET (in a pattern consistent with CS)	63	16
Late Gadolinium Enhancement on CMR (in a pattern consistent with CS)	67	15

The likelihood of cardiac sarcoidosis (CS) was assessed in a multidisciplinary team consisting of a pulmonologist, cardiologist, radiologist, nuclear specialist and nurse, predominantly based on the diagnostic criteria from the Heart Rhythm Society (HRS) consensus statement (17). Definite and probable CS were the gold standard for the diagnosis of CS.

Supplementary Table 2. Country of origin of study patients

Country of origin	Non-CS (n=153)	CS (n=103)
The Netherlands	146 (96.1)	95 (92.2)
Morocco	0	3 (2.9)
Curacao	0	2 (1.9)
Sri Lanka	2 (1.3)	0
Suriname	1 (0.7)	1 (1.0)
Colombia	1 (0.7)	0
Germany	0	1 (1.0)
Grenada	1 (0.7)	0
India	1 (0.7)	0
Syria	0	1 (1.0)
Unknown	1 (0.7)	0

The percentages of patients who originated from another country than the Netherlands did not significantly differ between the CS and non-CS group ($P = 0.189$).



Chapter 7

Presence of *Propionibacterium acnes* in granulomas associates with a chronic disease course in Dutch sarcoidosis patients

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Abstract

Several studies demonstrated that *Propionibacterium acnes* (*P. acnes*) may be involved in sarcoidosis pathogenesis. Presence of *P. acnes* was found in granulomas of the majority of Japanese sarcoidosis patients. However, presence of *P. acnes* in tissue has never been related to sarcoidosis phenotypes and clinical outcome. Therefore, the aims of our study were to demonstrate whether *P. acnes* can be detected in granulomas of Dutch sarcoidosis patients and to investigate whether its presence is related to a clinical phenotype and/or course of disease.

Sections of formalin fixed paraffin-embedded tissue blocks of 76 sarcoidosis patients were examined by immunostaining with a *P. acnes*-specific monoclonal antibody (PAB antibody) using a VENTANA BenchMark ULTRA. Clinical outcome status (COS) was determined and classified into two phenotype groups: A: resolved, minimal or persistent disease without treatment (COS 1-6) and B: persistent disease with need for treatment (COS 7-9).

P. acnes was detected in samples of 31 patients (41%) and located within granulomas in samples of 13 patients (17%). The frequency of *P. acnes* detected in granulomas at diagnosis was significantly higher in patients with phenotype B compared to patients with phenotype A (29% vs 0%, $P = 0.021$).

Presence of *P. acnes* in granulomas can be confirmed in Dutch sarcoidosis patients. It is intriguing that presence of *P. acnes* in granulomas is more frequently found in patients with chronic disease requiring treatment. This adds to the rationale that a subgroup of sarcoidosis patients might benefit from antibiotic therapy.

Introduction

Sarcoidosis is a multisystem inflammatory disorder of unknown etiology that is characterized by the presence of non-caseating granulomas. In over 90% of patients the lungs are involved (1), but the disease can also affect other organs (2). Patients with symptomatic organ involvement or risk of permanent damage can be treated with immunosuppressive drugs (3), which suppress disease activity but do not cure the disease. The cause of sarcoidosis is still puzzling scientists for more than a century. Several studies demonstrated that specific microorganisms, including mycobacteria and *Propionibacterium acnes* (*P. acnes*) could be involved in its disease pathogenesis (4–9). As *P. acnes* is a commensal, Koch's postulates cannot be applied, which makes it difficult to elucidate its etiologic role (10). A higher number of *P. acnes* genomes have been found in tissue from Chinese, Japanese, Italian, English and German sarcoidosis patients compared to tissue of controls, suggesting involvement in disease pathogenesis (11–13). Furthermore, an increased immune response to *P. acnes* among patients with sarcoidosis was found in different studies using Japanese or German patients (7,8,14). Remarkably, in recent work from our own group, we found a lower percentage of Dutch sarcoidosis patients than controls with an immunological response to *P. acnes* (15) which is conflicting with the abovementioned previous papers.

In addition to its role as possible antigen, *P. acnes* can also act as a mitogen which is demonstrated by the fact that *P. acnes* enhances immunogenicity of certain vaccines (16) and enhances cytotoxic activity toward different tumor cells (17–19). A potential mitogenic role of *P. acnes* in sarcoidosis has not previously been studied.

Based on both a possible antigenic as well as mitogenic role for *P. acnes*, we hypothesize that presence of *P. acnes* in tissue could be related to certain clinical phenotypes in sarcoidosis. We therefore examined the presence of *P. acnes* in tissue of Dutch patients with sarcoidosis using an existing *P. acnes* specific monoclonal antibody (PAB antibody) (20) and related results of immunostaining to clinical characteristics such as organ involvement and course of disease.

Methods and materials

Study patients

Unstained tissue blocks were requested from two sarcoidosis cohorts previously studied at the St Antonius Hospital (Nieuwegein, The Netherlands) (15,21). The diagnosis of sarcoidosis had been established according to the criteria of the American Thoracic Society/European Respiratory Society (22). Patients were included in the study when enough residual tissue was available and when presence of granulomas could be clearly detected in the hematoxylin and eosin (H&E) stained tissue sections. The study was

approved by the Medical research Ethics Committees United (MEC-U) of the St Antonius Hospital (R05-08A) and written consent was obtained from all patients.

Immunostaining

4 µM thick sections were cut from the formalin-fixed paraffin-embedded tissue sections which were immunohistochemically stained with the PAB antibody; a *P. acnes* specific monoclonal antibody that reacts with cell-membrane bound lipoteichoic acid of the bacterium (20). The PAB antibody was kindly provided by Prof. dr. Eishi and colleagues, Department of Human Pathology, Tokyo Medical and Dental University, Tokyo, Japan. We followed the protocol described by Negi *et al.* (20), but instead of the original manual procedures the sections were stained by the use of a VENTANA BenchMark ULTRA (Ventana Medical Systems, Inc., Tucson, AZ, USA) using ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems, Inc.). We modified the original protocol to optimize the sensitivity and specificity of the staining results for the VENTANA BenchMark ULTRA. Shortly, sections were de-paraffinized and rehydrated followed by antigen retrieval using the hot plate heating system of the machine instead of antigen retrieval by microwave. Because mineral oil (Liquid Coverslip, Ventana Medical Systems, Inc.) covering the tissue slides was found to inhibit the reaction with PAB antibody, before the primary antibody reaction with the PAB antibody, a washing step (3 times for 5 minutes each) in the EZ Prep buffer (Roche Nederland B.V, Woerden) was added under the Antibody Titration program selected, followed by washing 5 minutes with tap water and 5 minutes with reaction buffer (Roche Nederland B.V, Woerden, The Netherlands). The PAB antibody (crude mouse ascites fluid) was used in a concentration of 1:30.000 (diluted with DAKO REAL antibody diluent, S2022, DAKO, Glostrup, Denmark) and incubated for 16 minutes at room temperature. Instead of using peroxidase substrate diaminobenzidine (DAB) to develop the signal, ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems, Inc.) was used. Sections were counterstained with Mayer's hematoxylin. Detection of *P. acnes* in tissue was analyzed by a pulmonary pathologist (KS). The staining was considered positive when small round/dot like structures were seen. If such structures were detected, it was determined whether positive PAB staining was present in or outside the granulomas.

Identification of *P. acnes* related phenotypes

To determine possible *P. acnes* related phenotypes, organ involvement, age at diagnosis and Scadding stage at diagnosis and follow up was collected from medical records of sarcoidosis patients.

The Clinical outcome status (COS), a definition of clinical outcome in sarcoidosis established by The World Association of Sarcoidosis and Other Granulomatous disease (WASOG) (23), was determined 2 and 5 years after diagnosis. The disease status of patients was retrospectively examined and classified into resolved, minimal or persistent disease. Resolved was classified as patients showing no signs of disease anymore, so normalization of chest x-ray, pulmonary function test, laboratory tests etc. Minimal disease is defined as a disease burden of 25% or less compared to the maximum disease burden experienced by patients measured by for example pulmonary function test, Chest X-ray, biomarkers and skin lesions. For example, the worst pulmonary function test had to be improved by at least 75% to be considered minimal disease.

Furthermore, it was examined whether patients were ever treated, and if so whether they were still using medication or not (defined as no medication > one year). Patients in whom medication was increased the last year were considered worsening. Medication included all immunosuppressive systemic therapies used for sarcoidosis, including corticosteroids, DMARDS and anti-TNF α antibodies, except for non-steroid anti-inflammatory drugs. This results in 9 COS scores:

- 1: Resolved disease, never treated
- 2: Resolved diseases, no treatment > one year
- 3: Minimal disease, never treated
- 4: Minimal disease, no therapy > one year
- 5: Persistent disease, never treated
- 6: Persistent disease, no therapy > one year
- 7: Persistent disease, current therapy but no worsening in prior year and asymptomatic
- 8: Persistent disease, current therapy but no worsening in prior year and symptomatic
- 9: persistent disease, current therapy which worsened in the prior year

Patients who died in the period over which the COS was determined were placed in COS 9. To analyze whether there was a correlation between presence of *P. acnes* and COS, we classified the COS scores into two phenotype groups: A: resolved, minimal or persistent disease without treatment (COS 1-6) and B: persistent disease with need for treatment (COS 7-9).

Statistical analysis

Data was analyzed using IBM SPSS statistics version 24. An unpaired T-test was used to compare numerical data. Non-parametric tests were used for non-normally distributed data (Mann-Whitney U test). Categorical data were compared using the Chi-squared test. If

expected cell frequencies were below 5, Fisher's exact test was used for categorical data up to two categories. P-values <0.05 were considered significant.

Results

Characteristics of study patients and tissue samples

Formalin-fixed paraffin-embedded tissue blocks were available from 76 patients. Mean age of included patients was 44 years, 84% was Caucasian and 71% had extra pulmonary involvement. 68% and 67% were classified in phenotype group B 2 and 5 years after diagnosis respectively (Table 1).

Table 1. Characteristics of study patients

	Sarcoidosis (n=76)
Age^a	43.98±12.22
Male sex	42 (55)
Ever smoker	43 (59)
Caucasian	64 (84)
Medication at time of biopsy	6 (8)
Third-line therapy^b	27 (36)
COS group A/B (2 years follow up)	23/49 (32/68)
COS group A/B (5 years follow up)	15/31 (33/67)
Scadding stage at time biopsy (0/I/II/III/IV/unknown)	5/19/28/9/10/5 (7/25/37/12/13/7)
Extra pulmonary involvement	54 (71)
Skin	18 (24)
Eyes	7 (9)
Liver	8 (11)
Heart	13 (17)
Spleen	3 (4)
Bones	4 (5)
Nerve system	15 (20)
Central	6 (8)
Peripheral	1 (1)
SFN	10 (13)

Data is shown as absolute numbers with percentage in brackets.

^a Age is age at time of biopsy and is shown as mean ± SD. ^b Third-line therapy consisted of infliximab use at follow up. COS: Clinical outcome status, SFN: Small fiber neuropathy, Scadding stages: 0 = Normal chest radiograph; I = Bilateral hilar lymphadenopathy (BHL); II = BHL with pulmonary infiltrates; III = pulmonary infiltrates without BHL; IV = fibrosis.

***P. acnes* can be detected in tissue samples of Dutch patient with sarcoidosis**

Most tissue sections used for staining originated from the lung, followed by lymph node and skin samples. *P. acnes* was detected in tissue samples from 31 of the 76 sarcoidosis patients (41%) and was located in the granulomas in 13 samples of all patients (17%) (Table 2, Figure 1 and Figure 2). When *P. acnes* was not detected inside granulomas, it was mostly located directly adjacent to granulomas in histiocytes and in a few cases in granuloma-free areas of the tissue. Besides lung, lymph node and skin tissue, we also found presence of *P. acnes* in bone marrow and liver tissue.

Table 2. Detection of *P. acnes* and origin of tissue used

	n	Detection of <i>P. acnes</i> in:	
		Tissue	Granulomas
Total number of patients ^a	76	31 (41)	13 (17)
Total number of tissue sections	80	32 (40)	14 (18)
Lung	32	10 (31)	7 (22)
VATS	7	3 (43)	3 (43)
TBLB	25	7 (28)	4 (16)
Lymph node	25	12 (48)	2 (8)
lymphadenectomy	14	9 (64)	1 (7)
mediastinoscopy	5	1 (20)	0
EBUS-TBNA/needle	6	2 (33)	1 (17)
Skin	17	7 (41)	4 (24)
Other	6	3 (50)	1 (17)
Bone marrow	2	2 (100)	0
Liver	2	1 (50)	1 (50)
Nasal concha	1	0	0
Salivary gland	1	0	0

Data is shown as number of samples with percentages in brackets

^a Of 4 of 76 patients, 2 tissue sections of different organs were stained namely: Lymph node (negative) and lung (*P. acnes* positive in tissue and granulomas, liver (negative) and lymph node (*P. acnes* positive in tissue), skin (*P. acnes* positive in tissue and granulomas) and lung (negative), skin (*P. acnes* positive in tissue and granulomas) and lung (*P. acnes* positive in tissue and granulomas).

VATS: Video-assisted thoracoscopic surgery, TBLB: transbronchial lung biopsy, EBUS-TBNA: endobronchial ultrasound- transbronchial needle aspiration

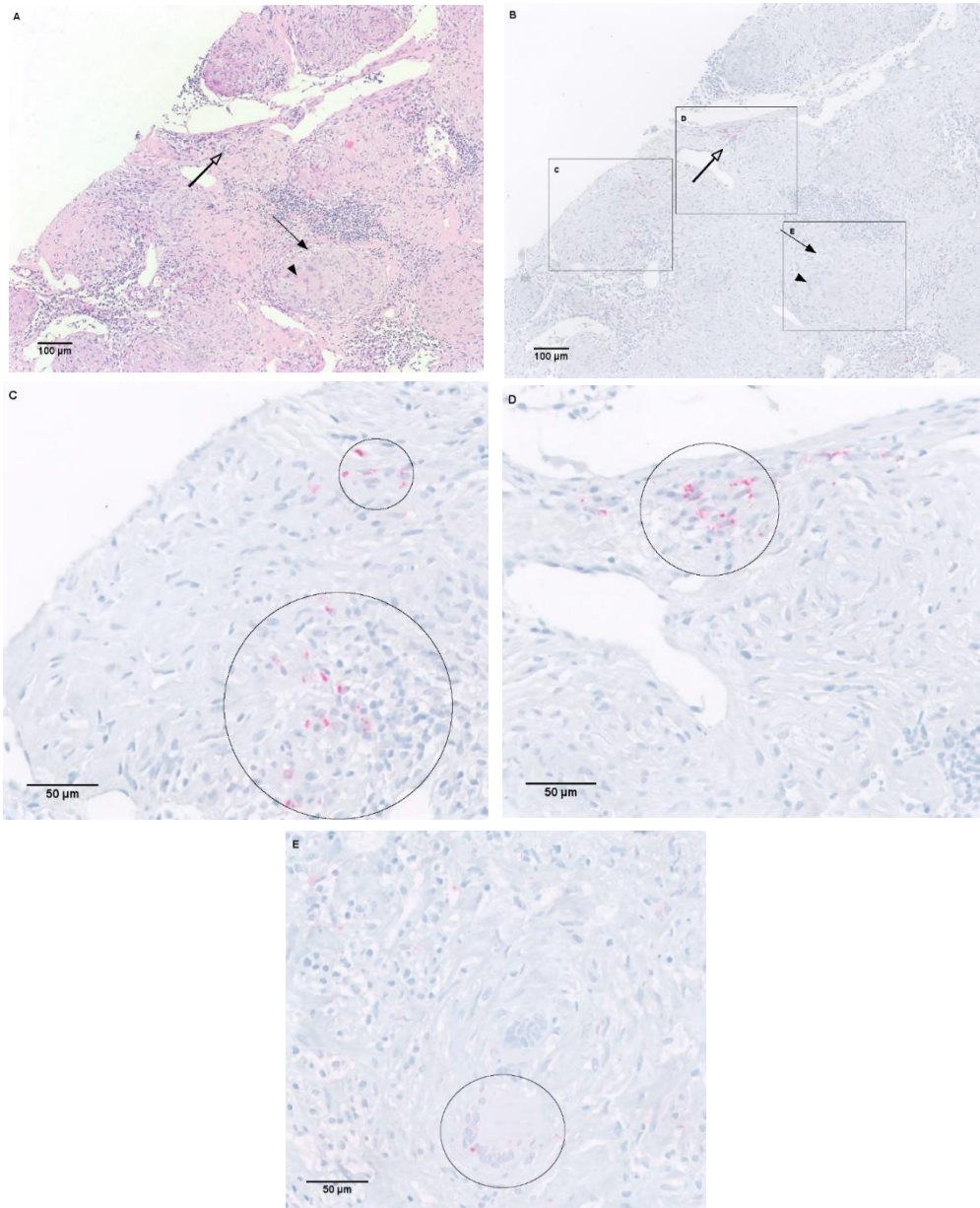


Figure 1. Presence of *P. acnes* in and around an immature granuloma of the lymph node
A. Hematoxylin and eosin (H&E) staining of a needle lymph node biopsy including several mature granulomas. Arrow: a mature granuloma including a multinucleated giant cell (arrow head). Open arrow: an immature granuloma. **B.** PAB staining corresponding with the area in picture **A**, including several areas with *P. acnes*-positive red signals (rectangle **C** and **D**) and a mature granuloma negative for *P. acnes* (rectangle **E**). **C.** Higher magnification of positive *P. acnes* staining (circles) around granulomas. **D.** Higher magnification of positive *P. acnes* staining (circle) in an immature granuloma. **E.** Higher magnification of another area including a multinucleated giant cell positive for *P. acnes*.

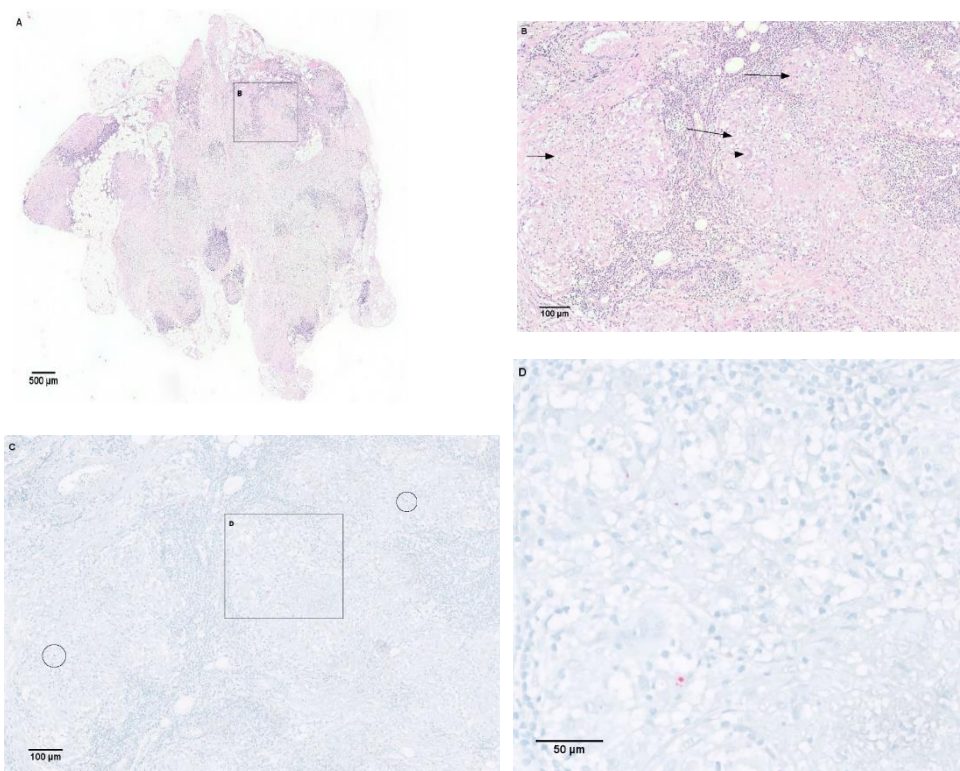


Figure 2. Presence of *P. acnes* in a mature granuloma in subcutaneous tissue of the lower eyelid
A. Overview of hematoxylin and eosin (H&E) staining of subcutaneous tissue of the lower eyelid. **B.** Higher magnification of area B from picture A, including mature granulomas (arrows) and multinucleated giant cells (arrow head). **C.** PAB staining of corresponding area of picture B, including several areas of *P. acnes*-positive signals (circles and rectangle D), in mature granulomas. **D.** Higher magnification of area D from picture C, with positive *P. acnes* staining in a mature granuloma.

No significant difference was observed in the frequency of *P. acnes* detected in tissue or granulomas between the different organs used in the study ($P = 0.583$ and $p = 0.490$, respectively). *P. acnes* was detected in tissue or granulomas in 3 (43%) and 3 (43%) respectively, of the 7 lung samples obtained by video-assisted thoracic surgery (VATS), and in 7 (28%) and 4 (16%) respectively, of the lung samples obtained by transbronchial lung biopsy (TBLB). No significant difference in presence of *P. acnes* in tissue or granulomas was observed between the different biopsy methods used for the lung samples ($P = 0.648$ and $P = 0.157$, respectively) and lymph node samples ($P = 0.167$ and $P = 0.588$, respectively).

Presence of *P. acnes* does not correlate with organ involvement

When relating positive *P. acnes* staining results to clinical characteristics, no relation with age, sex, ethnicity, organ involvement or Scadding stage was observed (Table 3A and 3B).

Presence of *P. acnes* correlates with clinical outcome

Presence of *P. acnes* in tissue was compared between the two COS groups defined two and five years after diagnosis. Two years after diagnosis, a trend ($P = 0.093$) towards a higher prevalence of *P. acnes* in tissue was seen in COS group B (47%, 23/49) compared to COS group A (26%, 6/23). When comparing presence of *P. acnes* specifically inside granulomas, also a trend ($P = 0.050$) towards a higher prevalence was seen in COS group B patients (25%, 12/49) compared to COS group A patients (4%, 1/23) (Figure 3A). COS defined after 5 years revealed a trend ($P = 0.072$) towards a higher prevalence of *P. acnes* in tissue in COS group B (55%, 17/31) compared to COS group A (27%, 4/15). A significantly higher percentage of patients within COS group B (29%, 9/31) were positively stained for *P. acnes* inside granulomas compared to COS group A patients (0, 0/15) ($P = 0.021$, Figure 3B).

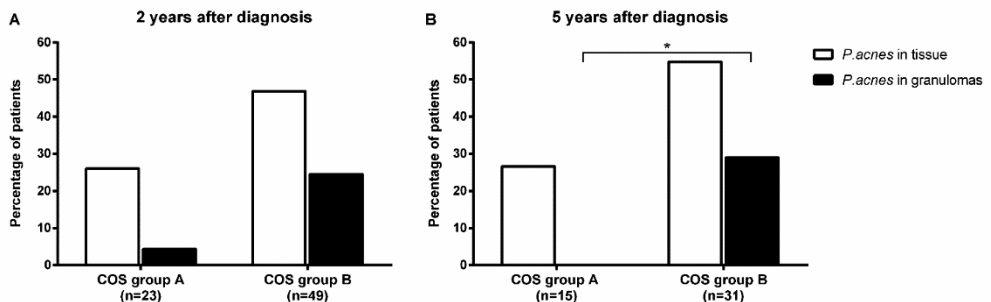


Figure 3. Frequency of *P. acnes* detected in tissue samples from sarcoidosis patients with a different clinical outcome status 2 years (A) and 5 years (B) after diagnosis

COS group A = Resolved, minimal or persistent disease without treatment (COS 1-6).

COS group B = Persistent disease with need for treatment (COS 7-9).

PAB: *P. acnes*-specific monoclonal antibodies that react with cell-membrane-bound lipoteichoic acid

Since COS group B can be a very heterogeneous group of patients, ranging from patients with remission of symptoms due to long-term corticosteroid treatment to severe therapy refractory patients requiring third-line medication, we also determined whether presence of *P. acnes* in tissue and granulomas was specifically related to patients requiring third-line medication at follow up. In a significant higher percentage of patients using infliximab, *P. acnes* was present in granulomas compared to non-third-line therapy requiring patients (33% vs. 8%, $P = 0.009$) (Figure 4).

Table 3A. Presence of *P.acnes* in tissue related to organ involvement and Scadding stage

	PAB staining		P*
	Negative (n=45)	Positive (n=31)	
Age	44.59±12.51	43.08±11.94	0.601
Male sex	23 (51)	19 (61)	0.380
White	38 (84)	26 (84)	1.000
Extra pulmonary involvement	32 (71)	22 (71)	0.989
Skin	12 (27)	6 (19)	0.461
Eyes	4 (9)	3 (10)	1.000
Liver	2 (4)	6 (19)	0.057
Heart	8 (18)	5 (16)	0.851
Spleen	2 (4)	1 (3)	1.000
Bones	3 (7)	1 (3)	0.641
Nerve system	11 (24)	4 (13)	0.214
Central	5 (11)	1 (3)	0.391
Peripheral	1 (2)	0	1.000
SFN	7 (16)	3 (10)	0.514
Scadding stage at diagnosis	(n=44)	(n=29)	
(0/I/II/III/IV)	3/13/19/5/4	0/10/13/2/4	0.585
(%)	(7/30/43/11/9)	(0/35/45/7/14)	
Scadding stage after 2 years	(n=39)	(n=24)	
(0/I/II/III/IV)	11/4/12/5/7	6/3/8/1/6	0.794
(%)	(28/10/31/13/18)	(25/13/33/4/25)	
Scadding stage after 5 years	(n=25)	(n=16)	
(0/I/II/III/IV)	5/2/4/6/8	3/1/4/2/6	0.878
(%)	(20/8/16/24/32)	(19/6/25/13/38)	

Age is age at time of biopsy and shown as mean ± SD

Chest X-rays to determine Scadding stages were missing from 3 sarcoidosis patients at diagnosis, from 13 patients 2 years after diagnosis and from 35 patients 5 years after diagnosis. Scadding stages: 0 = Normal chest radiograph; I = Bilateral hilar lymphadenopathy (BHL); II = BHL with pulmonary infiltrates; III = pulmonary infiltrates without BHL; IV = fibrosis.

SFN: Small fiber neuropathy, PAB: *P. acnes*-specific monoclonal antibodies that react with cell-membrane-bound lipoteichoic acid

* A P-value regarding age was calculated using an independent samples T-test. Other P-values were calculated by the use of a Fisher's exact test, except for sex (both p-values), extra pulmonary involvement in the *P. acnes* score group, skin, heart and nerve system involvement in the *P. acnes* score group and Scadding stages

Table 3B. Presence of *P. acnes* in granulomas related to organ involvement and Scadding stage

	PAB staining present in granulomas		P*
	No (n=63)	Yes (n=13)	
Age	44.76±12.17	40.18±12.26	0.222
Male sex	33 (52)	9 (69)	0.266
White	52 (83)	12 (92)	0.679
Extra pulmonary involvement	46 (73)	8 (62)	0.504
Skin	15 (24)	3 (23)	1.000
Eyes	6 (10)	1 (8)	1.000
Liver	6 (10)	2 (15)	0.619
Heart	12 (19)	1 (8)	0.446
Spleen	2 (3)	1 (8)	0.435
Bones	4 (6)	0	1.000
Nerve system	14 (22)	1 (8)	0.444
Central	6 (10)	0	0.582
Peripheral	1 (2)	0	1.000
SFN	9 (14)	1 (8)	1.000
Scadding stage at diagnosis	(n=61)	(n=12)	
(0/I/II/III/IV)	3/19/29/5/5	0/4/3/2/3	0.277
(%)	(5/31/48/8/8)	(0/33/25/17/25)	
Scadding stage after 2 years	(n=53)	(n=10)	
(0/I/II/III/IV)	14/6/19/5/9	3/1/1/1/4	0.408
(%)	(26/11/36/9/17)	(30/10/10/10/40)	
Scadding stage after 5 years	(n=34)	(n=7)	
(0/I/II/III/IV)	7/2/8/7/10	1/1/0/1/4	0.441
(%)	(21/6/24/21/29)	14/14/0/14/57	

Age is age at time of biopsy and shown as mean ± SD

Chest X-rays to determine Scadding stages were missing from 3 sarcoidosis patients at diagnosis, from 13 patients 2 years after diagnosis and from 35 patients 5 years after diagnosis. Scadding stages: 0 = Normal chest radiograph; I = Bilateral hilar lymphadenopathy (BHL); II = BHL with pulmonary infiltrates; III = pulmonary infiltrates without BHL; IV = fibrosis.

SFN: Small fiber neuropathy, PAB: *P. acnes*-specific monoclonal antibodies that react with cell-membrane-bound lipoteichoic acid

* A P-value regarding age was calculated using an independent samples T-test. Other P-values were calculated by the use of a Fisher's exact test, except for sex (both p-values), extra pulmonary involvement in the *P. acnes* score group, skin, heart and nerve system involvement in the *P. acnes* score group and Scadding stages

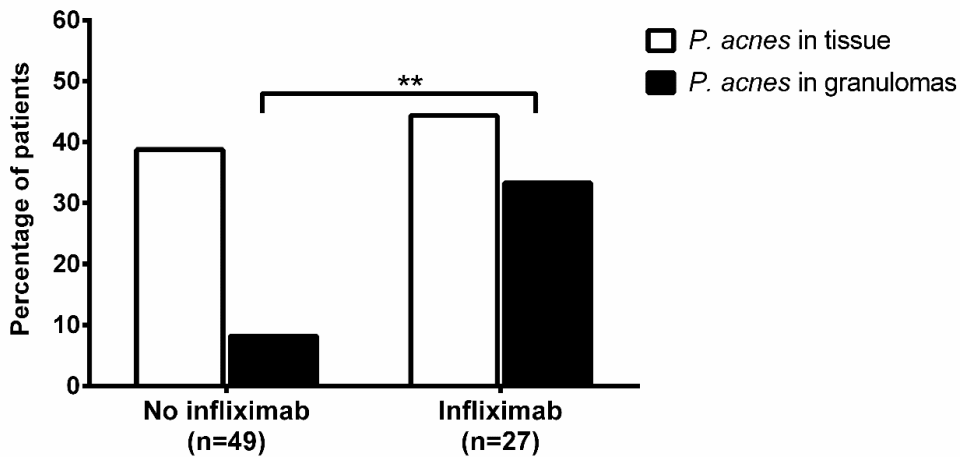


Figure 4. Frequency of *P. acnes* detected in biopsy samples from sarcoidosis patients with or without third-line therapy (infliximab) during follow up.

A significantly higher percentage of third-line therapy requiring patients (all using infliximab) showed presence of *P. acnes* in granulomas than patients who did not require third-line therapy ($P = 0.009$).

PAB: *P. acnes*-specific monoclonal antibodies that react with cell-membrane-bound lipoteichoic acid.

Discussion

In this study we demonstrated that *P. acnes* is present in granulomas of Dutch patients with sarcoidosis, which is in line with previous studies in Japanese and German sarcoidosis patients (20). Furthermore, to the best of our knowledge, our study is the first to show a relation between presence of *P. acnes* and clinical outcome in sarcoidosis. Regardless of whether the role of *P. acnes* might be antigenic or mitogenic, this data adds to the rationale that *P. acnes* might be involved in the disease pathogenesis of sarcoidosis.

Negi *et al.* (20) previously examined the presence of *P. acnes* in tissue samples from sarcoidosis patients, using the same PAB antibody as used in the current study. A remarkable difference was found in percentage of *P. acnes* positive sarcoidosis patients. They found *P. acnes* in granulomas in 57% of the 77 lung samples from Japanese sarcoidosis patients whereas we found *P. acnes* in granulomas in 22% of the 32 lung samples from Dutch sarcoidosis patients. This difference could be partially explained by the lower proportion of VATS samples (35% in their study compared to 22% in our study). Negi *et al.* described that *P. acnes* in granulomas was more frequently detected in VATS samples compared to TBLB samples, probably due to the larger size of VATS samples. Similar to the

lung samples, the percentage of Dutch patients with presence of *P. acnes* in granulomas of lymph node samples (8%) was lower compared to Japanese and German patients (88% and 89% respectively). Although the difference between Japanese and Dutch patients may be explained by difference in ethnicity, this is not a plausible explanation for the difference between German and Dutch patients. The difference in the detection frequency of *P. acnes* in granulomas can possibly be caused by a lower sensitivity of immunostaining method used in our study. Therefore, future studies will have to compare the detection sensitivity of *P. acnes* in granulomas using the different immunostaining methods. It is however important to state that the localization and pattern of *P. acnes* within and outside granulomas was comparable with the study of Negi *et al.* Specifically, we observed that presence of *P. acnes* outside granulomas was most frequently detected directly adjacent to granulomas but also in granuloma-free parts of the tissue in a few cases. Furthermore, the pattern within granulomas was comparable, with more intense dot like structures in immature granulomas and more sparsely- distributed staining or even no staining in mature granulomas.

A new and clinically relevant finding, in our opinion, is the fact that this is the first study that shows an association between presence of *P. acnes* and clinical outcome in patients with sarcoidosis. We observed that presence of *P. acnes* in tissue and in granulomas is more frequently found in patients with a chronic disease course requiring chronic treatment. Several studies demonstrated the mitogenic properties of *P. acnes* on different immune cells (16,24,25). Our study was not aimed to prove that *P. acnes* has a specific etiologic role in sarcoidosis. However, in our opinion, the results do suggest that at least a potential mitogenic role of *P. acnes* may contribute to sarcoidosis disease pathogenesis. It is tempting to speculate that presence of *P. acnes* in or around granulomas can enhance an ongoing inflammatory reaction in sarcoidosis, contributing to perpetuation of the inflammatory granulomatous response seen in some patients. If this holds true, it is interesting to see whether decreasing the bacterial load of *P. acnes* using antibiotics can be beneficial in a subgroup of patients with sarcoidosis.

A case report already described a good effect of clarithromycin on fever, joint pain, FDG uptake on PET-CT, CRP and s-IL2R levels in a sarcoidosis patient in whom *P. acnes* was present in granulomas (26). Moreover, a retrospective study described good responses on treatment with minocycline in *P. acnes* positive cutaneous sarcoidosis patients (27). When we examined severe therapy refractory patients for presence of *P. acnes*, we observed that patients requiring infliximab treatment at follow up, had more often presence of *P. acnes* in granulomas at diagnosis compared to patients without treatment or treated with first or second line treatment. If antibacterial treatment in sarcoidosis patients with presence of *P. acnes* in tissue and granulomas is beneficial, the requirement of 3rd line treatments (e.g. infliximab) may be prevented.

Currently, in a randomized controlled clinical trial (J-ACNES), the effect of antibacterial drugs in addition to standard corticosteroid therapy in cardiac sarcoidosis patients is examined (28). However, in this trial presence of *P. acnes* in myocardial tissue was not an inclusion criterion. Therefore, in future studies it would be interesting to have information on the presence or absence of *P. acnes* in these patients while investigating the effect of antibiotic therapy.

A limitation of the study was that the COS after five years could not be determined in all patients. However, since the proportion of patients within COS group A and B was quite similar after two and five years and the results regarding association with *P. acnes* staining as well, we assume that this analysis on a smaller group of patients has not introduced a bias. A disadvantage of using COS is the fact that disease status and medication use have been retrospectively assessed. Since we only scored whether medication was used or not, we have no information on patients who declined the use of medication while they actually needed it.

Another limitation is that we probably have a more severe patient group than other general hospitals, since the Antonius Hospital is a national referral center for ILD and Sarcoidosis. As a consequence we had very few patients in the resolved and minimal disease COS groups. For this reason we had not enough patients in every group to adequately analyze whether disease status alone, irrespective of use of medication, was associated with presence of *P. acnes* in tissue and granulomas. However, instead of disease status we think that need for treatment could be of more value regarding presence of *P. acnes*. If we want to further explore the use of antibacterial therapy, in our opinion, it is more relevant to focus on patients who actually need treatment.

In future studies, it would be interesting to add a second detection method, such as PCR, to the immunostaining. To accurately compare the results, development and use of a primer specific to LTA of the *P. acnes* bacteria would be valuable.

To conclude, this study confirms the presence of *P. acnes* in tissue and granulomas of respectively 40% and 17% of Dutch patients with sarcoidosis. Interestingly, the presence of *P. acnes* inside granulomas of Dutch sarcoidosis patients was associated with a chronic disease phenotype and requirement of treatment. Regardless of whether the role of *P. acnes* is antigenic or mitogenic, our data contribute to the premise that it is relevant to further explore antibacterial therapy as a treatment option for a subset of sarcoidosis patients.

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Chapter 8

Detection of *Propionibacterium acnes* in granulomas of patients with either hypersensitivity pneumonitis or vasculitis reveals that its presence is not unique for sarcoidosis

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Introduction

Granulomas are compact organized structures of different immune cells including macrophages, lymphocytes and plasma cells, thought to be formed when (foreign) antigens cannot be cleared. The differential diagnosis of a granulomatous lesion is broad and includes infectious etiologies, malignancy and inflammatory disorders like vasculitis, hypersensitivity pneumonitis and sarcoidosis (1).

Propionibacterium acnes (*P. acnes*) is a gram positive bacteria that is a commensal of the skin (2). Studies have demonstrated that *P. acnes* is also able to induce granulomas (3). Furthermore, *P. acnes* is suggested to be involved in the pathogenesis of sarcoidosis, a complex inflammatory disease mainly involving the lungs and lymph nodes, characterized by non-caseating granulomas (4).

Although presence of *P. acnes* has already been shown in granulomas of sarcoidosis patients (5), it is unknown whether this bacterium can also be found in granulomatous disorders with frequent involvement of the lungs other than sarcoidosis such as hypersensitivity pneumonitis (HP), Granulomatosis with polyangiitis (GPA) and Eosinophilic granulomatosis with polyangiitis (EGPA).

To investigate whether presence of *P. acnes* in granulomas is specific for sarcoidosis we examined granulomatous tissue of HP, GPA and EGPA patients for presence of *P. acnes*.

Methods

Tissue blocks were collected from patients with HP, GPA and EGPA who participated in our biobank study. Patients were included in the study when enough residual tissue was available and when granulomas could be detected in the haematoxylin stained tissue sections. Tissue blocks of 35 patients with HP and of 13 patients with (E)GPA were collected. Tissue blocks of all HP and 9 (E)GPA patients showed granulomas and had enough tissue left to be included in the study. The study was approved by the Medical research Ethics Committees United (MEC-U) of the St. Antonius Hospital (R05-08A) and written consent was obtained from all patients. Formalin fixed, paraffin embedded tissue sections were immunohistochemically stained with the PAB antibody; a *P. acnes* specific monoclonal antibody that reacts with cell-membrane bound lipoteichoic acid (LTA) of the bacterium (5). Full methods are described elsewhere (6). Difference in presence of *P. acnes* between the patients groups was compared using the Chi-squared test. An independent sample T-test was used to compare BAL lymphocytes between HP patients. Fisher's exact test was used to compare presence of *P. acnes* in granulomas of HP patients with and without an known inducing agent. P-values <0.05 were considered significant.

Results

In total, 35 patients with HP and 9 patients with (E)GPA were included in the study (Table 1.) Presence of *P. acnes* in tissue was found in 57.1% of HP patients (20/35) and in 33.3% of (E)GPA patients (3/9). More specifically, presence of *P. acnes* was observed inside granulomas of 25.7% HP patients (9/35) and of 11.1% (E)GPA patients (1/9). (Figure 1 and Figure 2). Results between the diagnostic groups were not statistically significant ($P = 0.272$ and $P = 0.659$ for presence of *P. acnes* in tissue and granulomas respectively). Moreover, the percentages of patients with presence of *P. acnes* in tissue or granulomas was comparable with a previously described Dutch sarcoidosis cohort (Figure 3).

Table 1. Characteristics of study patients

	HP (n=35)	(E)GPA (n=9)
Age (mean \pm SD)	58.2 \pm 9.2	51.6 \pm 14.7
Male sex	17 (48.6)	6 (66.7)
Origin of tissue		
Lung	34 (97.1)	6 (66.7)
Lymph node	1 (2.9)	0
Skin	0	1 (11.1)
Nose	0	2 (22.2)
Inducing agent		
Birds	14 (40.0)	NA
Farmer's lung	3 (8.6)	NA
Unknown	18 (51.4)	NA

Data is shown as absolute values with percentages in brackets. HP: hypersensitivity pneumonitis, (E)GPA: (Eosinophilic) granulomatosis with polyangiitis, NA: not applicable

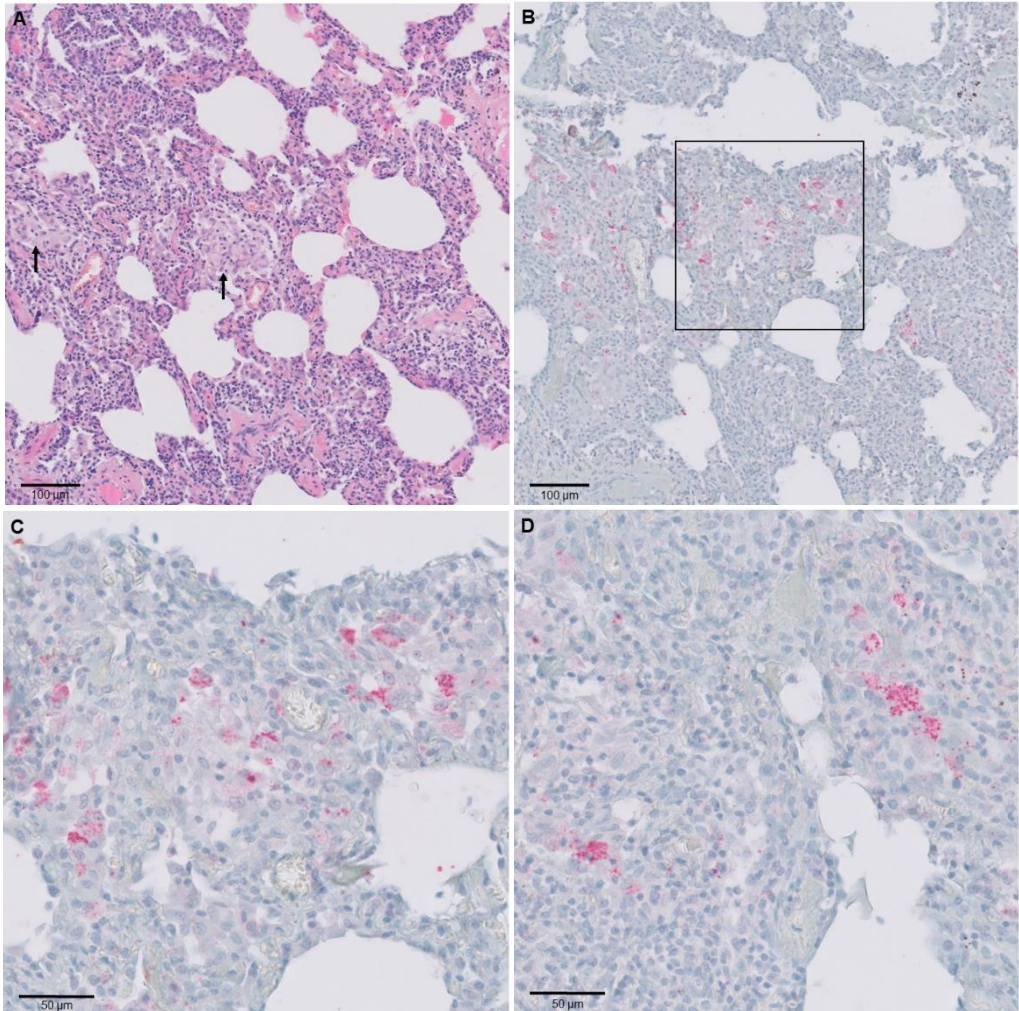


Figure 1. Presence of *P. acnes* in tissue and granulomas of an HP patient.

A. Hematoxylin and eosin stain (HE) stain of lung tissue. The black arrows show granulomas. **B.** Positive PAB staining (in red) in the granulomas corresponding with picture A. **C.** Higher magnification corresponding with the square in picture B. **D.** Area of positive PAB staining outside granulomas. HP: hypersensitivity pneumonitis, PAB: *P. acnes* specific monoclonal antibody that reacts with cell-membrane bound lipoteichoic acid (LTA) of the bacterium.

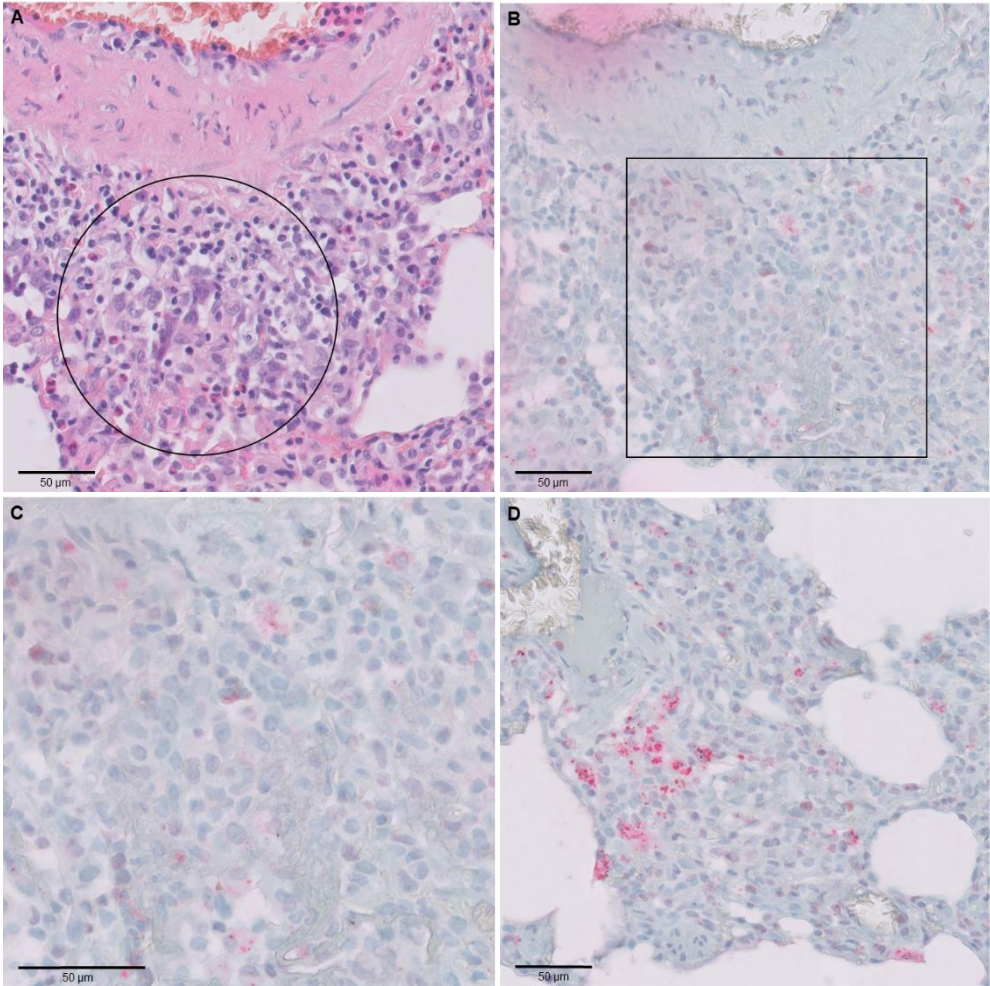


Figure 2. Presence of *P. acnes* in tissue and granulomas of an EGPA patient.

A. Hematoxylin and eosin stain (HE) stain of lung tissue, with an immature granuloma indicated by the circle. **B.** Positive PAB staining (in red) in the granuloma corresponding with picture A. **C.** Higher magnification corresponding with the square in picture B. **D.** Area of positive PAB staining outside granulomas.

EGPA: Eosinophilic granulomatosis with polyangiitis, PAB: *P. acnes* specific monoclonal antibody that reacts with cell-membrane bound lipoteichoic acid (LTA) of the bacterium.

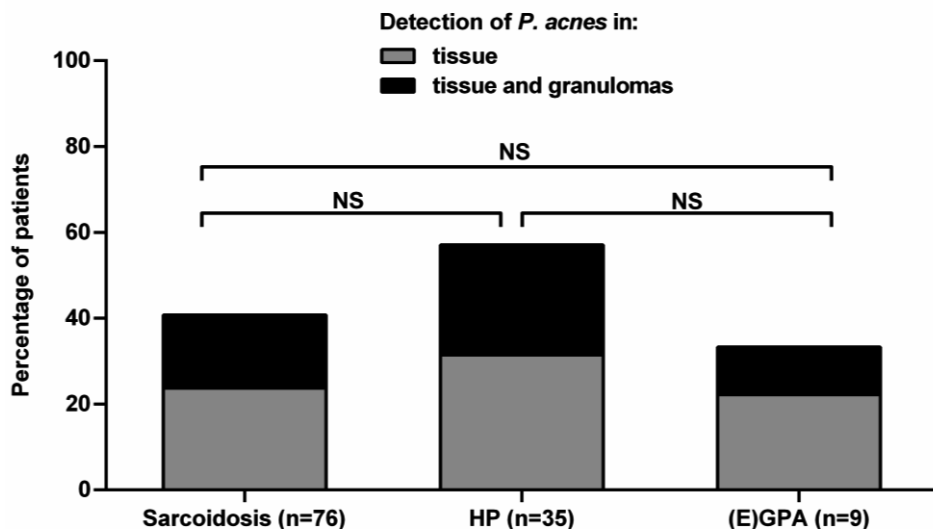


Figure 3. Percentage of patients with presence of *P. acnes* in tissue or in tissue and granulomas. The sarcoidosis group was previously described (6). No significant difference was observed between the three groups regarding *P. acnes* in tissue or *P. acnes* in granulomas ($P = 0.210$ and $P = 0.460$ respectively). HP: hypersensitivity pneumonitis, (E)GPA: (Eosinophilic) granulomatosis with polyangiitis, PAB: *P. acnes* specific monoclonal antibody that reacts with cell-membrane bound lipoteichoic acid (LTA) of the bacterium, NS: non-significant.

As presence of *P. acnes* appeared not to be specific to sarcoidosis and considering the known attributed mitogenic properties of this bacteria (7), we further explored such a role for *P. acnes* in our study by assessing BAL lymphocytosis in the patients with HP. Interestingly, a higher percentage of lymphocytes was indeed observed in HP patients stained positively for *P. acnes* in granulomas compared to HP patients without *P. acnes* staining in granulomas (70.3% vs 41.3%, $P = 0.018$) (Figure 4). The percentage of patients with presence of *P. acnes* in granulomas did not significantly differ between the HP group in which an inducing agent was known compared to the HP group in which the inducing agent was unknown (35.3% vs 16.7%, $P = 0.264$).

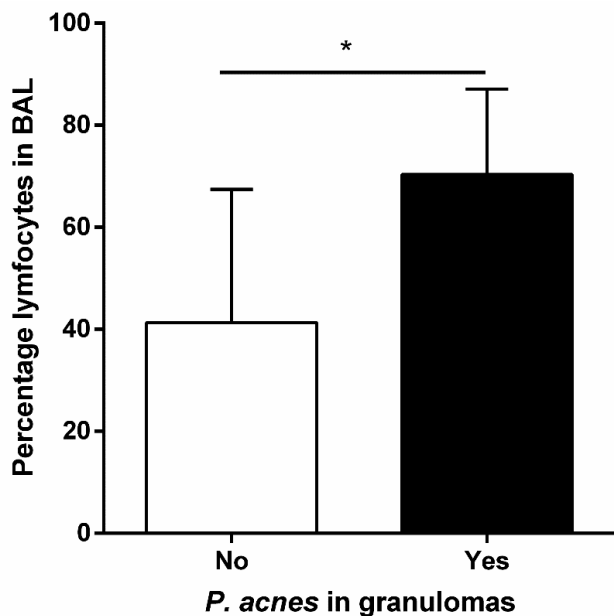


Figure 4. Lymphocyte percentage in BAL of HP patients with and without presence of *P. acnes* in granulomas.

A significantly higher percentage of lymphocytes was observed in HP patients stained positively for *P. acnes* in granulomas compared to HP patients without *P. acnes* staining in granulomas (70.3% vs 41.3%, $P = 0.018$).

BAL: Bronchoalveolar lavage, HP: hypersensitivity pneumonitis

Discussion

Up to now, considering granulomatous diseases, *P. acnes* has solely been related to the pathogenesis of sarcoidosis (8). Presence of *P. acnes* has been demonstrated in tissue and granulomas of Japanese, German and Dutch sarcoidosis patients (5,6). To the best of our knowledge, this is the first study that demonstrates that *P. acnes* can also be detected in tissue and granulomas of patients with HP and vasculitis. If presence of *P. acnes* is not disease specific, as our data suggests, one can debate whether *P. acnes* has an antigenic role in sarcoidosis pathogenesis. Regarding HP and (E)GPA pathogenesis no data is available suggesting an antigenic role for *P. acnes*. However, data on a possible mitogenic role for *P. acnes* have been described (7,9). The higher percentage of lymphocytes observed in BAL of HP patients with presence of *P. acnes* in granulomas, supports the hypothesis that *P. acnes* may indeed act as an mitogen by enhancing lymphocyte proliferation. Moreover, since we

observed presence of *P. acnes* in granulomas of HP patients in which a causal agent was previously identified it is unlikely that *P. acnes* act as a specific agent in HP.

The PAB antibody that has been used in this study reacts with cell-membrane bound lipoteichoic acid (LTA) of the *P. acnes* bacteria. LTA is a cell wall polymer of gram-positive bacteria and plays a role in bacterial growth, membrane homeostasis and virulence (10). Furthermore, LTAs have shown to be immunogenic (11) and activate the innate immune system via toll-like receptor 2 (TLR2) and NOD-like receptor family pyrin domain containing 6 (NLRP6) (12,13), key receptor families involved in the innate immune defense against invading pathogens. Interleukin-18 production can be stimulated following activation of NLRP6 (13). Results of several papers suggest that TLR2 and interleukin-18 are at least partly involved in granuloma formation (14,15) and *in vitro* and *in vivo* models have indeed shown that *P. acnes* is able to induce granulomas (3). It is therefore possible that *P. acnes* is not a specific trigger of sarcoidosis, HP and (E)GPA, but that its LTA contributes to inflammation and granuloma formation.

A limitation of the study is that no control group with healthy individuals was included in the study. It is however difficult to include an appropriate control group in the study, as healthy controls would usually not show granulomas in tissue. Another limitation was that we have not been able to analyze the disease course in HP patients due to the small sample size in combination with too much missing follow-up data. Furthermore, also the sample size of vasculitis patients from whom suitable tissue was available was too small to perform sub analysis on disease course or BAL. Consequently, we were unable to examine whether *P. acnes* is related to a chronic disease course in (E)GPA and HP as well. Due to the low samples sizes of the HP and EGPA group, the power to detect a difference with the sarcoidosis group was too small to conclude that those diseases and HP patients with and without a known inducing agent, do not really differ regarding presence of *P. acnes* in granulomas. Although the percentages of *P. acnes* in granulomas of the non-sarcoidosis group were quite comparable to the sarcoidosis group, further studies using a higher number of patients are needed to clarify whether there is really no difference in presence of *P. acnes* between those diseases. Last, in this study we only examined LTA of the *P. acnes* bacteria. It is plausible that LTAs of other gram positive bacteria can also be found in granulomas. Future studies will have to determine whether granulomas are uniquely related to LTAs of *P. acnes* or can be attributed to LTAs in general.

To conclude, we have shown that presence of *P. acnes* in granulomas is not unique for sarcoidosis but can also be found in patients with HP and EGPA. We hypothesize that *P. acnes* may be involved in the disease pathogenesis of those granulomatous diseases in a mitogenic way. Future studies are needed to determine the precise role of *P. acnes* and other LTAs in those granulomatous diseases.

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Chapter 9

Elevated Serum amyloid A levels are not specific for sarcoidosis but associate with a fibrotic pulmonary phenotype

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Abstract

Elevated SAA levels have been found in several inflammatory diseases, including sarcoidosis. SAA is suggested to be involved in sarcoidosis pathogenesis by involvement in granuloma formation and maintenance. We hypothesized that SAA serum levels would be higher in sarcoidosis compared to other non-infectious granulomatous and non-granulomatous diseases.

SAA levels were measured in serum from sarcoidosis, Hypersensitivity pneumonitis (HP) and (eosinophilic) granulomatosis with polyangiitis ((E)GPA) patients. Idiopathic pulmonary fibrosis (IPF) patients were included as non-granulomatous disease group.

SAA levels of patients with sarcoidosis (31.0 µg/mL), HP (23.4 µg/mL), (E)GPA (36.9 µg/mL) and IPF (22.1 µg/mL) were all higher than SAA levels of healthy controls (10.1 µg/mL). SAA levels did not differ between the diagnostic groups. When SAA serum levels were analyzed in sarcoidosis subgroups, fibrotic sarcoidosis patients showed higher SAA levels than sarcoidosis patients without fibrosis (47.8 µg/mL vs 29.4 µg/mL, $P = 0.005$).

To conclude, the observation that fibrotic sarcoidosis patients have higher SAA levels, together with our finding that SAA levels were also increased in IPF patients, suggests that SAA may next to granulomatous processes also reflect the process of fibrogenesis. Further studies should clarify the exact role of SAA in fibrosis and the underlying mechanisms involved.

Keywords: Sarcoidosis, SAA, fibrosis

Introduction

Serum amyloid A (SAA) is produced by the liver during the acute phase reaction (APR) which serves to eliminate foreign pathogens in a way that has not fully been elucidated yet (1). Elevated SAA levels have also been found in several inflammatory diseases, including rheumatoid arthritis, diabetes type II, Crohn's disease (2) and sarcoidosis (3–5). Sarcoidosis is a systemic disease which is characterized by formation of non-caseating granulomas through a complex immunological process. Lymph nodes, skin, eyes and lungs are the most frequent involved organs (6). The etiology of the disease is currently not completely clear, but probably results from the complex interplay between different etiologic agents and the immune system in predisposed individuals (7).

Multiple effects of SAA may relate to its contribution in sarcoidosis pathogenesis. First, SAA was found to contribute to Th17-cell proliferation and Th17 cytokine production (8). Th17-cells have been found to contribute in sarcoidosis pathogenesis (9,10) and in granuloma formation (11,12). Second, Chen *et al.* (13) studied the role of SAA in the regulation of granulomatous inflammation in sarcoidosis and found significant presence of SAA in granulomas of sarcoidosis patients which exceeded the intensity of SAA staining found in tissue from patients with tuberculosis, granulomatous polyangiitis or hypersensitivity pneumonitis (13). They therefore proposed that SAA might contribute to granuloma formation in sarcoidosis due to SAA overproduction as result of the intensity of the immune response in this disease. This accumulation of SAA might result in misfolding or aggregation of this protein and formation of complexes with other matrix proteins, leading to granuloma formation and entrapment of microbial antigens or autoantigens (13).

In our previous work we identified trigger related phenotypes in sarcoidosis based on immunological sensitization for mycobacterial antigens and presence of Propionibacterial antigens in granulomas (14,15). Based on its function as an acute phase protein upregulated by infections and contribution in bacterial clearance (1,16,17) together with its considered role as a biomarker in sarcoidosis, increased SAA levels may reflect underlying microbial antigens or autoantigens as triggers in a subgroup of sarcoidosis patients. This prompted us to further investigate the role of SAA in this heterogeneous disease. Based on the study of Chen *et al.* (13) we hypothesize that SAA serum levels will be higher in sarcoidosis compared to other non-infectious granulomatous and non-granulomatous diseases. Furthermore, we hypothesize that SAA levels in sarcoidosis patients with immunological sensitization for bacterial and auto-antigens will be higher compared to patients with immunological sensitization for inorganic triggers like metals or silica.

Material and methods

Study population

Two sarcoidosis cohorts previously studied in the St. Antonius hospital were included in the study (14,18). Sarcoidosis was diagnosed according to the criteria of the American Thoracic Society/European Respiratory Society (6). Hypersensitivity pneumonitis (HP) and (eosinophilic) granulomatosis with polyangiitis ((E)GPA) patients participating in our biobank study were included as granulomatous disease groups other than sarcoidosis. A cohort of patients with IPF (19) were included as non-granulomatous disease group. SAA levels of the granulomatous disease and non-granulomatous disease group were compared to a healthy control group.

Written consent was obtained from all patients and healthy controls. The study was approved by the Medical research Ethics Committees United (MEC-U) of the Antonius hospital (R05-08A).

SAA serum measurements and correlations

SAA levels were determined in serum from patients and controls through a SAA human ELISA kit (KHA0012, Invitrogen, Camarillo, CA) following the manufactures protocol. Data of C-reactive protein (CRP) and lung function at time of serum collection for SAA measurements were retrospectively collected from medical records. Correlations between SAA and CRP and lung function were determined for patients of whom data was available at time of serum collection used for SAA measurements.

Sarcoidosis patients with immunological sensitization to either bacterial, inorganic or autoantigens

Immunological sensitization to mycobacterial antigens, *Propionibacterium acnes* (*P. acnes*) catalase, vimentin and metals and silica were previously determined (14). Sarcoidosis patients in which sensitization was determined were divided in the following sub-groups: Bacterial sensitization (sensitization to mycobacterial antigens or *P. acnes* catalase), auto-antigen sensitization (sensitization to vimentin) and inorganic sensitization (sensitization to aluminum, beryllium, zirconium or silica). SAA serum levels of sarcoidosis patients with immunological sensitization to bacterial and autoantigens were compared to SAA levels of sarcoidosis patients with immunological sensitization to inorganic antigens.

Statistics

Study data were collected and managed using REDCap electronic data capture tools hosted at St. Antonius hospital. Data were analyzed using IBM SPSS statistics version 24. An unpaired T-test was used to compare numerical data between the groups. Non-parametric

tests were used for non-normally distributed data (Mann-Whitney U test). Categorical data were compared between the groups using the Chi-squared test. If expected cell frequencies were below 5, Fisher's exact test was used for categorical data up to two categories. Correlation between SAA and other variables was determined with Spearman's rho correlation. P-values <0.05 were considered significant.

Results

Demographics of study patients

SAA serum levels were measured in 215 sarcoidosis patients, 30 HP patients, 11 (E)GPA patients, 68 IPF patients and 200 healthy controls. Demographics can be found in Table 1.

SAA serum levels do not differ between patients with granulomatous and non-granulomatous diseases but are higher in general compared to healthy controls

Median SAA levels of sarcoidosis, HP, (E)GPA and IPF patients were all higher than the median SAA level of healthy controls. No differences in median SAA levels were observed between the different patient groups (Figure 1). When comparing the granulomatous diseases (sarcoidosis, HP, EAA, (E)GPA) with the non-granulomatous disease patients (IPF), no difference in median SAA levels was observed either (median 30.5 IQR 55.3 vs 22.1 IQR 55.5, $P = 0.737$).

SAA correlates with CRP and lung function

Significant moderate positive correlations were found between SAA and CRP serum levels. Significant weak negative correlations were observed between SAA and FEV1 percentage of predicted (Table 2). When analyzing the correlations per diagnostic group, only in sarcoidosis and IPF patients the correlation between SAA and CRP remained significant ($R = 0.384$ and $R = 0.598$ respectively, both $P < 0.001$). FEV1 showed only a weak negative correlation with SAA in sarcoidosis patients ($R = -0.191$, $P = 0.012$). Although no overall correlation was observed between SAA and DLCO, the sub analysis revealed a weak negative correlation in sarcoidosis patients ($R = -0.223$, $P = 0.005$) and a trend in IPF patients ($R = -0.265$, $P = 0.050$).

Table 1. Demographics of study patients

	HC (n=200)	Sar (n=215)	HP (n=30)	(E)GPA (n=11)	IPF (n=68)
Age ^a	46.6 ± 9.2	48.2 ± 11.8	59.6 ± 9.5	54.7 ± 13.9	69.5 ± 8.9
Sex (male)	92 (46)	117 (54)	15 (50)	7 (64)	60 (88)
Ethnicity (White)		190 (89)	30 (100)	11 (100)	67 (99)
Smoker (Never)		105 (49)	13 (43)	4 (36)	16 (24)
Medication	0	101 (47)	12 (40)	6 (55)	0
CRP (mg/L)		2.0 IQR 4.0	2.0 IQR 4.0	2.0 IQR 23.0	3.5 IQR 5.0
FVC % predicted		94.3 ± 20.3	78.5 ± 21.7	90.0 ± 21.2	80.1 ± 20.1
FEV1 % predicted		84.9 ± 21.5	81.2 ± 22.0	86.2 ± 20.1	82.9 ± 18.3
DLCO % predicted		73.5 ± 17.4	46.8 ± 13.8	58.9 ± 20.2	41.5 ± 11.4
Scadding stage					
0		44 (21)	NA	NA	NA
I		45 (21)			
II		50 (23)			
III		32 (15)			
IV		40 (19)			
Unknown		4 (2)			

Values are shown as absolute numbers with percentages in brackets.

^a Age, FVC % predicted, FEV1 % predicted and DLCO % predicted are shown as mean ± std. CRP, is shown as median IQR. Medication consisted of immunosuppressive medication including prednisone, methotrexate, azathioprine, infliximab, leflunomide, adalimumab, hydroxychloroquine and cellcept. CRP was available from 207 sarcoidosis patients, 27 HP patients, 9 (E)GPA patients and 68 IPF patients. FVC was available from 166 sarcoidosis patients, 27 HP patients, 11 (E)GPA patients and 59 IPF patients. FEV1 was available from 174 sarcoidosis patients, 26 HP patients, 11 (E)GPA patients and 60 IPF patients. DLCO was available from 160 sarcoidosis patients, 25 HP patients, 7 (E)GPA patients and 55 IPF patients.

HC: healthy control, Sar: sarcoidosis, HP: hypersensitivity pneumonitis, (E)GPA: (eosinophilic) granulomatosis with polyangiitis ((E)GPA), IPF: idiopathic pulmonary fibrosis, CRP: C-reactive protein, FVC: Forced vital capacity, FEV1: Forced expiratory volume in 1 second, DLCO: Diffusing capacity

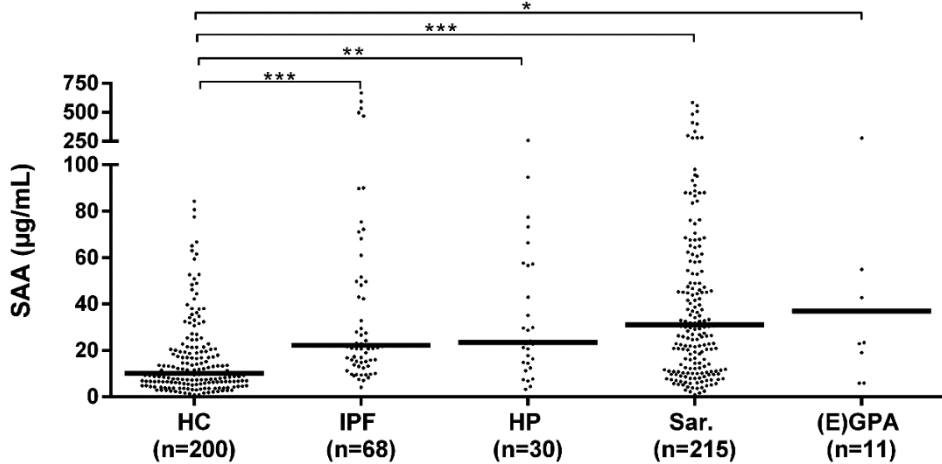


Figure 1. Serum SAA levels in patients and healthy controls.

Median SAA levels of IPF (22.1 IQR 55.5), HP (23.4 IQR 43.4), sarcoidosis (31.0 IQR 56.3) and (E)GPA patients (36.9 IQR 123.7) were all higher than the median SAA level of healthy controls (10.1 IQR 15.6). Median SAA levels did not significantly differ between the diagnostic groups.

HC: healthy control, Sar: sarcoidosis, HP: hypersensitivity pneumonitis, (E)GPA: (eosinophilic) granulomatosis with polyangiitis ((E)GPA), IPF: idiopathic pulmonary fibrosis.

P-values were calculated using Kruskal-Wallis test including pairwise comparisons

Table 2. Spearman’s rho correlations with SAA

	R	P
CRP (mg/L) (n=311)	0.408	< 0.001
FVC % predicted (n=263)	-0.084	0.173
FEV1 % predicted (n=271)	-0.154	0.011
DLCO % predicted (n=245)	-0.111	0.082

Correlations and P-values were determined by Spearman’s rho

CRP: C-reactive protein, FVC: Forced vital capacity, FEV1: Forced expiratory volume in 1 second, DLCO: Diffusing capacity

SAA levels are higher in patients using immunosuppressive medication

As part of the patients with sarcoidosis, HP and (E)GPA was using medication at time of SAA determination, we analyzed whether SAA levels differed between patients with and without medication. Overall, patients using immunosuppressive medication showed higher SAA levels than patients without medication. When considering the type of medication used, we only observed significant higher SAA levels in patients using prednisone (Table 3). When we compared the different diagnostic groups for SAA levels and the use of medication, we only observed significantly higher median SAA levels in sarcoidosis patients with medication compared to sarcoidosis patients without medication (44.4 µg/mL vs 26.3 µg/mL, P = 0.001). When we compared SAA levels per type of medication used, we again found significant higher SAA levels in sarcoidosis patients using prednisone. No difference was observed for the other types of medication used or for CRP levels.

Table 3. Median SAA levels in Sarcoidosis, HP and (E)GPA patients with and without immunosuppressive medication

Use of immunosuppressive medication	No (n=404)	Yes (n=119)	P
SAA (µg/mL)	16.9 IQR 30.2	43.8 IQR 72.5	<0.001
Prednisone	No (n=430)	Yes (n=93)	
SAA (µg/mL)	16.9 IQR 30.7	49.0 IQR 93.10	<0.001
MTX	No (n=488)	Yes (n=35)	
SAA (µg/mL)	20.6 IQR 40.0	25.9 IQR 56.4	0.536
Aza	No (n=515)	Yes (n=8)	
SAA (µg/mL)	20.6 IQR 40.0	32.2 IQR 32.7	0.232
Inf	No (n=516)	Yes (n=7)	
SAA (µg/mL)	20.7 IQR 40.0	35.0 IQR 87.6	0.531

MTX: Methotrexate, Aza: Azathioprine, Inf: infliximab

Sub analysis were performed only for medications used by 5 or more patients .

Leflunomide and hydroxychloroquine was used by 1 patient and adalimumab and cellcept by 2 patients

SAA levels do not differ between sarcoidosis patients with immunological sensitization to bacterial and autoantigens or inorganic antigens

The median SAA level of patients sensitized to bacterial and/or auto-antigens was 30.4 IQR 68.7 and to inorganic antigens 32.1 IQR 81.6 (Figure 2). SAA levels did not significantly differ between the groups (P = 0.719).

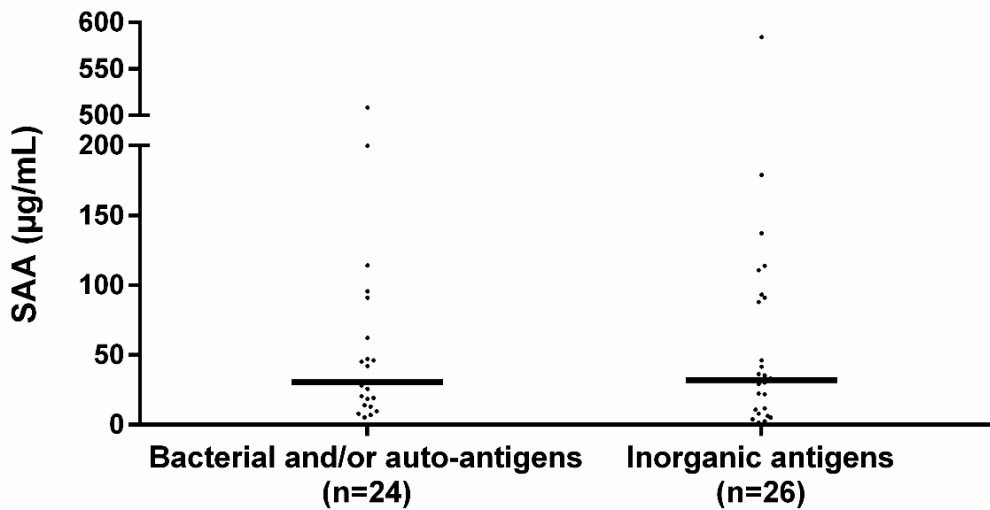


Figure 2. Serum SAA levels in sensitized sarcoidosis sub-groups.

Median SAA levels between bacterial sensitized and/or auto-antigen sensitized and inorganic agent sensitized sarcoidosis patients did not significantly differ.

Bacterial: sensitization to mycobacterial antigens or *P. acnes* catalase, Auto-antigen: sensitization to vimentin, Inorganic: sensitization to aluminum, beryllium, zirconium or silica.

P-values were calculated using Mann-Whitney U test.

SAA serum levels are higher in sarcoidosis patients with parenchymal fibrosis

Median SAA levels were compared between sarcoidosis patients with different Scadding stages. Comparing SAA levels between all Scadding stages showed a significant differences in median SAA levels between the 5 Scadding stages ($P = 0.025$, Table 4). Pairwise comparisons showed that Scadding stage IV patients had significantly higher median SAA levels compared to Scadding stage III patients (adj. $P = 0.012$). Furthermore, when SAA serum levels of Scadding stage IV (fibrosis) patients were compared with all other Scadding stages together (no fibrosis), significant higher median SAA levels were observed in the fibrotic group ($P = 0.005$). When we only examined sarcoidosis patients without use of immunosuppressive medication, median SAA levels were still significantly higher in the fibrotic group (48.9 vs 22.3, $P = 0.020$). No differences in median CRP levels were found between any of the Scadding stages.

Table 4. SAA levels among sarcoidosis patients with different Scadding stages

	Scadding stage					P
	0 (n=44)	I (n=45)	II (n=50)	III (n=32)	IV (n=40)	
SAA	27.8	32.7	31.3	21.6	47.8	0.025
(µg/mL)	IQR 43.5	IQR 67.7	IQR 48.7	IQR 39.1	IQR 76.4	
CRP	2.0	3.0	3.0	2.0	2.5	0.382
(mg/L)	IQR 4.0	IQR 7.0	IQR 4.0	IQR 6.0	IQR 5.0	
	0, I, II, III				IV	
SAA	29.4				47.8	0.005
(µg/mL)	IQR 51.3				IQR 76.4	
CRP	2.0				2.5	0.715
(mg/L)	IQR 5.0				IQR 5.0	

Values are shown as median IQR. CRP was missing from 2 Scadding stage II patients, 3 Scadding stage III patients and 2 Scadding stage IV patients.

P- values for multiple comparisons were calculated using Kruskal-Wallis test. Multiple comparisons were adjusted by the Bonferroni correction. P-values comparing 2 groups were defined by the Mann-Whitney U test.

Discussion

Although previous studies have suggested a possible role for SAA in the pathogenesis of sarcoidosis, our study shows this is not reflected by differences in serum levels between sarcoidosis and other pulmonary (granulomatous) diseases. SAA levels were elevated in patients with sarcoidosis, HP, (E)GPA and IPF compared to healthy controls. Within the group of sarcoidosis patients, SAA levels did not differ between patients with immunological sensitization to either bacterial and auto-antigens, or inorganic antigens. Important findings from our study are the increased SAA levels in patients with IPF and in sarcoidosis patients with pulmonary fibrosis compared to sarcoidosis patient without parenchymal fibrosis. Even though *in vitro* activated monocyte/macrophages are capable of producing SAA (20) and sarcoidosis is a monocyte/macrophage driven disease (21), this did not result in an overall increase in circulating SAA when compared to other inflammatory diseases. It may be that SAA produced in tissue does not necessarily contribute to the circulatory levels of SAA and vice versa. For instance, SAA was not observed in granulomas of GPA patients, while expression and serum levels were increased (13,22). Likewise, we observed elevated SAA levels in serum of HP patients while results of Chen *et al.* demonstrated limited to no SAA staining in granulomas of HP patients (13).

Chen *et al.* did observe that SAA patterns within granulomas of sarcoidosis patients were different from patients with other granulomatous diseases including chronic beryllium disease (CBD) and Crohn's disease. However, a lymph node of a patient infected with *Mycobacteria Tuberculosis* demonstrated patchy expression of SAA, localized mostly around the periphery of necrotizing granulomas (13). For this reason, combined with the fact that SAA is an acute phase protein upregulated by infections (1) and may have a role in clearance of bacterial antigens (16,17), we hypothesized that sarcoidosis patients sensitized to bacterial antigens had higher SAA levels than sarcoidosis patients sensitized to inorganic antigens such as metals and silica. The results of our study do not support this hypothesis. It is possible that blood is not the correct compartment to answer this question, and that immunohistochemical staining of SAA in granulomas may be required to determine whether there are different patterns between those subgroups of sarcoidosis patients and between sarcoidosis and other granulomatous disease patients.

Interestingly, we observed that sarcoidosis patients with a pulmonary fibrotic phenotype, had higher serum SAA levels than sarcoidosis patient without pulmonary fibrosis. Chen *et al.* showed that the extent of SAA staining correlated with the degree of collagen deposition (13). This suggests that SAA serum levels and tissue expression is associated with chronic inflammation and fibrosis. In line with the association between SAA and fibrosis, we observed a correlation between SAA and DLCO % predicted in sarcoidosis. In patients with systemic sclerosis (SSc), SAA was found to associate with pulmonary involvement and a negative correlation between SAA and DLCO was observed as well. Moreover, within SSc patients with different HRCT patterns, SSc patients with reticulation or honeycombing on HRCT, indicative for pulmonary fibrosis, had the highest serum SAA levels (23).

Comparable to our results, Vietri *et al.* showed that SAA levels in serum were higher in IPF patients than in healthy controls (24). They hypothesized that SAA is overproduced in response to fibrotic and hypoxic stimuli, since increased SAA levels are also observed in chronic obstructive pulmonary disease (COPD) (25) and obstructive sleep apnea (OSA) (26). Another possible explanation for the association between increased SAA levels and fibrosis may be that fibrosis is often accompanied by low-grade inflammation and tissue-barrier disruption (27). Since SAA contributes to (bacterial) antigen clearance, its production may be upregulated in response to the barrier disruption of fibrotic areas. A distinct, contrasting explanation is perhaps an anti-fibrogenic effect of SAA. This was observed in a mouse model of CCl₄-induced hepatic fibrogenesis in which liver fibrosis was enhanced after treatment with anti-SAA antibodies (28). Hypothetically, SAA production may be induced in response to fibrogenesis, to prevent further fibrosis formation.

We observed an overall correlation between CRP and SAA. After performing analysis in the diagnostic groups we only observed a significant correlation in sarcoidosis and IPF patients,

probably explained by the larger sample size of these patients groups. Although acute phase proteins such as CRP and SAA correlate quite well in several diseases (29–31), in our study SAA levels are higher in sarcoidosis patients with an pulmonary fibrotic phenotype compared to sarcoidosis patients without fibrosis, while CRP levels do not differ between these groups. This observation shows that determining SAA levels next to CRP levels can be meaningful in sarcoidosis patients, since those two acute phase proteins reflect different phases of disease.

An intriguing observation was the higher SAA level in patients using immunosuppressive medication at time of serum collection for SAA measurement. This effect was specifically seen in sarcoidosis patients using prednisone. Comparable to our observation, Yamada *et al.* also observed higher SAA levels in rheumatoid arthritis patients receiving oral prednisolone (32). An inducing effect of prednisolone on SAA production of HepG2 cells have been found (33). Interestingly, a major increase in SAA expression in human monocytes and macrophages was found after a combined treatment with lipopolysaccharide (LPS) and dexamethasone or methylprednisolone (20). In a previous study, we showed the presence of *C. acnes* lipoteichoic acid (LTA) in granulomas of sarcoidosis patients (15). Comparable to LPS of gram-negative bacteria, is LTA a cell wall polymer of gram-positive bacteria and plays a role in bacterial growth, membrane homeostasis and virulence (34). Furthermore, LTAs have shown to be immunogenic and activate the innate immune system via TLR2 and NLRP6 (35,36). It is possible that bacterial molecules including LPS and LTA together with glucocorticoids stimulate macrophages within granulomas to produce SAA, explaining the higher SAA levels observed in sarcoidosis patients using prednisone.

Based on the results of our study, it is not possible to determine whether medication itself has an effect on SAA levels or that SAA levels are increased in the more severe patient group needing immunosuppressive therapy. However, since we observed higher SAA levels in fibrotic sarcoidosis patients who did not use medication, there have to be other factors that determine the higher SAA levels we observed in fibrotic sarcoidosis patients compared to non-fibrotic sarcoidosis patients. It has been suggested that SAA is useful as biomarker in sarcoidosis, since it is not dramatically affected by immunosuppressive therapy (37). Further studies will have to clarify the association between immunosuppressive therapy and SAA, for example by performing SAA measurements before, during and after treatment.

Our study has some limitations. First, SAA levels were not measured at time of diagnosis for all patients. For this reason, we were not able to analyze whether increased SAA levels were predictive for disease course. We were not able to measure SAA levels of all patients at time of diagnosis since we made use of existing patient cohorts from which serum at time of diagnosis was not available. However, by making use of these patient cohorts, we were able

to analyze SAA levels between sensitization subgroups within the sarcoidosis patients, which had never been performed before. A second limitation was that a proportion of the patients were using immunosuppressive medication at time of SAA measurements. We were not able to track down whether increased SAA levels observed in the patients using medication was due to the medication itself or rather due to the more severe disease course of these patients. Further studies will have to reveal the effect of immunosuppressive medication on disease course, for instance by measuring SAA levels at diagnosis and during follow-up in one cohort of patients.

To conclude, increased SAA levels in serum is not specifically associated with sarcoidosis but can also be found in other granulomatous diseases such as HP and (E)GPA. In patients with sarcoidosis, the level of serum SAA is associated with the concomitant use of glucocorticoids. The fact that elevated SAA levels were observed in patients with IPF and were significantly higher in sarcoidosis patients with pulmonary fibrosis compared to patients without parenchymal fibrosis, suggests that SAA may also reflect the process of fibrogenesis. Further studies should clarify the exact role of SAA in fibrosis and the underlying mechanisms involved in the effects of glucocorticoids on SAA levels in sarcoidosis.

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Chapter 10

Summary and general discussion



The hallmark of sarcoidosis is the formation of non-caseating granulomas (1), which are compact organized structures of mature macrophages accompanied by other immune cells including lymphocytes and plasma cells. They are thought to be formed when phagocytes are not able to destroy certain particles. Granulomas are not specific for sarcoidosis since granulomas can be formed in response to infectious agents, foreign bodies, many inflammatory and autoimmune conditions (2). The diagnosis of sarcoidosis is therefore based on exclusion of granulomatous infectious diseases or other causes of granulomas (3). The presentation of sarcoidosis can be very heterogeneous and virtually any organ can be affected. The lungs and hilar and mediastinal lymph nodes are the most frequently involved (in about 90% of cases), followed by involvement of the skin, eyes and liver (4). Moreover, the course of the disease is unpredictable, meaning that it may either spontaneously resolve or become chronic (5). Due to the fact that various antigens are able to stimulate granuloma formation together with the heterogeneous presentation observed among sarcoidosis patients, it seems plausible that multiple antigens are able to trigger sarcoidosis development. This thesis addresses the question whether multiple potential triggers relate to different presentations or disease course of sarcoidosis, and aimed to identify trigger-related phenotypes.

Summary

Sarcoidosis has a complex disease pathogenesis, with involvement of both innate and adaptive immune responses. Several innate immune system receptors including NOD-like receptors and Toll-like receptors appear to be involved in the development of sarcoidosis as well as cellular players such as dendritic cells and macrophages. Furthermore, lymphocytes from the adaptive immune system including Th1, Th17, regulatory T cells, and B cells are likely to play a role in sarcoidosis pathogenesis as well. It is likely that sarcoidosis does not have one single cause but rather results from the complex interplay between different etiologic agents and the immune system in predisposed individuals. **Chapter 2** provides an overview of possible etiologic agents of sarcoidosis. Although sarcoidosis is a systemic disease, the intrathoracic lymph nodes and lungs are the most frequently involved organs. This has led to the hypothesis that the etiologic agent might be airborne. Both exogenous organic, including mycobacterial antigens and propionibacterial antigens, and inorganic components, like metals and silica, are potential candidates triggering the disease. More recent data also suggest that self-antigens, such as vimentin and serum amyloid A (SAA), could be involved in triggering sarcoidosis pathogenesis.

Chapter 3 describes in detail three patients in which the combination of exposure to inorganic substances and related immunological sensitization is, in our opinion, highly

suggestive for a causal relationship. These observations made in the ILD-clinic prompted us to further investigate the possibility of trigger-related phenotypes in sarcoidosis.

In **chapter 4** both occupational exposure and immunoreactivity to metals and silica was characterized in a group of sarcoidosis patients and controls. Standardized assessment of in-depth occupational exposure is not included in the current diagnostic work-up of sarcoidosis. When beryllium exposure is clinically suspected in patients with sarcoidosis, chronic beryllium disease (CBD) can be excluded by testing for beryllium immunoreactivity using a Lymphocyte Proliferation Test (LPT). This is important since CBD can be histopathological and clinical indistinguishable from sarcoidosis (6). Also other inorganic antigens such as aluminum, zirconium and silica are suggested to play a role in the pathogenesis of sarcoidosis (7–9). At present, however, a causal relationship between these antigens and sarcoidosis has not been established. First because studies using a control group are lacking and second because exposure is difficult to assess. Identification of possible etiologic antigens in sarcoidosis is important since it may lead to a more personalized treatment regime. For instance, avoiding exposure might be the first step in the treatment of sarcoidosis patients in whom metal or silica might trigger the disease pathogenesis. In patients with CBD, improved lung function and radiographic findings can indeed be observed when exposure is terminated or decreased (10,11).

We observed no differences in occupational groups or occupational exposure to metals and silica between the sarcoidosis and control patients. However, sensitization to the metals tested (aluminum, beryllium and zirconium) and silica was only observed in sarcoidosis patients. Taken together, significantly more sarcoidosis patients than controls had immunological sensitization to any of the antigens tested. This observation supports the concept that metals and silica might indeed be involved in the pathogenesis of sarcoidosis in a subgroup of patients. The finding of sarcoidosis patients with beryllium sensitization demonstrates that also in the Netherlands, patients with CBD can be identified within a group of biopsy proven sarcoidosis patients. Since immunological sensitivity to metal and silica, but not occupational exposure, differed between patients and controls, genetic variants may be involved in the ability to respond to the substances addressed in this study. This has already been shown in CBD where a Glu at position 69 in the HLA-DPB1 gene is found in approximately 90% of patients compared to about 40% of healthy controls (12,13). Moreover, occupational exposure was not associated with sensitization in our study. A possible explanation for this observation may be that even very low occasional exposures to potentially immune sensitizing substances can be relevant in genetic predisposed individuals (14,15) and that a simple occupational history is not detailed enough to identify these exposures.

Considerable research effort has focused on identification of triggers of sarcoidosis, including antigens of mycobacteria (16,17), *Propionibacterium acnes* (*P. acnes*) (18,19), vimentin (20,21), metals and silica (9,22). Although all these antigens can elicit non-caseating granulomas, results demonstrate that sarcoidosis probably has multiple triggers. Importantly, however, none of the above organic and inorganic antigens have been studied simultaneously in one group of sarcoidosis patients. In **chapter 5** we therefore describe peripheral T-cell responses to organic and inorganic antigens, simultaneously studied in one cohort of sarcoidosis patients. Antigen sensitization was analyzed in relation to clinical characteristics for identification of possible trigger-related phenotypes. We found no association between immunological sensitization towards the organic antigens and sarcoidosis. Quite unexpectedly, we found immunological sensitization towards propionibacterial antigens in a significantly lower percentage of sarcoidosis patients than controls. Sarcoidosis patients sensitized to antigens of *P. acnes*, were younger at diagnosis and showed a trend towards more often skin involvement than sarcoidosis patients without propionibacterial antigen sensitization. Immunological sensitization towards mycobacterial antigens was also found in a low percentage of patients with sarcoidosis suggesting that this is not a major causative antigen in Dutch patients. However, sarcoidosis patients sensitized for mycobacterial antigens had more often cardiac involvement than sarcoidosis patients without mycobacterial sensitization. Significant more sarcoidosis patients than control patients showed sensitization to metals and silica. Finally, we observed that sensitization to metals and silica is associated with development of fibrotic sarcoidosis 5 years after diagnosis. Our study therefore suggests a more prominent role for inorganic triggers such as metals and silica in sarcoidosis pathogenesis than previously thought. The data on mycobacteria and presence of cardiac sarcoidosis, the possible relation between sensitization to *P. acnes* and cutaneous involvement, and the association of fibrosis with metal and silica sensitization, also suggest that trigger-related phenotypes do exist in the heterogeneous population of sarcoidosis patients.

In **chapter 6** the possible association between a latent tuberculosis infection (LTBI) and cardiac involvement in sarcoidosis was further evaluated based on the data presented in chapter 5. About 5% of sarcoidosis patients have symptoms of cardiac sarcoidosis (CS), although the prevalence of CS is estimated to be 20-30% in autopsy and imaging studies (23). Identification of cardiac involvement is important since it is a major cause of death among sarcoidosis patients (24,25). Interestingly, in chapter 5 it was found that three of the five LTBI patients had cardiac involvement of their sarcoidosis. In the current chapter we used another cohort of CS patients to investigate whether an LTBI associates with cardiac

involvement in sarcoidosis. We observed that significantly more CS patients had an LTBI compared to non-CS patients. This is in concordance with the preliminary findings of chapter 5 where we observed that sarcoidosis patients sensitized for mycobacterial antigens had more often cardiac involvement compared to sarcoidosis patients without mycobacterial sensitization. If the association between LTBI and CS can be confirmed in future research, this could be of interest for clinical management. For instance, it could be relevant to screen sarcoidosis patients with an LTBI for CS, even though symptoms may not be present (yet).

In **chapter 7** the presence of *P. acnes* in granulomas of Dutch sarcoidosis patients was demonstrated using immunohistochemistry. Results of the staining were studied in relation to disease course. As *P. acnes* is a commensal, Koch's postulates cannot be applied, which makes it difficult to prove its etiologic role in sarcoidosis beyond doubt (26). However, a higher number of *P. acnes* genomes have been found in tissue from Chinese, Japanese, Italian, English and German sarcoidosis patients compared to tissue of controls, suggesting involvement in disease pathogenesis (27–29). Furthermore, an increased immune response to *P. acnes* among patients with sarcoidosis was found in different studies using Japanese or German patients (30–32). In addition to its role as possible antigen, *P. acnes* can also act as a mitogen which is demonstrated by the fact that *P. acnes* enhances immunogenicity of certain vaccines (33) and enhances cytotoxic activity toward different tumor cells (34–36). We showed in this chapter that *P. acnes* could be detected in tissue samples of a subgroup of Dutch patient with sarcoidosis. Moreover, the presence of *P. acnes* inside granulomas was associated with a chronic disease phenotype and requirement of treatment. Regardless of whether the role of *P. acnes* is antigenic or mitogenic, our data contribute to the premise that it is relevant to further explore antibacterial therapy and therapy against specific bacterial antigens as treatment options for a subset of sarcoidosis patients.

To further explore whether presence of *P. acnes* is unique for sarcoidosis, in **chapter 8** we examined granulomatous tissue of Granulomatosis with polyangiitis (GPA), Eosinophilic granulomatosis with polyangiitis (EGPA) and hypersensitivity pneumonitis (HP) patients for presence of *P. acnes*. Several studies have demonstrated that *P. acnes* is able to induce granulomas (37). Furthermore, *P. acnes* is suggested to be involved in the pathogenesis of sarcoidosis. Granulomas are however not specific for sarcoidosis, but are also observed in other diseases (38). More specifically, granulomas are seen in diseases other than sarcoidosis in which the lungs are frequently involved, including HP, GPA and EGPA (39). Although presence of *P. acnes* has already been shown in granulomas of sarcoidosis patients

(40,41), it is unknown whether this bacterium can be found in granulomas of those above mentioned granulomatous diseases.

Interestingly we found presence of *P. acnes* in tissue of HP and (E)GPA patients. More specifically, presence of *P. acnes* was also observed in granulomas of HP and (E)GPA patients. The percentage of patients with presence of *P. acnes* in tissue or granulomas was not different from the sarcoidosis cohort studied in chapter 7. These data suggest that presence of *P. acnes* in tissue and granulomas is not disease specific and the role of *P. acnes* as a specific antigenic trigger in sarcoidosis could therefore be questioned. Regarding the possible mitogenic effect of *P. acnes* observed among sarcoidosis patients in chapter 7, we wondered whether features pointing to a mitogenic effect of *P. acnes* could be observed in other inflammatory diseases as well. We therefore examined whether bronchoalveolar lavage (BAL) cell subsets differed between HP patients with and HP patients without presence of *P. acnes* in granulomas. Interestingly, HP patients who had presence of *P. acnes* in granulomas showed a higher percentage of lymphocytes in BAL than HP patients in whom presence of *P. acnes* was not detected in granulomas. This observation supports the rational that antigens of *P. acnes* might act as a mitogen by enhancing lymphocyte proliferation in HP. It is therefore possible that *P. acnes* is not a specific trigger of sarcoidosis, HP and (E)GPA, but that it contributes to granuloma formation and maintenance in a mitogenic way.

In **Chapter 9** SAA levels in serum of granulomatous diseases including sarcoidosis, HP, (E)GPA were compared with SAA serum levels of patients with idiopathic pulmonary fibrosis (IPF), as non-granulomatous group, and healthy controls. Serum amyloid A (SAA) is produced by the liver during the acute phase reaction (APR) which serves to eliminate foreign pathogens. Elevated SAA levels have also been found in several inflammatory diseases, including rheumatoid arthritis, diabetes type II, Crohn's disease (42) and sarcoidosis (43–45). Chen *et al.* (46) studied the role of SAA in the regulation of granulomatous inflammation in sarcoidosis and found presence of SAA in granulomas of sarcoidosis patients. They proposed that SAA might contribute to granuloma formation in sarcoidosis due to SAA overproduction as result of the intensity of the immune response in this disease. These abundant SAA levels likely result in misfolding or aggregation of SAA which could serve as a seed for further amyloid-like, protein aggregation leading to entrapment of microbial antigens or autoantigens resulting in granuloma formation (46). Moreover, being a TLR2 ligand (47,48), SAA might function as a feed forward loop to conserve granuloma formation. Although we observed higher serum SAA levels in serum of sarcoidosis, HP and (E)GPA patients than in serum of healthy controls, we did not observe a difference in SAA levels between the diagnostic groups. Moreover, we did also not observe

differences in SAA levels between the granulomatous and non-granulomatous diseases. Interestingly, however, we observed that sarcoidosis patients with a pulmonary fibrotic phenotype, had higher serum SAA levels than sarcoidosis patients without pulmonary fibrosis and that SAA levels negatively correlated with DLCO % predicted. This observation, together with our finding that SAA levels were not increased solely in granulomatous diseases but also in IPF patients, suggests that SAA can play a role in both granuloma formation as well as in fibrogenesis.

General discussion

Sarcoidosis is hallmarked by non-caseating granulomas (1) which are compact organized structure of immune cells thought to be formed in response to inability of phagocytes to destroy certain particles (2). Granulomas are not specific for sarcoidosis and in several other granulomatous diseases the underlying cause of the granulomas can be established. For instance, granulomas are observed in some infectious diseases including tuberculosis and histoplasmosis (49). When the cause of granulomas remains unknown, and the clinical and radiological features are compatible with sarcoidosis, the diagnosis of sarcoidosis is made based on exclusion of known granulomatous infectious diseases. Since sarcoidosis has a very heterogeneous presentation and multiple agents have been related to granuloma formation, it is plausible that the disease is rather a collection of several granulomatous diseases induced by different granuloma stimulating triggers. The cause of sarcoidosis is puzzling scientists for more than a century and considerable research effort has focused on the identification of triggers of sarcoidosis. However, multiple organic, inorganic and self-antigens have not been studied before in the same cohort of sarcoidosis patients. Moreover, relations between possible triggers and clinical data for the identification of new clinical phenotypes have not been described. In this thesis we therefore aimed to study the association between organic as well as inorganic antigens and sarcoidosis, and searched for disease phenotypes that correlated with the antigens studied.

Inorganic triggers

So far, the possible antigenic role of metal and silica in the disease pathogenesis of sarcoidosis was merely based on case reports and epidemiological studies. We were able to show that an immunological response to metals and silica associated with sarcoidosis in a study involving over 100 sarcoidosis patients and a non-sarcoidosis control group. Furthermore, we observed that immunological sensitization to metals or silica in sarcoidosis patients was associated with development of pulmonary fibrosis at follow up. This is in concordance with patients we do observe in clinical practice. Some of them are diagnosed with sarcoidosis and had high occupational exposure to metal or silica, such as workers in

metal industrial or plasterers. These subjects usually present with some atypical clinical as well as radiological features of sarcoidosis. This may include digital clubbing and pulmonary crackles on auscultation, indicative for the development of pulmonary fibrosis. Based on our findings it may now be informative to perform an LPT on different metals and silica, to examine the possible contributing agents of this clinical presentation of sarcoidosis. Since fibrosis is irreversible and extensive fibrosis will lead to a decline in lung function and a decreased diffusion capacity (50), it is of great importance that the fibrotic process will not be stimulated further. Moreover, it was indeed shown in patients with CBD that termination of exposure is beneficial in the context of disease progression (10,11). The avoidance of further exposure to the antigens for which sensitization is found, may therefore be relevant to prevent chronicity and/or further disease progression. Of note, we also showed that a positive LPT was present in patients without occupational metal and silica exposure, based on the job exposure matrices used. Several factors may be responsible for this observation. First, relevant exposures to initiate sarcoidosis may be originated from other sources than occupations (15), for instance certain recreational activities (51), living in an environment high in metal fumes (52,53) or wearing certain prosthetic implants (54). Second, it is possible that even very low amounts of exposures, which are not detected by the occupational questionnaire coupled to the job exposure matrix, are able to induce immunological sensitization and trigger sarcoidosis in genetic predisposed individuals. Genetic susceptibility has already been shown in CBD. Individuals expressing HLA-DPB1 alleles that are characterized by the presence of a glutamic acid at position 69 of the beta chain, are thought to develop beryllium sensitization upon beryllium exposure due to a conformational change in the HLA-DP/peptide complex induced by the Be^{2+} cation (55). Peptides of the plexin A protein may be the self-peptide present in the HLA-DP binding groove which after interaction with the beryllium cation are conformational changed and seen as foreign by HLA-DP specific CD4 T-cells (56,57).

It is possible that sensitization to other inorganic agents, including aluminum, silica and zirconium, are also induced by conformational changes in the MHCII-peptide binding complex. To study such immunological mechanisms, a larger group of patients with a positive LPT for these antigens is needed. Although it might be difficult to accurately screen all sarcoidosis patients on relevant exposure, it may be important in light of the disease course, to identify the source of exposure as soon as sensitization is determined.

Regarding the LPT, a stimulation index between 2.0 and 3.0 is considered weakly positive, making interpretation difficult. A weakly positive test does not prove, but rather suggests sensitization. However, when comparing LPT results using a stimulation index > 2.0 , we found a remarkable difference between patients and controls, with 27.6% of sarcoidosis patients showing an immunological response compared to only 4.2% of controls. Moreover,

we showed a significant correlation between the MELISA® LPT on beryllium and the official beryllium LPT following the ATS guidelines. It was shown that the specificity of this latter assay was quite high (0.969) (58), making the presence of false positive results less likely. A larger sarcoidosis and control cohort investigated for metal and silica sensitization is warranted to clarify the meaning of a weak positive LPT in sarcoidosis and to determine the specificity of the MELISA® LPT for metals and silica in sarcoidosis.

Propionibacterium acnes

Although we were able to show an association between sensitization to inorganic agents and sarcoidosis, we found no evidence for an association between sensitization to antigens of mycobacteria, vimentin or *P. acnes* and sarcoidosis in Dutch patients. Interestingly, we even observed that significant less sarcoidosis patients than control patients had a positive elispot test for *P. acnes* catalase. Since *P. acnes* is a commensal of the skin in the majority of the population (59) it is not surprising that sensitization to antigens of this bacterium can be found in healthy individuals. The finding that less sarcoidosis patients than controls showed sensitization to a specific antigen of this bacterium might have different explanations. First, in sarcoidosis patients a decreased peripheral cellular T-cell function to common recall antigens has been described, a phenomenon called T-cell exhausting and anergy (60). The appearance of peripheral T-cell anergy to common recall antigens at one hand and a prominent T-cell inflammatory reaction at affected sites at the other hand, is described as the immune paradox in sarcoidosis (61). This immune paradox might be caused by an unbalance in regulatory T-cells and memory T-cells, whereby the abundant presence of regulatory T-cells excessively suppress memory T-cell proliferation in response to recall antigens. Those regulatory T-cells were, on the other hand, found to ineffectively suppress TNF- α production. Since TNF- α is a major player in granuloma formation, this inability to effectively inhibit abundant TNF- α production may explain the granulomatous inflammation observed at diseased organs in sarcoidosis (60). Second, in addition to T-cell anergy in peripheral blood, it might be that *P. acnes* catalase specific T-cells are homed to inflammatory sites such as the lungs, lymph nodes and skin. In this scenario, blood may not be the most suitable compartment to study sensitization to *P. acnes*. It is however more invasive to study T-cell responses to *P. acnes* in other compartments, since this will require performance of bronchoalveolar lavages (BALs) or lymph node punctures. For this reason, we used immunostaining to examine whether *P. acnes* could be detected in tissue and granulomas of sarcoidosis patients.

Although presence of *P. acnes* was already shown in tissue and granulomas of Japanese and German patients with sarcoidosis (40), we showed for the first time that *P. acnes* is also present in tissue and granulomas of Dutch patients with sarcoidosis. In addition, we

observed that sarcoidosis patients in which *P. acnes* was detected in granulomas had a more chronic and severe disease course than sarcoidosis patients in which *P. acnes* was not demonstrated in granulomas. We however also observed that this *P. acnes* antigen was present in granulomas of patients with other diseases. Studies investigating the lung microbiome in sarcoidosis patients did not observe a difference in the microbiome composition and microbiome diversity between sarcoidosis and other ILD patients (62–64). Second, no difference in the abundance of *P. acnes* was found between sarcoidosis and other ILD patients (63). It could therefore be expected to find tissue presence of *P. acnes* not solely in sarcoidosis but in other ILDs as well.

Finally, our observation that a specific T-cell response to an antigen of *P. acnes* is not common in peripheral blood of sarcoidosis patients, and the finding that antigens of *P. acnes* are not unique for granulomas of sarcoidosis patients, do not support a role of *P. acnes* as specific antigenic trigger in sarcoidosis. Together with our observation that *P. acnes* in granulomas associated with a chronic disease course in sarcoidosis and with a higher lymphocyte percentage in BAL of HP patients, it seems likely to assume that lipoteichoic acids (LTAs) of *P. acnes* acts as a mitogen. Indeed, a mitogenic effect of *P. acnes* has been described previously (65).

Bacterial mitogenic effects

Although immunomodulatory effects have been found for *P. acnes* (33,36,66) it is plausible that such properties are not specially related to the *P. acnes* bacteria but that other commensal bacteria could exert similar effects. For example, *Corynebacterium pyruviciproducens* was found to have immunomodulatory effects since it enhanced macrophage proliferation, maturation and differentiation. Those effects on differentiation and maturation were especially found for macrophages with high levels of MHCII and co-stimulatory factors, which indicates that *Corynebacterium pyruviciproducens* promotes antigen presentation and interaction of macrophages with T- lymphocytes (67).

The PAB antibody that was used in our study reacts with cell-membrane bound LTA of the *P. acnes* bacteria. LTA is a cell wall polymer of gram-positive bacteria and plays a role in bacterial growth, membrane homeostasis and virulence (68). Furthermore, LTAs have shown to be immunogenic (69) and activates the innate immune system via TLR2 and NLRP6 (70,71). Interleukin-18 (IL-18) production can be stimulated following activation of NLRP6 (71). Interestingly, several papers have showed increased IL-18 levels in BAL and blood of sarcoidosis patients compared to controls (72) and it was shown that IL-18 plasma levels were significantly higher in sarcoidosis patients with disease progression and/or need of steroid treatment (73). As we observed that *P. acnes* was present in granulomas of patients

with a chronic disease course and need for treatment, it is plausible that LTAs cause an inflammatory response resulting in this chronic disease course.

Results of several papers suggest that TLR2 is at least partly involved in granuloma formation (46,74–77). It is possible that LTAs of other gram-positive bacteria are also able to stimulate the immune system via NLRP6 and TLR-2 and therefore contribute in inflammation and granuloma formation as well. First, *Corynebacterium tuberculo-stearicum* and *Staphylococcus epidermidis*, both commensals of the human skin (78), were indeed found to induce immune responses via TLR2 (79,80). Second, Corynebacteria have been associated with granulomatous diseases (81,82). It is therefore plausible that besides LTAs of the *P. acnes* bacteria also LTAs of other gram-positive bacteria can be found in granulomas of varying diseases. Future studies using antibodies against LTA of other gram-positive bacteria should indicate whether this is indeed the case.

Concluding, although our results suggest that *P. acnes* is rather a mitogen and not a specific antigen for sarcoidosis, identification of this bacterium in granulomas of sarcoidosis patients may be of importance. Since presence of antigens of this bacterium in granulomas was found to associate with a chronic medication requiring phenotype, and more specific, with the need for 3rd line medication, it seems valuable to evaluate whether this specific subgroup of patients can benefit from the use of antibacterial therapy or more specific therapy against bacterial LTAs.

Trigger-related phenotypes

The main aim of this thesis was to identify trigger-related phenotypes. In a heterogeneous group of sarcoidosis patients, which was studied simultaneously for sensitization to different antigens, we indeed observed trigger-related phenotypes. Two trigger-related phenotypes, namely pulmonary fibrosis related to inorganic agent sensitization, and a chronic disease course in patients with granulomas positive for *P. acnes*, are described extensively in the previous paragraphs. These 2 trigger-related phenotypes could possibly be explained by difficult clearance of inorganic agents leading to an ongoing inflammatory granulomatous reaction resulting in fibrosis (83), and the fact that *P. acnes* might act as a mitogen (65) resulting in an ongoing inflammatory reaction, contributing to perpetuation of the inflammatory granulomatous response.

We observed a third, more surprising, trigger-related phenotype, namely the association between a latent tuberculosis infection (LTBI) and cardiac involvement in sarcoidosis. An explanation for this trigger-related phenotype is more difficult to find. An interesting observation from literature however is that HLA-DQB1*0601 is both associated with cardiac involvement of sarcoidosis as well as susceptibility to Tuberculosis among Asian patients (84,85). It would be interesting to study whether such gene polymorphisms could also relate

an infection with *Mycobacterium tuberculosis* to cardiac involvement of sarcoidosis in Caucasian sarcoidosis patients.

The existence of molecular mimicry may be the underlying mechanisms explaining this association, and in more general, may underlie in the existence of trigger-related phenotypes. Molecular mimicry means that similar epitopes are shared on different proteins. For instance, bacteria and viruses can contain epitopes similar to epitopes present on the host's major histocompatibility complex (MHC). When an immune response is initiated to microbial antigens, tolerance to self-antigens can be broken and a cross-reaction to similar host proteins may develop, which could lead to tissue injury and auto-immune disease (86).

Tuberculosis reactive T-cells have indeed been found to recognize self-antigens (87,88). Moreover, an association between a historical mycobacterial infection and the development of auto-immune diseases have been observed in several studies (89,90). Molecular mimicry between mycobacterial heat shock proteins (HSPs) and host HSPs have been thought to play a role in the pathogenesis of sarcoidosis (91) and increased levels of anti-HSP antibodies were found in sera from sarcoidosis patients (92). Interestingly, Naruse *et al.* suggested that, among other genes, HSP70 may be a candidate gene for the development of cardiac sarcoidosis (84).

The cross-reactivity between pathogenic epitopes and host epitopes could be an explanation for the associations observed in this study including the association between cardiac involvement and an LTBI, and the possible association between skin involvement and sensitization to antigens of *P. acnes* catalase.

Serum amyloid A

The acute phase protein SAA was proposed to contribute to granuloma formation in sarcoidosis by overproduction resulting in misfolding or aggregation of SAA which could serve as a seed for further amyloid-like protein aggregation and entrapment of microbial antigens or auto-antigens. Based on its contribution to granuloma formation, we hypothesized that SAA levels would be higher in serum from patients with granulomatous diseases compared to patients with non-granulomatous diseases.

First, we observed that SAA levels were not significantly different between the patients with granulomatous diseases and patients with IPF, while both patient groups showed significant higher SAA levels than the healthy control group. Second, we observed that sarcoidosis patients with Scadding stage IV showed higher SAA levels than sarcoidosis patients without pulmonary fibrosis.

An association between SAA and fibrosis have been observed in other cases (93–95) in which the underlying cause of the elevated SAA levels can be related to an underlying

inflammatory process. However, we observed elevated SAA levels in IPF patients, a disease which not seems to be initiated in response to a primary immunopathogenic mechanism (96). Possibly, elevated SAA levels could be caused by, or could contribute to fibrotic processes as well. Indeed, SAA have been found to induce the production of matrix metalloproteases (97) which are thought to be critical players in the pathogenesis of lung fibrosis (98). Moreover, SAA was found to induce IL-6 production by human fibroblasts and monocytes (99,100). IL-6 seems to be involved in a fibrotic process by inducing production of collagen I (101) and blockage of IL-6 showed indeed attenuated collagen production and fibrosis in a cardiac rat model (102). Although IPF may not be initiated by a primary immunopathogenic mechanism, secondary inflammation may contribute in its pathogenesis (96). So possibly, SAA is involved in or contributes to a fibrotic process in several ways.

Due to the recruiting effect of microbial antigens on SAA production, we expected that SAA levels were higher in sarcoidosis patients with bacterial sensitization compared to sarcoidosis patients with inorganic sensitization. We were however not able to show that there was a difference between these subgroups of sarcoidosis patients. Multiple reason may explain the absence of an association between SAA levels and sensitization for bacterial agents in our study. First it could be that the subgroups studied were too small to detect a difference between the groups. Second, as previously mentioned, we showed that sensitization to inorganic agents was related to a fibrotic phenotype in sarcoidosis. Since we observed that SAA levels were also increased in IPF patients, it is possible that SAA is involved in fibrotic processes. For this reason SAA levels can be elevated in sarcoidosis patients with inorganic sensitization, but regarding its role as an acute phase protein, also in sarcoidosis patients sensitized for bacterial antigens.

Autophagy

As already mentioned, this thesis mainly focused on the identification of trigger-related phenotypes in sarcoidosis. The possible triggers studied in this thesis were, except for vimentin and SAA, all exogenous antigens. Beside specific antigens also genetic factors and cellular pathways are considered to be of at least equal importance regarding sarcoidosis pathogenesis. For instance, it is possible that granulomas are formed in response to antigens that not have been cleared due to a defect in underlying clearance mechanisms. Autophagy is an intracellular process whereby cytosolic components are degraded to maintain homeostasis and to provide substrates for energy generation. Autophagy is activated in response to several stimuli, including stress and nutrient shortage. It regulates various processes including metabolism and development, but also infection removal and antigen presentation (103). Autophagy is therefore involved in host defense as well as in

the regulation of inflammation (104) and contributes to sufficient elimination of exogenous substances including microbial antigens (105) metals and nanoparticles (106). Autophagy consist of multiple phases, with involvement of several genes such as mechanistic target of rapamycin (mTOR), NOD1 and NOD2 (103,107). Impaired autophagy processes have been related to multiple diseases including, but not limited to, liver diseases, diabetes, heart diseases, inflammatory bowel diseases and cancer (108).

Familial cases of sarcoidosis carrying variants in the NOD2 gene and other autophagy related genes have been described (109,110). However, also an unaffected familial member carried the NOD2 variant (109), suggesting that other factors such as epigenetics, or specific antigens have indeed to be present before sarcoidosis will develop.

Xenophagy is a specific type of autophagy used for the elimination of invading pathogens (104). Xenophagy was found to be involved in the clearance of several bacteria including mycobacteria and *P. acnes* (111). Interestingly, vimentin, another suspected antigen in the pathogenesis of sarcoidosis, is an essential contributor in the xenophagy process (111). Moreover, both surface-located vimentin as well as extracellular vimentin may be involved in the innate immune response to eliminate microbial infections and is a ligand for pattern recognition receptors (112). Adaptive Th17 immune responses to vimentin have been observed in peripheral blood of healthy individuals (113). It is possible that increased vimentin expression levels, for example induced by un-cleared microbial infections, lead to increased exposure and Th17 mediated pathologies. Th17-cells have been found to produce IFN- γ (114) and contribute in sarcoidosis pathogenesis (115,116).

The fact that vimentin is involved in the autophagy process and that both microbial antigens as well as metals and nanoparticles may induce autophagy, may explain our finding that some sarcoidosis patients were sensitized for vimentin as well as for bacterial or inorganic antigens. Possibly, vimentin is not a specific trigger in sarcoidosis, but vimentin-specific T-cells are rather a consequence of other processes such as infection or tissue damage, which may lead to expansion of auto-immune T-cells (117). This may also explain why vimentin reactive T-cells and auto-antibodies are not specific for sarcoidosis, but are also found in patients with IPF and systemic lupus erythematosus (118).

Besides bacterial and inorganic antigens, also SAA has found to be an inducer of autophagy (119). Taken together, all the antigens and proteins studied in this thesis for their possible relation in sarcoidosis pathogenesis, have been described in literature as inducers or contributors of autophagy. Several pathways are thought to be altered in sarcoidosis, of which many interact with autophagy (104). This shows once more that sarcoidosis is a multifactorial disease, which probably results from the interplay between genetic susceptibility, immunity and exposure to particular antigens. More specifically, it is possible that sarcoidosis will develop only when there is a coincidence of exposure to a particular

antigen in combination with specific variants in certain genes involved in pathways including both autophagy as well as immunity. In the absence of either the specific antigen exposure or the genetic variants, disease will probably never develop.

Future directions

Based on the results described in this thesis, it might be important to aim at future incorporation of screening for immunological sensitization to possible triggers in the diagnostic work-up of sarcoidosis patients. In view of this it is important to further investigate whether the possible trigger-related phenotypes described in this thesis have clinical relevance. In other words, we should bridge the step from identification of trigger-related phenotype to actual phenotype-based therapy in sarcoidosis. The new insights and hypotheses resulting from this thesis are summarized in Figure 1.

We observed that even sarcoidosis patients without JEM based metal and silica exposure demonstrated metal and silica sensitization. If low amounts of inorganic exposures are indeed able to initiate sensitization and sarcoidosis, it is difficult to determine based on exposure which patients should be further evaluated with an LPT. For this reason it seems more relevant to screen sarcoidosis patients for sensitization without determination of exposure beforehand. Moreover, since this will probably lead to a larger group of patients with a positive LPT, studies investigation genetic variants, including HLA-DPB1-Glu69, in inorganic agent sensitized patients can be initiated. The LPT is a complex test, which has some limitation since samples have to be fresh and both lymphocyte numbers as well as lymphocyte responses may be inhibited by the use of immunosuppressive medication. If certain genetic variants can be linked to metal and silica sensitization, genetic tests would be a more convenient way to screen sarcoidosis patients since such tests require only limited amounts of blood, immunosuppressive medication does not influence the results and DNA can be extracted from stored samples.

Since presence of *P. acnes* in granulomas was linked to the most severe clinical outcomes as defined by the COS-score, it could be relevant to evaluate the use of antibacterial therapy in this subgroup of patients. Some Japanese case reports already showed promising results of anti-bacterial therapy in sarcoidosis patients (120,121) and a trial with clarithromycin and doxycycline hydrochloride in Japanese cardiac sarcoidosis patients is ongoing (122). In this trial, treatment regimen was not based on the presence or absence of *P. acnes* in tissue or granulomas of patients. In future studies it would therefore be interesting to study the use of antibacterial therapy in sarcoidosis patients with and without presence of *P. acnes* in granulomas, to explore whether the identification of this subgroup, in light of treatment

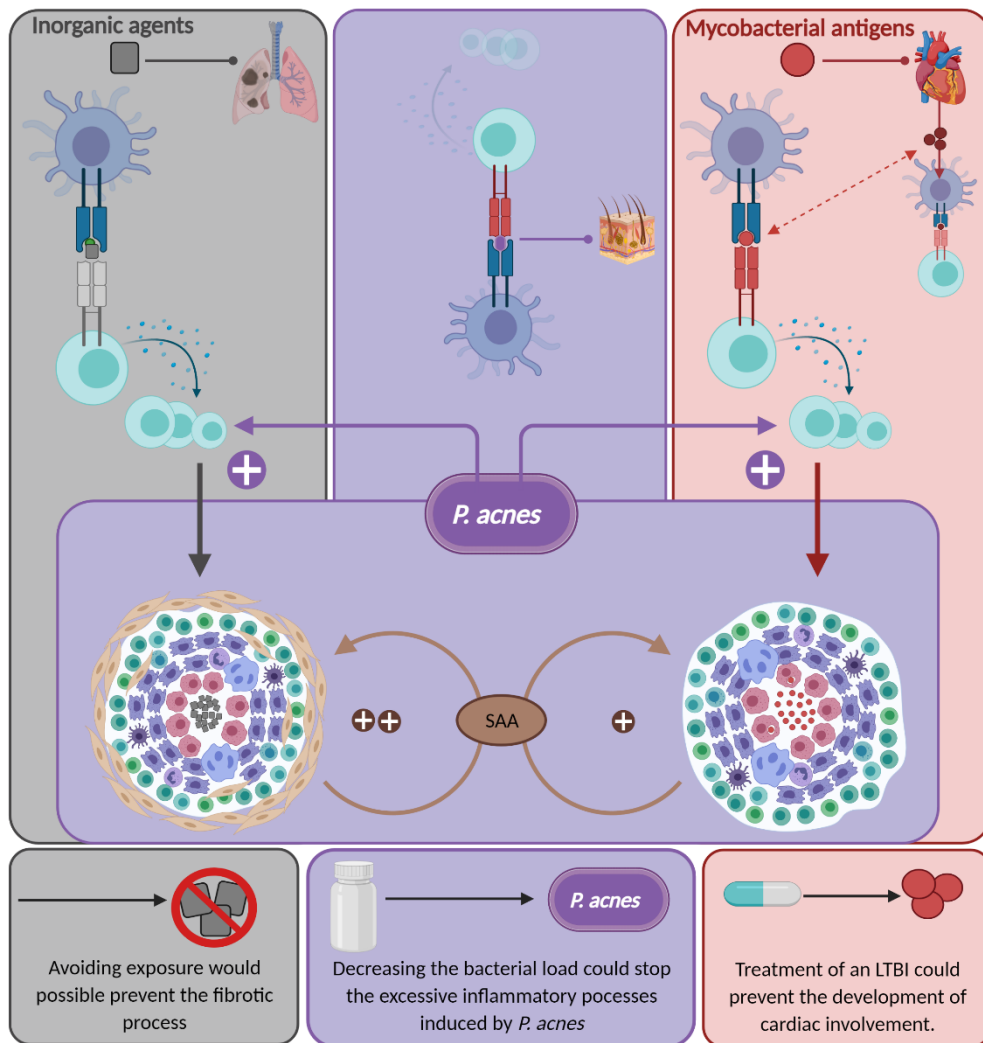


Figure 1. Schematic overview of the trigger related phenotypes observed in this thesis and possible future treatment options.

Inorganic agents that are not able to be cleared may, in susceptible individuals, lead to chronic inflammation and tissue damage resulting in fibrosis. Searching for certain genetic variants, including HLA-DPB1-Glu69, in inorganic agent sensitized patients could lead to identification of patients who could benefit from inorganic agent exposure avoidance. Mitogenic effects executed by *P. acnes* may increase the inflammatory and granulomas process by enhancing lymphocyte proliferation and cytokine secretion. Decreasing the bacterial load could decrease these excessive inflammatory processes observed in sarcoidosis. Cardiac sarcoidosis may be developed due to molecular mimicry between Tuberculosis antigens and cardiac proteins. Treatment of an LTBI may possible prevent the development of cardiac involvement in sarcoidosis.

responses, is valuable. At least in *P. acnes* induced granulomatous mice models, reduction of *P. acnes* by the use of anti-bacterial treatment showed improvement in the pulmonary lesions, including decreased granulomas and BAL cell counts (123).

Regarding the association found between an LTBI and presence of cardiac involvement in sarcoidosis, it could be considered to systematically explore cardiac involvement extensively in each patient with sarcoidosis and an LTBI, even though symptoms of cardiac involvement may not be present (yet). Furthermore, in current clinical practice, if a patient is diagnosed with an LTBI they can either choose follow-up without medication or start LTBI treatment using Isoniazid/Rifampicin. It could be beneficial for this group to start LTBI treatment instead of a wait-and-see policy directly after the diagnosis of LTBI is made.

In our study, we were not able to show that increased SAA serum levels were specifically associated with sarcoidosis or granulomatous diseases. SAA levels in serum can be increased due to the contribution of SAA in the acute phase response. Increased serum SAA levels can therefore be found in patients with several diseases. It would be interesting to examine the role of SAA in sarcoidosis pathogenesis in more detail. Antibodies to vimentin, another self-protein suspected to act as an auto-antigen in sarcoidosis, have been observed in bronchoalveolar lavage fluid (BALF) of a subgroup of sarcoidosis patients (124). If SAA act as an auto-antigen in sarcoidosis, it is plausible that antibodies against SAA in serum or BALF of sarcoidosis patients can be observed as well. Future studies should determine whether this is indeed the case.

In this thesis we focused solely on possible triggers of sarcoidosis. Although we studied multiple antigens simultaneously in one cohort of patients, we did not examine if it is a combination of particular antigens that together will trigger the development of sarcoidosis. Moreover, it is possible that sarcoidosis will develop only when there is a coincidence of exposure to particular antigens in combination with genetic variants in specific genes involved in pathways including autophagy and immunity. To thoroughly explore such hypotheses, a study should be conducted which combines different aspects thought to contribute in sarcoidosis pathogenesis, including triggers, auto-antibody analysis and whole exome sequencing. Stratification of patients will demand a large cohort with different genetic backgrounds, heterogeneous disease presentations and diverse disease outcomes and therefore requires international collaborations.

If such future studies reveal that genetic variants associate with particular antigens, personalized treatment options can be further explored. For instance, antibiotic treatment in combination with mTOR inhibitors such as rapamycin, could be effective in sarcoidosis

patients with a bacterial inducing antigen in combination with genetic variants in the autophagy process. Moreover, non-affected family members of sarcoidosis patients carrying the same genetic variants may be advised to avoid exposure to particular antigens, to prevent disease development.

To conclude, sarcoidosis is a complex and heterogeneous granulomatous disease that seems the result of an interplay between genetic variants in immune and non-immune related processes and specific antigens. The results of this thesis strengthen the hypothesis that the disease can develop in response to a variety of antigens, which can differ from patient to patient. Studying both inducing antigens as well as defects in underlying pathways and genetic variants in MHC and other immune related genes, especially those involved in autophagy, innate as well as adaptive immunity, could probably lead to the identification of more personalized based treatments and better prediction of disease course and dangerous phenotypes.

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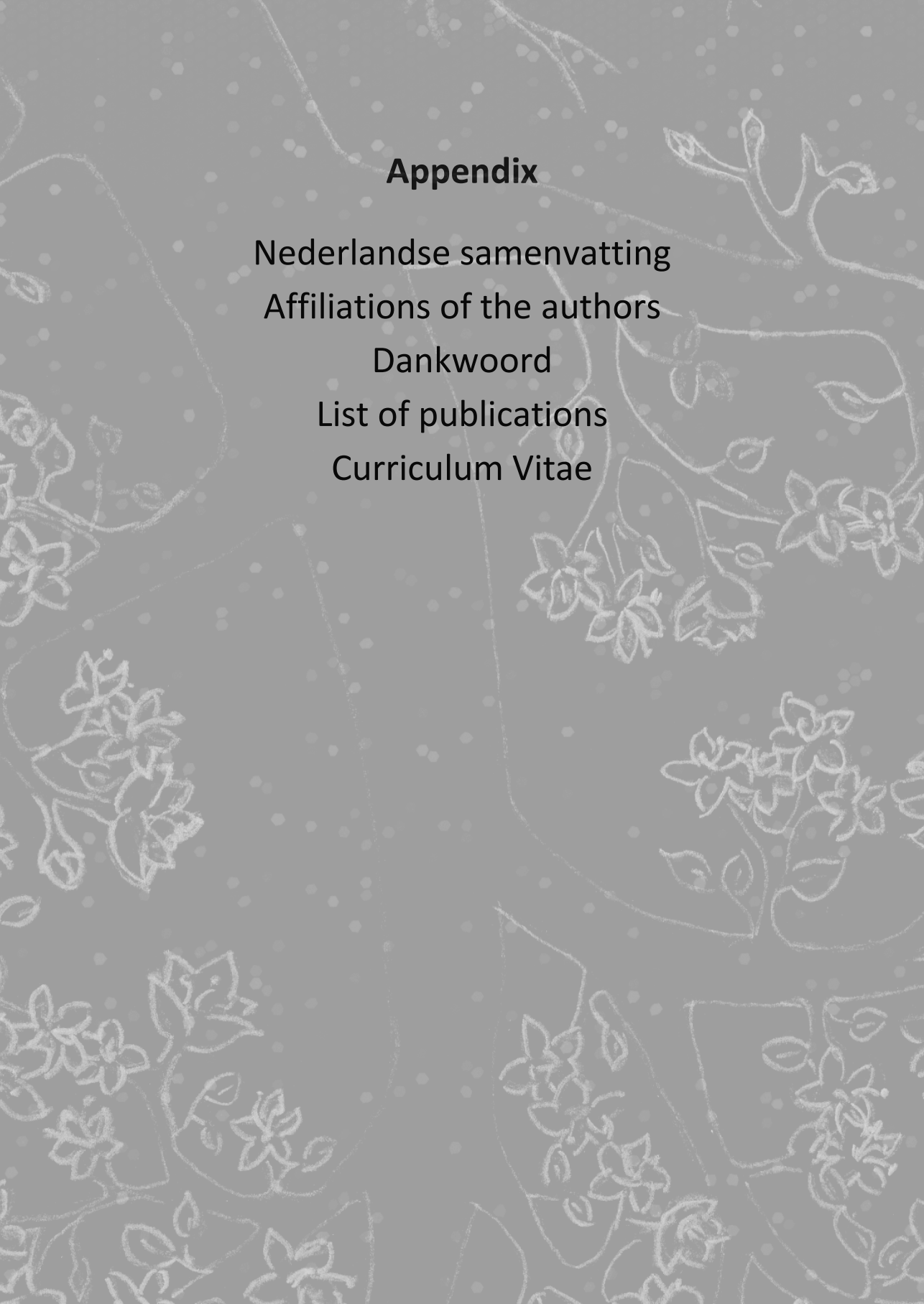
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Appendix

Nederlandse samenvatting

Affiliations of the authors

Dankwoord

List of publications

Curriculum Vitae

Nederlandse samenvatting



Inleiding

Sarcoïdose is een systemische ziekte die gekenmerkt wordt door de vorming van granulomen; opeenhopingen van verschillende witte bloedcellen welke betrokken zijn bij ons afweersysteem. De granulomen die gezien worden bij sarcoïdose zijn meestal niet-verkazend, wat betekend dat er geen necrose (weefsel afsterving) plaats vindt in het granuloom. Granulomen worden doorgaans gevormd wanneer het witte bloedcellen niet lukt bepaalde onderdelen van bijvoorbeeld bacteriën of lichaamsvreemde stoffen op te ruimen. Een granuloom bakent deze deeltjes dan af zodat verdere verspreiding voorkomen wordt.

Sarcoïdose ontstaat meestal bij mannen met een leeftijd tussen de 30 en 50 jaar en bij vrouwen met een leeftijd tussen de 40 en 60 jaar. Het is een zeldzame ziekte waarvan wordt geschat dat er in Nederland om en nabij 7000 mensen aan lijden (ongeveer 40 per 100.000 mensen). Sarcoïdose kan zich per patiënt anders presenteren. De longen en intra thoracale lymfeklieren (klieren in de borstholte) zijn het vaakst aangedaan, maar eigenlijk kan elk orgaan betrokken raken in de ziekte. Sarcoïdose patiënten kunnen geclassificeerd worden op basis van de mate van betrokkenheid van de longen en intra thoracale lymfeklieren middels de Scadding stadia. Deze Scadding stadia worden bepaald middels een Röntgen foto van de longen en rijken van 0 tot en met 5, waarbij er bij stadium 0 geen betrokkenheid is van de intra thoracale lymfeklieren en longen, bij Scadding stage 1 zijn de intra thoracale lymfeklieren aangedaan maar de longen niet, Scadding stage 2 geeft aan dat zowel de intra thoracale lymfeklieren als de longen aangedaan zijn, bij Scadding stage 3 zijn de intra thoracale lymfeklieren niet aangedaan maar de longen wel, en bij Scadding stage 4 is er sprake van littekenvorming in de longen, ook wel fibrose genoemd. Naast de heterogene presentatie die sarcoïdose kent, kan ook het beloop van de ziekte per patiënt verschillen. Een scoringssysteem dat het beloop van de ziekte aangeeft is de zogenaamde clinical outcome status (COS). Deze wordt doorgaans bepaald 5 jaar nadat de diagnose gesteld is en geeft onder andere aan of de ziekte verdwenen of nog steeds actief is, of de patiënt medicatie gebruikt of ooit een medicamenteuze behandeling heeft gehad. Deze status zegt dus niet alleen iets over de lymfeklier en long betrokkenheid, maar geeft een algemeen uitkomst beeld van de ziekte weer.

De oorzaak van sarcoïdose is nog niet geheel bekend. Er wordt gedacht dat een samenspel tussen veranderingen in onze eigen genen (erfelijk materiaal), het afweersysteem en deeltjes die granuloomvorming kunnen initiëren kan resulteren in sarcoïdose. Omdat het beeld dat bij sarcoïdose gezien wordt kan lijken op infectieuze granulomateuze ziekten zoals lepra en tuberculose, wordt gesuggereerd dat bepaalde bacteriën, waaronder

mycobacteriën en *Propionibacterium acnes* (*P. acnes*), een oorzaak van sarcoïdose kunnen zijn. Omdat de longen vaak aangedaan zijn in sarcoïdose, wordt ook vermoed dat het inademen van anorganische deeltjes, bestaande uit bijvoorbeeld metaal of silica, sarcoïdose zou kunnen veroorzaken. Daarnaast wordt in recentere studies gesuggereerd dat sarcoïdose het resultaat is van een auto-immuun reactie tegen lichaamseigen eiwitten, waaronder vimentine, een eiwit dat onderdeel is van het cytoskelet verantwoordelijk voor de vorm en stevigheid van onze cellen. Ook serum amyloïde A (SAA), een eiwit dat door het lichaam geproduceerd wordt als reactie op infecties en andere ontstekingsprocessen, zou een rol kunnen spelen bij de granuloom vorming in sarcoïdose. Deze bacteriën, metaal en silica deeltjes en lichaamseigen eiwitten zouden een granulomateuze reactie kunnen uitlokken, wat in samenspel met andere factoren uiteindelijk kan resulteren in sarcoïdose. Deze granuloom initiërende deeltjes worden daarom ook wel “triggers” van sarcoïdose genoemd.

Doel van dit proefschrift

Omdat sarcoïdose een erg heterogene presentatie kent en meerdere stoffen (ook wel antigenen genoemd) granulomen kunnen veroorzaken, is het mogelijk dat sarcoïdose niet één enkele homogene ziekte is, maar eerder een verzameling van meerdere granulomateuze ziekten geïnduceerd door verschillende granuloom stimulerende antigenen. Zoals hierboven beschreven zijn er afgelopen jaren al verschillende mogelijke triggers van sarcoïdose onderzocht. Een relatie tussen anorganische stoffen en sarcoïdose is tot op heden enkel gebaseerd op case reports en epidemiologische studies waarbij enkel naar blootstelling is gekeken. In dit proefschrift hebben we daarom een groep sarcoïdose patiënten onderzocht op zowel blootstelling als immuunreacties tegen anorganische stoffen waaronder metalen en silica.

Ondanks dat er al veel onderzoek gedaan is naar mogelijke triggers van sarcoïdose, zijn mogelijke bacteriële, auto-immuun en anorganische triggers van sarcoïdose nog nooit tegelijkertijd in hetzelfde cohort van sarcoïdose patiënten onderzocht. Daarnaast is er nog nooit onderzoek gedaan naar een relatie tussen mogelijke triggers en ziekteverschijnselen zoals orgaan betrokkenheid, Scadding stage en COS. In dit proefschrift hebben we daarom in dezelfde patiëntengroep onderzoek gedaan naar de mycobacterie, *P. acnes* bacterie, vimentine, metalen en silica. Daarnaast hebben we gekeken of er een verband is tussen deze mogelijke triggers en ziekte verschijnselen, om zogenoemde trigger-gerelateerde fenotypes te ontdekken.

Momenteel is er nog geen genezende behandeling voor sarcoïdose. Patiënten die vanwege het risico op orgaan beschadiging medicatie nodig hebben worden behandeld met immuun

onderdrukkende middelen om zo de ontsteking te onderdrukken en symptomen te verminderen. Meer inzicht in triggers van sarcoïdose en mogelijke trigger-gerelateerde fenotypes is daarom van belang. Deze kennis zou namelijk kunnen leiden tot een meer gepersonaliseerde behandeling van de ziekte. Patiënten bij wie bacteriën een mogelijke rol spelen in het ziekte proces kunnen bijvoorbeeld baat hebben bij antibacteriële therapie, terwijl het vermijden van relevante blootstelling meer effect zal hebben bij patiënten waarbij metalen en silica sarcoïdose hebben getriggered. Het identificeren van trigger-gerelateerde-fenotypes zou er daarnaast voor kunnen zorgen dat het ziekteverloop beter te voorspellen is, en dat er wellicht ook beter voorspeld kan worden welke patiënten behandeld zouden moeten worden en bij wie het minder noodzakelijk is.

Hoofdstuk 2: Etiologie en immuun gerelateerde pathologie van sarcoïdose: Nieuwe inzichten.

In dit review wordt een overzicht gegeven van belangrijke spelers van het afweersysteem, ook wel immuunsysteem genoemd, die mogelijk betrokken zijn in de pathogenese van sarcoïdose. Daarnaast wordt een overzicht gegeven van tot op heden gesuggereerde triggers van sarcoïdose, die ook genoemd worden in de inleiding van deze samenvatting. Het aangeboren immuunsysteem zorgt voor de eerste verdedigingen tegen pathogenen. Micro-organismen bevatten een groot scala aan moleculaire patronen waardoor ze te onderscheiden zijn van lichaamseigen cellen. Deze moleculaire patronen worden pathogen-associated molecular patterns (PAMPs) genoemd. Naast deze PAMPs kunnen eiwitten die geproduceerd en uitgescheiden worden tijdens weefselschade, herkend worden door het aangeboren immuunsysteem. Deze eiwitten worden ook wel danger-associated molecular patterns (DAMPs) genoemd. Het aangeboren immuunsysteem herkent deze DAMPs en PAMPs door middel van pattern recognition receptors (PRRs), zoals bijvoorbeeld toll-like receptors (TLRs) en nucleotide-binding oligomerization domain (NOD) like receptors (NLRs). Wanneer deze receptoren geactiveerd worden zal er een immuun respons geïnitieerd worden. Meerdere studies hebben afwijkingen in TLRs en NLRs gevonden in sarcoïdose patiënten. Aangetaste capaciteit om PAMPs te kunnen herkennen kan resulteren in een verminderde verdediging tegen pathogenen. Dit zou granuloomvorming kunnen triggeren en zo kunnen leiden tot een verhoogde sarcoïdose vatbaarheid. In tegenstelling tot het aangeboren immuunsysteem, is het verworven immuunsysteem erg specifiek. Het omvat antigeen presenterende cellen (APCs), T-cellen en B-cellen met het vermogen om antilichamen te produceren. Elke T-cel heeft een specifieke T-cel receptor die in staat is om verwerkte antigenen, gepresenteerd in major histocompatibility complex (MHC) moleculen

op APCs, te herkennen. Als reactie op herkende lichaamsvreemde antigenen, zullen T-cellen zich delen en cytokines produceren, die bijdragen aan het doden van de indringers. Door de blijvende immuun stimulatie in sarcoïdose, hopen T-cellen zich op rond de granulomen. Het is niet geheel bekend waarom een overactieve T-cel reactie plaats vindt in sarcoïdose, maar het zou zo kunnen zijn dat T-cellen reageren op antigenen maar dat signalen, moleculen en cellen die dit proces na verloop van tijd weer af moeten remmen ontbreken of inefficiënt zijn in sarcoïdose.

Hoofdstuk 3: Klinische observaties: is metaal of silica geassocieerde sarcoïdose een nieuw trigger-gerelateerd fenotype?

Voor sommige anorganische stoffen, zoals bepaalde metalen, is al beschreven dat zij granuloom vorming kunnen veroorzaken. Het meest beschreven metaal in deze context is beryllium. Chronische beryllium ziekte of berylliose is een granulomateuze ziekte die gerelateerd is aan beryllium blootstelling, en die klinisch vaak niet te onderscheiden is van sarcoïdose. Wanneer iemand die hier genetisch gevoelig voor is in aanraking komt met beryllium, zal er een immuunrespons volgen waarbij er beryllium geheugen cellen gevormd worden. Deze aanwezigheid van zulke geheugencellen kan vastgesteld worden door witte bloedcellen buiten het lichaam bloot te stellen aan beryllium. Wanneer er geheugen cellen aanwezig zijn, zullen deze na blootstelling aan beryllium delen en cytokines produceren. Dit fenomeen wordt immunologische sensibilisatie genoemd aan kan aangetoond worden met verschillende laboratorium testen zoals een elispot test of een lymfocyten proliferatie test (LPT). De diagnose chronische beryllium ziekte wordt gesteld wanneer er aanwezigheid is van beryllium blootstelling, niet-verkazende granulomen en beryllium sensibilisatie. In dit hoofdstuk hebben wij een patiënt beschreven bij wie de diagnose sarcoïdose gewijzigd kon worden in chronische beryllium ziekte op basis van werkzaamheden in een ijzergieterij, niet verkazende granulomen en een positieve beryllium LPT. Daarnaast hebben wij 2 patiënten beschreven die zich presenteerden met een radiologisch beeld niet geheel typerend voor sarcoïdose. Op basis van de longbiopten die niet verkazende granulomen lieten zien werd toch de diagnose sarcoïdose gesteld. Bij beide patiënten werd in het longbiopt lichaamsvreemd materiaal gevonden, mogelijk veroorzaakt door werkzaamheden als, respectievelijk, logistiek medewerker in de haven van een staalfabriek en stukadoor, met daarbij blootstelling aan metaal en silica. Een LPT toonde bij de ene patiënt aluminium sensibilisatie en bij de andere sensibilisatie voor silica. Wanneer de diagnostische criteria om chronische beryllium ziekte vast te stellen gevolgd worden, is het aannemelijk (maar

niet bewezen) dat aluminium dan wel silica een rol hebben gespeeld bij het ontstaan van sarcoïdose bij deze laatste twee patiënten.

Hoofdstuk 4: Sensibilisatie tegen metalen en silica is geassocieerd met sarcoïdose in Nederlandse patiënten.

De relatie tussen anorganische stoffen en sarcoïdose is tot op heden enkel beschreven in epidemiologische studies die alleen naar blootstelling hebben gekeken, of in beschrijvingen van enkele patiënten zoals in hoofdstuk 3. Om deze reden hebben we in hoofdstuk 4 zowel metaal en silica blootstelling als metaal en silica sensibilisatie onderzocht in een groep sarcoïdose patiënten en dit vergeleken met een controlegroep. Metaal en silica blootstelling werd in 256 sarcoïdose patiënten en in 73 controles bepaald middels een complete beroepsgeschiedenis gekoppeld aan een job exposure matrix (JEM). Een JEM is een soort schema dat per beroepstitel aangeeft of er in dat beroep blootstelling plaats vindt aan bepaalde stoffen. Met een LPT werd een deel van deze patiënten (n=33) en controles (n=19) onderzocht op sensibilisatie tegen aluminium, beryllium, zirkonium en silica. We zagen geen verschil tussen het percentage sarcoïdose patiënten en controles dat blootstelling had aan metalen of silica o.b.v. de resultaten van de vragenlijsten gekoppeld aan de JEM. Echter, sensibilisatie tegen metalen of silica werd alleen gevonden in sarcoïdose patiënten en niet in de controles, wat met 21.2% tegenover 0% een significant verschil was. Deze observatie ondersteunt de hypothese dat metalen en silica bijdragend zijn in de ontwikkeling van sarcoïdose in een subgroep van de patiënten.

Omdat sensibilisatie wel verschilde tussen de sarcoïdose en controlegroep, maar blootstelling niet, is het goed mogelijk dat veranderingen in bepaalde genen in sarcoïdose patiënten er toe leidt dat sensibilisatie tegen metalen en silica ontstaat. Zulke genetische veranderingen zijn al aangetoond in chronische beryllium ziekte, waarbij 90% van de patiënten met chronische beryllium ziekte een specifieke verandering had in een aminozuur van het HLA-DPB1 gen, terwijl dit in ongeveer 40% gezonde controles gezien werd.

Een opvallende bevinding in onze studie was dat er ook sarcoïdose patiënten waren die op basis van onze vragenlijst gekoppeld aan de JEM geen blootstelling hadden aan metalen en silica, maar wel sensibilisatie lieten zien. Het zou kunnen zijn dat door genetische vatbaarheid, een hele kleine mate van blootstelling die door deze JEM niet opgepikt wordt, al kan leiden tot sensibilisatie en uiteindelijk sarcoïdose.

Hoofdstuk 5: Uit het gelijktijdig testen van sarcoïdose patiënten op sensibilisatie tegen verschillende antigenen blijkt een associatie met anorganische antigenen gerelateerd aan een fibrotisch fenotype van sarcoïdose.

In dit hoofdstuk hebben we in een cohort van 201 sarcoïdose patiënten en 51 controles sensibilisatie tegen antigenen van de Mycobacterie, de *P. acnes* bacterie en vimentine onderzocht. In 105 van deze sarcoïdose patiënten en in 24 van de controles hebben we ook naar sensibilisatie tegen metalen en silica gekeken. Daarna hebben we gekeken of sensibilisatie voor bepaalde antigenen gerelateerd was aan bepaalde ziekteverschijnselen, om zo trigger-gerelateerde fenotypes te identificeren.

We vonden geen verschil in het percentage sarcoïdose patiënten en controles dat gesensibiliseerd was voor mycobacteriële antigenen of vimentine. Opvallend was dat 3 van de 5 sarcoïdose patiënten die gesensibiliseerd waren voor mycobacteriële antigenen betrokkenheid van het hart hadden, zogenaamde cardiale sarcoïdose. Een verrassende bevinding was dat een lager percentage van de sarcoïdose patiënten sensibilisatie voor *P. acnes* antigenen liet zien (3.5% vergeleken met 15.7% van de controles). Sarcoïdose patiënten met *P. acnes* sensibilisatie waren jonger ten tijde van diagnose dan sarcoïdose patiënten zonder *P. acnes* sensibilisatie. Daarnaast leek het zo te zijn dat deze patiëntengroep vaker huid betrokkenheid had, al was dit net niet significant verschillend van de groep zonder *P. acnes* sensibilisatie. In tegenstelling tot deze organische antigenen, zagen we wel een associatie tussen de geteste anorganische antigenen en sarcoïdose. Een hoger percentage van de sarcoïdose patiënten liet namelijk sensibilisatie zien tegen aluminium, beryllium, silica of zirkonium in vergelijking met de controlegroep (27.6% versus 4.2%). Daarnaast hadden sarcoïdose patiënten met sensibilisatie tegen een van deze anorganische stoffen vaker longfibrose ontwikkeld 5 jaar na de diagnose dan sarcoïdose patiënten zonder sensibilisatie tegen de geteste anorganische stoffen. De resultaten van deze studie suggereren dat anorganische triggers zoals metalen en silica een grotere rol spelen in sarcoïdose dan tot op heden is gedacht. Daarnaast blijkt uit de relaties tussen mycobacteriële antigenen en cardiale betrokkenheid, *P. acnes* antigenen en huid betrokkenheid en anorganische antigenen en fibrose, dat het inderdaad mogelijk is om trigger-gerelateerde fenotypes te identificeren in een heterogene groep sarcoïdose patiënten.

Hoofdstuk 6: Een latente tuberculose infectie is geassocieerd met cardiale betrokkenheid in patiënten met sarcoïdose

In dit hoofdstuk hebben we de mogelijke associatie tussen mycobacteriële antigenen en cardiale betrokkenheid in sarcoïdose uit hoofdstuk 5 verder onderzocht. Cardiale betrokkenheid komt bij ongeveer 5% van de sarcoïdose patiënten voor, hoewel uit autopsies en radiologische studies blijkt dat dit een onderschatting kan zijn en dat het werkelijke percentage waarschijnlijk tussen de 20 en 30% ligt. Identificatie van cardiale betrokkenheid is belangrijk omdat dit een belangrijke doodsoorzaak is bij sarcoïdose. In dit hoofdstuk hebben we gekeken naar een latente tuberculose infectie (LTBI). Bij een LTBI is iemand in het verleden ooit besmet met *Mycobacterium tuberculosis*, hetgeen blijkt uit sensibilisatie in het bloed, zonder dat tekenen te tonen van een actieve tuberculose infectie. Een groep cardiale sarcoïdose patiënten (n=103) werd onderzocht op het hebben van een LTBI en vergeleken met sarcoïdose patiënten zonder cardiale betrokkenheid (n=153). We zagen dat een significant hoger percentage van de cardiale sarcoïdose patiënten een LTBI had dan de niet-cardiale sarcoïdose patiënten (6.8% vergeleken met 0.7%). Als toekomstige studies deze associatie kunnen bevestigen, kan dat van klinisch belang zijn. Het kan bijvoorbeeld relevant zijn om sarcoïdose patiënten bij wie een LTBI vastgesteld wordt, direct te screenen op cardiale betrokkenheid, ook al zijn er nog geen signalen die wijzen op cardiale sarcoïdose. Daarnaast zou het behandelen van de LTBI wellicht kunnen voorkomen dat er cardiale betrokkenheid op treedt.

Hoofdstuk 7: Aanwezigheid van *P. acnes* in granulomen associeert met een chronisch ziekte beloop in Nederlandse sarcoïdose patiënten

In dit hoofdstuk hebben we onderzocht of we *P. acnes* kunnen detecteren in weefsel en granulomen van Nederlandse sarcoïdose patiënten. Daarnaast hebben we gekeken of aanwezigheid van *P. acnes* in weefsel en granulomen gerelateerd is aan het ziekteverloop van sarcoïdose.

Het is lastig om een etiologische rol van *P. acnes* in sarcoïdose aan te tonen omdat deze bacterie bij de meeste mensen als commensaal op de huid voorkomt. Echter, DNA van de *P. acnes* bacterie is vaker gevonden in weefsel van Chinese, Japanse, Italiaanse, Engelse en Duitse sarcoïdose patiënten dan in controles. Dit suggereert dat *P. acnes* op z'n minst betrokken is in het ziekteproces. Daarnaast is er een verhoogde immuunrespons tegen *P. acnes* gevonden in verschillende studies met Japanse en Duitse sarcoïdose patiënten. In hoofdstuk 5 konden wij dit echter niet aantonen in ons cohort van sarcoïdose patiënten uit

Nederland. Wellicht is bloed niet de juiste plek om te zoeken naar een mogelijke rol van *P. acnes* in het ontstaan van sarcoïdose. Daarom hebben wij in dit huidige hoofdstuk weefsel van sarcoïdose patiënten gebruikt om door middel van een andere methode naar de rol van *P. acnes* in sarcoïdose te kijken. Granulomateus weefsel van 76 sarcoïdose patiënten werd gekleurd met een antilichaam tegen een deeltje van de celwand van de *P. acnes* bacterie, om zo de aanwezigheid van deze bacterie zichtbaar te maken. Daarna hebben we gekeken of de sarcoïdose patiënten bij wie *P. acnes* gedetecteerd was een ander ziektebeloop hadden dan de sarcoïdose patiënten zonder *P. acnes* in het weefsel of granulomen. We zagen dat bij een subgroep van deze Nederlandse sarcoïdose patiënten (41%) *P. acnes* aanwezig was in het onderzochte weefsel. Bij 17% van de sarcoïdose groep was *P. acnes* ook specifiek aantoonbaar in granulomen. Daarnaast zagen we dat sarcoïdose patiënten met *P. acnes* in granulomen vaker een chronisch ziektebeloop en noodzaak voor immunosuppressieve behandeling hadden, dan de patiënten waar *P. acnes* niet detecteerbaar was in granulomen. Een mogelijke verklaring voor de relatie tussen aanwezigheid van *P. acnes* en een chronisch ziektebeloop kan zijn dat *P. acnes* mitogene effecten heeft, hetgeen ook beschreven is in andere studies. Een mitogeen is een deeltje of stof die cellen specifiek aanzet tot delen. Dus naast dat *P. acnes* sarcoïdose zou kunnen initiëren, zou het ook als een mitogeen in brede context kunnen werken en zo een inflammatoire reactie en de presentatie van antigenen aan T-cellen in stand kunnen houden.

Hoofdstuk 8: Detectie van *P. acnes* in granulomen van patiënten met hypersensitiviteits pneumonitis of vasculitis onthult dat *P. acnes* niet specifiek is voor granulomen van patiënten met sarcoïdose.

Omdat we in hoofdstuk 7 veronderstelden dat *P. acnes* een mitogene rol zou kunnen hebben en wellicht geen specifieke trigger is van sarcoïdose, hebben we in hoofdstuk 8 verder onderzocht of de aanwezigheid van *P. acnes* in granulomen specifiek is voor sarcoïdose.

Verschillende studies hebben al laten zien dat *P. acnes* granuloom vorming kan triggeren. Bovendien wordt er gesuggereerd dat de *P. acnes* bacterie betrokken is bij de pathogenese van sarcoïdose. Granulomen zijn echter niet specifiek voor sarcoïdose en komen ook voor bij andere ziektes. *P. acnes* is al aangetoond in granulomen van sarcoïdose patiënten maar het is niet bekend of deze bacterie ook aanwezig is in granulomen van patiënten met andere granulomateuze ziekten zoals hypersensitiviteits pneumonitis (HP), Granulomatose met polyangiitis (GPA) en Eosinofiele Granulomatose met Polyangiitis (EGPA). Granulomateus

weefsel van patiënten met HP, GPA en EGPA werd daarom onderzocht op de aanwezigheid van de *P. acnes* bacterie door gebruik te maken van dezelfde methode als in hoofdstuk 7. We zagen aanwezigheid van de *P. acnes* bacterie ook in weefsel en granulomen van een deel van de HP, GPA en EGPA patiënten. Het percentage van de patiënten waarbij aanwezigheid van de *P. acnes* bacterie gevonden werd in weefsel of granulomen verschilde niet van het percentage sarcoidose patiënten uit hoofdstuk 7 waarbij *P. acnes* in weefsel of granulomen gevonden werd. Deze resultaten suggereren dat aanwezigheid van *P. acnes* in weefsel en granulomen niet ziekte specifiek is en maken de rol van *P. acnes* als specifieke trigger in sarcoidose twijfelachtig. Met betrekking tot het mitogene effect van de *P. acnes* bacterie, waargenomen in hoofdstuk 7, vroegen we ons af of er in andere inflammatoire ziekten ook kenmerken te zien waren die konden wijzen op een mogelijk mitogeen effect van de *P. acnes* bacterie. Om deze reden hebben we gekeken naar subgroepen van immuuncellen in bronchoalveolaire lavage (BAL) van HP patiënten met en zonder aanwezigheid van *P. acnes* in granulomen. We zagen dat HP patiënten met aanwezigheid van *P. acnes* in granulomen een hoger lymfocyten percentage in de BAL hadden dan HP patiënten zonder *P. acnes* in granulomen. Deze bevinding ondersteunt de hypothese dat *P. acnes* kan werken als een mitogeen, waardoor ook in HP proliferatie van lymfocyten versterkt wordt. Het is daarom mogelijk dat *P. acnes* geen specifieke trigger is in sarcoidose, HP en (E)GPA maar dat het als mitogeen bijdraagt in de vorming en in stand houding van granulomen.

Hoofdstuk 9: Serum Amyloïde A bloed waardes zijn verhoogd in patiënten met granulomateuze en fibrotische ziektes

In dit hoofdstuk hebben we Serum Amyloïde A (SAA) waardes in bloed van patiënten met granulomateuze ziektes waaronder sarcoidose, HP en (E)GPA vergeleken met SAA bloed waardes van patiënten met idiopathische pulmonaire fibrose (IPF), een niet granulomateuze ziekte.

Verhoogde waardes van het acute fase eiwit SAA zijn gevonden in verscheidene inflammatoire ziekten zoals reumatoïde artritis, ziekte van Crohn en ook in sarcoidose. Daarnaast wordt in de literatuur gesuggereerd dat SAA een regulerende rol heeft in de vorming en in stand houding van granulomen in sarcoidose.

Ondanks dat we hogere SAA concentraties vonden in bloed van sarcoidose, HP en (E)GPA patiënten in vergelijking met gezonde controles, zagen we tussen de verschillende granulomateuze ziektes geen verschillen in SAA concentraties. Bovendien werd geen verschil gevonden in SAA concentraties tussen de patiënten met granulomateuze ziekten

enerzijds en patiënten met IPF anderzijds. Daarnaast zagen we dat sarcoïdose patiënten met pulmonale fibrose (Scadding stadium IV) hogere SAA concentraties bloed hadden dan sarcoïdose patiënten zonder pulmonale fibrose. Deze observatie, samen met de bevinding dat SAA waarden niet alleen verhoogd zijn in granulomateuze ziektes maar ook in patiënten met IPF, doet vermoeden dat SAA naast granulomateuze processen ook betrokken is in fibrotische processen.

Conclusies

De oorzaak van sarcoïdose houdt wetenschappers al meer dan een eeuw bezig. Sarcoïdose wordt gekarakteriseerd door granulomen, echter granulomen zijn niet specifiek voor sarcoïdose maar ook te vinden bij andere ziekten zoals tuberculose, Hypersensitiviteits Pneumonitis (HP), Granulomatose met Polyangiitis (GPA) en Eosinofiele Granulomatose met Polyangiitis (EGPA). Wanneer de oorzaak van aanwezige granulomen niet bekend is en het radiologische en klinische beeld past bij sarcoïdose, wordt de diagnose sarcoïdose gesteld door andere ziekten uit te sluiten (per exclusionem). Omdat sarcoïdose een heterogene presentatie kent en verscheidene antigenen in relatie zijn gebracht met granuloom vorming, is het mogelijk dat sarcoïdose niet een enkele homogene ziekte is maar eerder een verzameling van meerdere granulomateuze ziektes geïnduceerd door verschillende triggers.

Het doel van dit proefschrift was om zowel organische als anorganische antigenen te onderzoeken in hetzelfde cohort, en daarnaast te onderzoeken of er trigger-gerelateerde fenotypes van sarcoïdose te identificeren zijn.

De suggestie dat metalen en silica een rol spelen in de pathogenese van sarcoïdose was tot dusver gebaseerd op epidemiologische studies en casus beschrijvingen. In hoofdstuk 4 en 5 hebben we aangetoond dat immunologische sensibilisatie tegen metalen en silica geassocieerd is met sarcoïdose op basis van onderzoek in een grote groep sarcoïdose patiënten die vergeleken werd met een controle groep. Daarnaast zagen we dat sensibilisatie tegen metalen en silica gerelateerd was aan het ontwikkelen pulmonale fibrose in sarcoïdose patiënten 5 jaar na de diagnose. Omdat fibrose onomkeerbaar is en grote mate van fibrose kan leiden tot een verlaagde longfunctie en diffusie capaciteit, is het belangrijk dat een fibrotisch proces niet verder gestimuleerd wordt. In patiënten met chronische beryllium ziekte is het inderdaad aangetoond dat het stoppen van blootstelling aan beryllium bevorderlijk kan zijn met het oog op voorkomen van verdere ziekteprogressie. Het vermijden van verdere blootstelling aan metalen en silica zal daarom ook een chronisch

beloop en progressie kunnen voorkomen in sarcoïdose patiënten die metaal of silica gesensibiliseerd zijn.

We hebben aan de andere kant ook laten zien dat sarcoïdose patiënten met metaal en silica sensibilisatie niet altijd blootstelling aan metalen en silica hebben op basis van de vragenlijsten gekoppeld aan de JEM. Meerdere oorzaken kunnen ten grondslag liggen aan deze bevinding. Ten eerste kan relevante blootstelling aan metalen en silica plaats hebben gevonden op basis van niet werk-gerelateerde activiteiten, zoals bepaalde hobby's, het wonen in een omgeving met een hoge mate van metaal gassen of het hebben van bepaalde implantaten. Daarnaast is het mogelijk dat zelfs een hele lage mate van blootstelling, die niet opgepikt wordt door de JEM, al kan leiden tot sensibilisatie en sarcoïdose bij genetisch vatbare patiënten. Zulke genetische vatbaarheid is al beschreven bij CBD. Het is mogelijk dat bepaalde genetische veranderingen ook een rol spelen bij sensibilisatie tegen andere anorganische antigenen zoals aluminium, silica en zirkonium. Verder onderzoek zal dit uit moeten wijzen.

Ondanks dat we een associatie aan konden tonen tussen anorganische antigenen en sarcoïdose, hebben we geen associatie gevonden tussen sensibilisatie tegen antigenen van de *P. acnes* bacterie, de mycobacterie en vimentine en sarcoïdose in een Nederlandse patiëntengroep. We vonden zelfs dat een lager percentage sarcoïdose patiënten dan controles gesensibiliseerd was voor *P. acnes* antigenen. Omdat de *P. acnes* bacterie als commensaal voorkomt op de huid bij het merendeel van de mensen, is het niet verassend dat sensibilisatie tegen deze bacterie bij gezonde personen gevonden kan worden. Het lage percentage sarcoïdose patiënten dat sensibilisatie tegen de *P. acnes* bacterie liet zien, kan mogelijk verklaard worden door de verlaagde perifere T-cel functionaliteit tegen vaak terugkerende antigenen welke is beschreven in sarcoïdose. Deze verminderde T-cel functionaliteit in bloed enerzijds en een buitensporige T-cel inflammatie in aangedane organen anderzijds is beschreven als de immuun paradox in sarcoïdose. Daarnaast zou het zo kunnen zijn dat *P. acnes* specifieke T-cellen vanuit het bloed naar aangedane weefsels zijn getrokken waardoor er geen sensibilisatie in bloed gevonden wordt. Bloed lijkt daarom niet het meest geschikt om sensibilisatie tegen de *P. acnes* bacterie te onderzoeken. Echter, het is vrij invasief om sensibilisatie tegen *P. acnes* in andere organen te onderzoeken omdat dit verrichting van bijvoorbeeld een BAL of lymfeklier punctie zou vereisen. Om deze reden hebben we met een immunohistochemische kleuring gekeken of we *P. acnes* konden aantonen in reeds aanwezig weefsel en granulomen van sarcoïdose patiënten. We hebben in hoofdstuk 7 voor de eerste keer laten zien dat de *P. acnes* bacterie aanwezig is in weefsel en granulomen van Nederlandse sarcoïdose patiënten. Daarnaast bleek de subgroep van de sarcoïdose patiënten waarbij *P. acnes* aangetoond kon worden in granulomen een

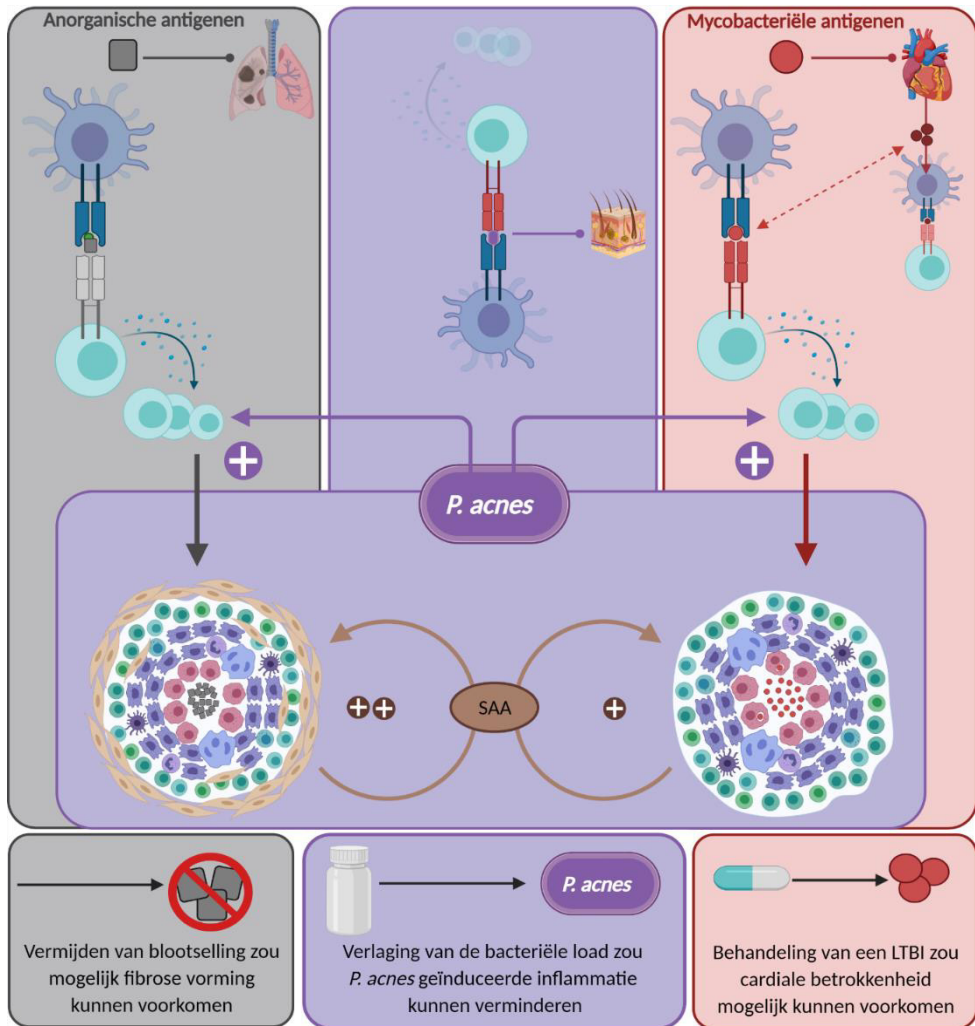
chronischer en ernstiger ziektebeloop te hebben dan de sarcoïdose patiënten waarbij we *P. acnes* niet hebben gedetecteerd in granulomen. In hoofdstuk 8 zagen we dat aanwezigheid van *P. acnes* in granulomen niet specifiek is voor sarcoïdose en dat dit ook gevonden kan worden bij patiënten met andere granulomateuze ziektes. Deze bevinding, samen met de afwezigheid van sensibilisatie tegen *P. acnes* antigenen in bloed, is niet ondersteunend voor een rol van *P. acnes* als specifieke trigger in sarcoïdose. De observatie dat *P. acnes* in granulomen van sarcoïdose patiënten associeerde met een chronisch ziektebeloop en de observatie dat HP patiënten met *P. acnes* in granulomen een hoger lymfocyten percentage hebben in de BAL duiden meer op een mitogene rol van *P. acnes*, zoals al eerder is beschreven in de literatuur. Ondanks dat onze resultaten meer wijzen op een mitogene rol van *P. acnes* dan op een rol als specifieke trigger in sarcoïdose zou het identificeren van *P. acnes* in granulomen van sarcoïdose patiënten van belang kunnen zijn. Er kan dan bijvoorbeeld onderzocht worden of deze subgroep van sarcoïdose patiënten gebaat is bij het gebruik van antibacteriële therapie.

Het voornaamste doel van dit proefschrift was om te onderzoeken of er trigger-gerelateerde fenotypes van sarcoïdose te identificeren zijn. In een heterogene groep sarcoïdose patiënten zagen we inderdaad dat bepaalde antigenen gerelateerd waren aan de betrokkenheid van organen of aan een ander ziektebeloop. Twee van deze trigger-gerelateerde fenotypes, namelijk *P. acnes* en een chronisch ziekte beloop, en anorganische antigenen en pulmonale fibrose, zijn hierboven al uitgebreid beschreven. Een verklaring voor de relaties tussen deze triggers en fenotypes zou verklaard kunnen worden door de mitogene werking van *P. acnes* waardoor een inflammatoire reactie aanhoudt, en het door het lichaam moeilijk kunnen verwijderen van anorganische deeltjes waardoor inflammatie, schade en fibrose zou kunnen ontstaan. We zagen in hoofdstuk 5 een derde, meer verrassend, trigger-gerelateerd fenotype namelijk de relatie tussen een LTBI en cardiale betrokkenheid in sarcoïdose. Sarcoïdose patiënten waarbij een LTBI gevonden wordt zouden mogelijk baat kunnen hebben bij een Tuberculose behandeling, om zo cardiale betrokkenheid te voorkomen. Een verklaring voor de associatie tussen een LTBI en cardiale sarcoïdose zou moleculaire mimicry kunnen zijn. Moleculaire mimicry houdt in dat dezelfde epitopen aanwezig kunnen zijn op meerdere eiwitten. Een epitootop is het gedeelte van een molecuul of antigeen, dat door cellen van het immunsysteem herkend wordt. Bacteriën en virussen kunnen bijvoorbeeld dezelfde epitopen bevatten als epitopen die voorkomen op onze eigen immuun cellen. Wanneer een immuunreactie tegen deze microbiële antigenen in gang wordt gezet kan door een kruis reactie de tolerantie tegen onze eigen epitopen verbroken worden waardoor een auto-immuun reactie ontstaat. Een voorbeeld hiervan is reumatische hart ziekte als gevolg van acute reuma. Hierbij ontstaat een auto-immune

reactie als vervolg op infectie met de *Streptococcus pyogenes* bacterie, waarbij T-cellen zowel streptokokken peptiden als peptiden in ons eigen hart herkennen en aanvallen. Zulke cross-reacties zouden ook aanwezig kunnen zijn tussen de mycobacterie en hart weefsel. Verder onderzoek is nodig om dit aan te kunnen tonen.

Gebaseerd op de resultaten beschreven in dit proefschrift lijkt het belangrijk om het vaststellen van immunologische sensibilisatie tegen mogelijk triggers van sarcoïdose op te nemen in de work-up van toekomstige sarcoïdose patiënten. Het is van belang om verder te onderzoeken of de trigger-gerelateerde fenotypes beschreven in dit proefschrift klinisch relevant zijn. In andere woorden, we zullen een brug moeten slaan tussen de identificatie van trigger-gerelateerde fenotypes en een op fenotype gebaseerde behandeling van sarcoïdose. De nieuwe inzichten en hypothesen gebaseerd op de bevindingen in dit proefschrift zijn samengevat in Figuur 1.

Concluderend, sarcoïdose is een complexe ziekte en mogelijk het resultaat van de wisselwerking tussen genetische varianten in immuun cellen en andere processen enerzijds en specifieke antigenen anderzijds. De resultaten beschreven in dit proefschrift versterken de hypothese dat sarcoïdose kan ontstaan als reactie op verscheidene antigenen welke van patiënt tot patiënt kunnen verschillen, en zijn mogelijk een eerste stap in de richting van antigeen gerelateerde behandeling in subgroepen van sarcoïdose patiënten.



Figuur 1 geeft een schematisch overzicht van de trigger-gerelateerde fenotypes waargenomen in dit proefschrift en bijbehorende toekomstige behandel opties.

Anorganische antigenen die niet geklaard kunnen worden zouden, in genetisch vatbare patiënten, kunnen leiden tot chronische ontsteking en weefselschade, wat kan resulteren in fibrose. Het vermijden van blootstelling zou voor deze patiënten meer baad kunnen hebben dan het ophogen van immuun suppressieve medicatie. Mitogene effecten van de *P. acnes* bacterie, zoals het versterken van lymfocyten proliferatie en cytokine productie, zouden inflammatie en granuloomvorming in stand kunnen houden. Het verlagen van de bacteriële load zou deze extensieve ontstekingsprocessen kunnen verminderen. Cardiale betrokkenheid zou kunnen ontstaan door mimicry tussen tuberculose antigenen en cardiale eiwitten. Behandeling van een latente tuberculose infectie zou mogelijk cardiale betrokkenheid kunnen voorkomen.

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List of publications



Appendix. List of publications

Beijer E, Roodenburg C, Schimmelpennink MC, Grutters JC, Meek B, Veltkamp M. Elevated Serum amyloid A levels are not specific for sarcoidosis but associate with a fibrotic pulmonary phenotype. *Cells*. 2021; 10(3):585.

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Curriculum Vitae



Elsje Dinanda Beijer was born on May 8th 1991 in Zutphen. She finished high school at Stedelijk Dalton college Zutphen in 2009. In this year she started studying Nutrition & Health at the Wageningen University and obtained her bachelor degree in 2012. She continued with the master specialization Molecular nutrition and Toxicology at the Wageningen University in 2012. During her master she performed research in the department of Nutrition & Pharmacology on receptor(s) possible involved in the anti-inflammatory effects of the fatty acid ethanol-amide DHEA. Thereafter she moved from Wageningen to Utrecht for an internship at the Utrecht institute of pharmaceutical sciences about the effects of probiotics on the allergic/immune response in a co-culture model consisting of intestinal epithelial cells and PBMCs. In 2014 she received her master's degree (MSc). A triggered interest for the immune system and research lead to the start of this PhD project at the Interstitial Lung Diseases Centre of Excellence at the St Antonius Hospital in Nieuwegein. Parts of her research were awarded with a best presentation and a young investigator award at the annual WASOG conferences in 2018 and 2019.

