Löfgren's Syndrome

Genetic associations, clinical course and outcome

Bekir Karakaya

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Löfgren's syndrome: genetic associations, clinical course and outcome

Syndroom van Löfgren: genetische associaties, klinisch beloop en uitkomst
(met een samenvatting in het Nederlands)

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For all the Karakaya's ...

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Ya Hu

The Anatomy of a Struggle

"Ya" is a sudden human cry ("O!"), while "Hu" is the breath of the lungs, signifying the inner self. Here, the Arabic calligraphy of "Ya Hu" visually traces the body's journey through struggle, its shapes reflecting the progression of acute illness — a metaphor for the soul's passage from turmoil to peace.

Chapter 1

General introduction

Introduction

Sarcoidosis is a systemic inflammatory disorder of unknown cause, characterized by the formation of non-caseating epithelioid cell granulomas and a heterogeneous clinical course.[1] Löfgren's syndrome is a well-defined, distinct phenotype of sarcoidosis. This thesis focuses on Löfgren's syndrome, in particular genetic factors that are predisposing for the disease and those that associate with manifestations of its symptoms. As **chapter 2** contains an extensive description of Löfgren's syndrome in terms of diagnosis, disease pathogenesis and patient management, this introduction chapter will succinctly refer to the general background, subsequently leading to the aim of this thesis.

Löfgren's syndrome

Löfgren's syndrome was first described in 1946 by Sven Löfgren, a Swedish pulmonologist. Initially, he used the term 'The bilateral hilar lymphoma syndrome' for patients having erythema nodosum with bilateral hilar lymphadenopathy.[2] In the following years, he published several reports about these patients. Eventually, he recognized a group of patients that were characterized by acute onset of symptoms, usually with fever, bilateral hilar lymphadenopathy on chest radiography, erythema nodosum and/or bilateral ankle arthritis or distinct periarticular inflammation.[3-6] This group was defined as Löfgren's syndrome.

Löfgren's syndrome and "non-Löfgren's syndrome" sarcoidosis share several common characteristics, such as the formation of noncaseating granulomas in effected organs and an elevated $CD4^+/CD8^+$ T-cell ratio in bronchoalveolar lavage fluid (BALF).[7] However, the crucial difference between the two types of sarcoidosis is that the Löfgren's syndrome phenotype has an acute and more homogenous presentation, while non-Löfgren's syndrome sarcoidosis displays an insidious onset with a heterogeneous presentation.[7]

Prognosis of patients with Löfgren's syndrome is very good compared to patients with non-Löfgren's syndrome sarcoidosis, especially in those who are *HLA-DRB1*03* positive, where 95% recover within 2 years.[8]

Diagnosis and incidence

The diagnosis of Löfgren's syndrome is established on clinical and radiological findings. Patients have an acute onset of disease with bilateral hilar lymphadenopathy (BHL) on chest radiography. At presentation, patients can also have erythema nodosum (EN), arthritis, or both. The combination of an acute onset, BHL, EN, and

bilateral ankle arthritis has a high sensitivity and specificity for diagnosis, up to 93 and 99%, respectively.[9] Histopathological confirmation is not required for diagnostic purposes in patients with typical clinical features of Löfgren's syndrome. Erythema nodosum is significantly more common in female Löfgren's syndrome patients, whereas arthritis is more common in male Löfgren's syndrome patients.[10] In earlier reports, Löfgren's syndrome was believed to occur more in female patients. This may be attributed to the observed gender bias due to the incorporation of EN as a diagnostic criterion in several studies.[11,12]

Global incidence of Löfgren's syndrome varies greatly, constituting up to half of sarcoidosis patients in Scandinavian countries,[13] the Netherlands[14] and Spain,[15] while being far less common in the UK,[14] the USA[16] and Asia,[17,18] with less than 1% of the sarcoidosis patients presenting with Löfgren's syndrome.

Genetics

The etiopathogenesis of sarcoidosis remains largely unknown, although multiple reports have indicated genetic inheritance, infectious transmission and shared exposure to environmental agents as main causes. The hypothesis can be formulated that genetically predisposed patients who are exposed to certain environmental triggers are likely to develop the disease. There have been reports suggesting a seasonal clustering of Löfgren's syndrome. Increased incidence in springtime has been reported in the northern as well as southern hemisphere, suggesting a common environmental trigger.[19-21]

Human leukocyte antigen (HLA)-region

The immunological features underlying the pathogenesis of sarcoidosis has led to many studies addressing the human leukocyte antigen (HLA) region. HLA has a central role in antigen presentation and many various alleles in the genetic region on chromosome 6 are associated with disease risk and course. Associations between *HLA-DRB1*05*, *HLA-DRB1*11* and *HLA-DRB1*14* and risk for sarcoidosis are shown,[14,22] whereas for *HLA DRB1*15* associations are shown with extrapulmonary involvement[23] and development of chronic disease.[13]

For Löfgren's syndrome the strongest association is shown with *HLA-DRB1*03*, which is associated with development of the disease but also with a good prognosis, resolving within 2 years.[8] In non-Löfgren's syndrome sarcoidosis patients *HLA-DRB1*03* is also associated with a good prognosis.[24] These associations have led to many studies searching for the disease-initiating antigen(s). In HLA-DR3 positive patients, accumulation of TCR-restricted, differentiated $CD4^+$ T cells in the lungs supports the notion of specific antigen recognition.[25,26] Higher frequencies of $V\alpha2.3^+CD4^+$ T cells correlate with acute disease onset and a more rapid disease

resolution.[27] Molecular modeling of the TCR complex Vα2.3/Vβ22, showed an ideal fit of a peptide derived from cytoskeletal protein vimentin into the peptide-binding cleft.[28] Vimentin has on separate occasions been eluted from HLA molecules on alveolar macrophages of HLA-DR3 positive sarcoidosis patients, and also been observed to induce T-cell IFN-γ responses in HLA-DR3 positive patients. Recent studies showed that Löfgren's syndrome specific TCR's recognized a peptide derived from the NAD-dependent protein deacetylase (NDPD) of an airborne mold species, *Aspergillus nidulans*.[29] This peptide stimulated $CD4^+$ T cells from the BAL of the majority of *HLA-DRB1*03* positive Löfgren's syndrome patients and increased IgG antibody responses to *Aspergillus nidulans* NDPD were detected in the serum of *HLA-DRB1*03* positive Löfgren's syndrome patients.[29]

Single Nucleotide Polymorphisms (SNP)

Genetic associations between sarcoidosis, Löfgren's syndrome and non-Löfgren's syndrome, are not only confined to the HLA-region. Several single nucleotide polymorphisms (SNP) in non-HLA regions are shown to be associated with increased disease risk for sarcoidosis and phenotype. A SNP is a single nucleotide variation at a specific genomic position that occurs in at least 1% of the population, representing common genetic diversity. In contrast, a mutation is any alteration in the DNA sequence, which may be rare, spontaneous, or pathogenic, and does not require a minimum frequency in the population.

ANXA11

The best known and most highly replicated genetic association in sarcoidosis is found in the gene for annexin A11 (*ANXA11*) at the locus rs1049550, first discovered in a German cohort of sarcoidosis patients.[30] *ANXA11* is known to be involved in calcium signaling, cell cycle, vesicle trafficking, and apoptosis.[31,32] A change in *ANXA11* could result in a dysfunctional Annexin A11 influencing cell processes, like cell trafficking, which in turn can influence granuloma formation and maintenance in sarcoidosis patients.[33]

MIF

Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine which plays a role in T cell and macrophage activation and migration.[34,35] MIF is involved in antigen-specific immune responses. The interaction between antigen-presenting cells and T cells, leading to T cell activation and cytokine production, plays a key role in the immunopathogenesis of sarcoidosis by driving the formation and maintenance of granulomas.[36]

Associations have been found between a genetic variation in macrophage migration inhibitory factor (*MIF*), the -173C allele, which is associated with increased MIF

production,[37] and chronic inflammatory and auto-immune diseases, like sarcoidosis and systemic sclerosis.[38] In sarcoidosis patients with erythema nodosum (EN) the frequency of the *MIF* -173C allele has been found to be significantly higher than in patients with EN due to other causes or controls, indicating a role for MIF in the clinical presentation of sarcoidosis.[39]

CCR2 and CCR5

Within the C-C chemokine receptor 2 (*CCR2*) gene a combination of single nucleotide polymorphisms (SNPs), the *CCR2* haplotype 2, has been found to be associated with Löfgren's syndrome in Dutch, Spanish, and Swedish patients, independently of *HLA-DRB1*03*.[40,41] CCR2 is involved in cellular trafficking and granuloma formation, and important for generating efficient immune responses.

The CC chemokine receptor 5 (CCR5) plays a crucial role in T-cell function, and polymorphisms in the *CCR5*-encoding gene are known to influence its expression. While genetic studies on *CCR5* variants have not demonstrated an association with sarcoidosis susceptibility,[42] a *CCR5* haplotype has been linked to persistent lung involvement in Dutch and British sarcoidosis patients.[43] Additionally, in Löfgren's syndrome, *CCR5* promoter polymorphisms have been associated with female patients.[44]

Aims and thesis outline

As described above, certain genes are related to sarcoidosis, but have not been thoroughly studied in patients with Löfgren's syndrome until now. Furthermore, functional implications of mutations in genes have not yet been elucidated in Löfgren's syndrome.

In this thesis, we aim to contribute to the search for genetic and/or functional defects predisposing to Löfgren's syndrome and the difference in these defects between Löfgren's syndrome and non-Löfgren's syndrome patients. Besides assessment of genetic predisposition for Löfgren's syndrome, an important focus is the relationship between genetic background and manifestations of symptoms and prognosis by a long-term follow-up in a large Dutch cohort of patients with Löfgren's syndrome.

Chapter 2 will provide a comprehensive overview of disease pathogenesis, diagnosis, as well as patient management. Furthermore, reflections on future scientific challenges, emphasizing the concept of Löfgren's syndrome as a disease in its own right are given.

Chapter 3 will validate the association of tag-SNPs for *HLA-DRB1*03* and *HLA-DRB1*15*. Also, the association between the tag SNPs and bronchoalveolar lavage fluid (BALF) characteristics of Löfgren's syndrome and non- Löfgren's syndrome patients will be evaluated.

In **Chapter 4** the long-term follow-up data of patients with Löfgren's syndrome will be shown, including data about prognosis and treatment.

In **Chapter 5** a case control study will be performed for the association of *ANXA11* rs1049550 in patients with Löfgren's syndrome and sarcoidosis compared to controls. In addition, a meta-analysis will also be performed for the association between *ANXA11* rs1049550 and different phenotypes of sarcoidosis: Löfgren's syndrome and chronic sarcoidosis.

In **Chapter 6** the association of *MIF* -173C allele with susceptibility to Löfgren's syndrome or with clinical manifestations of symptoms of Löfgren's syndrome will be investigated.

In **Chapter 7** the association of *CCR5* rs1799987 with susceptibility to Löfgren's syndrome will be evaluated. Next, the effect of this SNP to the CCR5 expression and function by measuring calcium influx reaction to simulation will be determined.

Chapter 8 will give a summary and discuss the results of this thesis, and will give directions for future research.

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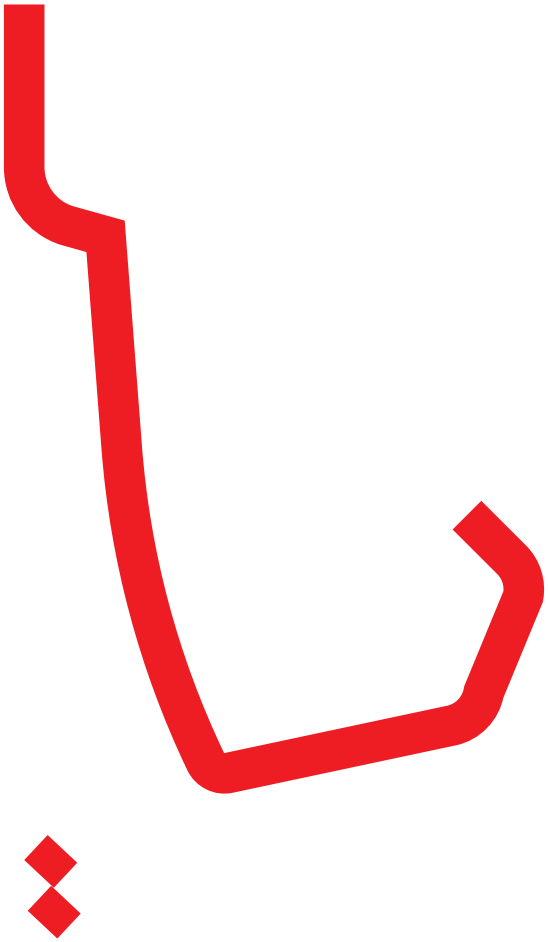

The Cry

The Onset. The letter Ya (O!) serves as the initial hook.

Anatomically, this stroke represents the clavicle and the spine, but symbolically, it captures the struggling person's internal—the sharp, unseen burden of pain and the mental weight of anticipating the unknown. It is the silent "cry" of the psyche before the body even shows the first mark.

Chapter 2

Löfgren's syndrome: diagnosis, management and disease pathogenesis

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Abstract

Löfgren's syndrome (LS), first described in 1946 by Swedish Professor of Medicine Sven Löfgren, is a clinically distinct phenotype of sarcoidosis. Patients typically experience an acute disease onset, usually with fever, and characteristic symptoms of bilateral hilar lymphadenopathy, erythema nodosum and/or bilateral ankle arthritis or periarticular inflammation. LS patients are well documented to have a good prognosis, which is especially true for *HLA-DRB1*03*$^{+}$ individuals. The presence of this allele correlates closely with accumulation of clonal CD4^{+} T cell populations in the lung, suggestive of local antigen recognition. Moreover, LS differs markedly from "non-LS" sarcoidosis in terms of immune cell activation, differentiation and regulation, which may influence clinical outcome and spontaneous disease resolution.

This review offers an overview of the clinical characteristics, genetic background and immunological characteristics of LS, as well as patient management, and reflections on future scientific challenges, emphasising the concept of LS as a disease in its own right.

Keywords: Löfgren's syndrome, HLA-DRB1*03, erythema nodosum, arthritis, bilateral hilar lymphadenopathy, bronchoalveolar lavage, CD4^{+} T cells, sarcoidosis

Introduction

Löfgren's syndrome (LS) and "non-LS" sarcoidosis share several common characteristics, most notably the formation of noncaseating granulomas in organs engaged by disease, as well as an elevated $CD4^+/CD8^+$ T cell ratio in bronchoalveolar lavage fluid (BALF).[1-4] However, the LS phenotype is both more acute in its presentation and more homogeneous in its clinical manifestation.[5-6] In contrast to non-LS patients, who commonly display an insidious onset and slower progression, often culminating in a chronic disease state, LS patients, and especially those carrying the *HLA-DRB1*03* allele (hereafter designated as *HLA-DR3*), often experience spontaneous recovery.[7] These striking differences in disease presentation and outcome suggest the involvement of altered or additional genetic and immunological pathways in LS, which have yet to be fully understood. In this review, we highlight the unique clinical, genetic and immunological profile of LS, along with current approaches in diagnostics, treatment and clinical follow-up. We conclude by discussing future research perspectives that may further enhance our understanding of this intriguing disease.

Clinical Aspects

In 1946, Swedish pulmonologist Sven Löfgren described 178 patients presenting with erythema nodosum (EN).[8] Fifteen of these patients had bilateral hilar lymphadenopathy with a negative or weak tuberculin test, and in two of these patients sarcoidosis was histologically proven. Löfgren coined the term "the bilateral hilar lymphoma (BHL) syndrome", and conducted further investigations on a large cohort of 212 patients.[5,9-11] These patients had BHL, a negative or weak-to-moderate tuberculin reaction, and were clinically determined to have an acute stage pulmonary sarcoidosis. Among them, Löfgren defined three groups based on symptomatic presentation: patients presenting with EN, with symptoms other than EN (e.g. cough, dyspnea, joint pains, fever), or those where the disease was detected after routine chest radiographic examination. Patients presenting with EN constituted the largest (113 patients; 53.3%) and most uniform group with regards to clinical aspects such as articular symptoms and fever. Patients presenting with an acute onset, EN, BHL on chest radiography, fever and/or bilateral ankle arthritis or distinct periarticular inflammation were later all defined as having LS (Table 1).[4]

Table 1. Löfgren's syndrome, diagnosis and management

Löfgren's syndrome

Diagnosis based on
- Clinical symptoms
  o Erythema nodosum and/or ankle arthritis or periarticular inflammation, usually fever
- Chest X-ray
  o BHL, parenchymal involvement (usually mild) may be present

Additional evaluation
- Extrapulmonary localizations
  o Peripheral lymph nodes, uveitis, parotitis, central nerve palsy can occur
- Laboratory tests
  o No specific laboratory measurements required for diagnosis
  o ACE elevated in 50% of patients
  o Hypercalcemia may occur
- Bronchoscopy
  o BAL is not routinely recommended in initial work-up
  o $CD4^+/CD8^+$ ratio is usually elevated, with a low $CD103^+CD4^+/CD4^+$ ratio.
- Imaging
  o HRCT and FDG-PET are not routinely recommended
- Histopathology
  o Biopsy is not routinely recommended in initial work-up
- Additional investigation that may be helpful with differential diagnosis
  o TBC: Tuberculin skin test, interferon gamma assay, biopsy of hilar or mediastinal nodes with EBUS, peripheral nodes, trans-bronchial biopsies
  o Malignancy: biopsy of hilar or mediastinal nodes with EBUS, peripheral nodes
  o Infection: cultures of BALF

Treatment
- In general no need for treatment
- Treat only disabling symptoms of EN and arthritis
  o EN: NSAID's, colchicine, potassium iodide, hydroxychloroquine
  o Arthritis: NSAID's, colchicine, low-mild doses of oral corticosteroids

Follow-up
- With *HLA*-typing
  o *HLA-DRB1*03*-positive patients do not need long term follow-up
  o *HLA-DRB1*03*-negative patients are recommended to follow-up for at least 2 y
- Without *HLA*-typing
  o Follow-up of patients is recommended at least until resolution of complaints and BHL

Abbreviations: ACE, angiotensin-converting enzyme; BALF, bronchoalveolar lavage fluid; BHL, bilateral hilar lymphadenopathy; EBUS, endobronchial ultrasound; EN, erythema nodosum; FDG-PET, fluorodeoxyglucose-positron emission tomography; HRCT, high-resolution computed tomography; NSAID, nonsteroidal anti-inflammatory drug; TBC, tuberculosis.

Symptoms

Bilateral Hilar Lymphadenopathy

In Löfgren's original cohort as well as confirmed later by other studies, most patients (79-81%) present with chest radiographic stage I (hilar nodal enlargement only, Figure 1), while 16-23% have stage II disease (hilar enlargement and parenchymal disease). On average, 2% of patients show no abnormalities on chest radiography at presentation. BHL can present with or without additional parenchymal involvement.[6,10-12]

A B

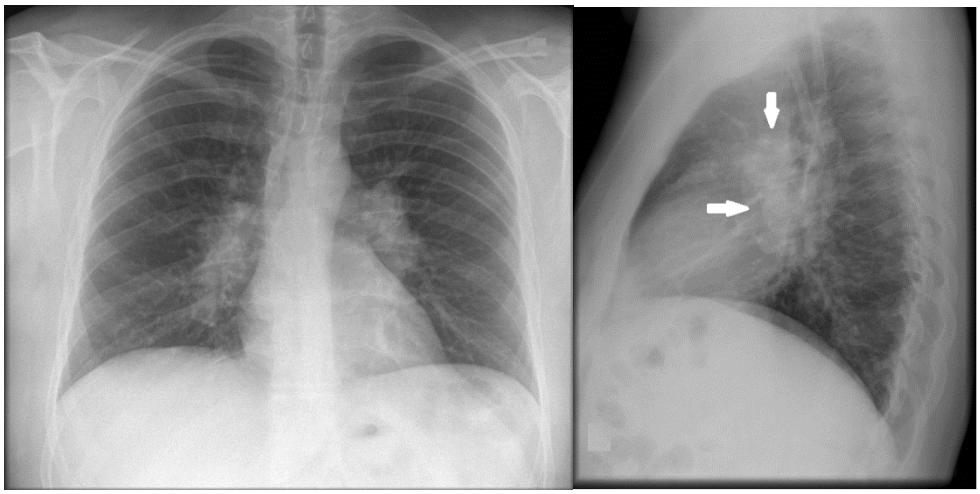

Figure 1. Bilateral hilar lymphadenopathy. (**A**) Posteroanterior view of chest radiography, showing bilateral hilar lymphadenopathy. (**B**) Lateral view of chest radiography, showing enlarged hilar lymph nodes, as indicated by arrows.

Erythema nodosum

EN can occur at any age, with a peak incidence between 20 and 40 years. The most common identifiable causes are sarcoidosis,[13] infections, malignancies, drugs and pregnancy,[14] though roughly 50% of cases have an idiopathic origin. At the time of Löfgren's discovery, EN was most commonly associated with tuberculosis, which shows symptomatic and histological similarities to sarcoidosis. EN most commonly presents in the first half of the year, especially during the spring months.

EN occurs three to six times more frequently in women than in men, which is also true in LS.[6,9] In Löfgrens' description of 113 patients with acute primary pulmonary sarcoidosis with EN, 107 were women. Löfgren also noted that in approximately 30% of these women, EN occurred primarily during pregnancy or lactation period.[9]

EN is the most common form of panniculitis, which is characterized by an acute onset of erythematous, tender nodules, classically located on the bilateral extensor surfaces of the lower limbs (Figure 2), but can also be seen on the ankles, lower thighs and arms. The nodules are rounded, slightly elevated and 1 to 6 cm in diameter. Nodules develop over several days and may follow a prodrome of fatigue, fever,

malaise, arthralgia, or symptoms of an upper respiratory infection, after a period of 1 to 3 weeks. Usually, acute bouts of EN are associated with fever, fatigue, malaise, arthralgia, headache, abdominal pain, vomiting, cough, or diarrhea. The symptoms typically reach their maximum after 1 to 2 weeks and nodules resolve spontaneously in 1 to 6 weeks; all in all, EN can take up to 12 weeks to fully resolve.[15,16]

Histopathological investigation of EN reveals a septal panniculitis without vasculitis. EN nodules show histological variation over time, with early lesions distinguished by septal edema and neutrophilic infiltrates, and as EN progresses, older lesions characteristically show signs of fibrosis, septal granulation tissue, and infiltration of lymphocytes and multinucleated giant cells.[14] Lesions undergo spontaneous regression, and despite a striking fibrotic process, this occurs without ulceration or permanent scarring.

Direct immunofluorescence of EN lesions shows deposits of immunoglobulins and complement factors in and around blood vessels of connective tissue septa. In patients with EN secondary to tuberculosis and sarcoidosis, immune complexes have been found to be elevated in serum,[17] which could explain the accompanying symptoms of arthralgia, fever and malaise. Based on these findings, EN is believed to be the end stage of an immunological process that can be initiated by a wide variety of antigenic stimuli.[15]

Figure 2. Erythema Nodosum on the shins of a patient with Löfgren's syndrome.

Arthritis

Arthritis involvement in sarcoidosis can be divided into two types: an acute, transient form and a persistent or chronic variant. The most common form of polyarthritis develops acutely and often as one of the first symptoms of the disease. The ankles are the predominantly involved joints, showing evidence of remarkable periarticular inflammation, but knees and wrists can also be affected. Patients with arthritis due to sarcoidosis have bilateral hilar lymphadenopathy on chest radiography, and in approximately 30 to 40% of patients, these symptoms are accompanied by EN. Of note, patients presenting with acute arthritis due to other causes usually do not have visible abnormalities on chest radiography.[18-20] Histopathological biopsies of the joint commonly reveal an unspecific, mild synovitis.[19,21] Imaging of the joints by magnetic resonance imaging or ultrasonography typically shows periarticular inflammation with subcutaneous and soft tissue edema, and small amounts of joint and tenosynovial fluid without evidence of synovial thickening or synovitis.[22,23] Interestingly, a seasonal clustering of disease onset in the spring is observed in patients who present with acute arthritis as their only symptom of sarcoidosis.[6,20] These symptoms then resolve in 6 to 12 weeks.[20,24-26]

In his original report, Löfgren noted that out of 212 patients with BHL, 119 patients had articular symptoms; 101 of these patients presented with EN and the remaining 18 patients without EN.[10] In a study on patients referred to the rheumatologist with sarcoid arthritis as the initial symptom, 51% were males. However, in this study patients were not separated by arthritis with or without EN;[20] where such distinction has been made, 73 to 79% in the “arthritis only” group has been found to be males.[6,19] It was long debated whether patients with BHL and arthritis without EN constituted a variant of LS or a wholly different entity of sarcoidosis, but today, due to the marked similarities in disease evolution and genetic background, patients with either EN, arthritis or both are all diagnosed with LS.[20,25]

Extra-pulmonary manifestations in LS

In Löfgren’s report, extrapulmonary sarcoidosis was described in 26 patients (12%), seven of these with BHL only and 19 with BHL and parenchymal involvement. The eyes were the most common sites of extrapulmonary manifestations, while a few had parotitis engagement or facial palsy.[11] Other affected sites included the nasopharynx and thyroid gland. In a Spanish cohort, granulomatous skin lesions (13% of patients), hepatomegaly (6%), ocular involvement (5%), splenomegaly (2%), salivary gland hypertrophy (1%), and central nervous system involvement (1%) were all observed as extrapulmonary manifestations of LS.[12]

Incidence

LS usually arises in individuals between ages 25 and 40 years; for women, a second peak is observed at 45 to 60 years of age.[9,27] LS is believed to occur predominantly in women, although the observed gender bias may be due to the incorporation of EN as a diagnostic criterion in several studies.[12,28] In a study of 150 Swedish patients diagnosed with LS, half were women.[6]

Globally, LS shows a broad variation in incidence, most likely attributable to a large genetic influence on disease development and marked differences in the genetic background across ethnic groups.[9,12] Approximately 50% of Spanish sarcoidosis patients are diagnosed with LS;[29] in Scandinavia[30] and the Netherlands,[31] LS patients constitute roughly a third of all sarcoidosis cases. LS is less common in the United Kingdom[31] and the United States,[32] with only 0.9 and 0.7% of sarcoidosis patients presenting with LS, respectively. Moreover, LS is extremely rare in Asia; in Japan, only 12 case reports on patients with LS have been published,[33] while 22 LS patients are described in a solitary report on Chinese Han patients.[34]

Diagnosis

The diagnosis of LS is based on clinical and radiological findings. Patients have an acute onset of disease with bilateral hilar lymphadenopathy on chest radiography. At presentation, patients can have EN, arthritis, or both. The combination of an acute onset, BHL, EN and bilateral ankle arthritis has a high sensitivity and specificity for diagnosis, up to 93 and 99% respectively.[20]

Similar to non-LS sarcoidosis patients, about half of all LS patients show elevated levels of angiotensin-converting enzyme (ACE) in serum, thus complicating use of ACE as an indicator for confirmation or exclusion of LS diagnosis.[12] Sedimentation rate can be elevated in up to 84% of patients[20] and hypercalcemia is seen in approximately 2% of LS patients.[12]

Patients commonly present with a cough and dyspnea, and show no obvious impairment upon pulmonary function tests. No correlation has been found between lung function at presentation and persistence of disease activity or risk of recurrence.[6,12] In LS patients, BHL with or without pulmonary involvement can be found, but advanced radiographic stages III and IV have never been described at presentation.

A recent study on sarcoidosis patients, however not subclassified into LS or non-LS, with stage I disease established by chest radiography, showed parenchymal changes on high-resolution computed tomography (HRCT) in 28 out of 51 patients

(55%). A 2-year follow-up with chest radiography showed no significant difference between patients with or without the parenchymal involvement and resolving disease.[35] Also, Mana et al performed gallium-67 scintigraphy in 142 patients, which provided additional information in six patients diagnosed with stage 0 disease (i.e. normal chest radiography), and in seven patients where parenchymal involvement was not suspected by chest radiography alone.[12] Though usually used for HRCT, gallium-67 scans and fluorodeoxyglucose-positron emission tomography are not recommended routinely in LS.

The cellular profile in BALF is similar in LS and non-LS sarcoidosis, distinguished by lymphocytosis primarily constituted by $CD4^+$ T cells, which manifests in an elevated $CD4^+/CD8^+$ ratio (>3.5) in up to 74% of patients.[36-38] Analysis of integrin CD103 expression on $CD4^+$ T lymphocytes in interstitial lung diseases (ILDs) showed that the ratio of $(CD103^+CD4^+)/CD4^+$ was lower in sarcoidosis compared with other ILDs. In stage I sarcoidosis patients, including LS patients, the $(CD103^+CD4^+)/CD4^+$ ratio was significantly lower compared with advanced radiographic stage sarcoidosis.[37,39]

As shown by Mana et al, histological confirmation of granulomatous inflammation, especially biopsies of skin lesions other than EN, and peripheral lymph nodes could verify LS diagnosis in 63% of patients.[12] Biopsies are currently not required for diagnostic purposes in patients with typical clinical features of LS. However, in the case of atypical symptomatic or radiological findings, or a patient history suggesting the differential diagnosis, for example tuberculosis, lymphoma or malignancies, a histopathological confirmation is warranted.[1] The same applies to patients with a prolonged disease course, where validation is of particular importance before initiation of corticosteroid treatment. The biopsy should be obtained from the affected organ most easily accessed, for example non-EN skin lesions, peripheral lymph nodes, lacrimal glands, liver or conjunctiva. In patients with a high, a priori risk of tuberculosis, a tuberculin skin test or an interferon-γ (IFNγ) release assay should be performed to exclude *Mycobacterium tuberculosis* infection. When there is a suspicion of lymphoma or malignancy, the mediastinal or hilar lymph nodes can be approached with EBUS or through mediastinoscopy. In case of parenchymal involvement, transbronchial biopsies provide a valid alternative.[12]

Treatment and Follow-Up

The highly favorable prognosis of LS patients, and especially those positive for HLA-DR3, more or less eliminates the need for treatment. However, several studies report a minority of patients, ranging from 5 to 19%, to have been treated with oral corticosteroids. Treatment indications vary from intolerance to nonsteroidal anti-inflammatory drugs (NSAIDs) to disabling complaints, such as facial nerve palsy or pronounced ankle arthritis.

A retrospective study conducted on LS patients in Sweden showed no difference in prognosis between treated and untreated HLA-DR3-positive patients, while amongst the HLA-DR3-negative patients treated with oral corticosteroids, 80% had nonresolving disease, whereas only 37% experienced nonresolving disease in the untreated group. Among HLA-DR3-negative patients, the nonresolving disease was seen primarily in patients who were positive for *HLA-DRB1*04* (DR4) or *HLA-DRB1*15* (DR15).[7] Either HLA-DR3-negative patients are more prone to develop chronic disease regardless of corticosteroid treatment, or oral corticosteroids actively interfere with normal, protective – or at least beneficial – immune processes, thereby resulting in a prolonged disease course.

In LS, symptoms caused by EN and arthritis can be treated when considered disabling. Due to the spontaneous regression of EN nodules, NSAIDs may be a sufficient aid in inducing analgesia and promoting resolution. If lesions persist, some patients may also respond to colchicine or hydroxychloroquine on a twice daily dosage.[14] Patients with symptomatic arthritis can also be treated with a short course of low-to-moderate corticosteroid doses (15-40 mg).[40]

Follow-up after disease remission is usually not necessary; however, recurrence of disease has been observed in 3 to 6% of patients with LS, where symptoms return years after full clinical recovery.[7,41] Remarkably, this appears restricted to HLA-DR3-positive individuals,[7] suggesting a link to antigen presentation in an individual with a certain genetic constitution.

Genetic Background

Human Leukocyte Antigen Association

The human leukocyte antigen (HLA) genetic region is central regarding antigen presentation and contains the strongest genetic link with LS. HLA class I antigen *HLA-B8* was the first described genetic association with acute sarcoidosis,[42,43] and the combination of HLA-B8 with HLA-DR3, a class II antigen, was later confirmed to form a risk haplotype.[44-46] HLA-B8/DR3 is part of the so-called “8.1 ancestral haplotype“ (*HLA A*0101: Cw*0701: B*0801: DRB1*0301: DQA1*0501: DQB1*0201*), which is common in Europeans and associated with several autoimmune diseases.[47] The haplotype *HLA-A*01:B*08:DRB1*03* was found in 20% of Swedish sarcoidosis patients and all patients with this haplotype showed resolving disease.[27] In Sweden and the Netherlands, HLA-DR3 is relatively common and LS more frequent,[30,31] while HLA-DR3 and LS are both extremely rare in Japan. In the 12 Japanese LS patients described thus far, no one was positive for HLA-DR3, but five out of nine HLA-typed patients (55.6%) were positive for *HLA-DRB1*12* (DR12), suggesting an association

between LS and HLA-DR12 in Japanese patients and perhaps a different disease-triggering antigen (Figure 3).[33]

LS is usually a self-limiting disease, with 81 to 92% of patients recovering within 2 years,[7,12,20] most patients even in the first year.[11,12] Especially HLA-DR3-positive LS patients seem to have a favorable prognosis, with recovery within 2 years in 95% of the cases. In contrast, HLA-DR3-negative patients have a less favorable disease course, with only 51% of patients experiencing resolving disease in the same time interval.[7] No differences could be discerned between the HLA-DR3-positive and -negative groups in terms of radiological staging or BALF CD4/CD8 ratio. A strong correlation with a good prognosis in Caucasians has also been noted for *HLA-DQB1*0201*,[45,48] which, as previously stated, is part of the 8.1 ancestral haplotype and in strong linkage disequilibrium with HLA-DR3.

The HLA class III region comprises a set of immune regulatory genes, such as tumor necrosis factor-α (TNFα). A nucleotide substitution of a G for an A at position 308 in the promoter of the *TNFA2* allele associates with the *HLA-A*01, -B8-, DR3* haplotype and with elevated TNFα production.[49] Moreover, this allele is more frequent in LS patients.[50] The link to the ”8.1 ancestral haplotype” complicates independent assessment of the functional role of the *TNFA2* allele[51] and its effect on disease pathology remains unclear, as exemplified by a study where *TNFA2* did not associate with higher levels of TNFα release from mononuclear cells.[52]

Using a genome-wide association approach in the Immunochip platform, a comprehensive genetic study was recently conducted on LS and non-LS phenotypes, including 1,048 Swedish patients (384 with LS) and 2,086 controls, and with results being replicated in four independent cohorts.[53] Within the HLA region, 727 gene variants specific for LS were identified, along with 68 for non-LS. Also, 17 gene variants within the HLA region associated with both LS and non-LS. Notably, interleukin (IL-)17 immune response pathways were found preferably in LS, in support of recent functional studies.[54,55] Altogether, LS patients exhibit unique genetic susceptibility that is distinct from non-LS sarcoidosis.[53]

Non-HLA Gene Involvement

Genes associated with LS susceptibility or presentation is not confined to the HLA region. The major histocompatibility complex 2 transactivator (*MHC2TA*) gene regulates the expression of HLA class II molecules on the surface of immune cells, and specific *MHC2TA* alleles are associated with LS, but not with non-LS. Interestingly, this association also appears to be independent of HLA-DR3.[56]

A combination of single nucleotide polymorphisms (SNPs) within the C-C chemokine receptor 2 *(CCR2)* gene (*CCR2* haplotype 2) has been found to associate with LS in Dutch, Spanish and Swedish patients, independently of HLA-DR3.[57,58] The association could not be replicated in a German cohort, although a positive association between sarcoidosis and the chromosomal region for *CCR2* was found.[59] CCR2 is involved in cellular trafficking and granuloma formation, and important for generating efficient immune responses, for example in *Mycobacterium tuberculosis* infection. However, the biological relevance of *CCR2* gene variants remains a matter for speculation. Interestingly, a recent report demonstrated that Th17 cells could switch from CCR6 to CCR2 expression, subsequently promoting a GM-CSF/IFNγ-driven phenotype.[60] Although the impact of this mechanism in the context of human disease is not yet understood, the heightened CCR6 expression and reduced IFNγ production observed in LS[54] could potentially be attributable to *CCR2* SNPs that disrupt such a chemokine receptor switch.

In a German study, an association of LS with certain SNPs in the *CCR5* promoter (*CCR5* HHC haplotype) was found to be restricted to females,[28] further reinforcing genetic, immunological and hormonal influence on gender-specific variations in symptomatic presentation. Remarkably, in Dutch and British sarcoidosis populations, the *CCR5* HHC haplotype was not associated with disease susceptibility, but instead linked to parenchymal involvement.[61] Linkage disequilibrium between the *CCR2* haplotype 2 and *CCR5* HHC haplotype has also been documented,[61-63] and elucidation of the independent contribution of *CCR2* and/or *CCR5* to disease susceptibility or development in LS thus requires further investigation.

In a Dutch cohort of LS patients, a genetic variation in macrophage *migration inhibitory factor (MIF*), the -173C allele, which is associated with increased MIF production, did not contribute to disease susceptibility; allele frequencies were equal in healthy controls and LS patients. However, subgrouping based on symptoms at disease presentation revealed an increased *MIF* -173C allele frequency in patients with EN only, and a correspondingly reduced frequency in patients with only arthritis.[64] MIF is a key regulator of innate and adaptive immune responses, and the effect of this particular polymorphism is observed after the initiation of the sarcoid reaction, thereby affecting the clinical presentation. In this context, it is interesting to note that specific alleles in the genes encoding immunomodulatory cytokine transforming growth factor β (TGF-β) isoforms TGF-β2 and TGF-β3 were observed more frequently in LS patients compared with chronic sarcoidosis in a German cohort. As certain *TGF-β* alleles are believed to influence development of fibrosis, the identified SNPs in LS supposedly contribute protection against chronic disease.[65]

A recent large-scale network analysis investigated the interaction between transcription factor (T-bet), messenger RNA (mRNA), and microRNA (miRNA) in sarcoidosis BALF cells, concluding that while T-bet appeared to have the most influence over cytokine and chemokine receptor systems, miRNAs acted as "fine-tuners" of inflammatory gene expression. Moreover, altered miRNA patterns in progressing and regressing disease suggest a potential role for miRNA dysregulation in chronic sarcoidosis that ought to be further explored in functional studies.[66]

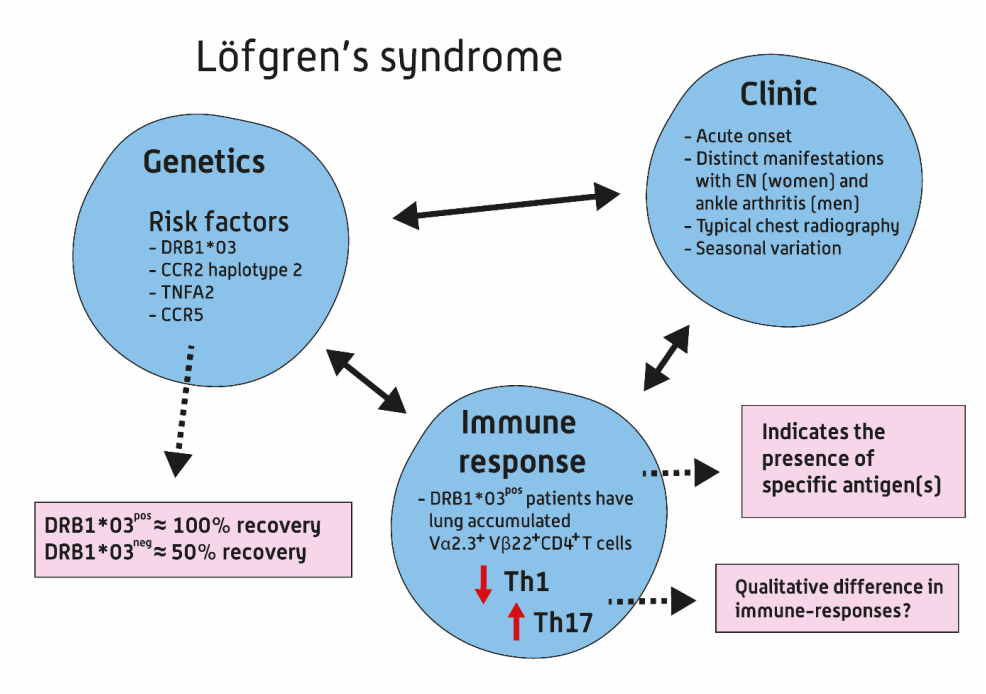

Figure 3. Genetic, clinical and immunological features of LS.
The LS patient group is quite distinct from other sarcoidosis patients as revealed by their clinical manifestation, genetics and immunological profile. Their specific genetic background, with strong links to especially *HLA-DRB1*0301*, is in addition connected to a favorable prognosis. The clinical picture is characteristic with an acute onset, usually fever, bilateral ankle arthritis (especially men) and/or erythema nodosum (mostly women), and bilateral hilar lymphadenopathy on chest radiography. The immune response is characterized by lung-accumulated $CD4^+$ Th expressing the TCR variable gene segments Vα2.3/Vβ22. The immune response in LS patients moreover seems to produce a broader range of cytokines and altogether display a less pronounced Th1, but a more marked Th17 immune response. LS: Löfgren's syndrome, TCR: T-cell receptor; Th: helper T cell.

Gene-Environment Interaction

The appearance of LS patients in the clinic shows a notable seasonal clustering during the spring months not observed for non-LS sarcoidosis and indicates the influence of an environmental factor in the etiology of the disease. Furthermore, seasonal variation was observed primarily in HLA-DR3-positive patients.[7,20,67,68] Structured studies of gene-environment interactions in sarcoidosis are scarce, but a significant interaction between *HLA-DRB1*1101* and exposure to insecticides has been reported.[69] Several reports also show a significant negative association between smoking and LS, possibly due to alterations in the number, type and functionality of inflammatory cells in the lung caused by cigarette smoking.[7,20] In general, gene-environment interactions that may specifically influence LS development is an area in need of further investigation.

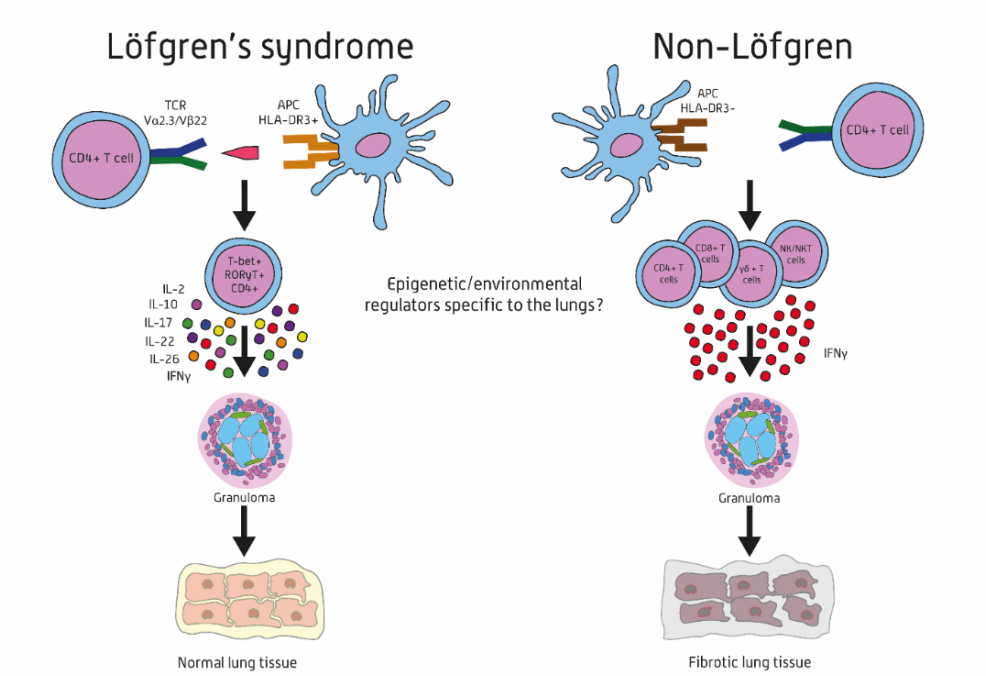

Figure 4. Proposed model of CD4⁺ T cell activation and differentiation following antigen presentation in LS and non-LS.
Correlation of clonally expanded, TCR-restricted effector T cells and presence of a particular HLA type indicates specific antigen recognition. Although the triggering antigen remains unknown, CD4⁺ T cells in the lungs of LS patients display a more variable cytokine profile in response to stimulation, with a relative decrease in IFNγ production compared with non-LS, and an increase in IL-10, IL-17A, IL-22, IL-2 and IL-26. Moreover, simultaneous expression of Th1 and Th17 transcription factors T-bet and RORγT, respectively, is more pronounced in LS than non-LS patients, and a higher percentage of T-bet⁺RORγT⁺ double-positive cells has been shown to correlate with resolving disease. Although the Th1/Th17 profile has been

described as pathogenic in inflammatory diseases such as rheumatoid arthritis and multiple sclerosis, IL-17A and IL-22 are produced in the healthy gut mucosa, and IL-17A depletion has been shown to exacerbate symptoms of Crohn's disease. It is, therefore, likely that the observed T-bet$^+$RORγT$^+$ expression is an inherent trait of pulmonary CD4$^+$ T cells, and that in LS, higher frequencies of these cells contribute to a more targeted, antigen-specific response while simultaneously providing tissue protection, ultimately leading to dissipation of granulomas, and disease resolution. In non-LS patients, a stronger IFNγ-driven response with the contribution of several immune cell compartments may be insufficient to clear granulomas, resulting in prolonged inflammation, and eventually development of pulmonary fibrosis. Differences in clinical disease presentation between LS and non-LS may thus result from a combination of genetic, epigenetic or micro-environmental, and immune-regulatory factors that ultimately contribute to a resolution or progression. APC: antigen-presenting cell; HLA-DR3: *HLA-DRB1*03*; IFN: interferon; IL: interleukin; LS: Löfgren's syndrome; TCR: T-cell receptors; Th: helper T cell.

Immunological Profile

Effector T-cell responses

In sarcoidosis, accumulation of effector CD4$^+$ T cells, either by migration to the lungs or through local expansion, suggests activation in response to antigen(s) present in the pulmonary compartment.[70] A majority of CD4$^+$ T cells in the lung, regardless of underlying disease and also in healthy individuals, typically exhibit an effector memory phenotype and possess limited proliferative capacity.[71] Nonetheless, investigation of T-cell proliferation in sarcoidosis in response to recall antigens, such as purified protein derivative, instead revealed an enhanced proliferative response early in the disease course, that is, in patients with acute disease, lower chest radiographic stage, and LS, compared with patients with a disease duration exceeding 3 months.[72]

Sarcoidosis has traditionally been regarded as a T helper (Th) 1-driven disease, characterized by excessive IFNγ, IL-12 and TNFα production in the lungs.[73] Both the IL-12 and IL-18 receptors and Th1-associated chemokine receptors CCR5 and CXCR3 are highly expressed on BALF CD4$^+$ T cells, while Th2 receptors CCR4 and CXCR4 are markedly reduced.[74] For CCR5, this profile has been found to be most pronounced in early disease stages, while expression is reduced upon the development of pulmonary fibrosis.[75] The release of CXCR3 ligands, such as CXCL9, CXCL10 and CXCL11 by activated alveolar macrophages,[76] may also contribute to the accumulation of CXCR3$^+$ Th1 effector T cells specifically in the lung.

However, the matter of effector T-cell subsets is complicated by separating patients into LS and non-LS, or stratification by HLA type. For example, HLA-DR3-positive sarcoidosis patients showed increased mRNA expression of TGF-β and reduced expression of IFNγ and TNFα compared with HLA-DR3-negative patients,[36,77]

indicative of reduced effector cell activity, or, alternatively, a more efficient self-limiting immune response.[36] Moreover, recent years' increased understanding of T-cell plasticity and compartmentalization has shifted our concept of sarcoidosis to a Th1/Th17 disorder.[78,79] Ramstein et al recently published a comprehensive study on two sarcoidosis cohorts, demonstrating the presence of Th17-polarised "Th17.1" cells in BALF of sarcoidosis patients. These cells, defined by their $CXCR3^+CCR6^+$ chemokine receptor expression, were able to exert Th1 and Th17 functions simultaneously and also responsible for the bulk of IFNγ production in the lungs.[80] In addition to these findings, $CXCR3^+CCR6^+$ $CD4^+$ T cells in BALF coexpressing Th1 and Th17 transcriptional regulators T-bet and RORγT were found to be increased in LS patients compared with non-LS.[54] Analysis of cytokine production further demonstrated the discrepancy between the two patient groups on a functional level. IFNγ was indeed the dominant cytokine produced by BALF T cells in both patient groups, but LS patients displayed significantly lower IFNγ levels and more pronounced production of IL-17A, IL-10 and IL-2, as well as a tendency toward elevated IL-22.[54] This suggests a broader cytokine profile to be beneficial in terms of disease resolution. Previous studies have also shown IL-17A levels in BALF to be elevated in LS compared with non-LS in response to mycobacterial protein mKatG,[55] and multifunctional $CD4^+$ T cells to be more prominent in LS (Figure 4).[81]

While IL-10 has a known immune-regulatory role, the effects of IL-17A and IL-22 appear to depend largely on the local tissue compartment. In the lungs, reduced levels of IL-22 has been observed in chronic sarcoidosis and idiopathic pulmonary fibrosis (IPF).[82] In the gut, another mucosal organ, these cytokines are produced under homeostatic conditions and contribute to maintaining a tolerogenic environment.[83,84] As indicated by the findings mentioned above, this might also be the case in lungs of LS patients.

Regulatory T-Cell Responses

A common trait of inflammatory and autoimmune diseases is not only a tissue-specific accumulation of effector cells, but also a disruption of the balance between effector and regulatory T cells (Tregs). In sarcoidosis, the presence of dysfunctional Tregs has been reported on several occasions, with observations of either lower levels of $FoxP3^+$ $CD4^+$ T cells,[85,86] or increased numbers but with reduced functional capacity, as measured in terms of for example IL-10 production,[36] survival[87] or suppression of effector cytokine secretion.[88]

Broos et al recently demonstrated that expression of coinhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) was reduced specifically on Th17 cells and Tregs in sarcoidosis,[89] which may shift the balance between disease-driving elements

and immune tolerance. Interestingly, the CTLA-4 blockade has also resulted in induction of a sarcoidosis-like granulomatous disease state in patients treated for malignancy.[90,91] Conversely, sarcoidosis has repeatedly been described in patients receiving IFNα therapy, which boosts the immune response.[92-96] Altered expression of programmed death protein-1 (PD-1), another negative regulator of effector T-cell responses, has also been observed in BALF and peripheral blood of sarcoidosis patients.[89,97] Similarly to the CTLA-4 blockade, anti-PD-1 treatment has been found to induce sarcoid-like granulomas in mediastinal lymph nodes and skin.[98] All of these observations stress the importance of delicate immune regulation in the lung in the context of sarcoidosis development and progression.
Upon T-cell receptor (TCR) ligation, inducible costimulator (ICOS) expression is upregulated and acts to potentiate proliferation and differentiation of T cells.[99] Specifically, the interaction of ICOS with its ligand ICOS-L increases production of IL-10.[100] In a recent report,[86] the highest expression of ICOS was found on BALF Tregs from LS patients, corresponding well to the overall more pronounced IL-10 production observed in this group.

T-cell immunoglobulin mucin (TIM) domain molecules have been suggested to influence T-cell activity, partly through their negative regulation of Th1 responses. In non-LS, expression of both TIM-1 and TIM-3 was found to be reduced, while LS differed only marginally compared with healthy controls.[77] This could be one mechanism through which IFNγ responses are enhanced in non-LS, thus driving a chronic inflammatory state.
Butyrophilin-like 2 (BTNL2) is a coinhibitory receptor separate from the CD28-CTLA-4 axis, with the capacity to negatively regulate T-cell activation by promoting de novo FoxP3 expression.[101] Polymorphisms in the *BTNL2* gene have been shown to influence the risk of sarcoidosis, but the overrepresented SNP variants and *BTNL2* haplotypes differ between patient groups when LS and non-LS sarcoidosis patients are considered separately.[102] Notably, *BTNL2* polymorphisms are known to associate with multiple autoimmune conditions such as rheumatoid arthritis (RA),[103,104] Crohn's disease,[104-106] systemic lupus erythematosus (SLE),[104] multiple sclerosis (MS),[107] Grave's disease,[108] type 1 diabetes,[104] as well as granulomatous lung disease tuberculosis.[105] As with *CCR2*, however, several of these links may be deceptive due to linkage disequilibrium of *BTNL2* with risk-conferring HLA alleles, most notably HLA-DR14 and HLA-DR15,[102] both of which are associated with chronic sarcoidosis.[109] Nevertheless, *BTNL2*'s role in Treg versus Th17 development is worthy of further exploration, especially in LS patients.[101,110]

The concept of "classical" Tregs in human disease is complicated by findings of T cells that exhibit regulatory functions but lack FoxP3 expression.[111] Conversely,

FoxP3-expressing cells with the potential to produce pro-inflammatory cytokines have been described.[112,113] Also, CD4+ T cells may, particularly in a tissue-specific context, express other markers signifying a regulatory role, e.g., CD103,[114,115] calling for a more thorough functional and phenotypical definition of regulatory cells involved in disease progression.

Non-T Cell Immune Responses

Although CD4+ T cells are acknowledged as the primary players in sarcoidosis immunopathology, other immune cells may also be involved, and are of particular interest regarding defining differences between patient groups.
CD1-restricted invariant natural killer T (iNKT) cells have been shown to possess an immune-regulatory role and to be protective against Th1-driven inflammation in animal models.[116] In sarcoidosis patients, Ho et al, found a decrease in iNKT cell levels compared with healthy individuals in all patient groups, except LS.[117] The authors speculate that iNKT cells may aid in the regulation of CD4+ T cell activity in LS, whereas a deficiency in their numbers or function in non-LS contributes to exaggerated IFNγ responses.

In a study focused on granulocytes in sarcoidosis, the percentage of neutrophils was found to be lower in LS patients compared with non-LS. Moreover, expanded neutrophil and NK cell populations associated with an unfavorable disease outcome, advanced radiographic stage and a requirement for corticosteroid therapy.[118] Neutrophils contribute to alveolar injury in several pulmonary diseases, for example IPF and hypersensitivity pneumonitis,[119] and their amplified numbers could contribute to the increased risk of fibrosis observed in non-LS patients. NK cells, on the other hand, have been suggested to stimulate TNFα release by alveolar macrophages, thereby promoting progressive disease with potential for corticosteroid resistance.[120] Also, a direct correlation has been found between percentages of T cells and IgG-secreting cells in BALF.[121] B cells have been implicated in the disease process through elevated levels of antibody-producing cells having undergone somatic hypermutation and affinity maturation. This increase was normalized following treatment with TNFα blockers.[122] Although this particular study did not distinguish between LS and non-LS, it is reasonable to assume that pulmonary B cell responses may reflect the differences observed for CD4+ T cells in the two conditions. B cells are also highly efficient antigen-presenting cells (APCs), further supporting their involvement in antigen uptake and recognition, as well as interaction with T cells in the pulmonary compartment. Given that EN is considered to result from deposition of immune complexes,[17,123] further exploration of patient subgroups as well as gender differences with regards to B cell and antibody production is warranted.

While alveolar macrophages constitute the vast majority of cells in BALF, professional APCs such as dendritic cells (DCs) are scarce. Reduced DC numbers and/or functionality has been reported both in peripheral blood[124,125] and lymph nodes[126] of sarcoidosis patients. In contrast, a local expansion of mature myeloid DCs (mDCs) has been detected in the vicinity of pulmonary granulomas.[127] Other studies have reported BALF mDCs in sarcoidosis to express lower-than-normal levels of costimulatory molecules CD83 and CD86, as well as CD1a.[128] Such cells are reported to be less efficient APCs, but potent producers of IL-1, IL-6 and TNFα,[129] cytokines that all could contribute to sarcoid inflammation.[130,131] The discrepancy in HLA distribution and proposed patterns of antigen presentation between LS and non-LS patients strongly suggests DC subset diversity to influence disease outcome.

Löfgren's Syndrome: An Autoimmune Form of Sarcoidosis?

Disease etiology in sarcoidosis as a whole remains elusive, though several infectious, environmental, and autoimmune components have been considered. The strong association with certain HLA-DR haplotypes, as well as the induction of disease, observed in anti-CTLA-4,[90] anti-PD-1,[98] IFN-,[94] and highly active antiretroviral therapy-treated patients,[132] are all in line with excessive immune responsiveness, as in autoimmunity, rather than a poorly controlled infection. Still, the underlying cause may be an infectious agent, and the immunological process observed a result of molecular mimicry and transient autoimmunity, as reported for conditions such as psoriasis.[133] It is also possible that different antigens are involved in the pathogenesis of LS and non-LS.

In HLA-DR3-positive patients, accumulation of TCR-restricted, differentiated CD4+ T cells in the lungs supports the notion of specific antigen recognition,[134,135] as these cells are neither found in healthy individuals carrying this *HLA* allele nor in sarcoidosis patients with other HLA types (the sole exception being a slight expansion in *HLA-DRB3*0101*$^{+}$ patients[70]). Higher frequencies of Vα2.3^{+} CD4^{+} T cells correlate with acute, nonchronic disease, and more rapid disease resolution.[136] Moreover, their levels normalize after clinical recovery, suggesting that these cells influence clearance of disease-initiating antigen(s).[7,136,137] Although rare, clinical recurrence of LS[7] might be considered a form of disease "flare", possibly induced by reactivation of memory T cells by an environmental or microbial trigger, as well documented for autoimmune diseases such as SLE and MS.
More recently, Vα2.3 was shown to associate with preferentially β-chain variable segment Vβ22, enabling sequencing of the TCR and modeling of the TCR-HLA complex. This revealed a high degree of clonality, in line with antigenspecific expansion, and an ideal fit of a peptide derived from cytoskeletal protein vimentin into the peptide-binding cleft.[70] Vimentin has previously been identified as an autoantigen

in RA[138] and SLE,[139] and the above-mentioned peptide has on separate occasions been eluted from HLA molecules on alveolar macrophages in BALF of HLA-DR3-positive sarcoidosis patients.[140,141] When used to stimulate T cells in an enzyme-linked immunospot assay, this peptide also induced IFNγ production in a subset of HLA-DR3-positive patients.[142] It is also reasonable to assume, given recent findings of cytokine distribution in LS,[54] that the T cells involved in antigen recognition might also respond with other forms of cytokine production, such as IL-17A and IL-10, which has not yet been investigated. Other candidate autoantigens have been identified by protein bead array technology, with different autoantibody profiles observed in LS and non-LS. Of these, mitochondrial ribosomal protein L43 was the target most specific for LS, though overall reactivity frequency was fairly low.[143]

Future Perspectives

The clinical, genetic and immunological findings in the unique LS patient group highlight with growing authority the distinction between the two forms of sarcoidosis. Historically, LS has primarily been a research focus in Northern Europe. The homogeneity of the patient group in question makes LS an ideal platform for detailed efforts at understanding disease origin, which can, if successful, be further applied to the more heterogeneous and globally prevalent non-LS population. Genetically, LS and non-LS overlap to a surprisingly limited degree,[53] clearly indicating that despite certain clinical similarities, LS constitutes a separate disease entity. Considering sarcoidosis patients as one group may mask relevant immunological phenotypes, and future studies should, whenever possible, strive to routinely separate LS and non-LS sarcoidosis patients into individual groups. Moreover, subgrouping based on organ involvement could further aid in proper diagnosis, as well as an understanding of the underlying immunological and genetic traits that drive specific clinical phenotypes in LS and non-LS. For example, it is known that HLA-DR4 and HLA-DR15 are strongly associated with the presence of extrapulmonary symptoms,[144] and in the case of HLA-DR4, especially with ocular manifestations.[145] Also, both clinical and genetic differences suggest the disease process in LS differ between males and females, arguing for more studies henceforth to distinguish between the two.

With new technology offering higher sensitivity and specificity, identification of new diagnostic and prognostic markers for either phenotype should only be a matter of time. Unpublished mass cytometry data from our group, accounting for concomitant expression of >30 unique T cell markers in multiparameter analysis, show significant differences between LS and non-LS BALF $CD4^+$ T cells (Ylva Kaiser, MSc; Tadepally Lakshmikanth, PhD; Yang Chen, PhD; Jaromir Mikes, PhD; Anders Eklund, MD, PhD; Petter Brodin, MD, PhD; Adnane Achour, PhD; Johan Grunewald, MD, PhD. manuscript submitted for publication, March 14, 2017). Intriguingly, molecules

involved in immune regulatory pathways, for example CTLA-4, ICOS and PD-1 that have been covered by other studies, were more abundantly expressed in LS than non-LS. Similarly, expression of CD127 (IL-7R), which has been linked to a genetic risk of developing sarcoidosis,[146] was significantly more frequent in non-LS. The role of antigen-specific B cells and autoantibody production in relation to clinical parameters and T cell responses is also under current investigation (Ylva Kaiser, MSc, Andrew J. Kinloch, PhD, Anders Eklund, MD, PhD, Marcus R. Clark, MD, PhD, Johan Grunewald, MD, PhD, in preparation), with preliminary findings further supporting the conclusion that LS should be considered a disease in its own right. Finally, although $CD4^+$ T cells constitute the driving force in both LS and non-LS sarcoidosis, other immune cells should not be forgotten. In the search for disease-specific antigens and immunological mechanisms, collaborative efforts to simultaneously study function and interaction of T cells, B cells, DCs, NK and NKT cells as well as alveolar macrophages, among others, might hold the answer to the riddle.

Concluding Remarks

LS patients represent an intriguing subset within the spectrum of sarcoidosis phenotypes, specifically characterized by their self-limiting disease course. Patients can be well diagnosed and normally require no treatment. Studies specifically targeting LS separately from other sarcoidosis patients have gradually provided us with an enhanced understanding of immunological pathways, and consistent subgrouping of patients is crucial to unravel the complexity of pulmonary immune responses in sarcoidosis. Moreover, studies conducted on LS alone might in the future be utilized to optimize management also of chronic sarcoidosis. Although much has already been learned, the fundamental question of which immunological mechanisms drives disease resolution or disease progression remains, and will continue to demand the attention of future clinical and translational research.

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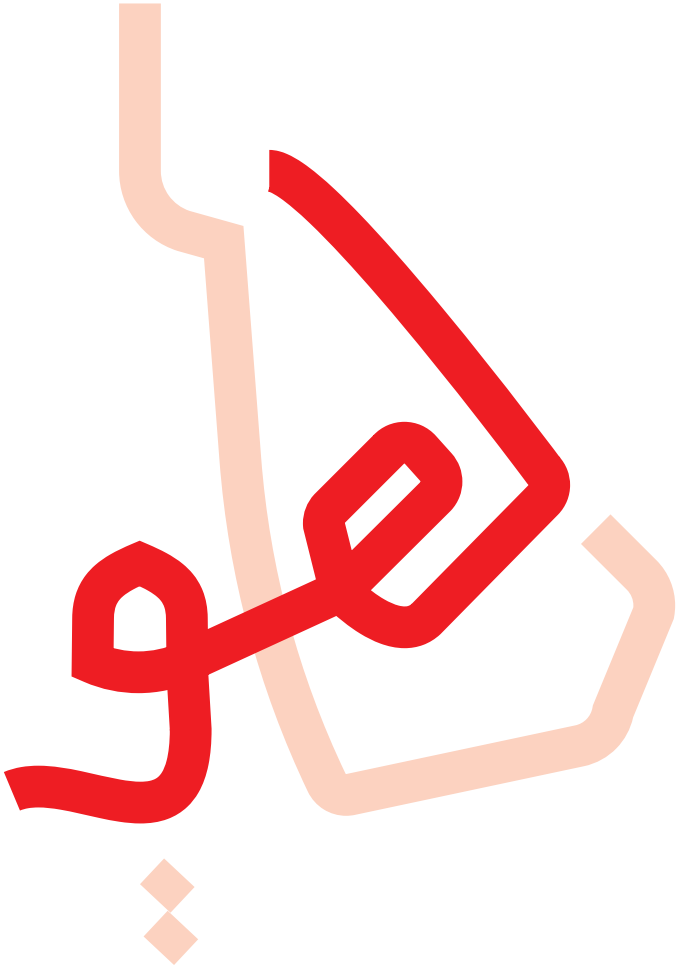

The Breath

The Inner Void. The addition of the Hu loop creates the negative space of the lungs.

This represents the Batin (Hidden) aspect of the syndrome: the silent swelling of the hilar lymph nodes deep within the chest, invisible to the eye yet felt in the breath.

Chapter 3

Bronchoalveolar lavage characteristics correlate with HLA tag SNPs in patients with Löfgren's syndrome and other sarcoidosis

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ABSTRACT

Objectives

Genetic susceptibility for sarcoidosis and Löfgren's syndrome (LS) has been associated with prognosis. Human Leukoxcyte Antigen (*HLA*)-*DRB1*03* is over-represented in LS, and is associated with a good prognosis, whereas *HLA-DRB1*15*-positive patients have a more chronic course of sarcoidosis. These *HLA-DRB1* types can be easily tagged by single nucleotide polymorphisms (SNPs). Our aim was to evaluate the association between these tag SNPs and bronchoalveolar lavage (BAL) characteristics.

Methods

In 29 patients both complete *HLA-DRB1** locus genotyping and SNP tagging was performed in parallel. *HLA-DRB1* type was inferred from the presence of *03 tag rs2040410 allele A and referred to as *03. *HLA-DRB1*15* was inferred from the presence of tag SNP rs3135388 allele A and referred to as*15.
For BAL analysis, 122 patients with LS and 165 patients with non-LS sarcoidosis were included. BAL lymphocyte subsets were analyzed by flow cytometry.

Results

The presence of tag SNPs completely corresponded with *HLA-DRB1*03/*15* genotypes in all 29 patients in whom both *HLA-DRB1** genotyping and SNP tagging was performed.

In all patients together, $*03^{+}/*15^{-}$ patients showed higher $CD4^{+}/CD8^{+}$ ratio than $*03^{-}/*15^{+}$ (P = 0.004) and $*03^{-}/*15^{-}$ (P = 0.001).
LS patients with $*03^{+}/*15^{-}$ had lower BAL lymphocyte count compared to $*03^{-}/*15^{+}$ patients (P = 0.011). Non-LS sarcoidosis patients with $*03^{+}/*15^{-}$ patients showed a decreased $CD103^{+}CD4^{+}/CD4^{+}$ ratio compared to $*03^{-}/*15^{+}$ patients (P = 0.045) and $*03^{-}/*15^{-}$ patients (P = 0.018).

Conclusion

We found that *HLA-DRB1*03* and *HLA-DRB1*15* can be approximated by genotyping of tag SNPs and corresponds with the degree of lymphocytosis and cell phenotypes in BAL in both LS and non-LS sarcoidosis patients.

Keywords: Lung, T-cells, MHC

Introduction

Sarcoidosis is a multi-system granulomatous disorder with a wide variation in clinical manifestation and disease outcome and is mediated primarily by $CD4^+$ T helper (Th) cells. The course and prognosis may correlate with the mode of onset and extent of the disease. Löfgren's syndrome (LS), first described by Sven Löfgren, is an acute form of sarcoidosis that is associated with a favorable prognosis.[1-5]

Genetic variation in the human leukocyte antigen (in humans, HLA) region has been associated with the clinical course of sarcoidosis patients. *HLA-DRB1*03* allele is associated with a spontaneously resolving course of the disease and is associated with Löfgren's syndrome.[6-9] In more than 90% of the *HLA-DRB1*03* positive Löfgren patients the disease resolves within 2 years.[6]

By contrast, *HLA-DRB1*03* negative patients commonly have non-resolving disease. In line with this, non-Löfgren sarcoidosis patients carrying *HLA-DRB1*15* have an increased risk for a chronic course of the disease.[10-12] Frequencies of different HLA genotypes vary between ethnicities. For example, *HLA-DRB1*03* is extremely uncommon in Japan, whereas in a Dutch cohort *DRB1*03* was found in 40% of the sarcoidosis patients.[9]

Furthermore, *HLA-DRB1*15* was found to be a risk factor for sarcoidosis in a white population, but not in a black sarcoidosis population.[13] *HLA-DRB1* typing can be used for risk stratification in sarcoidosis patients. However, full *HLA-DRB1* typing requires multiple steps using either sequence-specific oligonucleotides, polymerase chain reaction (PCR) primers or even sequencing, which makes this method laborious and expensive. In the last decade studies have shown that particular *HLA-DRB1* types can be tagged by single nucleotide polymorphisms (SNPs) in linkage disequilibrium with DRB1 genotypes.[14] In contrast to full *HLA-DRB1* genotyping, SNP tagging is a simple procedure. Bakker et al. found that SNP rs2040410 allele A and SNP rs3135388 have been associated with *HLA-DRB1*0301* and *HLA-DRB1*1501*, respectively. Furthermore, in patients with diabetes type I SNP rs204010 was found to identify *HLA-DRB1*0301* with great sensitivity and specificity, while SNP rs3135388 has been associated with systemic lupus erythematosus and multiple sclerosis.[15] Tagging is less time-consuming and less expensive compared with complex full HLA-analysis. In other systemic diseases, tag SNPs have also been investigated in order to capture HLA genotypes. For example, in a Japanese cohort with patients with type I diabetes tag SNP rs3129888 captured haplotype *HLA-DRB1*0802* with high sensitivity and specificity. To our knowledge, we have performed the first use of these tag SNPs in sarcoidosis. For this reason, we validated the *HLA-DRB1* tag SNPs in patients with LS.

Analysis of bronchoalveolar lavage can be used to support the diagnosis of sarcoidosis by demonstrating increased total cell count, lymphocytosis and an increased $CD4^+/CD8^+$ ratio.[16,17] More recent studies have proved that a decreased $CD103^+CD4^+/CD4^+$ ratio in the BAL is an additional reliable tool in the diagnostic work-up of sarcoidosis patients.[18,19]

Previous reports have described characteristics of BAL in different clinical phenotypes of sarcoidosis, particularly LS versus non-LS sarcoidosis patients.[20] However, variability between patients is considerable. A few studies have investigated the role of *HLA-DRB1*03* on BAL outcomes.[21,22]

However, in most of these studies no subanalysis was performed on patients with LS. Furthermore, to our knowledge, the influence of *HLA-DRB1*1501* on the BAL outcomes has not been described. This is of particular interest because *HLA-DRB1*1501* associates with a worse prognosis and clinically it is most important to identify these patients. We investigated the $CD103^+CD4^+/CD4^+$ ratio in BAL and compared this in different HLA genotypes. This ratio has not yet been described in different HLA genotypes. Next to validation of the tag SNPs for *HLA-DRB1*03* and *-DRB1*15*, we investigated if these tags correlate with BAL cell phenotypes in patients with (LS) and non-LS.

Material and methods

All patients were diagnosed in accordance with the American Thoracic Society/ European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders (ATS/ERS/WASOG) consensus statement on sarcoidosis.[23] Patients with LS presented with the classic symptoms of: acute onset with bilateral hilar lymphadenopathy, fever, erythema nodosum (EN) and/or bilateral ankle arthritis.[5]
A total of 126 LS patients were included in our cohort: 122 patients with BAL and HLA typing/tag alleles and four patients with only HLA typing/tag alleles.
In the first part of our study, the association of *HLA-DRB1*03* and **15* with the tag alleles was examined in 29 unrelated Dutch patients with LS. The tagging was confirmed with high resolution HLA typing, i.e. *HLA-DRB1*0301* and *HLA-DRB1*1501*.

For the second part of the study, BAL and presence of the *03 and *15 tag alleles were analysed in 122 patients with LS (23 patients from the above-mentioned LS cohort), 165 patients with non-LS and 53 healthy controls. All included patients visited St Antonius ILD Center of Excellence, a tertiary referral center for interstitial lung disease.

We accepted a maximum duration of 4 months between diagnosis and performing BAL in LS-patients and non-LS patients. Data from patients were collected retrospectively from medical charts and the following parameters were recorded: gender, age at diagnosis, scadding (chest X-ray) stage, corticosteroid use and smoking status. At the time of BAL collection 10 LS patients used corticosteroids, nine oral and one by inhalation. Regarding non-LS patients 17 patients used corticosteroids; 4 oral and 13 by inhalation at the time of BAL. The study was approved by the Medical research Ethics Committees United (MEC-U) of the St. Antonius Hospital (R05-08A), and all subjects gave written informed consent.

Genotyping for HLA-DRB1, SNP tags and bronchoalveolar lavage

Genomic DNA was extracted from peripheral blood (PB) of each individual using standard methods. In 29 patients, *HLA-DRB1* locus was genotyped using PCR reverse sequence-specific oligonucleotides (SSO#) methodology (LABType® SSO; One Lambda Inc. Canoga Park, CA, USA).

In all subjects, tag SNP rs2040410A and rs3135388A were used to capture *HLA-DRB1*0301* and *HLA-DRB1*1501*, respectively.[15] For rs2040410 genotyping, a restriction fragment length polymorphism (RFLP) assay was performed. Briefly, we amplified a 228 base pairs (bp) PCR product (forward primer: 5'- GTCTTTGGCTGGAGGCATTG-3'; reverse primer: 5'-GACTCATGGCTTGCCCCATA-3') and the product was digested by the restriction enzyme BsrGI (New England Biolabs, Ipswich, MA, USA) for 16 h at 37°C. The products were separated on 2% agarose gel. The band sizes were as follows for each genotype: AA = 228, AG = 49, 179 and 228, and GG = 49 and 179 bp. To identify genotype rs3135388 tag, a custom designed Taqman SNP genotyping assay was performed on an ABI 7500Fast analyser (Applied Biosystems, Foster City, CA, USA) according to standard methodology. From this point forward in our report, the tagging alleles for *HLA-DRB1*03* and *HLA-DRB1*15* are abbreviated as *03 and *15, respectively.
All patients and healthy subjects underwent bronchoscopy and BAL procedure with a flexible bronchoscope according to the guidelines of the ERS,[24,25] as described previously.[18] To determine lymphocyte subsets in peripheral blood and BAL cellular fraction, flow cytometry was performed as described previously.[18]

Statistics

SPSS version 24 and Graphpad prism software version 6.05 were used for the data analysis. Data are expressed as median; upper (maximum) and lower (minimum) values. The non-parametric Mann-Whitney *U*-test and Kruskal-Wallis test were computed to test for differences in medians. The χ2 test was used to compare proportions.

Results

In a total of 29 patients both full *HLA-DRB1* genotypes and tag SNPs rs2040410 and rs3135388 were determined. Characteristics of the patients are shown in Table 1.

Table 1. Characteristics of patients with LS, non-LS, and healthy controls

		LS (*n* = 126)	**Non-LS** (*n* = 165)	***P***	**Healthy Subjects** (*n* = 53)
Age		34 (±15)	39 (±17)	0.003	22 (±21)
Gender male		48 (38%)	94 (57%)	0.001	29 (55%)
Caucasian race		124 (98%)	152 (93%)	0.025	
Tag *DRB1*03* positive		89 (70%)	26 (16%)	<0.001	17 (33%)
Tag *DRB1*15* positive		31 (24%)	48 (29%)	NS	10 (19%)
Smoking	Never	80/125 (63%)	87/164 (53%)	NS	26/49 (53%)
	Current	20/125 (16%)	34/164 (21%)		19/49 (39%)
	Ex	25/125 (20%)	43/164 (26%)		4/49 (8%)
Scadding stage	0	3/104 (3%)	6/145 (4%)	<0.001	
	I	93/104 (89%)	68/145 (47%)		
	II	8/104 (8%)	43/145 (30%)		
	III	-	24/145 (17%)		
	IV	-	4/145 (4%)		

Age is median ± IQR LS = Löfgren's syndrome, non-LS = non-Löfgren's syndrome, Bronchoalveolar lavage (BAL) was performed in 122/126 patients with LS. Furthermore, smoking history was available from 125 patients with LS, 164 non-LS, and 49 healthy subject. Scadding stage was available from104 patients with LS and 110 patients with non-LS.
P-value is based on the comparison between LS and non-LS.

Table 2 shows the validation of the association between the *HLA-DRB1* genotypes and the tags *03 and *15 in Dutch patients. The tag alleles and the corresponding *HLA-DRB1* alleles overlapped completely.

*HLA-DRB1*03* positivity corresponded with presence of the A allele of the tag SNP rs2040410, while *HLA-DRB1*15* positivity corresponded with the A allele of the tag SNP rs3135388.

Table 2. Validation of association between *HLA-DRB1* genotyping and tag SNPs rs2040410 and rs3135388

DRB1*03	**rs2040410**	**Allele A**	***DRB1*15***	**rs3135388**	**Allele A**	***DRB1*03***	***DRB1*15***	
	positive	negative		positive	negative		positive	negative
positive	16	0	positive	13	0	positive	4	12
negative	0	13	negative	0	16	negative	9	4

BAL outcomes in all patients

BAL was performed in a total of 122 patients with LS, 165 patients with non-LS and 53 healthy controls. All BAL samples were obtained and analysed in our hospital.

We compared the BAL results of all patients (both LS and non-LS), subdividing patients on the basis of HLA tags *03 and *15 (Table 3 and Figures 1 and 2).

Table 3. Bronchoalveolar lavage (BAL) and peripheral blood (PB) findings of all patients categorized in four *HLA-DRB1* genotypes: $*03^{+}/*15^{-}$, $*03^{-}/*15^{+}$, $*03^{-}/*15^{-}$, $*03^{+}/*15^{+}$

Sarcoidosis patients		$*03^{+}/*15^{-}$		$*03^{-}/*15^{+}$		$*03^{-}/*15^{-}$		$*03^{+}/*15^{+}$	***P***
(LS and non-LS)	*n*	Median (upper-lower value)	*n*	Median (upper-lower value)	*n*	Median (upper-lower value)	*n*	Median (upper-lower value)	
Cells/mL	51	19.1 (6.7-75.6)	32	16.9 (3.5-44.0)	71	21.8 (5.6-68.0)	8	17.8 (11.6-28.6)	NS
Lymphocytes (%)	100	25.2 (0.0-70.3)	58	31.3 (1.0-73.0)	114	30.0 (1.6-95.1)	15	29.8 (7.7-48.8)	NS
Neutrophils (%)	100	1.0 (0.0-15.2)	58	1.0 (0.0-30.2)	114	1.1(0.0-34.9)	15	1.4 (0.2-4.2)	NS
$CD4^{+}$ (%)	82	80.0 (39.0-93.0)	38	74.1 (47.0-93.0)	86	74.5 (28.0-94.0)	14	85.5 (64.0-91.0)	^{A}0.010
									^{B}0.001
									^{C}0.003
									^{D}0.004
$CD8^{+}$ (%)	82	12.0 (2.0-50.0)	38	17.9 (3.0-41.0)	86	17.0 (4.0-62.0)	14	9.5 (3.0-28.0)	^{A}0.005
									^{B}0.002
									^{C}0.003
									^{D}0.002
$CD4^{+}/CD8^{+}$ ratio	82	6.6 (0.8-39.0)	38	4.26 (1.3-31.0)	87	4.5 (0.5-22.0)	14	8.9 (2.4-30.0)	^{A}0.004
									^{B}0.001
									^{C}0.003
									^{D}0.002
$CD103^{+}CD4^{+}/CD4^{+}$ ratio	34	0.050 (0.00-0.52)	21	0.10 (0.01-0.44)	49	0.090 (0.01-0.62)	7	0.040 (0.00-0.61)	^{B}0.024
PB $CD4^{+}/CD8^{+}$ ratio	76	2.2 (0.4-15.5)	37	1.8 (0.0-9.0)	82	1.62 (0.60-16.3)	14	1.6 (0.6-3.4)	^{A}0.005
									^{B}0.003

*03 and *15 were typed using tag single nucleotide polymorphisms (SNPs). Median (upper-lower value)
LS = Löfgren's syndrome, non-LS = non-Löfgren's syndrome, PB:Peripheral Blood
^{A} =$*03^{+}/*15^{-}$ *versus* $*03^{-}/*15^{+}$.
^{B}= $*03^{+}/*15^{-}$ *versus* $*03^{-}/*15^{-}$.
^{C}= $*03^{+}/*15^{+}$ *versus* $*03^{-}/*15^{-}$.
^{D} =$*03^{-}/*15^{+}$ *versus* $*03^{+}/*15^{+}$.

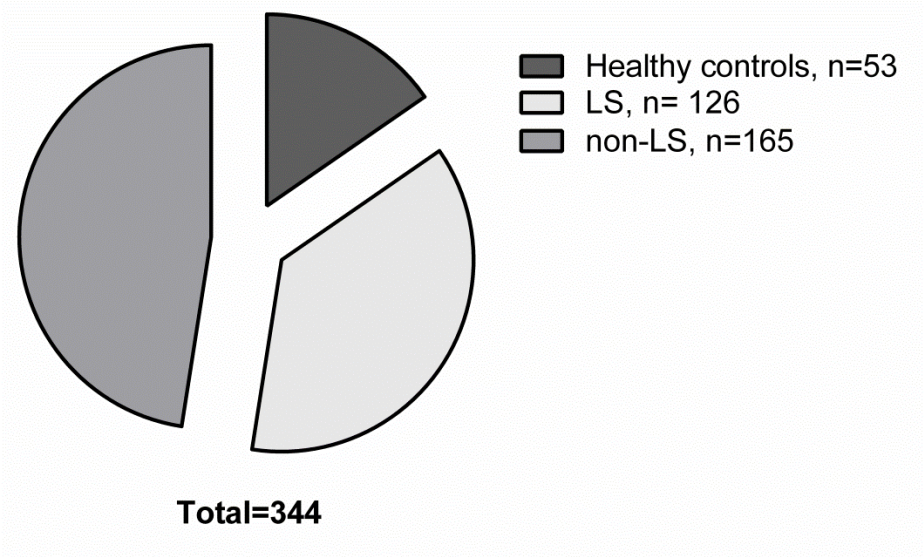

Figure 1. Overview of patients and healthy controls

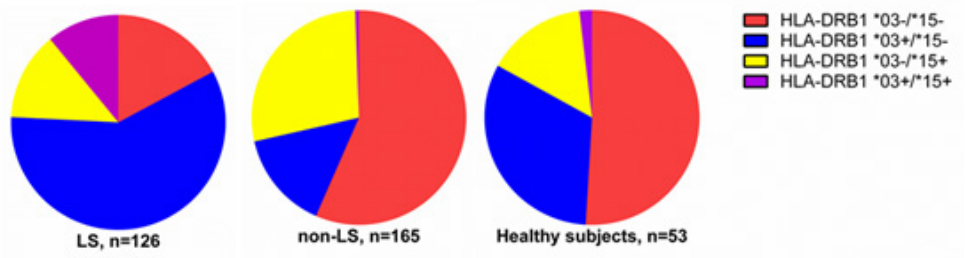

Figure 2. HLA genotypes in LS, non-LS and healthy subjects respectively

In all patients (LS and non-LS), no significant differences were found in % of BAL lymphocytes and % of neutrophils between carriers and non-carriers. Significant differences were observed in % of lymphocyte subsets and ratios (Figure 3a). The highest BAL $CD4^+/CD8^+$ ratio was found in $*03^+/*15^+$ patients (median ratio = 8.9; 2.36-30.00) (Figure 3b). We found no statistically significant difference between $*03^+/*15^+$ and $*03^+/*15^-$. The BAL $CD4^+/CD8^+$ ratio in $*03^+/*15^-$ patients (median ratio = 6.6; 0.78-39.00) was significantly higher compared to $*03^-/*15^+$ patients ($P = 0.004$) and $*03^-/*15^-$ patients ($P = 0.002$). Patients with $*03^-/*15^+$ had the lowest BAL $CD4^+/CD8^+$ratio (median ratio = 4.26; 1.29-31.00).

The median $CD103^+CD4^+/CD4^+$ ratio was decreased (reference value < 0.20[18]) in all groups (Figure 3c). Patients with $*03^-/*15^-$ had a higher $CD103^+CD4^+/CD4^+$ ratio compared to $*03^+/*15^-$ ($P = 0.024$).

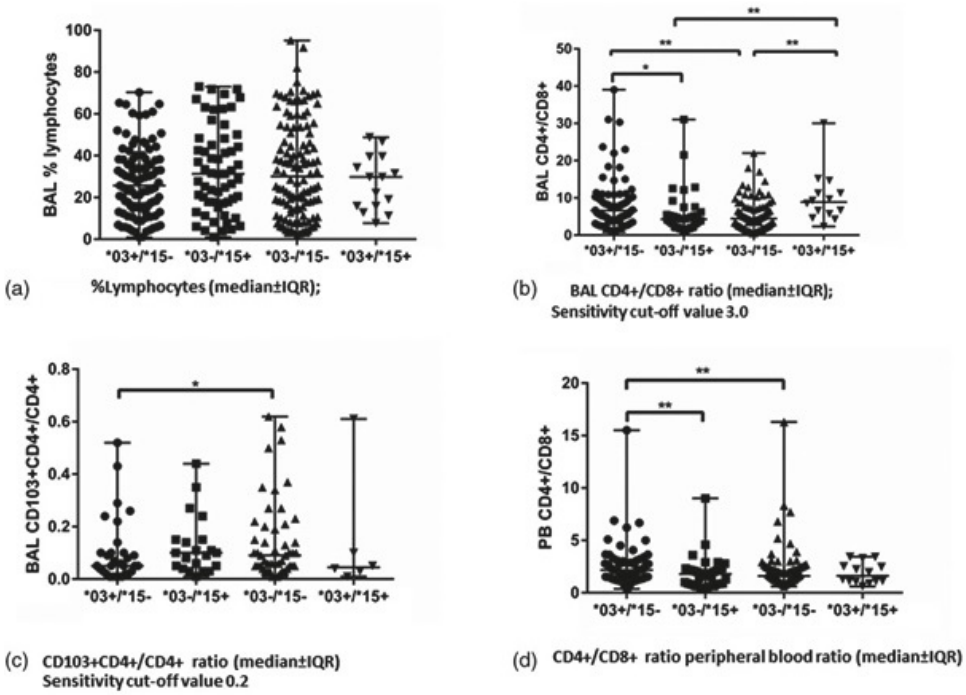

Figure 3. Bronchoalveolar lavage (BAL) cell percentage of lymphocytes and cell phenotype ratios in all patients stratified per carriership of DRB1*03 and *15 tag. *$P \leq 0.05$; **$P \leq 0.01$;

A. %Lymphocytes (median ± interquartile range (IQR));
B. BAL $CD4^+/CD8^+$ ratio (median ± IQR); Sensitivity cut-off value 3.0[18]
C. $CD103^+CD4^+/CD4^+$ ratio (median ± IQR) Sensitivity cut-off value 0.2[18]
D. $CD4^+/CD8^+$ ratio (median ± IQR) peripheral blood

The $CD4^+/CD8^+$ ratio in peripheral blood was highest in the $*03^+/*15^-$ patients (median ratio = 2.2; 0.38-15.50), which was significantly higher compared to $*03^-/*15^+$ patients ($P = 0.005$) and $*03^-/*15^-$ patients ($P = 0.003$) (Figure 3d).

We performed a subanalysis to determine the influence of smoking on the BAL outcomes. Comparable results were demonstrated in non-smokers; however, in smokers only the BAL $CD4^+/CD8^+$ ratio was significantly higher in $*03^+/*15^-$ (median ratio = 6.0; 2.7-30.3) compared to $*03^-/*15^+$ (median ratio = 1.84; 1.62-2.06), $P = 0.022$. In addition, no significant differences were found between different *HLA* genotypes in smokers in the BAL $CD103^+CD4^+/CD4^+$ and PB $CD4^+/CD8^+$ ratios.

The group of patients using oral corticosteroids was too small to perform a subanalysis, therefore we excluded these patients. In this group (without patients using oral corticosteroids) the results of BAL outcomes in four different *HLA-DRB1* genotypes were comparable to the whole group.

BAL: LS *versus* non-LS

Results from BAL analysis of patients with LS are shown in Table 4 and Figure 4. LS patients with $*03^{+}/*15^{-}$ showed a significantly lower percentage of lymphocytes (median ratio = 23.8; 0.0-70.3), compared to $*03^{-}/*15^{+}$ patients, who showed the highest percentage of lymphocytes (median ratio = 42.0; 11.3-71.8; P = 0.011) (Figure 4a).

Patients with $*03^{+}/*15^{-}$ and $*03^{+}/*15^{+}$ showed a higher $CD4^{+}/CD8^{+}$ ratio in lavages, a higher $CD4^{+}/CD8^{+}$ ratio in PB and a lower $CD103^{+}CD4^{+}/CD4^{+}$ ratio in lavages compared to $*03^{-}/*15^{+}$ and $*03^{-}/*15^{-}$ patients, but this difference was not statistically significant (Figures 4b-d).

Table 4. Bronchoalveolar lavage (BAL) and peripheral blood (PB) findings of LS and non-LS categorized in four human leukocyte antigen (*HLA)-DRB1* genotypes: $*03^{+}/*15^{-}$, $*03^{-}/*15^{+}$, $*03^{-}/*15^{-}$, $*03^{+}/*15^{+}$

	$*03^{+}/*15^{-}$		$*03^{-}/*15^{+}$		$*03^{-}/*15^{-}$		$*03^{+}/*15^{+}$		*P*
LS	*n*	Median (upper-lower value)	*n*	Median (upper-lower value)	*n*	Median (upper-lower value)	*n*	Median (upper-lower value)	
Cells/mL	39	18.5 (7.5-75.6)	4	27.8 (12.0-30.7)	13	23.7 (7.5-53.9)	7	16.9 (11.6-24.0)	NS
Lymphocytes (%)	75	23.8 (0.0-70.3)	11	42.0 (11.3-71.8)	22	24.4 (4.1-69.9)	14	26.0 (7.7-48.8)	^{A}0.011 ^{B}0.020
Neutrophils (%)	75	1.1 (0.0-15.2)	11	0.9 (0.0-4.70)	22	1.2(0.0-31.2)	14	1.4 (0.2-4.2)	NS
$CD4^{+}$ (%)	67	81.0 (47.0-93.0)	8	79.5 (73.0-90.0)	18	82.0 (56.0-94.0)	13	86.0 (64.0-91.0)	NS
$CD8^{+}$ (%)	67	11.0 (2.0 36.0)	8	16.9 (4.0-23.0)	18	12.0 (5.0-22.0)	13	9.0 (3.0-16.0)	NS
$CD4^{+}/CD8^{+}$ ratio	67	7.6 (1.3-39.0)	8	4.7 (3.2-21.5)	19	7.0 (3.3-18.0)	13	9.3 (4.3-30.0)	NS
$CD103^{+}CD4^{+}/CD4^{+}$ ratio	23	0.05 (0.0-0.5)	4	0.07 (0.03-0.10)	12	0.06 (0.01-0.20)	6	0.04 (0.00-0.10)	NS
PB $CD4^{+}/CD8^{+}$ ratio	62	2.1 (0.4-6.9)	8	2.1 (0.00-2.3)	16	2.0 (1.2-7.7)	13	2.0 (0.6-3.4)	NS
Non-LS*	n	Median (upper-lower value)	n	Median (upper-lower value)	n	Median(upper-lower value)			
Cells/mL	12	21.3 (6.7-54.4)	28	16.7 (3.5-44.0)	58	21.6 (5.6-68.0)			NS
Lymphocytes (%)	25	27.6 (1.1-64.5)	47	26.5 (1.0-73.0)	92	32.8 (1.6-95.1)			NS
Neutrophils (%)	25	0.8 (0.10-9.20)	47	1.0 (0.1-30.2)	92	1.1 (0.0-34.9)			NS
$CD4^{+}$ (%)	15	71.0 (39.0-93.0)	30	73.5 (47.0-93.0)	68	74.0 (28.0-93.0)			NS
CD8+ (%)	15	17.0 (3.0-50.0)	30	19.5 (3.0-41.0)	68	20.0 (4.0-62.0)			NS
$CD4^{+}$/CD8+ ratio	15	3.5 (0.78-30.3)	30	3.8 (1.3-31.0)	68	3.8 (0.5-22.0)			NS
$CD103^{+}CD4^{+}/CD4^{+}$ ratio	11	0.03 (0.01-0.43)	17	0.11 (0.01-0.44)	37	0.1 (0.01-0.62)			^{A}0.045 ^{C}0.018
PB CD4+/$CD8^{+}$ ratio	14	2.6 (1.3-15.5)	29	1.5 (0.3-9.0)	66	1.5 (0.6-16.3)			^{A}0.019 ^{C}0.011

*03 and *15 were typed using tag single nucleotide polymorphism (SNPs). Median (upper-lower value)
LS:Löfgren's syndrome, non-LS:non-Löfgren's syndrome, PB:Peripheral Blood
Our cohort included only one $*03^{+}/*15^{+}$ non-LS patient, therefore this group was to small and was excluded from analysis
A =$*03^{+}/*15^{-}$ *versus* $*03^{-}/*15^{+}$.
B = $*03^{-}/*15^{+}$ *versus* $*03^{+}/*15^{+}$.
C= $*03^{+}/*15^{-}$ *versus* $*03^{-}/*15^{-}$.

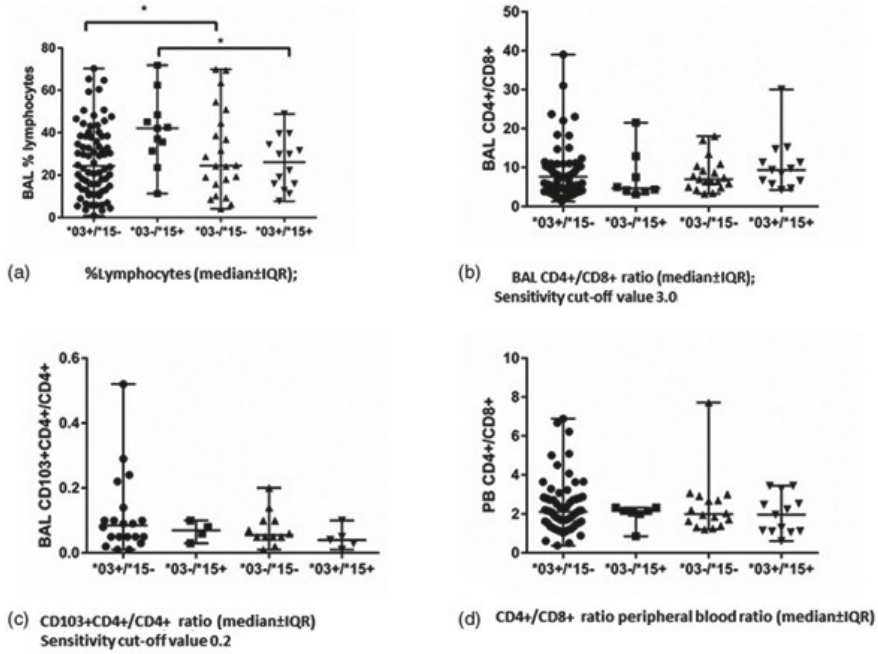

Figure 4. Bronchoalveolar (BAL) cell percentage of lymphocytes and cell phenotype ratios in LS stratified per carriership of DRB1*03 and *15 tag. *$P \leq 0.05$

A. %Lymphocytes (median ± interquartile range (IQR));
B. BAL $CD4^+/CD8^+$ ratio (median ± IQR); Sensitivity cut-off value 3.0[18]
C. $CD103^+CD4^+/CD4^+$ ratio (median ± IQR) Sensitivity cut-off value 0.2[18]
D. $CD4^+/CD8^+$ ratio (median ± IQR) peripheral blood

BAL outcomes of non-LS patients comparing distinct genotypes are shown in Table 4 and Figure 5. No significant differences were found when comparing the percentages of lymphocytes or neutrophils in the BAL of different *HLA-DRB1* genotypes in non-LS patients. Patients with $*03^+/*15^-$ had a lower $CD103^+CD4^+/CD4^+$ ratio (median ratio = 0.030; 0.01-0.43) than $*03^-/*15^+$ patients (median ratio = 0.11; 0.01-0.44) and $*03^-/*15^-$ patients (median ratio = 0.10; 0.01-0.62), $P = 0.045$ and $P = 0.011$ respectively (Figure 5c).

Furthermore, the $CD4^+/CD8^+$ ratio in the peripheral blood of $*03^+/*15^-$ patients was higher (median ratio = 2.6, 1.3-15.5), than in the $*03^-/*15^-$ patients (median ratio 1.5; 0.6-16.3; $P = 0.011$) (Figure 5d).

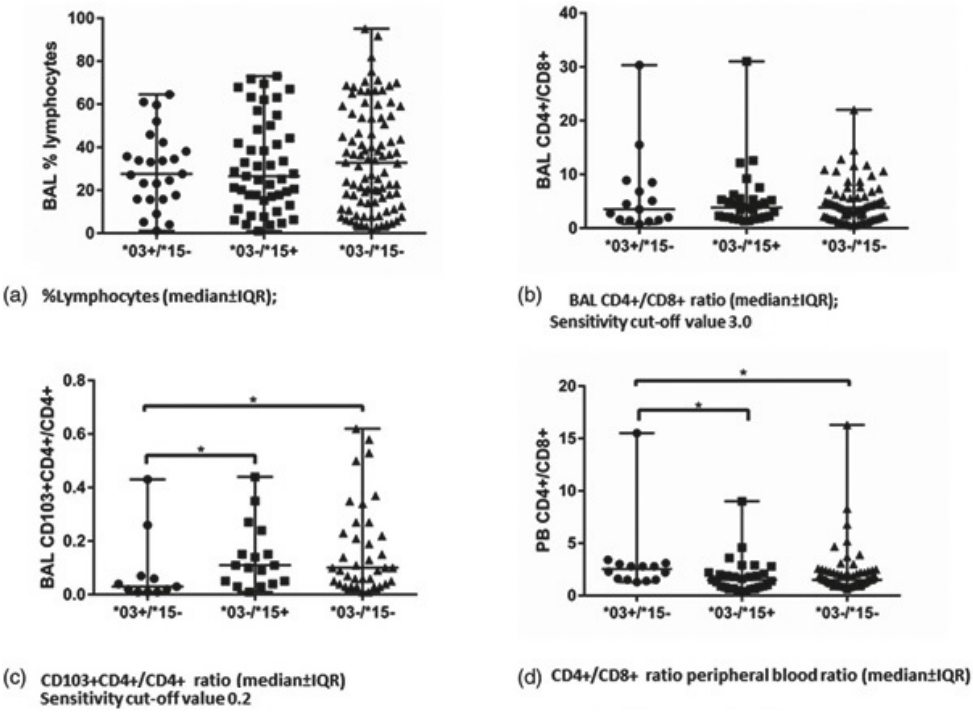

Figure 5. Bronchoalveolar lavage (BAL) cell percentage of lymphocytes and cell phenotype ratios (median ± interquartile range (IQR)) in non-LS stratified per carriership of DRB1*03 and *15 tag. * $P \leq 0.05$

A. %Lymphocytes (median ± IQR);
B. BAL $CD4^+/CD8^+$ ratio (median ± IQR); Sensitivity cut-off value 3.0[18]
C. $CD103^+CD4^+/CD4^+$ ratio (median ± IQR) Sensitivity cut-off value 0.2[18]
D. $CD4^+/CD8^+$ ratio (median ± IQR) peripheral blood

Discussion

Most sarcoidosis patients have a good prognosis, although a clear proportion of patients develop chronic and/or progressive disease. It is a clinical challenge to distinguish patients who will develop a chronic course from patients who are more likely to spontaneously resolve disease. Usually, the only way is to monitor all patients on a regular basis to identify the few who develop chronic disease. Detailed analysis of BAL in combination with *HLA-DRB1*03* and **15* typing may aid in the early identification of these patients. We studied whether clinically informative HLA types associate with BAL cell characteristics.

Regarding the goals of our study, we confirmed the association of *HLA-DRB1*03* with the A allele of rs2040410 and *-DRB1*15* with the A allele of rs3135388.[15] The SNPs tagging *HLA-DRB1*03* and *HLA-DRB1*15* have each been used before independently in other diseases diabetes and multiple sclerosis respectively.[26,27] To

our knowledge, this is the first study that validated a tag SNP with HLA typing in sarcoidosis patients. Several studies have shown that *HLA-DRB1*03*-positive LS patients have a favorable prognosis, with recovery within 2 years in 95% of the cases. In contrast, only half of the *HLA-DRB1*03*-negative LS patients experience a resolving course of disease. Furthermore, more than half of the non-resolving *HLA-DRB1*03* patients are *HLA-DRB1*15*-positive.[6] This association of *HLA-DRB1*15* with chronic disease has been confirmed by others[10,28]

Due to its complexity, complete HLA typing is not suitable for daily clinical practice. However, the use of SNP tagging is simple and affordable, and allows easy identification of patients with a good prognosis and patients who will develop a chronic course of disease.

Over decades various studies have investigated the characteristics of BAL and its relation to prognosis of disease in different phenotypes of sarcoidosis. Drent et al. demonstrated that patients with an acute presentation of sarcoidosis with arthritis and erythema nodosum had significantly higher proportions of lymphocytes and $CD4^+$/$CD8^+$ ratio than patients with respiratory and general constitutional symptoms.[20] This study and others have implied a possible beneficial role of $CD4^+$ T-lymphocytes.[29,30]

In the whole group, the $CD4^+$/$CD8^+$ ratio was higher in $*03^+$ patients compared to $*03^-$ patients in our combined cohort. In LS patients a similar difference was observed, which did not reach statistical significance, due probably to the small group of patients in the analyses. In our LS cohort a higher lymphocyte percentage was found in the patients with $*03^-/*15^+$ versus $*03^+/*15^-$.
A few papers have described BAL characteristics comparing *HLA-DRB1*03*-positive and -negative sarcoidosis patients.

Idali et al.[31] did not observe differences in the BAL lymphocyte percentage between *HLA-DRB1*03*-positive and -negative patients. However, they did not make a distinction between LS and non-LS patients, nor did they include other HLA genotypes than *HLA-DRB1*03*. In line with their findings, in our combined cohort there are also no differences in lymphocyte percentages between $*03^+$ and $*03^-$ patients. A striking finding is that in LS patients the $*03^-/*15^+$ patients had significantly higher lymphocyte percentages compared to $*03^+/*15^-$ patients.

In a cohort of 118 sarcoidosis patients, Planck et al.[22] also observed a decreased lymphocyte percentage and an increased $CD4^+$/$CD8^+$ ratio in BAL from *HLA-DRB1*03*-positive patients compared to those negative for *HLA-DRB1*03*. Although their cohort included 43% LS patients, LS and non-LS patients were not analyzed

separately, nor was the contribution of the *HLA-DRB1*15* allele studied. Similar findings regarding lymphocyte percentage and $CD4^+/CD8^+$ ratio in *HLA-DRB1*03*-positive patients were reported in a more recent study that included LS patients.[32] Unfortunately, other HLA genotypes were not studied. Our results show that besides a low lymphocyte percentage, a high $CD4^+/CD8^+$ ratio provides a good prognosis.

In addition, a more recent paper published by Kinloch et al. found lower lymphocyte counts in combination with a higher $CD4^+/CD8^+$ ratio in BAL fluid of *HLA-DRB1*03*-positive patients.[21] However, none of the above-mentioned studies had investigated the $CD4^+/CD8^+$ ratio in the peripheral blood.

In our cohort a total of 15 patients were positive for both *03 and *15. The effect of carriage of *HLA-DRB1*15* in -*DRB1*03*$^+$ patients is not known; one allele could be dominant over the other or the combined carriage could cancel the effect. No hard conclusions can be made, due to low patient numbers in this group. However, a significantly higher percentage of lymphocytes was found in $*03^-/*15^+$ patients compared to $*03^+/*15^+$ patients. This finding suggests that the influence of *03 is dominant over the influence of *15 in patients with LS. In essence, this is implicated by the studies from Grunewald, and is therefore most likely.[12,22]

Braun et al., who analysed $CD4^+$ T cells from the BAL of a variety of fibrotic lung diseases, suggested that $CD103^+$ cells are terminally differentiated effector T cells that might be involved in the process of lung fibrosis.[33]

Several authors have reported that patients with a more advanced radiological stage of sarcoidosis show a higher proportion of $CD4^+$ T lymphocytes expressing CD103 and have a higher $CD103^+CD4^+/CD4^+$ ratio.[18,19] Our current findings support this by showing that sarcoidosis patients with $*03^+/*15^-$, who are generally known as having a favorable prognosis, have a decreased $CD103^+CD4^+/CD4^+$ ratio compared with $*03^-/15^+$ and $*03^-/*15^-$ patients. Our data suggest that a decreased $CD103^+CD4^+/CD4^+$ ratio predicts a benign course of disease.
Sarcoidosis has traditionally been regarded as a Th1-driven disease, characterized by excessive interferon (IFN)-γ, interleukin (IL)-12, and tumor necrosis factor (TNF)-α production in the lungs.[34] By separating patients into LS and non-LS or stratifying by HLA type, differences in effector T cell subsets were seen by Moller and co-workers.[34]

Our data show that the type of lymphocytes is important in redirecting the inflammation towards a self-limiting or chronic disease. Further studies need to be conducted to elucidate the complex interactions between genetics, T cell function and clinical behavior of the disease.

Whether an inflammatory immune response results in a self-limiting or a chronic relapsing-remitting type of disease is dependent on multiple factors. Important factors are type and quantity of the antigens, the extent of antigen presentation in terms of tissue and duration, the context of antigen presentation and genetic composition. The net balance determines whether the CD4 response is balanced with a beneficial CD4 T regulatory component, or dominated by proinflammatory Th17.1 cells.

Regarding genetic composition, DR3 is generally a good prognostic factor for LS, irrespective of the second DR allele. Detailed immunological analysis of LS cohorts revealed that $V\alpha2.3^{+}V\beta22^{+}CD4^{+}$ T cells are expanded in these patients.[32]. Their phenotype and cytokine profiles in lavages of LS patients demonstrate profiles to be less skewed towards a proinflammatory Th17.1 cells,[35] and it is speculated these cells may recognize autoantigens such as vimentin.[21]

The current study points out that DR3 positivity does not guarantee an inflammatory process to become selflimiting, and a proportion of the patients develop sarcoidosis. Considering the lower $CD4^{+}/CD8^{+}$ ratios particularly observed in sarcoidosis patients, the capacity to establish a more Th1-like response and involve CD8 T cells in the inflammatory response may mark a relapsing-remitting type of response.

Whether the DR3 non-LS patients are incapable of producing $V\alpha2.3^{+}V\beta22^{+}CD4^{+}$ T cells, or vimentin is not involved in the inflammatory response, remains to be determined. Furthermore, studies aiming to identify the triggers in sarcoidosis[36] will help to identify the Th phenotype and establish their role in the immunological puzzle that results in a self-limiting or a relapsing-remitting type of disease, along with DR type.

Due to its retrospective design, not all parameters were available for all patients. Furthermore, 23% of the patients were current smokers. Smoking increases the total cell count and reduces the lymphocyte percentage and $CD4^{+}/CD8^{+}$ ratio in BAL.[20] However, the percentage of smokers was similar in both groups, LS and non-LS. Also, the duration between BAL and diagnosis in LS patients, a maximum of 4 months, could have possibly influenced the BAL outcomes, because LS is known for its acute inflammation.

The strength of our study is that for confirmation of tag SNPs full *HLA-DRB1** typing was performed in a laboratory that regularly participates in external proficiency testing in order to ensure the quality of the laboratory. Furthermore, we separated the patients into four groups ($*03^{+}/*15^{-}$, $*03^{-}/*15^{+}$, $*03^{-}/*15^{-}$, $*03^{+}/*15^{+}$) in order to

study the influence of both clinical course and associated tag SNPs.
In conclusion, in this study we show that *HLA-DRB1*03* and *DRB1*15* can be perfectly approximated by genotyping tag SNPs, which can be used easily in daily clinical practice to distinguish between patients who will develop a chronic course or not. Secondly, we found a significantly higher $CD4^+/CD8^+$ ratio in $*03^+/*15^-$ patients in the whole group. We also found a lower lymphocyte percentage in $*03^+/*15^-$ LS patients, and a decreased $CD103^+CD4^+/CD4^+$ ratio in $*03^+/*15^-$ non-LS patients.

Our results indicate that a phenotype and HLA markers of favorable disease associate with a low lymphocyte percentage, high $CD4^+/CD8^+$ ratio and low $CD103^+CD4^+/CD4^+$ ratio in BAL.

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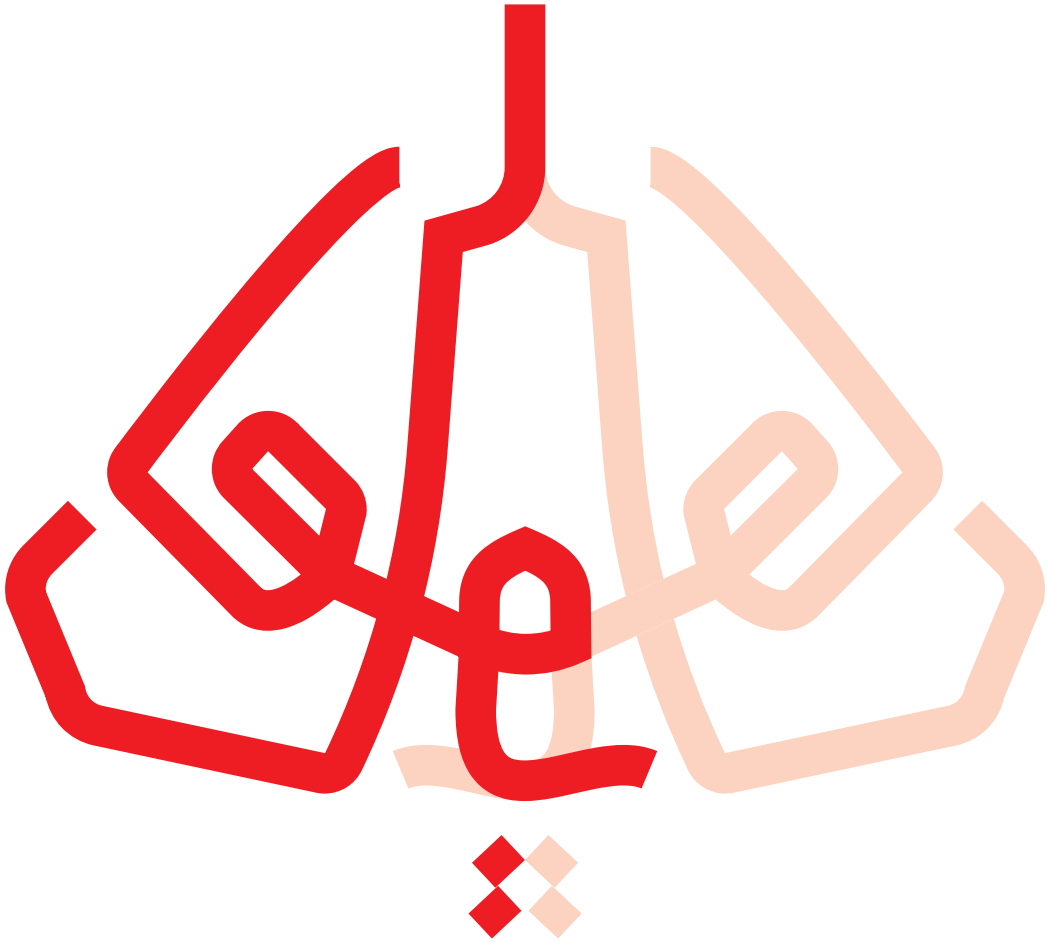

The Heat

The Reaction. The form turns Red, signaling the arrival of the Zahir (Apparent) symptoms.

This is the "Heat" phase—the fever, the Erythema Nodosum and inflammation wrapping itself around the joints. The loop tightens, mimicking the ankle's stiffness and the skin's inflammatory flush.

Chapter 4

Disease relapse rate from long-term follow-up data in Löfgren's syndrome

Bekir Karakaya, Marcel Veltkamp, Coline H.M. van Moorsel, Jan C. Grutters

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*Supplemental data (unpublished) is added to the end of the chapter

To the Editor:

Sarcoidosis is a systemic inflammatory disorder of unknown cause, characterized by the formation of noncaseating epithelioid cell granulomas and a heterogeneous clinical course.[1] A well-defined phenotype of sarcoidosis is Löfgren's syndrome (LS) which manifests as acute onset, bilateral hilair lymphadenopathy, erythema nodosum and/or bilateral ankle arthritis or periarticular inflammation.[2] LS is most commonly seen in North and Western Europe and is strongly associated with the presence of *HLA-DRB1*03*.[3] Patients with LS usually have a very good prognosis, up to 95% of patients who are *HLA-DRB1*03* positive (+) experience resolving disease within 2 years.[4] In contrast, *HLA-DRB1*03* negative (-) patients have a less favorable disease course, with only 51% of patients experiencing resolving disease within 2 years. Furthermore, presence of *HLA-DRB1*15* is a risk factor for non-resolving disease in LS.[4]

In patients with LS with resolving disease, a relapse rate of 3-8%[4,5] was found in a follow-up period of up to 5 years, however no long-term follow-up data is available. The aim of the current study was to determine the long-term relapse rate in a large cohort of Dutch patients with LS. Furthermore, *HLA-DRB1*03* and *HLA-DRB1*15* were determined to investigate a possible association with long-term outcome.

From 1959 to 2020, 380 patients were diagnosed with LS in our hospital. These 380 patients were invited for a reevaluation, which 207 patients (54%) accepted; they underwent clinical evaluation, laboratory tests, lung function test and chest X-ray. Medical records were reviewed for clinical data. Patients were classified as having resolving disease when there were no signs of disease 2 years after onset and as having nonresolving disease when there were signs of ongoing disease 2 years after onset. Tag SNPs rs2040410A and rs3135388A were used to capture *HLA-DRB1*03* and *HLA-DRB1*15*, respectively as previously described.[6] A descriptive analysis was performed expressing the results as medians and interquartile ranges (IQR) for continuous variables, and absolute values and percentages for categorical variables. A Mann–Whitney *U* test was performed for the comparison between continuous variables and the chi-square test for the comparison of categorical variables.

Baseline characteristics are presented in Table 1, showing that the majority of patients with LS were *HLA-DRB1*03*$^{+}$ (71%) and female (60%). Within 2 years after presentation, 93% of patients with LS demonstrated a spontaneous resolution of disease, with a higher percentage of resolution in the *HLA-DRB1*03*$^{+}$ patients compared with *HLA-DRB1*03*$^{-}$ patients (100% vs 74%, respectively; $P<0.00001$). Nonresolving disease only occurred in 10 patients who were all negative for *HLA-DRB1*03* and of whom 5 were *HLA-DRB1*15*$^{+}$.

During the long-term follow-up period (median, 10.8 yr; IQR 4.3-20.6 yr) 11% of the cohort experienced relapsing disease after a median period of 5.5 years (IQR, 3.0-10.5 yr; Figures 1A and B) with 50% of the patients relapsing more than 5 years after the initial diagnosis.

Interestingly, in the group of *HLA-DRB1*03*$^{+}$ patients who experienced a relapse, only 38% relapsed as LS, whereas this was 89% in the *HLA-DRB1*03*$^{-}$ patients (P = 0.018). Furthermore, significantly more relapsing *HLA-DRB1*03*$^{-}$ were *HLA-DRB1*15*$^{+}$ compared with relapsing *HLA-DRB1*03*$^{+}$ patients (P = 0.004). *HLA-DRB1*03*$^{-}$ patients who developed chronic disease after a relapse were also more often positive for *HLA-DRB1*15*. Positivity for *HLA-DRB1*15* was significantly associated with the form of relapsing disease and was significantly more common in patients developing chronic disease after relapse.

For 132 patients we have complete data of treatment, and resolving and relapsing disease. Of these patients, 32% received oral steroid treatment upon diagnosis. There was no significant difference in treatment between *HLA-DRB1*03*$^{+}$ and *HLA-DRB1*03*$^{-}$ patients. In patients treated with steroids, significantly more *HLA-DRB1*03*$^{-}$ patients had nonresolving disease, as expected, because all *HLA-DRB1*03*$^{+}$ patients had resolving disease (Table 1). Interestingly, among the *HLA-DRB1*03*$^{-}$ patients treated with oral steroids, 7 out of 13 (54%) had a resolving disease course, whereas among the nontreated *HLA-DRB1*03*$^{-}$ patients, 17 out of 19 (89%) had resolving disease (P = 0.022) (Figure 2). No difference was found in the proportion of treated and nontreated relapsing patients.

Data on long-term follow-up are scarce in patients with LS. Previously, Grunewald and Eklund[4] described a large cohort of patients with LS with a follow-up period of at least 2 years without describing the maximum follow-up period. They found that only 1% of *HLA-DRB1*03*$^{+}$ patients had nonresolving disease and 4% had relapsing disease, whereas 49% of the *HLA-DRB1*03*$^{-}$ patients had non-resolving disease and none had relapsing disease. The percentage of *HLA-DRB1*03*$^{+}$ in our patients with LS was similar, but relapsing disease in our *HLA-DRB1*03*$^{+}$ patients was much higher (9%) after a median period of 7.0 years (IQR, 3.5-16.0 yr) of follow-up. As depicted in Figures 1A and 1B, our data suggest that relapse may occur at any point in time during follow-up and that patients remain at increased risk for sarcoidosis for at least 10 years after diagnosis.

Furthermore, in comparison with patients from Sweden described by Grunewald and Eklund,[4] particularly *HLA-DRB1*03*$^{-}$ patients showed more often (74% vs 51% in

Sweden) resolving disease and a much higher frequency (16% vs 0% in Sweden) of relapsing disease. In line with this, in our cohort, fewer *HLA-DRB1*03*$^{-}$ patients treated with oral steroids showed nonresolving disease compared with the Swedish cohort (46% vs 80%, respectively). Although this suggests that treatment may be a beneficial option in some patients, our number of treated *HLA-DRB1*03-* patients is low and stems mainly from the period before 1990.

Of our relapsing *HLA-DRB1*03*$^{-}$ patients 67% carried the *HLA-DRB1*15* allele, which makes it even more remarkable that we found that more *HLA-DRB1*03*$^{-}$ patients had clinical signs of LS during relapsing compared with *HLA-DR1*03*$^{+}$ patients. Because of the strong association between *HLA-DR1*03*$^{+}$ and LS one would expect the opposite.

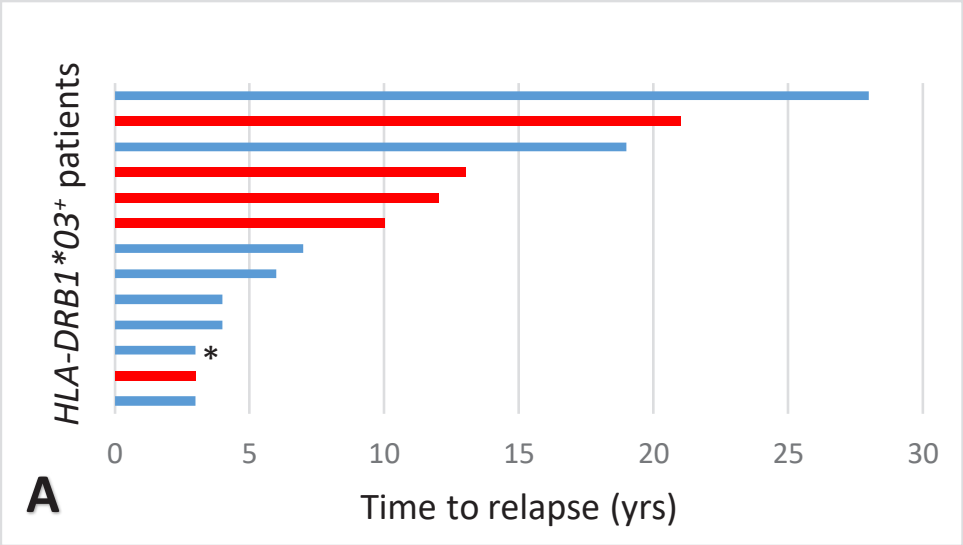
The strength of the study involves the high number of patients with clinical reevaluation and long-term follow-up data. However, this study has its limitations as only 54% of invited patients participated which may have caused inclusion bias toward relapsing and nonresolving disease. Therefore, it could be that the prevalence of nonresolving disease or relapse in Dutch patients with LS is lower than reported in our study. Furthermore, all associations are unadjusted.

To conclude, this is the first study describing long-term follow-up in LS. In our cohort of Dutch patients with LS, we found a relapse rate as high as 11%, with 50% of relapses occurring more than 5 years after the initial diagnosis. Furthermore, our data suggest that the type of *HLA-DRB1* influences not only the evolution towards resolving disease but also the risk of relapse and even the accompanying clinical phenotype at the time of relapse. *HLA-DRB1* tagging,[6] which is simple and inexpensive to perform, could be used in clinical practice to inform patients with LS about both their short- and long-term prognosis.

Table 1. Baseline characteristics and disease course in subjects ever diagnosed with Löfgren's syndrome (LS).

	All patients	*DRB1*03*$^{+}$	*DRB1*03*$^{-}$	***P* value**
Demographics	207	146 (71)	61 (29)	
Year of diagnosis				
1959-1990	48 (23)	32 (22)	16 (26)	
1991-2000	60 (29	51 (35)	9 (15)	
2001-2010	57 (28)	36 (25)	21 (34)	
2011-2020	42 (20)	27 (18)	15 (25)	
Median age at diagnosis, yr (IQR)	34.4 (20.8-67.9)	34.4 (20.8-64.1)	34.5 (20.8-67.9)	0.96
Sex, m/f	83/124 (40/60)	63/83 (43/57)	20/41 (33/67)	0.17
Smoking history	198	142 (72)	56 (28)	
Current smoker	43 (22)	35 (24)	8 (14)	0.13
Ex-smoker	55 (28)	42 (30)	13 (23)	0.48
Never smoker	100 (51)	65 (46)	35 (63	0.04
Fever (n = 165), n (%)	99 (60)	78 (64)	21 (49)	0.08
EN (n = 201)	162 (81)	117 (81)	45 (79)	0.71
Periarticular ankle arthritis (*n = 199*)	151 (76)	109 (77)	42 (72)	0.46
Both EN and ankle arthritis (*n = 196*)	110 (56)	82 (59)	28 (50)	0.28
*HLA-DRB1*15+*	42 (20)	17 (12)	25 (41)	< 0.00001
Disease course >2 years available	*n* = 153	*n* = 114	*n* = 39	
Resolving disease	143 (93)	114 (100)	29 (74)	< 0.00001
Non-resolving disease	10 (7)	0	10 (26)	< 0.00001
Non-resolving disease, *HLA-DRB1*15+*			5 (50)	
Longterm follow-up available	*n* = 201	*n* = 144	*n* = 57	
Median Follow-up, years (IQR)	10.8 (4.3-20.6)	11.4 (3.8-20.6)	9.4 (5.6-20.7)	0.64
Relapsing disease	22 (11)	13 (9)	9 (16)	0.18
Relapsing disease, *HLA-DRB1*15+*	7 (32)	1 (8)	6 (67)	< 0.004
Median time to relapse, yr (IQR)	5.5 (3-10.5)	7.0 (3.5-16.0)	4.0 (3.0-6.5)	0.39
Relapse as LS	13 (59)	5 (38)	8 (89)	0.018
Relapse as LS, *HLA-DRB1*15+*	5 (38)	0	5 (63)	0.024
Chronic after relapse	11 (50)	5 (38)	6 (67)	0.19
Chronic after relapse, *HLA-DRB1*15+*	6 (55)	1 (20)	5 (83)	0.036
Data treatment available*	*n* = 132	*n* = 100	*n* = 32	
Treated	42 (32)	29 (29)	13 (41)	0.219
Non-resolving disease	6 (14)	0	6 (46)	< 0.0001
Relapsing disease	7 (17)	5 (1)	2 (15)	0.881

Definition of abbreviations: EN = Erythema nodosum; LS = Löfgren's syndrome.
Data are presented as n (%) unless otherwise noted. *P* value for comparison between *HLA-DRB1*03* positive ($^{+}$) and negative ($^{-}$) patients.*Treatment with oral steroids upon diagnosis.



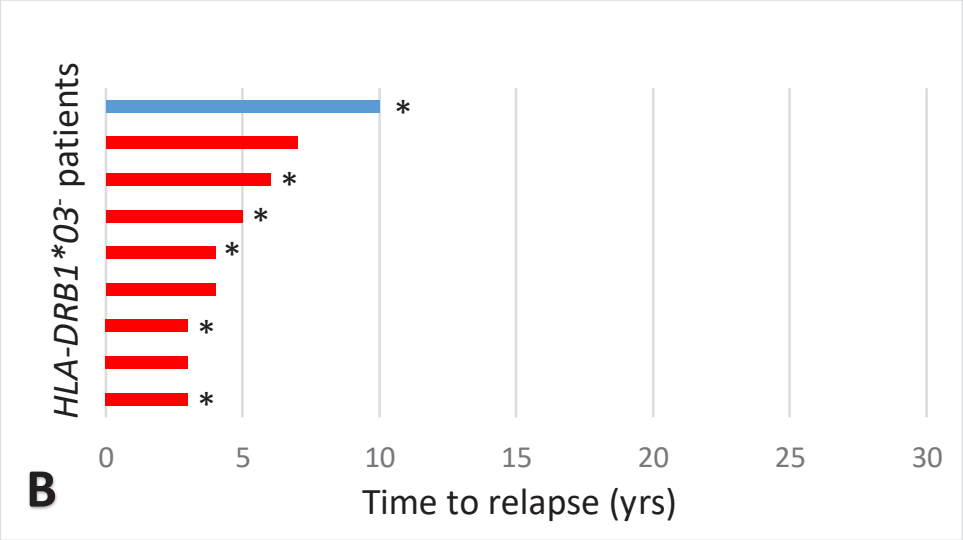
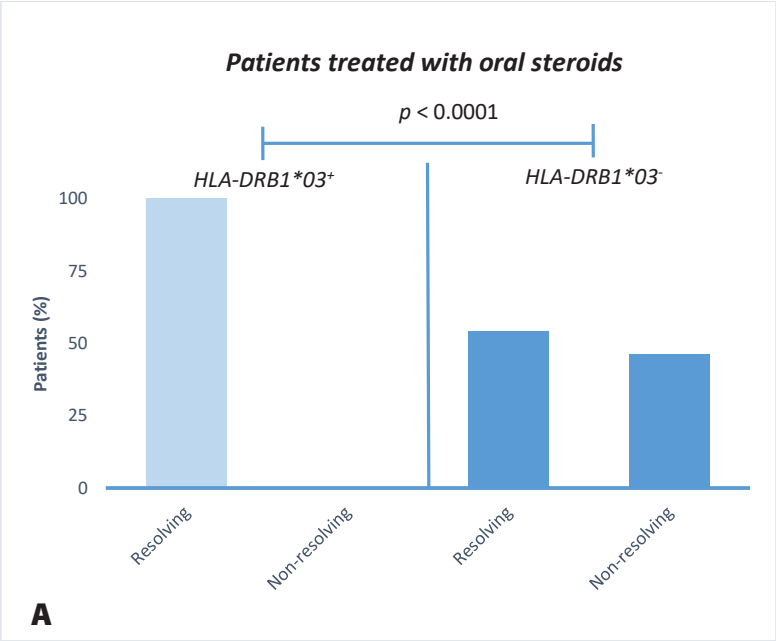


Figure 1. (**A** and **B**) Time to relapse after initial diagnosis of Löfgren's syndrome (LS) and form of relapsing disease for each relapsing *HLA-DRB1*03*$^{+}$ patient (**A**), and relapsing *HLA-DRB1*03*$^{-}$ patient (**B**). Relapse is shown as LS (red) or sarcoidosis (blue). * *HLA-DRB1*15*$^{+}$



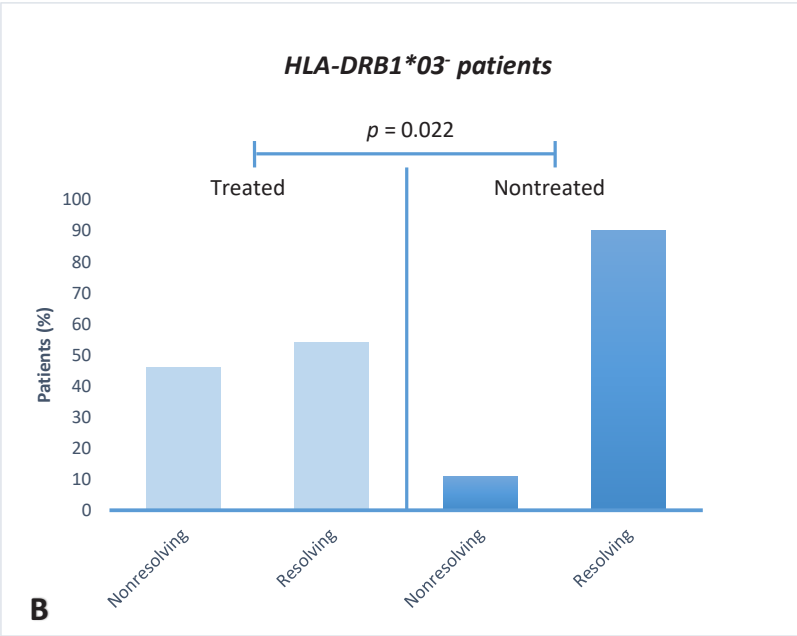


Figure 2. (***A***) Disease course of *HLA-DRB1*03*$^{+}$ and *HLA-DRB1*03*$^{-}$ patients, showing the relative numbers for resolving and non-resolving disease. (***B***) Disease course in *HLA-DRB1*03-* patients treated (n = 13) or non-treated (n = 19) with oral steroids

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Supplemental data

In the following paragraph new and unpublished data from our cohort with patients with Löfgren's syndrome will be present.

Month of onset

Interestingly, a high incidence of disease onset in the first months of the year was observed. In terms of absolute patient numbers and the month of presentation, we observed a peak in January and the spring months. However, in relative numbers (the percentages) we saw that about 40% (n = 82) of the patients were having disease onset in March (12.6%), April (15.0%) and May (12.1%) (Figure 1). No difference in month of disease onset between *HLA-DRB1*03* positive and *HLA-DRB1*03* negative patients was found (Figure 2). This was in line with earlier publications, where even in the southern hemisphere patients with acute onset and erythema nodosum presenting mostly in the spring season.[1-3]

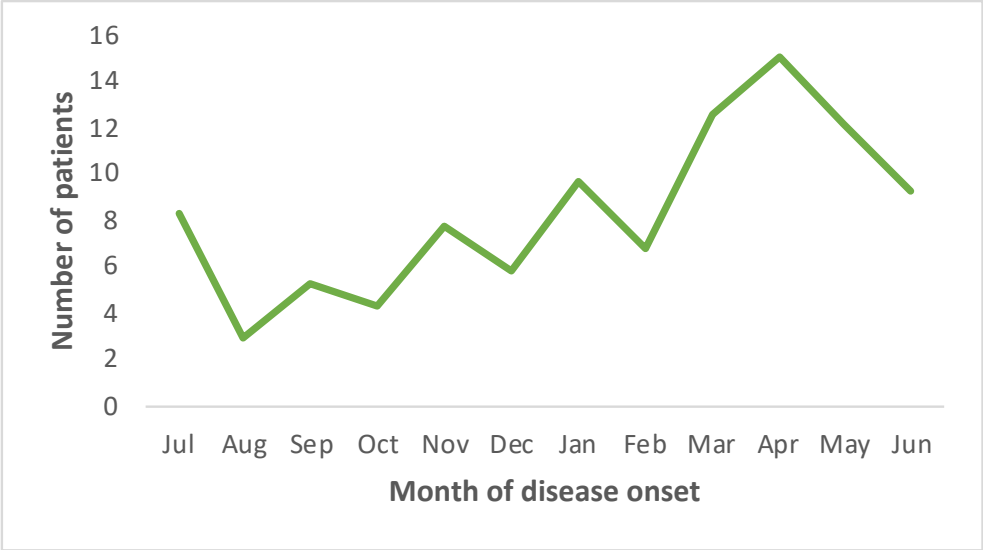
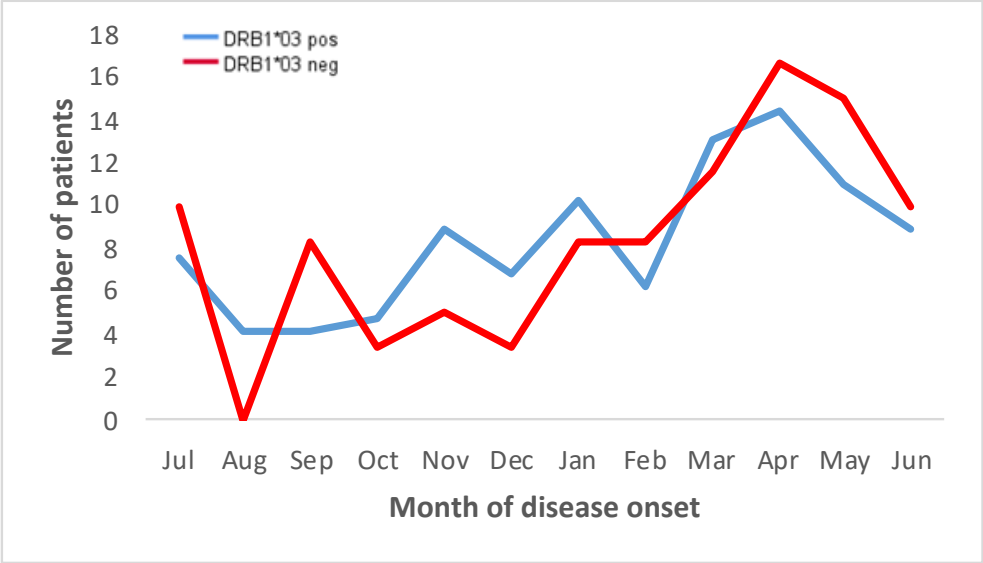


Figure 1. Month of disease onset in all patients



Figures 2. Month of disease onset in *HLA-DRB1*03* positive (blue line) and *HLA-DRB1*03* negative (red line) patients.

Hypothesizing that encountering antigens in the first years of life can lead to sensitivity to developing disease in the later years, we collected the month of birth of patients. We saw a peak of birth rates in the spring months. It is noteworthy that in the period 1960-1994, the birth rates in the Netherlands generally peaked in the spring months. Further research is needed to clarify if encountering antigens in early life has any effect on developing sarcoidosis in later years.

Treatment

Treatment and follow-up data are presented in Figure 3. Data were not complete for all patients due to loss to follow-up. After presentation 38 (22%) patients were hospitalized on the respiratory ward, with a median admission of 21.0 days, and 137 patients (78%) were followed up via the outpatient clinics. Hundred and twenty-three patients received no treatment or analgesic, like NSAID's, because of joint pain. Steroids were given to 54 patients with a variety of treatment schemes (i.e solely oral steroids, starting intravenously and continuing oral steroid, several days to years: 3 patients received corticosteroids for more than 52 weeks, of which one patient 572 weeks), with a median treatment duration of 19 weeks. Six patients were treated with DMARD's (1 patient received DMARD's as first line treatment), and 4 patients with TNF-α blockers (2 of these patients were already on treatment with TNF-α blockers because of colitis ulcerosa and rheumatoid artritis). Six patients were treated with the then current Tuberculosis treatment regime, including corticosteroids, diagnoses of these patients was made in 1959, 1963, 1966, 1974, 1977 and 1980. These data

are particularly illustrative of the changes and development of knowledge about optimal care strategies for patients with Löfgren's syndrome over the years.

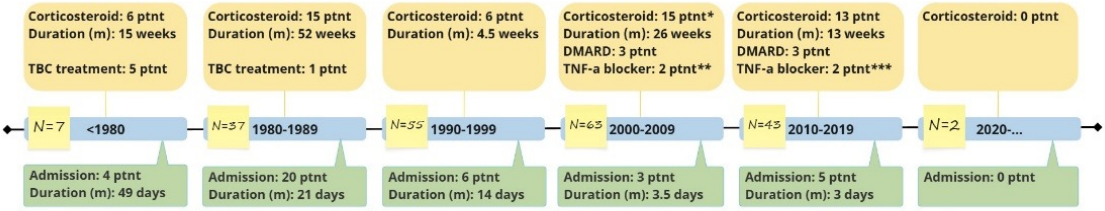


Figure 3. Timeline showing treatment regimens over the years
The timeline is showing choice of treatment and hospital admission ot the patients with Löfgren's syndrome in the studied cohort.
m=median.
*In the period 2000-2009, 1 patient received corticosteroids for 572 weeks. Without this patient the median duration of corticosteroid treatment was 24 weeks.

**both patients were treated with a TNF-α blocker due to progressive disease despite of treatment with a DMARD.

***both patients were treated with a TNF-α blocker prior to developing Löfgren's syndrome, one with diagnosis of colitis ulcerosa and the other with diagnosis of rheumatoid arthritis.

Fatigue

About 89% of the patients with Löfgren's syndrome in our cohort indicated to have troublesome fatigue at time of diagnosis. The median duration of fatigue before improvement or resolution was 7 months. Importantly, 49 patients still suffered from fatigue at a median follow-up of 11 years. Comparing *HLA-DRB1*03* positive and negative patients, there was no difference regarding fatigue and duration. Interestingly, after a follow-up period of more than 9 years more *HLA-DRB1*03* negative patients still had fatigue ($p < 0.001$, table 1). No difference was seen between male and female patients regarding fatigue symptoms (Table 2). These data show that a significant amount of patients have troublesome fatigue for a long period of time and that after a long follow-up period, especially *HLA-DRB1*03* negative patients, experience persistent fatigue.

Table 1. Fatigue in *HLA-DRB1*03* positive and negative patients

	Total (n = 186)	***DRB1*03*⁺** (*n* = 135)	***DRB1*03*⁻** (*n* = 51)	***P* value**
Fatigue	166 (89%)	117 (87%)	49 (96%)	NS
Sex m/f	63/103	49/68	14/35	NS
Resolving in, m (IQR) (n = 171)	7.0 (3-12)	7.0 (3-12)	8.3 (4.3-12)	NS
Still fatigue	49 (30%)	25 (22%)	24 (52%)	< 0.001
Follow-up, yr (IQR))	11.0 (3.3-18.8)	11.0 (3-19)	9.0 (5.3-17.8)	NS

m=months, yr=years

Table 2. Fatigue in male and female patients

	Total (*n* = 186)	**Male** (*n* = 74)	**Female** (*n* = 112)	***P* value**
Fatigue	166 (89%)	63 (85%)	103 (92%)	NS
Resolving in, m (IQR) (n = 171)	7.0 (3-12)	7.0 (3-12m)	7.0 (3.3-12)	NS
Still fatigue	49 (31%)	17 (27%)	32 (33%)	NS
Follow-up, yr (median)	11.0 (3.3-18.8)	8.0 (3-18)	13.0 (4.8-19)	0.045

m=months, yr=years

All patients filled out at follow-up a CIS-20 and SF-36 questionnaire. No differences were found in both questionnaires when *HLA-DRB1*03* positive patients were compared to *HLA-DRB1*03* negative patients. Comparing males to females, we found for females a higher score in the CIS-20 and a lower score in the next dimensions of the SF-36 questionnaire: role limitations due to physical problems, role limitations due to emotional problems, social functioning and pain. These data seem to indicate that female patients suffer more from fatigue and functional impairment compared to males, but several studies showed that in general, healthy or not, females scores lower for all or some dimensions in the SF-36 compared to males.[4,5] Furthermore Myalgic Encephalomyelitis/Chronic Fatigue Syndrome is more frequent in females than in males.[6] Up to 90% of sarcoidosis patients[7] reports severe life-altering fatigue affecting the overall quality of life.[8] Recent studies show associations between HLA and Myalgic Encephalomyelitis/Chronic Fatigue Syndrome.[9,10] Our data show that especially *HLA-DRB1*03* negative patients still suffer from fatigue after a long follow-up time. These patients need extra care and attention like non-Löfgren's syndrome sarcoidosis patients. Further research is needed to elucidate the association between HLA and fatigue in sarcoidosis patients. A recent study showed that a 12 week online mindfulness-based cognitive therapy (eMBCT) improved fatigue in patients with sarcoidosis.[11] Given the chronicity of fatigue in especially *HLA-DRB1*03* negative patients with Löfgren's syndrome, we suggest that they may also benefit from eMBCT.

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The Synthesis

The Mirror. The full form reveals the bilateral symmetry of Löfgren's Syndrome.

Red (Spirit) and Black (Pain) interlock along the central axis. The "Ankle Knot" is now complete, showing the condition not as a broken part but as a unified, symmetrical embrace of the body's struggle.

Chapter 5

ANXA11 rs1049550 Associates with Löfgren's Syndrome and Chronic Sarcoidosis Patients

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ABSTRACT

Objectives

Sarcoidosis is an immune mediated granulomatous disease commonly affecting the lungs. Genome wide association studies identified many genomic regions that are shared among multiple immune mediated diseases. However, *ANXA11* gene polymorphism rs1049550 is exclusively associated with sarcoidosis, making it a key gene of interest for sarcoidosis disease pathogenesis. However, sarcoidosis is a heterogeneous disease and contradictory findings for *ANXA11* have been reported for disease phenotypes. We performed a case–control association study to investigate if *ANXA11* associates with benign (Löfgren's syndrome (LS)) or chronic sarcoidosis and performed a meta-analysis on previously reported findings.

Methods

A total of 262 sarcoidosis patients, of which 149 had LS and 113 chronic sarcoidosis, and 363 controls were genotyped for rs1049550. Meta-analysis included allele findings for rs1049550 from 6 additional studies.

Results

We found a significantly lower T allele frequency in sarcoidosis patients than in healthy controls (0.30 vs. 0.41, respectively, odds ratio (OR) 0.61, 95% confidence interval (CI) 0.48–0.77, $p = 3 \cdot 10^{-5}$). In LS the T allele frequency of 0.33, and in chronic sarcoidosis the T allele frequency of 0.26 were significantly lower than in healthy controls (OR 0.69, 95% CI 0.52–0.92, $p = 0.01$ and OR 0.51, 95% CI 0.36–0.70, $p = 4 \cdot 10^{-5}$, respectively). Meta-analysis including previously published European, African American and Asian cohorts confirmed the association of rs1049550 with sarcoidosis and resulted in a pooled OR of 0.70 (CI 0.66–0.75, $p = 3.58 \cdot 10^{-29}$).

Conclusion

Presence of the T allele of rs1049550 in *ANXA11* is protective for sarcoidosis, including benign and chronic phenotypes of the disease.

Keywords: sarcoidosis; Löfgren's syndrome; *ANXA11*; SNP

Introduction

Sarcoidosis is a systemic inflammatory disorder of unknown cause with a wide clinical spectrum. The disease is characterized by the formation of non-caseating epithelioid cell granulomas. It commonly affects the lungs and intrathoracic lymph nodes, but any organ can be involved. The clinical course and prognosis is heterogeneous. Löfgren's syndrome (LS) is a well-defined phenotype of sarcoidosis manifesting as acute onset, bilateral hilair lymphadenopathy (BHL), erythema nodosum, and/or bilateral ankle arthritis or periarticular inflammation.[1] Patients with LS usually have a very good prognosis in contrast with the non-LS patients of whom about one third develop chronic sarcoidosis.[2]

Sarcoidosis is a complex disease, suggested to be the result of an interaction between an environmental trigger and a patient's genetic make-up. Genome wide association studies (GWAS) showed that multiple genes associate with sarcoidosis, and most of these gene regions are shared among multiple immune mediated diseases. However, the association between *ANXA11* and sarcoidosis, which was for the first time described in a German cohort by Hofmann et al.[3] is unique for sarcoidosis. Several studies have replicated these results and showed an association with the *ANXA11* Single Nucleotide Polymorphism (SNP) rs1049550 and sarcoidosis.[4-9] All studies found that carriage of the minor T allele conferred protection for sarcoidosis, as the minor T allele frequency was lower in sarcoidosis patients than in controls. However, subsequent subgroup analyses investigated whether this SNP associated with specific disease phenotypes of sarcoidosis, resulting in some contradictory findings when groups were formed according to radiological Scadding stages. Where one study found a lower T allele frequency in Scadding stage II–IV patients,[5] two other studies[8,10] found a higher T allele frequency in Scadding stage II–IV patients when compared to a group of patients with lower Scadding stages.

Furthermore, two studies described findings in a sarcoidosis subgroup consisting of LS patients. Mrazek et al.[5] found a higher TT frequency in LS patients compared to non-LS patients, and Morais et al.[7] found no difference between LS patients and controls regarding rs1049550. However, both studies had limited power and thus wide confidence intervals, as the numbers of LS patients in both studies were relatively small, with 39 and 55 patients, respectively.

Most genes identified by GWAS do not only associate with sarcoidosis, but have also been identified in studies for immune-mediated disorders, such as inflammatory bowel disease and Crohn's disease.[11] *ANXA11* is currently the only gene identified by GWAS that predisposes to sarcoidosis but not to other immune mediated diseases.[12] *ANXA11* may therefore provide the key to understanding sarcoidosis-specific

disease processes. However, while the association between sarcoidosis in general and rs1049550 has been confirmed in several studies, the protective role of the rs1049550 T allele for clinical phenotypes of sarcoidosis remains to be determined. Prior to further experimental studies into the role of *ANXA11* in sarcoidosis, it is essential to determine for which phenotypes the T allele may protect. The aim of this study was therefore, first, to investigate the association of the *ANXA11* SNP rs1049550 with sarcoidosis in general and corroborate previous findings by adding new results in a meta-analysis. Second, to analyze clinical sub-phenotypes LS and chronic sarcoidosis to provide new evidence about the effect of rs1049550 T allele. These data will provide insights on the role of *ANXA11* polymorphism in sarcoidosis in general and specific disease phenotypes and may influence experimental design and choice of therapy in the future.

Materials and Methods

Subjects

A total of 262 Dutch sarcoidosis patients from St. Antonius Hospital, Nieuwegein, the Netherlands, were included in the study. All patients were diagnosed in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis.[13] LS was defined as presenting with the classic symptoms: acute onset with bilateral hilar lymphadenopathy, fever, erythema nodosum (EN), and/or bilateral ankle arthritis.[1]

Thoracic involvement was classified using the Scadding criteria:[14] stage 0, no lung involvement; stage I, lymphadenopathy without parenchymal involvement; stage II, lymphadenopathy with parenchymal involvement; stage III, parenchymal involvement only; and stage IV, pulmonary fibrosis.
Our group consisted of 149 patients with LS and 113 patients with chronic sarcoidosis. Patients with chronic disease had evidence of disease after at least 4 years of follow-up. Three hundred and sixty-three healthy Caucasian subjects were included as controls in this study.
Written consent was obtained from all subjects, and authorization was given by the Medical research Ethics Committees United (MEC-U) of the St. Antonius Hospital, Nieuwegein (approval number R08-37A).

Genotyping

Genomic DNA was extracted from peripheral blood of each individual using standard methods. *ANXA11* rs1049550 was genotyped with a pre-designed taqman SNP genotyping assay (Assay ID C_7881261_1) and the Quantstudio® 5 real-time PCR system (both ThermoFisher Scientific, Waltham, MA, USA).

Meta-Analysis

For meta-analysis, we included studies with (a) a case–control study design, (b) diagnosis of sarcoidosis according to internationally accepted criteria, and (c) reporting genotype or allele frequencies. For the study by Levin et al.,[6] we switched the C and T alleles (see discussion). For the study by Feng et al.,[8] we used published frequencies for the first meta-analysis and excluded the study in a second meta-analysis.

Most of the studies included in the first meta-analysis also analyzed phenotypes of sarcoidosis. We also performed a meta-analysis for the phenotypes of sarcoidosis, resolving and chronic sarcoidosis, LS and non-LS, by using the data provided in the studies. In our LS patients, there were 7 patients with chronic disease. By excluding these 7 patients from our LS patients, we had a group of patients with resolving disease, which we included for the meta-analysis. We did not perform a meta-analysis with the Scadding stage data due to the variation in selected Scadding stages. However, the data about Scadding stages and *ANXA11* rs1049500 are presented in Supplementary Table S1 and Supplementary Figure S1.

Statistical Analysis

Allele and genotype frequencies were calculated for SNP rs1049550 and tested for Hardy–Weinberg equilibrium (HWE). Differences in allele frequencies were calculated with the Pearson's goodness-of-fit Chi-square test. Three genetic models (additive, dominant, and recessive) were assessed with a logistic regression analysis on genotype results using SNPStats[15] test for Hardy-Weinberg equilibrium and linkage disequilibrium. Analysis of association is based on linear or logistic regression according to the response variable (quantitative or binary disease status, respectively. The odds ratio (OR) and confidence interval (95% CI) were calculated, and a p value < 0.05 was considered statistically significant.

Furthermore, when presenting data from previous studies, the OR, 95% CI, and p-value were calculated from the data described in the original publication; when these data were not available the OR, 95% CI, and p-values were copied from the original article.

Meta-analyses were performed using the allele contrast in the web tool META-Genyo.[16] Heterogeneity in the data was evaluated with I^2 statistics and Cochran's Q test was low for the allele contrast. The fixed-effect estimate method inverse variance was used. Publication bias was investigated by Egger's regression test.

Results

Sarcoidosis Patients and Subgroups

In total, 262 sarcoidosis patients and 363 healthy controls were included. The patient cohorts consisted of 149 patients with LS and 113 non-LS patients with chronic sarcoidosis and Scadding stage II–IV (Table 1). Patients with LS were more often female and chronic sarcoidosis patients were more often male and slightly older.

Table 1. Baseline characteristics of patients with Löfgren's syndrome and chronic sarcoidosis.

		Controls	Sarcoidosis All	Löfgren's Syndrome	Chronic Sarcoidosis
N		363	262	149	113
Age *yrs*		40	38	36	42
Female *n (%)*		185 (50.1)	130 (49.6)	94 (63.1)	36 (31.8)
Scadding Stage			*n* = 228	*n* = 115	*n* = 113
n (%)	0		5 (2.2)	5 (4.3)	
	I		101 (44.3)	101 (87.8)	
	II		38 (16.7)	9 (7.8)	29 (25.7)
	III		13 (5.7)		13 (11.5)
	IV		71 (31.1)		71 (62.8)

Association of ANXA11 rs1049550 with Sarcoidosis

Genotyping results are shown in Table 2. For all groups, no deviation from Hardy–Weinberg equilibrium was observed ($p > 0.05$). A significantly decreased minor T allele frequency was observed in the total group of sarcoidosis patients compared with controls (0.30 vs. 0.41, OR 0.61, 95% CI 0.48–0.77, $p = 3 \cdot 10^{-5}$).

Subgroup analysis showed that comparison of LS with controls yielded a significantly lower minor T allele frequency of 0.33 in LS with an OR 0.69 (95% CI 0.52–0.92, $p = 0.01$). Additionally, comparison of chronic sarcoidosis patients with controls also yielded a significantly lower minor T allele frequency of 0.26 with an OR 0.51 (95% CI 0.36–0.70, $p = 4 \cdot 10^{-5}$; Table 2). Furthermore, no significant difference was found between the LS and chronic sarcoidosis groups.

In addition to the allelic analysis, the association between *ANXA11* rs1049550 and total group of sarcoidosis patients, LS, and chronic sarcoidosis patients was assessed for underlying genetic model (Table 3). The additive and recessive (CC+CT vs. TT) models provide comparable significant results in sarcoidosis and its phenotypes. No significant results were retrieved for the dominant (CC vs. CT+TT) model in LS.

Table 2. *ANXA11* rs1049550 genotype and allele frequencies of patients with Löfgren's syndrome and chronic sarcoidosis.

		Controls	Sarcoidosis All	Löfgren's Syndrome	Chronic Sarcoidosis
N		363	262	149	113
Genotype					
n (%)	CC	130 (35.8)	125 (47.7)	66 (44.3)	59 (52.2)
	CT	167 (46.0)	118 (45.0)	69 (46.3)	49 (43.4)
	TT	66 (18.2)	19 (7.3)	14 (9.4)	5 (4.4)
Allele					
n (%)	C	427 (58.8)	368 (70.2)	201 (67.4)	167 (73.9)
	T	299 (41.2)	156 (29.8)	97 (32.6)	59 (26.1)
OR *			0.61	0.69	0.51
95% CI			0.48–0.77	0.52–0.92	0.36–0.70
p			$3 \cdot 10^{-5}$	0.01	$4 \cdot 10^{-5}$

* comparison of the patients group with controls, OR, CI and *p*-value are related to the allelic model

Table 3. Analysis of genetic models possibly underlying the association between *ANXA11* rs1049550 and sarcoidosis.

		Additive		Dominant (CC vs. CT+TT)		Recessive (CC+CT vs. TT)	
	n	**OR, 95% CI***	***p* Value**	**OR, 95% CI***	***p* Value**	**OR, 95% CI***	***p* Value**
Controls	363						
Sarcoidosis	262	0.61, 0.48–0.77	< 0.0001	0.61, 0.44–0.85	0.0029	0.35, 0.21–0.60	< 0.0001
Löfgren's syndrome	149	0.69, 0.52–0.92	0.01	0.70, 0.48–1.03	0.074	0.47, 0.25–0.86	0.0095
Chronic Sarcoidosis	113	0.51, 0.36–0.71	< 0.0001	0.51, 0.33–0.78	0.002	0.21, 0.08–0.53	0.0001

* comparison of the patients group with controls.

Meta-Analysis

For meta-analysis, we included six previous studies[3-8] describing the association between *ANXA11* rs1049550 and sarcoidosis and the current findings (Table 4 and Figure 1). One study[9] that did not describe the genotype frequencies was added to Table 4 to provide a complete overview of studies performed, but could not be included in the meta-analysis. The meta-analysis showed that sarcoidosis patients have a significantly lower T allele frequency in comparison to controls, with a pooled OR of 0.70 (95% CI 0.66–0.75, $p = 3.58 \cdot 10^{-29}$). There was no significant heterogeneity across the studies, and no significant publication bias was detected by Egger's test, p = 0.52. As the T allele frequency of the control cohort in Feng and co-authors[8] deviated significantly from that in public databases, we also performed a meta-analysis without that study, obtaining a pooled OR of 0.71 (95% CI 0.67–0.76, $p = 2.03 \cdot 10^{-24}$).

Table 4. Previous and present *ANXA11* rs1049550 association studies with sarcoidosis.

Author	Year	Country	Ethnicity	Case/Control, N	Case T allele freq	Control T allele freq	OR, 95% CI, *P* value*	Population T allele frequency gnomAD
Hofmann S et al.[3]	2008	Germany	Caucasian	1636/1811	0.33	0.41	0.70, 0.63-0.77, $1 \cdot 10^{-12}$	European: 0.42
Li et al.[4]	2010	Germany	Caucasian	349/313	0.35	0.45	0.65, 0.52-0.81, $1 \cdot 10^{-4}$	European: 0.42
Mrazek et al.[5]	2011	Czech	Caucasian	245/254	0.35	0.42	0.77, 0.59-0.99, 0.04	European: 0.42
Levin et al.[6#]	2013	USA	European American	446/350	0.36	0.42	0.76, 0.62-0.93, 0.008	European: 0.42
			African American	1232/893	0.15	0.18	0.83, 0.70-0.97, 0.02	African/African American: 0.20
Morais et al.[7]	2013	Portugal	Caucasian	208/197	0.33	0.45	0.61, 0.46-0.81, $6 \cdot 10^{-4}$	European : 0.42
Feng et al.[8]	2014	China	Chinese-Han	412/418	0.29	0.40	0.60, 0.49-0.73, $8 \cdot 10^{-7}$	East Asian : 0.66[§]
Sikorova et al.[9]	2020	Greece	Caucasian	103/100	Not available	Not available	0.59, 0.39-0.89, 0.01[†]	European: 0.42
Karakaya et al.		Netherlands	Caucasian	262/363	0.30	0.41	0.61, 0.48-0.77, $3 \cdot 10^{-5}$	European: 0.42

* Odds ratio (OR), 95% confidence interval (CI), and p-values are calculated from the data provided in the original articles. [#] For correct presentation, the C and T allele are switched. [§] The gnomAD database shows that the T allele in East Asian is the major allele. Within the gnomAD database, the 1 KG shows different East Asian populations: Han Chinese in Beijing have a T allele frequency of 0.6373; Southern Han Chinese have a T allele frequency of 0.6619. [†] The genotype data were not available for the study by Sikorova et al.; values were copied from the original article.

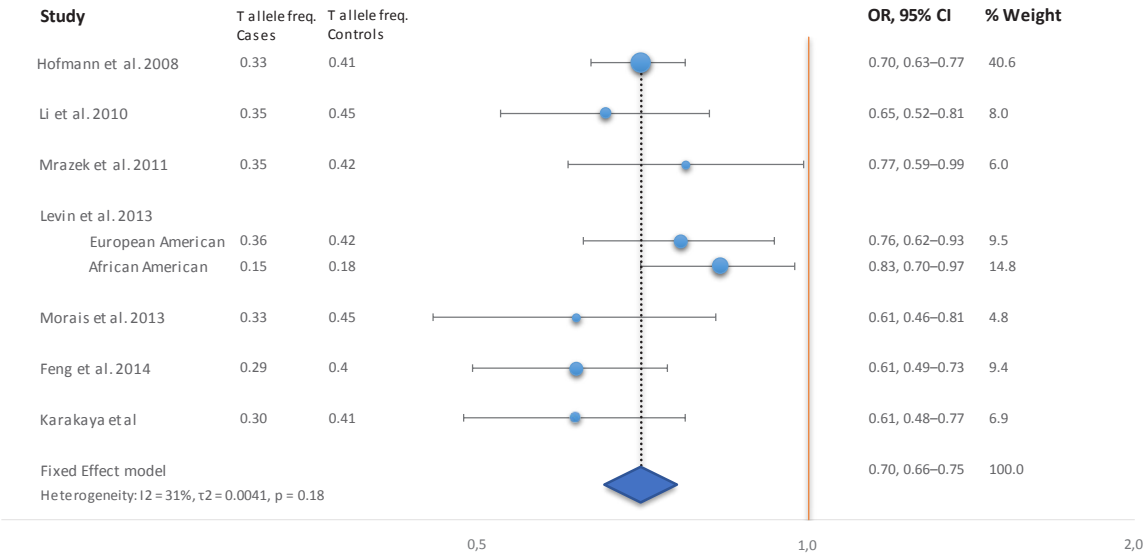


Figure 1. Meta-analysis for the studies of *ANXA11* with sarcoidosis. Forest plot of the odds ratio for *ANXA11* rs1049550 T allele in sarcoidosis cases versus controls. For correct presentation, the C and T allele are switched for the studies of Levin et al. Dotted line represents the odds ratio from the combined analysis. The result of the meta-analysis is presented as a diamond at the bottom that covers the combined OR in the middle and the CI at the tips. OR: Odds ratio, 95% CI: 95% confidence interval.

Sarcoidosis Phenotype Studies

In Table 5, we show the comparisons made in previous studies[4-7] and the present study between phenotypes of sarcoidosis: four studies for chronic and resolving disease and three studies for LS. A summary of all studies[4-10] is provided in the Supplementary Figure S1 for comparison of reported ORs and in Supplementary Table S1 for Scadding stage data. All phenotype studies found lower T allele frequency for phenotypes when compared with controls, however in some studies this was not significant.

Combining previous studies and new data, we performed meta-analyses for the following phenotypes of sarcoidosis: resolving disease and chronic disease, LS and non-LS. Levin et al.[6] and Mrazek et al.[5] did not provide genotype data, so these studies were not incorporated in the meta-analysis for the phenotypes of sarcoidosis.

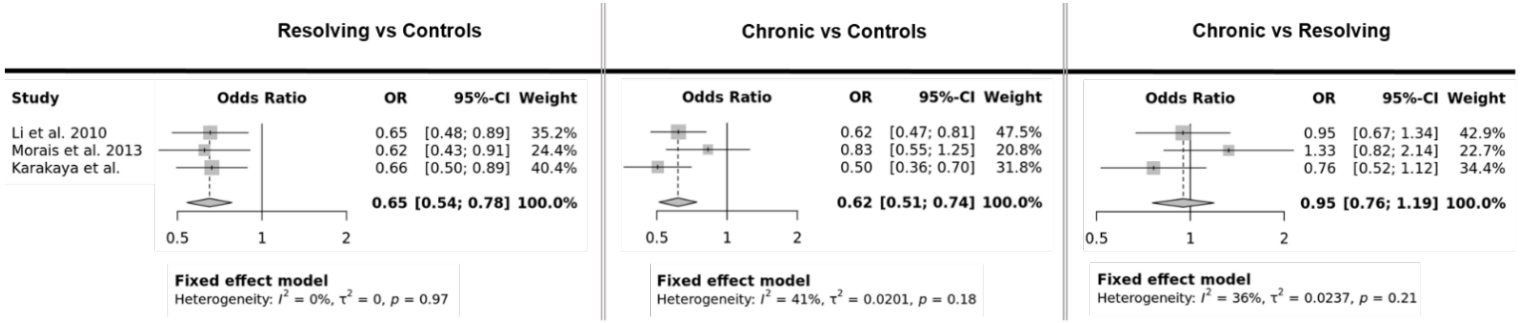
Pooled data show a significantly decreased T allele frequency in resolving disease, chronic disease, LS, and non-LS when compared with controls, with an OR of 0.65 (95% CI 0.54–0.78, $p = 4.7 \cdot 10^{-6}$), an OR of 0.62 (95% CI 0.51–74, $p = 3.8 \cdot 10^{-7}$), an OR of 0.69 (95% CI 0.55–0.88, $p = 0.0025$), and an OR of 0.54 (95% CI 0.43–0.68, $p = 1.1 \cdot 10^{-7}$), respectively (Figure 2A, B). Comparison between resolving and chronic disease, or LS and non-LS phenotypes, did not yield significant results.

Comparison between Scadding stages and controls showed similar results (Supplementary Figure S1 and Table S1), however comparing Scadding stages with each other showed contradictory findings. It is important to note that the compared Scadding stages differed from each other in the studies, i.e., where one study included patients with Scadding stage II–IV in the higher stage group, the other included only patients with Scadding stage IV.

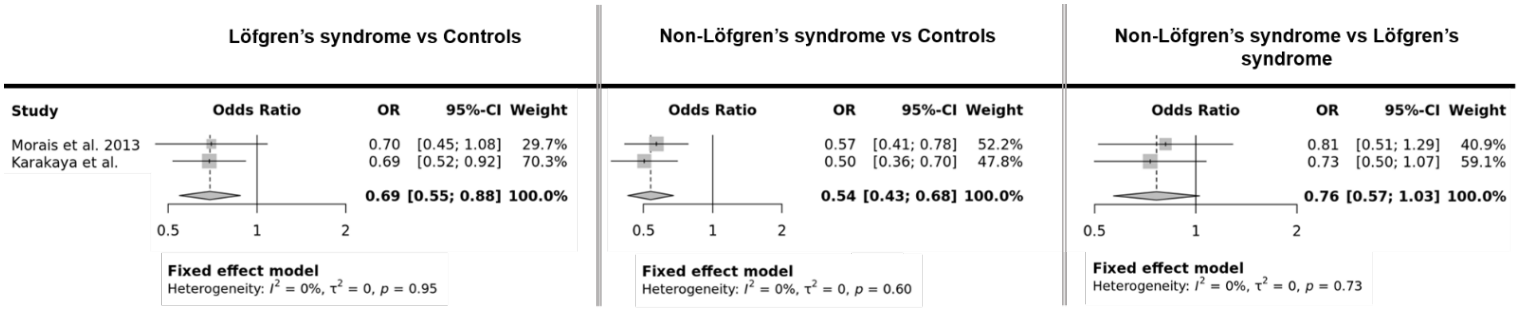
Table 5. Studies reporting an association of *ANXA11* rs1049550 with sarcoidosis disease phenotypes.

Study	T allele frequency			OR, 95% CI, *P* value*		
	Controls	**Resolving**	**Chronic**	**Resolving vs. Controls**	**Chronic vs. Controls**	**Chronic vs. Resolving**
Li et al, 2010[4]	0.45 (n = 313)	0.35 (n = 117)	0.34 (n = 176)	0.65, 0.48–0.89, 0.007	0.62, 0.47–0.81, $5 \cdot 10^{-4}$	0.95, 0.67–1.34, 0.76
Levin et al. 2013[6#]	0.18 (n = 893)	n = 304	n = 650	0.82, 0.64–1.06, 0.13[§,†]	0.79, 0.65–0.95, 0.02[§,†]	
Morais et al. 2013[7]	0.45 (n = 197)	0.34 (n = 86)	0.40 (n = 62)	0.62, 0.43-0.91, 0.01	0.83, 0.55–1.25, 0.37	1.33, 0.82–2.14, 0.24
Karakaya et al.	0.41 (n = 363)	0.32 (n = 142)	0.26 (n = 113)	0.66, 0.50–0.89, 0.005	0.51, 0.36–0.70, $4 \cdot 10^{-5}$	0.76, 0.52–1.12, 0.65
	Controls	**Löfgren's syndrome**	**Non-Löfgren's syndrome**	**Löfgren's syndrome vs. Controls**	**Non-Löfgren's syndrome vs. Controls**	**Non-Löfgren's syndrome vs. Löfgren's syndrome**
Morais, 2013[7]	0.45 (n = 197)	0.36 (n = 55)	0.32 (n = 145)	0.70, 0.45–1.08, 0.11	0.57, 0.42–0.78, $5 \cdot 10^{-4}$	0.81, 0.51–1.29, 0.38
Karakaya et al.	0.41 (n = 363)	0.33 (n = 149)	0.26 (n = 113)	0.69, 0.52–0.92, 0.01	0.51, 0.36–0.70, $4 \cdot 10^{-5}$	0.73, 0.50–1.07, 0.11
Mrazek, 2011[5]	TT frequency: 0.15 (n = 254)	TT frequency: 0.21 (n = 39)	TT frequency: 0.07 (n = 147)			0.31, 0.11–0.84, 0.02[†]

* Odds ratio (OR), 95% confidence interval (CI), and p-values are calculated from the data provided in the original articles. [#] Study population: African Americans. For correct presentation, the C and T allele are switched. [§] The original article states that the additive genetic model was used to estimate the OR, and the OR is adjusted for sex and percent African ancestry. [†] Original data were not available; values are copied from the original article.



(**A**)



(**B**)

Figure 2. Meta-analysis for the studies of *ANXA11* rs1049550 and phenotypes of Sarcoidosis. (**A**) Forest plot of the results for resolving and chronic sarcoidosis patients. (**B**) Forest plot of the results for Löfgren's syndrome and non-Löfgren's syndrome patients. Dotted line represents the odds ratio from the combined analysis. OR: Odds ratio, 95% CI: 95% confidence interval.

Discussion

In the present study, we investigated if *ANXA11* rs1049550 associates with sarcoidosis and with disease phenotypes. Meta-analysis shows that the SNP rs1049550 associates with sarcoidosis resulting in a pooled OR of 0.70 (95% CI 0.66–0.75); $p = 3.58 \cdot 10^{-29}$. Furthermore, we show that this association is independent of sarcoidosis phenotype, because in both LS and chronic sarcoidosis a similar association was present.

The association between rs1049550 and sarcoidosis was described in a German cohort for the first time by Hofmann et al.,[3] who performed the first genome-wide association study (GWAS) in sarcoidosis. Afterwards, rs1049550 was studied in other populations of sarcoidosis and phenotypes of the disease.[4-10] However, due to corrigenda and contradictory findings, especially regarding the clinical phenotypes that were studied, the results have been difficult to oversee. The GWAS report was followed by a correction of the notation of the SNP, reversing the minor allele from c.688C into c.688T.[17] Following the GWAS study, several studies investigated this association and correctly used the T allele as the minor allele. However, in a study by Levin et al.,[6] the C allele was again described as the minor allele in an European American and an African American population. This description not only contradicted previous findings, but also with reported allele frequencies in public databases.[18] Most confusing, the authors concluded that their results were in line with the study by Hofmann et al.,[3] which would mean that the alleles were switched. We have therefore chosen to switch these alleles in our meta-analysis and forest plots, resulting in the T allele being the minor allele. In 2014, a meta-analysis[19] was performed on six studies, including the study of Levin et al.,[6] but without correction for the proper minor allele and thus with an inversely calculated OR.

Furthermore, to determine if control cohorts conferred to established allele frequencies in ethnic cohorts, we checked rs1049550 in the genome aggregation database (gnomAD).[18] We observed that the T allele frequency varies considerably between populations, with the lowest frequency in African/African Americans (0.1994), with a slightly higher frequency in South Asian (0.3592) and in a European population (0.4206). However, in East Asian populations of gnomAD (v3.1.2),[18] the frequency of the T allele is reported to be considerably higher at 0.6633. Within gnomAD, the 1000 genome (1 KG) project shows specific East Asian findings: for Southern Han Chinese and Han Chinese in Beijing, there are reported T allele frequencies of 0.6619 and 0.6373, respectively. Surprisingly, Feng et al.[8] report a Chinese control population from Xinxiang (a city about 600 km to the southwest of Beijing) with a T allele frequency of 0.40. As this is far from the database report of East Asian frequencies, further research is needed to determine the correct control

frequency and compare this with a population of sarcoidosis patients from the same region. Although genetic studies in different ethnic populations can be difficult to compare, the case–control meta-analysis for rs1049550 yielded a strong significant result. For our meta-analysis, we incorporated the reported findings of Feng et al.;[8] if we exclude these findings a highly similar result was found.

In several studies, associations between rs1049550 and phenotypes of sarcoidosis were investigated (Table 5 and Figure 2). These phenotypes are partly, but not completely, overlapping. LS is usually non-chronic/resolving with Scadding stage 0 or I, whereas chronic pulmonary disease predominantly involves Scadding stages II and up. Most studies comparing disease phenotypes with controls found that the T allele protected against the phenotype of sarcoidosis. The T allele frequencies in resolving and in chronic disease were significantly lower than in controls[4,6] (Table 5 and Figure 2A). Furthermore, no difference was found between resolving (acute) and chronic sarcoidosis.[4,7]

LS is one of the best studied phenotypes of sarcoidosis. This phenotype is characterized by specific genetic associations, like with HLA-DR3,[20] CCR5,[21] and CCR2,[22] that are not all shared with non-LS sarcoidosis. Data for *ANXA11* in LS are scarce; only two studies mention patients with LS; Mrazek et al.[5] included 39 LS patients. Data about the T allele frequency in LS patients were not provided, but they reported a significantly higher frequency of the TT genotype when compared to non-LS patients. However, TT genotype frequency in LS patients was still lower than in controls, but no comparison was made. Morais et al.[7] did not find any difference between LS (n = 55) and non-LS patients in T allele frequency. We therefore analyzed rs1049550 in a large cohort of 149 Dutch LS patients, all with Scadding stage 0 or I except for nine patients with Scadding stage II. Our results demonstrated a similar association of rs1049550 with LS, as seen in sarcoidosis patients, which is a protective effect of the minor T allele. Furthermore, we showed that there is no difference between LS and chronic sarcoidosis. A meta-analysis with the pooled data from Morais et al.[7] and our data shows a lower T allele frequency in LS patients compared to controls, but no difference when compared to non-LS patients. Therefore, we can conclude that the association of *ANXA11* rs1049550 applies for sarcoidosis in general and the clinical phenotypes of LS and chronic disease. Presumably, the association may be found in all sarcoidosis cohorts, regardless of phenotype.

In the past years, several studies found genetic commonalities between sarcoidosis and other immune modulated diseases, such as Crohn's disease.[23] However, for *ANXA11*, no associations with other immune modulated diseases are published. The *ANXA11* rs1049550 polymorphism leads to an amino-acid substitution of arginine

to cysteine, at position 230 (p.(R230C)) of the Annexin A11 protein, within a highly conserved domain responsible for $Ca2^{+}$-binding properties.[24] Annexin A11 is known to be involved in calcium signaling, cell cycle, vesicle trafficking, and apoptosis[25,26] The effect of this amino-acid change is not well defined; although Hofmann et al.[3] showed no difference in *ANXA11* mRNA expression between control and diseased lung tissue, they showed a downregulation of *ANXA11* mRNA expression in $CD8^{+}$T and $CD19^{+}$B cells after stimulation. Furthermore, *ANXA11* mRNA expression in bronchoalveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMC) was not associated with rs1049550 genotype.[5] However, the Open Targets Genetics[27] database shows imputed results for rs1049550, and finds a strong association with sarcoidosis and differential expression of ANXA11 for the lead variant. This strongly suggest that ANXA11 is indeed the quantitative trait for rs1049550. Moreover, this differential expression is observed in monocytes.[28]

Levin et al.[6] found a significant SNP–SNP interaction between rs1049550 and a *HLA* SNP. This may indicate an interplay between HLA molecules, by presenting antigens, and ANXA11 by influencing the inflammatory response. Both ANXA11 and HLA molecules are among the few proteins detectable in human B cell exosomes. In BAL fluid of sarcoidosis patients, an increased level of exosomes was shown, especially expressing major histocompatibility complex class I and II proteins.[29] It is hypothesized that exosomes play a role in B cell activation thereby lowering the threshold for T cell activation.[29] It has been suggested that the amino-acid change caused by rs1049550 results in a dysfunctional Annexin A11 which can influence cell processes, thereby altering cell trafficking and apoptosis, which in turn can influence granuloma formation and maintenance, respectively, in sarcoidosis patients.[30] Further hypothesizing that without apoptosis the inflammatory and granulomatous inflammation will continue. There is one important aspect to take into account; the minor T allele, which would affect the Annexin A11 function, is actually less frequent in sarcoidosis and therefore its presence protects against sarcoidosis.

Now that we established that *ANXA11* rs1049550 associates with sarcoidosis in general, regardless of disease phenotype, more research is needed to elucidate the effects of the gene variant on Annexin A11 function and how this effects granuloma formation and maintenance in sarcoidosis patients. On the subject level, we found that the genetic additive or recessive (CC+CT vs. TT) model may underlie the association with sarcoidosis and its phenotypes, suggesting that future experimental studies should study differences between TT and CC genotypes. It would be of great interest to investigate ANXA11 in cell types known to be involved in granuloma formation, such as macrophages and monocytes. Other interesting cells would be $CD4^{+}$, $CD8^{+}$, and $CD19^{+}$ T cells, which are also known to play an important role in sarcoidosis.

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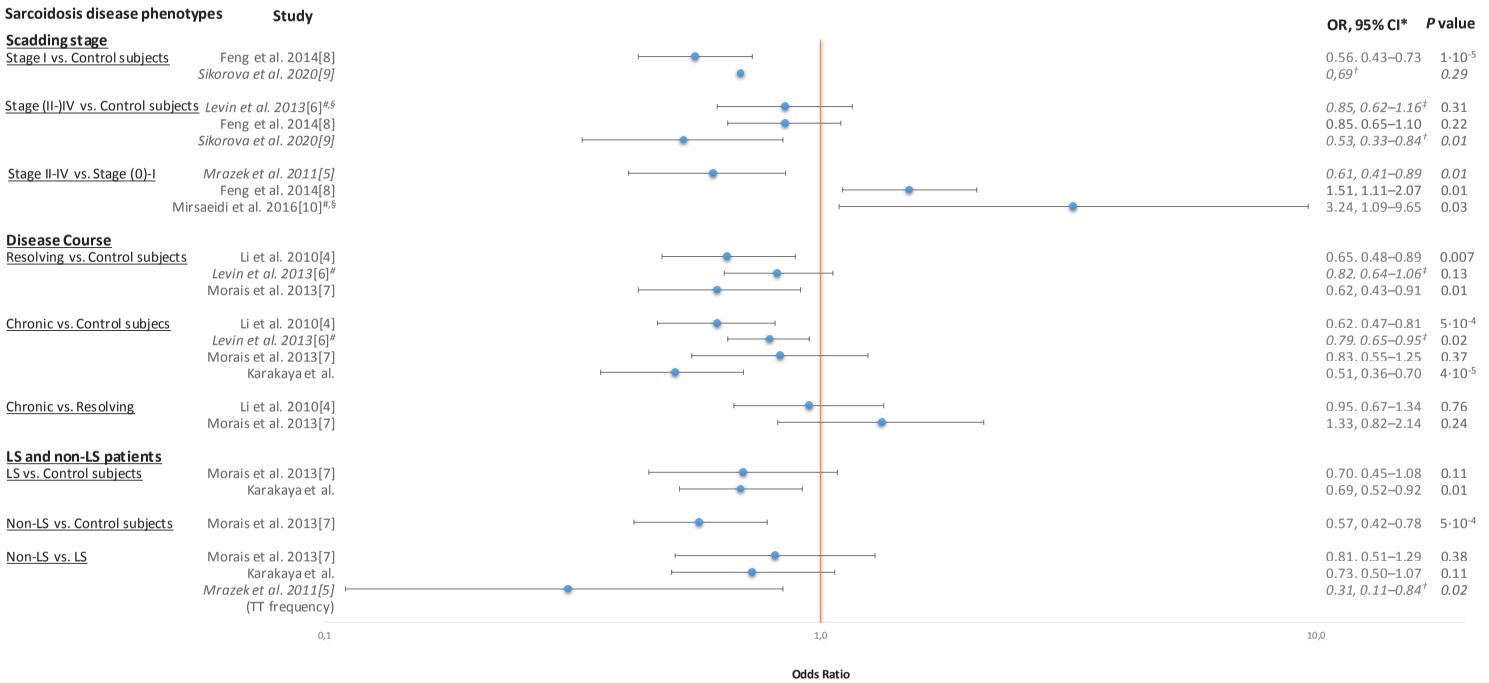
Supplementary Table S1. Studies reporting an association of *ANXA11* rs1049550 with sarcoidosis disease phenotypes.

Study	T allele frequency			OR, 95% CI, *p* value*		
	Control	**Stage (0)-I**	**Stage II-IV**	**Stage (0)I vs. Controls**	**Stage II-IV vs. Controls**	**Stage II-IV vs. Stage (0)-I**
Mrazek et al. 2011[5]	0.42 (*n* = 254)	0.42 (*n* = 110)	0.30 (*n* = 117)			0.61 (0.41–0.85) *p* = 0.01
Levin et al. 2013[6*]	0.18 (*n* = 893)		Stage IV: (*n* = 188)		stage IV: 0.85, 0.62–1.16, *p* = 0.31[§,†]	
Feng et al. 2014[8]	0.40 (*n* = 418)	0.28 (*n* = 196)	0.37 (*n* = 167)	0.56, 0.43–0.73, $p = 1 \cdot 10^{-5}$	0.85, 0.65–1.10, *p* = 0.22	1.51, 1.11–2.07, *p* = 0.01
Mirsaeidi et al. 2016[10*]		0.10 (*n* = 35)	Stage IV: 0.26 (*n* = 17)			3.24, 1.09–9.65, *p* = 0.03
Sikorova et al. 2020[9]	*n* = 100	*n* = 29	*n* = 68	0.69, *p* = 0.29	0.53, 0.33–0.84, *p* = 0.01[†]	
	Control	**Resolving**	**Chronic**	**Resolving vs. Controls**	**Chronic vs. Controls**	**Chronic vs. Resolving**
Li et al. 2010[4]	0.45 (*n* = 313)	0.35 (*n* = 117)	0.34 (*n* = 176)	0.65, 0.48–0.89, *p* = 0.007	0.62, 0.47–0.81, $p = 5 \cdot 10^{-4}$	0.95, 0.67–1.34, *p* = 0.76
Levin et al. 2013[6#]	0.18 (*n* = 893)	*n* = 304	*n* = 650	0.82, 0.64–1.06, *p* = 0.13[§,†]	0.79, 0.65–0.95, *p* = 0.02[§,†]	
Morais et al. 2013[7]	0.45 (*n* = 197)	0.34 (*n* = 86)	0.40 (*n* = 62)	0.62, 0.43–0.91, *p* = 0.01	0.83, 0.55–1.25, *p* = 0.37	1.33, 0.82–2.14, *p* = 0.24
Karakaya et al.	0.41 (*n* = 363)	0.32 (*n* = 142)	0.26 (*n* = 113)	0.66, 0.50–0.89, *p* = 0.005	0.51, 0.36–0.70, $p = 4 \cdot 10^{-5}$	0.76, 0.52–1.12, *p* = 0.65
	Control	**Löfgren's syndrome**	**Non-Löfgren's syndrome**	**Löfgren's syndrome vs. Controls**	**Non-Löfgren's syndrome vs. Controls**	**Non-Löfgren's syndrome vs. Löfgren's syndrome**
Morais, 2013[7]	0.45 (*n* = 197)	0.36 (*n* = 55)	0.32 (*n* = 145)	0.70, 0.45–1.08, *p* = 0.11	0.57, 0.42–0.78, $p = 5 \cdot 10^{-4}$	0.81, 0.51–1.29, *p* = 0.38
Karakaya et al.	0.41 (*n* = 363)	0.33 (*n* = 149)	0.26 (*n* = 113)	0.69, 0.52–0.92, *p* = 0.01	0.51, 0.36–0.70, $p = 4 \cdot 10^{-5}$	0.73, 0.50–1.07, *p* = 0.11
Mrazek, 2011[5]	TT frequency: 0.15 (*n* = 254)	TT frequency: 0.21 (*n* = 39)	TT frequency: 0.07 (*n* = 147)			0.31, 0.11–0.84, *p* = 0.02[†]

*Odds ratio (OR), 95% confidence interval (CI) and *p*-values are calculated from the data provided in the original articles.
[#]Studypopulation: African Americans. For correct presentation the C and T allele are switched.
[§]The original article states that the additive genetic model was used to estimate the OR, and the OR is adjusted for sex and percent African ancestry.
[†]Original data were not available, values are copied from the original article.



Supplementary Figure S1. Forest plot of the results for *ANXA11* rs1049550 T allele associations phenotypes of sarcoidosis.
§*Odds ratio (OR), 95% confidence interval (CI) and *p*-values are calculated from the data provided in the original articles.
†Original data were not available, values are copied from the original article.
#Studypopulation: African Americans. For correct presentation the C and T allele are switched.
§only Scadding stage IV patients were analyzed.
‡The original article states that the additive genetic model was used to estimate the OR, and the OR is adjusted for sex and percent African ancestry.

Deconstruction visual

Chapter 6

Macrophage Migration Inhibitory Factor (MIF) -173 polymorphism is associated with clinical erythema nodosum in Löfgren's syndrome

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ABSTRACT

Objectives

Macrophage migration inhibitory factor (MIF) has been shown to be a key regulator in innate and adaptive immune responses. A single nucleotide polymorphism in the 5' region of the *MIF* gene, *MIF* -173*G/C, is associated with increased MIF protein production, in vivo and in vitro. Associations have been shown between the minor *MIF* -173C allele and sarcoidosis patients with erythema nodosum (EN). Löfgren's syndrome is an acute and usually self-remitting phenotype of sarcoidosis. It is defined as having an acute onset with bilateral hilar lymphadenopathy (BHL), fever, erythema nodosum (EN) and/or arthritis.

The aim of this study was to investigate whether *MIF* -173G/C associates with the susceptibility to and the clinical manifestations, i.e. arthritis or EN, of Löfgren's syndrome.

Methods

A total of 171 patients with Löfgren's syndrome and 313 controls were genotyped for a single nucleotide polymorphism at position -173 of the *MIF* gene (SNP rs755622), using a PCR and a restriction enzyme technique.

Results

There were no significant differences found in the *MIF* -173C allele frequencies between patients with Löfgren's syndrome and controls. In patients with Löfgren's syndrome with only EN, a significantly increased frequency of the C minor allele was observed compared to patients with arthritis only (p = 0.0095; OR 3.08, CI: 1.28-7.39).

Patients with only EN compared to patients with EN and arthritis showed a significantly increased frequency of the minor C allele (p = 0.044; OR 1.97, CI: 1.01-3.85). But patients with only arthritis compared to patients with EN and arthritis did not show a significant difference in C allele frequency (p = 0.270; OR 0.64, CI: 0.29-1.42)

Conclusions

The *MIF* -173C allele is associated with erythema nodosum in Löfgren's syndrome, but not with susceptibility to sarcoidosis. This indicates a role for MIF after antigen presenting to the T cell has taken place and the sarcoid inflammatory response has begun.

Keywords*:* Sarcoidosis, Löfgren's Syndrome, Erythema Nodosum, Macrophage Migration Inhibitory Factor (MIF), Single Nucleotide Polymorphism (SNP)

Introduction

Macrophage migration inhibitory factor (MIF) has been shown to be a key regulator in innate and adaptive immune responses. It is constitutively expressed and stored in intracellular pools in a broad variety of cells of the immune system, like T-cells and macrophages.[1,2] MIF is also expressed by cells and tissues, that are in direct contact with the host's natural environment such as the lung[3] and the skin.[4] It is rapidly released after exposure to microbial products and proinflammatory mediators.[5]

Once released, MIF contributes to an excessive inflammatory response directly by promoting the production or expression of pro-inflammatory molecules, like cytokines such as TNF-α, IFN-γ, IL-1β, IL-2, IL-6 and IL-8, and indirectly by antagonizing the anti-inflammatory effects of glucocorticoids.[6,7] The magnitude of this effect varies with the concentration of both glucocorticoids and MIF.[8,9]
Recent studies showed an important role for MIF in the pathogenesis of acute and chronic inflammatory and autoimmune diseases.[10,11] Increased levels of MIF have been detected in the synovial fluids of patients with rheumatoid arthritis and serum of patients with juvenile idiopathic arthritis and sarcoidosis.[12,13] Associations between MIF and other pulmonary diseases, like asthma,[14] lung cancer[15] and IPF,[16] have also been described.

A single nucleotide polymorphism in the 5' region of the *MIF* gene, *MIF* -173*C, is associated with increased MIF protein production, in vivo and in vitro.[11] Associations have been shown between the minor *MIF* -173C allele and rheumatoid arthritis, juvenile idiopathic arthritis, systemic sclerosis,[17] systemic lupus erythematosus[18] and sarcoidosis. In sarcoidosis patients with erythema nodosum (EN) the frequency of the *MIF* -173C allele have been found to be significantly higher than in patients with EN due to other causes or controls, indicating a role for MIF in the clinical presentation of sarcoidosis.[19]
Sarcoidosis is a multisystemic granulomatous disease of which the cause remains unknown. Several reports support the hypothesis that sarcoidosis results from exposure of genetically susceptible hosts to specific environmental agents, thereby inducing an immune response mediated by macrophages and lymphocytes.[20] Erythema nodosum due to sarcoidosis is usually seen as part of the Löfgren's syndrome, the best defined phenotype of sarcoidosis. Löfgren's syndrome, the acute form of sarcoidosis presents with bilateral hilar lymphadenopathy, erythema nodosum (EN) and/or articular inflammation or arthritis.[21] It is seen in about one third of the sarcoidosis patients and has a good prognosis.[22] Associations have been found between variations in several genes encoding for molecules with important functions in the immune system, such as TNF-α, IFN-γ and HLA-molecules,[23-25] and Löfgren's syndrome. In particular *HLA-DRB1*03:01* has been strongly associated with Löfgren's syndrome and its outcome.[26]

The aim of this study was to investigate whether polymorphisms of the macrophage migration inhibitory factor (*MIF*) associate with the susceptibility to and the clinical manifestations, arthritis or EN, of Löfgren's syndrome.

Materials and methods

Subjects

A total of 171 unrelated Caucasian patients, from 2 hospitals in the Netherlands, were included in the study. All patients were diagnosed in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis. All patients presented with the classic symptoms of Löfgren's syndrome: acute onset with bilateral hilar lymphadenopathy, fever, erythema nodosum and/or bilateral ankle arthritis.

Presence of EN and arthritis was collected from medical records for all patients.
Three hundred and thirteen healthy Caucasian subjects were included as controls in this study, matched by sex and ethnicity with the Löfgren's syndrome patients.
Written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein and Leiden University Medical Center.

Genotyping

Genomic DNA was extracted from peripheral blood of each individual using standard methods. We genotyped 171 patients and 313 controls for *MIF* -173G/C (SNP rs755622). Primers were used to amplify a 374 bp PCR product for rs755622 (forward primer: 5'-CTGGCGACTAACATCGGTGA-3'; reverse primer: 5'-ACATCGGCATGATGGCAGAA-3'), which was digested by AluI restriction enzyme (New England Biolabs) overnight at 37°C. Restriction product was separated on a 2% agarose gel, the G allele consisted of 2 fragments of 74- and 300-bp, the C allele consisted of 3 fragments of 74-, 94-and 206-bp.

Statistical analysis

Allele and genotype frequencies were calculated for the -173G to C polymorphism and tested for Hardy-Weinberg equilibrium (HWE) in controls. Case-control association studies were analyzed by χ^2 test using contingency tables of genotype and allele frequencies. Hardy-Weinberg equilibrium (HWE), Odds ratios and confidence intervals (CI) were calculated with an online tool, available at http://ihg.gsf.de/ihg/snps.html. A p value <0.05 was considered significant. The power of the study was calculated using the software Quanto.

A power of >80% was estimated for the analysis between the full set of patients and controls (considering $p = 0.05$ and a reference OR = 1.70) and for the analysis between subtypes of patients (considering $p = 0.05$ and reference OR = 3.3).

Results

Patient characterization

All 171 patients had an acute onset of the disease, with BHL, EN and/or periarticular inflammation or arthritis of the ankles. Thirtyfour patients (20%) had only EN, while 43 patients (25%) had only arthritis and 94 patients (55%) had both of the characteristics of the disease. Hundred and six patients (62%) were female. No significant differences were found in clinical manifestations between males and females (see Table 1).

Table 1. Baseline Characteristics of Controls and Patients with Löfgren's syndrome

	n	%	Sex		Age
			Male	Female	Mean years
Controls	313		118 (37%)	199 (63%)	40
Löfgren's syndrome patients	171		65 (38%)	106 (62%)	35
EN only	34	20	12 (18%)	22 (21%)	
Arthritis and EN	94	55	35 (54%)	59 (56%)	
Arthritis only	43	25	18 (28%)	25 (23%)	

EN only: patients having only Erythema Nodosum (EN), Arthritis only: patients having only arthritis, Arthritis and EN: patients having both arthritis and EN.

Controls versus Löfgren's syndrome.

No evidence of departure from Hardy-Weinberg equilibrium in controls was seen ($p > 0.05$).

There were no significant differences for the genotype distribution and allele frequencies between controls and Löfgren's syndrome patients.

Löfgren's syndrome patients

We further examined the SNP in the group of Löfgren's syndrome patients, and looked if there were differences between patient groups with different clinical presentation.

Erythema nodosum versus arthritis

A significantly increased frequency of the minor C allele was observed in the patients with only EN compared to patients with only arthritis (p = 0.0095; OR 3.08, CI: 1.28-7.39) (Table 2). The frequency of the three possible genotypes differed significantly between patients with EN and patients without EN (p = 0.048). The genotype distribution of GC + CC genotypes versus GG genotype was significantly increased in patients with only EN (p = 0.015; OR 3.45, CI 1.24-9.61) (Table 3 and Figure 1).

Table 2. Allele frequencies of *MIF* -173 gene polymorphism in controls and Löfgren's syndrome patients and the clinical subgroups of Löfgren's syndrome patients.

	Controls	**Löfgren's syndrome patients**	**Patients with only EN[1,2]**	**Patients with arthritis and EN[2]**	**Patients with only arthritis[1]**
	(*n* = 313)	(*n* = 171)	(*n* = 34)	(*n* = 94)	(*n* = 43)
Allele (2N)					
G (%)	501(80%)	286(84%)	50(74%)	159 (85%)	77(90%)
C (%)	125(20%)	56(16%)	18(26%)	29(15%)	9(10%)

[1]Allele C was significantly increased in patients with only EN compared to patients with only arthrits: p = 0.0095; OR 3.08, CI: 1.28-7.39

[2]Allele C was significantly increased in patients with only EN compared to patients with arthritis and EN: p = 0.044; OR 1.97, CI: 1.01-3.85

Table 3. Genotype frequencies of *MIF* -173 gene polymorphism in controls and Löfgren's syndrome patients and the clinical subgroups of Löfgren's syndrome patients.

	Controls	**Löfgren's syndrome patients**	**Patients with only EN[1]**	**Patients with arthritis and EN**	**Patients with only arthritis[1]**
	(*n* = 313)	(*n* = 171)	(*n* = 34)	(*n* = 94)	(*n* = 43)
Genotype					
GG (%)	205(65%)	121(71%)	19(56%)	67(71%)	35(81%)
GC (%)	91(29%)	44(26%)	12(35%)	25(27%)	7(16%)
CC (%)	17(5%)	6(3%)	3(9%)	2(2%)	1(2%)

[1]Distribution of GC + CC genotypes versus GG genotype: in patients with only EN compared to patients with only arthritis: p = 0.015; OR3.45, CI 1.24-9.61

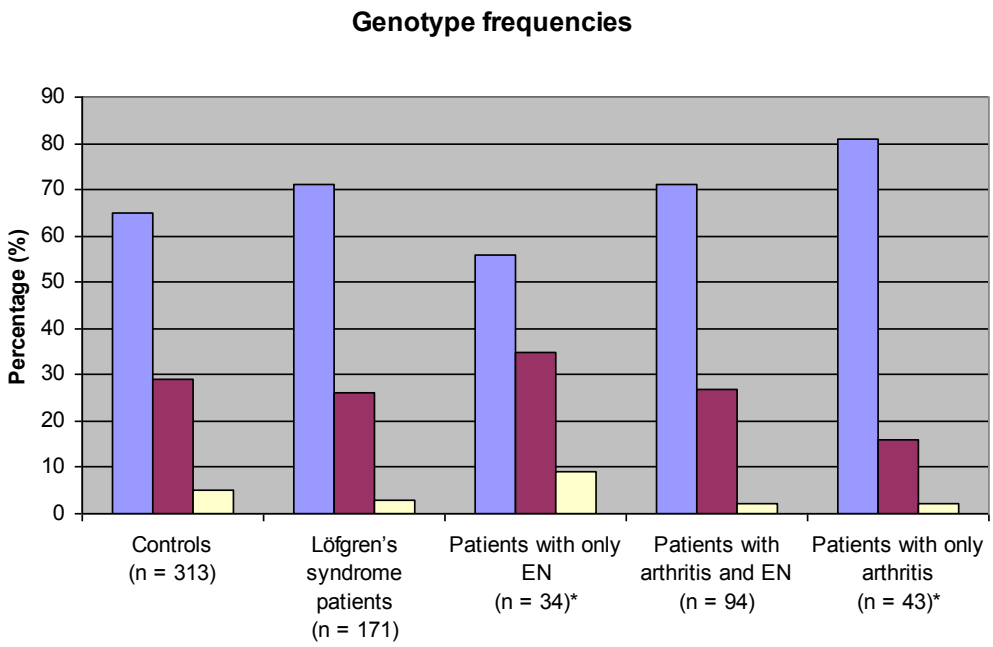

Figure 1. Genotype distribution in controls, Löfgren's syndrome patients and the clinical subgroups of Löfgren's syndrome patients, i.e. patients with only erythema nodosum, patients with arthritis and erythema nodosum and patients with only arthritis. Genotype GG: blue bar, GC: red bar, CC: yellow bar.
*Distribution of GC + CC genotypes versus GG genotype: in patients with only EN compared to patients with only artritis: p = 0.015; OR3.45, CI 1.24-9.61.

Erythema nodosum versus arthritis and erythema nodosum

We also examined patients who had both EN and arthritis, and patients who had only one of the two characteristics. Patients with only EN compared to patients with EN and arthritis showed a significantly increased frequency of the minor C allele (p = 0.044; OR 1.97, CI: 1.01-3.85) (Table 2).

When we examined the frequency of the genotypes we found no significant difference between patients with only EN compared to patients with EN and arthritis (p = 0.111) The genotype distribution of GC + CC genotypes versus CC genotype was not significantly different in patients with EN compared to patients with EN and arthritis (p = 0.101; OR 1.96, CI 0.87-4.41) (Table 3 and Figure 1).

Patients with only arthritis compared to patients with EN and arthritis did not show a significant difference in C allele frequency (p = 0.270; OR 0.64, CI: 0.29-1.42) (Table 2 and Figure 1). The genotype distribution of GC + CC genotypes versus CC genotype was not significantly different in patients with only arthritis compared to patients with EN and arthritis (p = 0.208; OR 0.57, CI 0.23-1.38) (Table 3 and Figure 1).

Discussion

In Löfgren's syndrome patients we found that *MIF* -173C allele is associated with the clinical manifestation of the syndrome and not with susceptibility to Löfgren's syndrome. The *MIF* -173C allele is known to cause higher expression of MIF.[11,27] Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine which plays a role in T cell and macrophage activation and migration.[28,29] MIF is involved in antigen-specific immune responses; neutralizing anti-MIF antibodies inhibited T cell proliferation and interleukin-2 production in vitro, suppressing antigen-driven T cell activation and antibody production in vivo.[30] It is well described that interaction between antigen-presenting cells and T cells, resulting in activation of these T cells and subsequently production of cytokines, like IL-2, TNF-α and IFNγ, play a cardinal role in the immunopathogenesis of sarcoidosis by initiating the formation and maintenance of granulomas. In sarcoidosis patients MIF was found in culture supernatants of cutaneous granulomas[31] and elevated levels of MIF were found in serum and BALF,[12,32] indicating a role for MIF in the immunologic process of sarcoidosis.

In the study of Amoli et al.[19] the *MIF* -173C polymorphism was associated with sarcoidosis when patients had EN, however it was not mentioned how many patients had Löfgren's syndrome and if the patients had arthritis or not.
In our study no association was seen by comparing controls to all patients with EN, irrespective of the presence of arthritis. However, comparing controls to Löfgren's syndrome patients who had only EN, so without arthritis, an increased C allele frequency was found, although this was not significant (results not shown). But, analyses between the different subphenotypes of Löfgren's syndrome, showed that the patients with only EN had a significantly increased frequency of the *MIF* -173C allele compared to the patients with only arthritis. These results are an addition to the results published by Amoli et al.[19] and could indicate that MIF has a role in a specific group of sarcoidosis patients with EN.

EN is a nonspecific cutaneous reaction and presumed to represent a delayed type hypersensitivity reaction to numerous antigens.[33] MIF was first discovered as a cytokine having a role in the delayed type hypersensitivity reaction, assuming a role for MIF in the pathogenesis of EN.

While other studies show that the *MIF* -173C allele is increased with different forms of arthritis, like rheumatoid arthritis and juvenile idiopathic arthritis,[34,35] in the present study Löfgren's syndrome patients with arthritis show a decreased *MIF* -173C allele. A possible explanation could be that sarcoidosis is a very complex disease with a variety of cytokines and chemokines, produced after the initiation of the disease,

which contributes to granuloma formation.[36] Associations between sarcoidosis and Human leukocyte antigen (HLA) are well described,[37-39] as well as for other auto-immune diseases, like rheumatoid arthritis and juvenile idiopathic arthritis.[40-42] Earlier studies show even different HLA patterns in different phenotypes of sarcoidosis, like between Löfgren's syndrome patients and non-Löfgren's syndrome patients.[43] For an inflammatory process, the type of antigen and antigen-presenting cell decides, through cytokines, which direction the response will go and what the effect of a cytokine will be,[44-46] which can result in different roles for the same cytokine. This might be the case for the effect of MIF in different forms of arthritis and diseases with arthritis, like rheumatoid arthritis and sarcoidosis.

It is also interesting to note that in other studies there was no association found between *MIF* -173C and auto-immune diseases, like cutaneous vasculitis,[47] characterized by tissue infiltration with macrophages, and giant cell arteritis,[48] characterized by granuloma forming, both characteristics also found in sarcoidosis. Suggesting that different pathogenic mechanisms may be implicated in the development of the inflammatory disease in these diseases.
In Löfgren's syndrome we see that patients with EN have an increased *MIF* -173C allele and patients with arthritis have a decreased *MIF* -173C allele, indicating a role for MIF after antigen presenting to the T cell has taken place and the sarcoid inflammatory response has begun.

We can conclude that a variant of *MIF* that encodes for a higher protein expression level might have an effect on the subphenotypes of Löfgren's syndrome, i.e. associates with the cutaneous manifestation of EN.

These results can contribute in analyzing phenotypes of sarcoidosis. Further research, ie analysing MIF levels in serum and bronchoalveolar lavage fluid, has to be done to elucidate the role of MIF in the inflammatory process of Sarcoidosis.

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The Cooling

The Retreat: The Red begins to fade, marking the withdrawal of the Zahir (Apparent) symptoms.

The resolution phase is characterized by the subsiding of fever and the gradual fading of the bruised, inflamed lesions of Erythema Nodosum from the skin. The previously constrictive sensation around the ankles diminishes, indicating the restoration of mobility and the reduction of the body's inflammatory response.

Chapter 7

A polymorphism in C-C Chemokine Receptor 5 (CCR5) associates with Löfgren's syndrome and alters receptor expression as well as functional response

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Abstract

Objectives

CC-chemokine receptor 5 (CCR5) and polymorphisms in *CCR5* gene are associated with sarcoidosis and Löfgren's syndrome. Löfgren's syndrome is an acute and usually self-remitting phenotype of sarcoidosis. We investigated whether the single nucleotide polymorphim (SNP) rs1799987 is associated with susceptibility for Löfgren's syndrome and has an effect on CCR5 expression on monocytes and function of CCR5.

Methods

A total of 106 patients with Löfgren's syndrome and 257 controls were genotyped for rs1799987. Expression of CCR5 on monocytes was measured by flowcytometry. We evaluated Calcium influx kinetics following stimulation upon N-formylmethionylleucyl-phenylalanine (fMLP) and macrophage inflammatory protein-1α (MIP-1α) on monocytes by measuring the median fluorescence intensity (MFI).

Results

The frequency of the G allele of rs1799987 was significantly higher in Löfgren's syndrome than in healthy controls (p = 0.0015, confidence interval (CI) 1.22-2.32, odds ratio (OR) 1.680). Patients with a GG genotype showed higher CCR5 expression on monocytes, than patients with the AA genotype (p = 0.026). A significantly (p = 0.027) lower count of patients with the GG genotype showed a Calcium influx reaction to simulation upon MIP-1α, compared with patients with the AA genotype.

Conclusion

The rs1799987 G allele in *CCR5* gene is associated with susceptibility to Löfgren's syndrome and with quantitative and qualitative changes in CCR5, potentially effecting the inflammatory response.

Keywords: sarcoidosis; Löfgren's Syndrome; C-C Chemokine Receptor 5 (CCR5); calcium influx; calcium kinetics; single nucleotide polymorphism (SNP)

Introduction

Sarcoidosis is a systemic inflammatory disorder of unknown cause with a wide clinical spectrum.[1] It commonly affects the lungs and intrathoracic lymph nodes, and is characterized by the formation of non-caseating epithelioid cell granulomas. During granuloma formation, there is a tight collaboration between macrophages, dendritic cells, and lymphocytes, orchestrated by cytokines and chemokines, which are potent chemoattractants for these cell types to sites of inflammation.[2,3]
Chemokine receptors belong to the G-protein-coupled receptors (GPCR) superfamily and are divided into four classes, named by the type of chemokine (CC, CXC, CX3C, or C) with which they interact.[4]

Signaling via G-protein-coupled receptors (GPCRs) is frequently linked to ion channels, which may induce temporary changes in cytoplasmic ion concentrations important in regulation of many functions of, for example, macrophages, such as innate host defense and secretory responses, including cytokine production. An important and frequently studied GPCR is C-C chemokine receptor 5 (CCR5), for which a role has been suggested in many different diseases like MS, HIV, and cancer, as well as autoimmune diseases like IBD, rheumatoid arthritis, and sarcoidosis.[5-10]

Several polymorphisms have been described in the *CCR5* gene, of which rs1799987 at position -2459 (A > G) (also known as 303 A > G, 59029 A > G) promoter polymorphisms is of particular relevance. In human immunodeficiency virus (HIV)-infected patients, rs1799987 minor G allele is associated with slower progression of the disease.[9]

In sarcoidosis, a haplotype (human haplotype C (HHC) ACT<u>G</u>TGC) of *CCR5* polymorphisms, including rs1799987 A > G (the underlined G in the Haplotype), was found to be associated with persistent lung involvement in both Dutch and British patients.[11] In a German study, two variants in the *CCR5* gene, other than rs1799987, which were also part of this HHC haplotype, were shown to be associated with Löfgren's syndrome.[12] Löfgren's syndrome is a self-limiting benign form of sarcoidosis, which presents with bilateral hilar lymphadenopathy, erythema nodosum (EN), and/or articular inflammation or arthritis.[13] However, in contrast with sarcoidosis, the disease is characterized by an acute onset, which can be invalidating for a limited period of time.

Apart from being a chemokine receptor, CCR5 also is a co-receptor, next to CD4, for HIV to enter the target cell.[14] Binding of HIV to $CD4^+$ T cells promotes a chronic immune activation, which in turn upregulates CCR5 expression, creating a vicious circle driving HIV replication and progression of HIV infection. The rs1799987 G allele

results in reduced expression of CCR5, thereby slowing the disease progression in HIV-infected persons.[15]

We hypothesize that a decreased CCR5 expression contributes to a less intense inflammatory response and, therefore, a more benign course of disease as is present in patients with Löfgren's syndrome. To better understand the role of rs1799987 in patients with Löfgren's syndrome, we genotyped rs1799987 and measured the CCR5 expression on monocytes. Further, to explore if this polymorphism has functional consequences, we studied the kinetics and magnitude of changes in intracellular calcium of in vitro activated monocytes.

Materials and Methods

Subjects

A total of 106 unrelated Caucasian sarcoidosis patients, from two hospitals in the Netherlands (St. Antonius Hospital, Nieuwegein and Leiden University Medical Center, Leiden), were included in the study. All patients were diagnosed in accordance with the consensus of the ATS/ERS/ WASOG Statement on sarcoidosis.[16] All patients presented with the classic symptoms of Löfgren's syndrome: acute onset with bilateral hilar lymphadenopathy, fever, erythema nodosum, and/or bilateral ankle arthritis.

Two hundred and fifty-seven healthy Caucasian subjects were included as controls in this study, matched by sex and ethnicity with the Löfgren's syndrome patients. Written informed consent was obtained from all subjects, and authorization was given by the Ethics Committees of the St. Antonius Hospital, Nieuwegein and of Leiden University Medical Center. There was no significant difference in age or sex between Löfgren's syndrome patients (mean age 34.8 years, 37.7% male) and controls (mean age 36.6 years, 35.4% male).

Genotyping

DNA was extracted from whole blood samples and the SNP analysis was performed using a custom GoldenGate Genotyping Assay (Illumina Inc, San Diego, USA) performed in accordance with the manufacturer's recommendations. We genotyped the *CCR5* polymorphism at position -2459 (promoter region, SNP rs1799987) for 106 patients and 257 controls.

Flow Cytometry

CCR5 Expression on Peripheral Blood Monocytes

Cryopreserved PBMCs from 21 Löfgren's syndrome patients were thawn and resuspended in phosphate-buffered saline (PBS). The cells were stained with CCR5-PECy7 (Ebioscience, San Diego, CA, USA), CD14-PerCP (monoclonal Peridinin-Chlorophyll-Protein, PerCP-labelled antibody, Becton Dickinson, San Jose, USA), and CD16 PE (Phycoerythrin labeled antibody, Becton Dickinson). Mouse IgG1 kappa Isotype Control PE-Cy7 (Ebioscience) was used as negative control. The cells were measured on a FACS-Calibur (BD Biosciences, San Jose, CA, USA) and data analysis was performed using FlowJo software (v10.7, Ashland, OR, USA). Gating strategies for differentiation between classical, intermediate, and non-classical monocytes were performed as described before.[17] Monocytes were first gated according to their size and granularity characteristics in a FSC-SSC plot and then for CD14 expression. The percentage of CCR5 positive cells as well as the CCR5 median fluorescence intensity (MFI) expression levels were determined on $CD14^+$ monocytes.

Ca-Influx Assay

Sodium heparinized whole blood from the same 21 Löfgren's syndrome patients, mentioned in the previous paragraph, was lysed and cells were loaded with fluo4-AM (F14201, Invitrogen, Carlsbad, USA), which was dissolved in DMSO (Sigma-Aldrich Co, St Louis, USA) to a final concentration of 5 µM for 30 min. After centrifugation, the cells were stained with CD14-PerCP antibody (Becton Dickinson) and resuspended in 300 µl assay buffer composed of 1 mM CaCl2.2H20, 5 mM Glucose-Hydrate, 5 mM KCl, 1 mM Na2HPSO4.2H20, 0.5 mM MgSO4.7H20, 145 mM NaCl, and 10 mM HEPES (pH 7.4). Cells then were stimulated with 3.8 pM MIP-1α (HPC1105, R&D systems, Minneapolis, USA) or 5 nM fMLP (n-Formyl-Met-Leu-Phe F3506, Sigma-Aldrich Co, St. Louis, USA) after baseline recording for 40 s and Ca-influx in time in $CD14^+$ monocytes was recorded for a total of 200 s.

Quantification of Changes in $[Ca2^+]_i$ after Stimulation

To quantify the changes of the $[Ca^{2+}]_i$, the median fluorescence intensity (MFI) of the fluo4 signal was measured after stimulation of the monocytes with fMLP or MIP-1α (macrophage inflammatory protein-1α). The monocytes were first gated on CD14 positivity followed by FSC-SSC scatter characteristics. In addition, because the variation in response to fMLP or MIP-1α differs in especially the start of the response to the stimuli in time and the duration of the $[Ca^{2+}]_i$ (Figure 1), we also used another approach to analyze the changes in $[Ca^{2+}]_i$. We measured the area under the curve and corrected this for the time (AUC/time) of the curve for three timeframes:

Frame 1: Baseline, the $[Ca^{2+}]_i$ before stimulation with any chemoattractant (fMLP or MIP-1α). Mean time of 16.9 s (SD = 4.1 s).

Frame 2: MIP-1α, the increase in $[Ca^{2+}]_i$ upon stimulation MIP-1α. Mean time 21.0 seconds (SD = 2.8 s).

Frame 3: fMLP, the increase in $[Ca^{2+}]_i$ upon stimulation with fMLP. Mean time 49.1 seconds (SD = 10.9 s).

To see the net effect of stimulation with fMLP and MIP-1α, we subtracted the calcium influx baseline values from the values after stimulation with the ligand.

Formula 1.

A. MIP-1α (AUC/time) — Baseline MIP-1α (AUC/time)
B. fMLP (AUC/time) — Baseline fMLP (AUC/time)

We used fMLP as a positive control. The height of the Ca-influx after fMLP was different between the samples, so we also analyzed the difference in Ca-influx after MIP-1α compared with the possible maximum response after fMLP. To express the magnitude of the $[Ca^{2+}]_i$ response to MIP-1α relative to the fMLP response, and thereby to reduce inter-patient differences in the potency of the monocytes, we used the following formula:

Formula 2.

$$\frac{\text{MIP-1}\alpha\ (\text{AUC/time}) - \text{Baseline MIP-1}\alpha\ (\text{AUC/time})}{\text{fMLP(AUC/time)} - \text{Baseline fMLP(AUC/time)}}$$

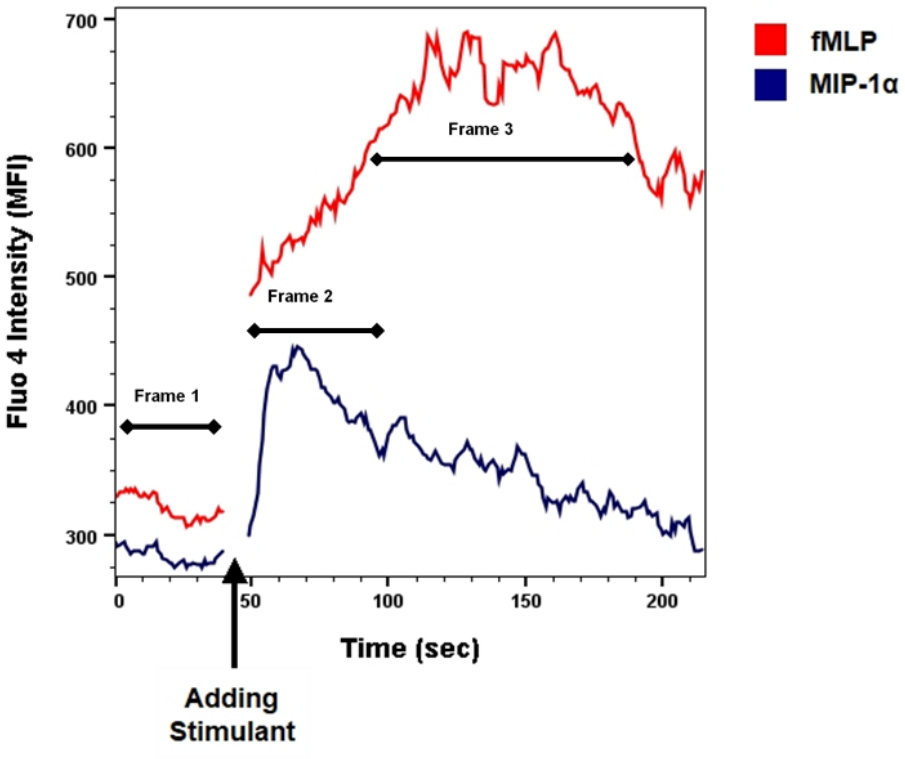

Figure 1. Flow cytometric analyses of kinetic changes in $[Ca^{2+}]_i$ in monocytes after stimulation with fMLP (5.0 nmol) or MIP-1α (3.8 pmol), in one representative patient. The arrow indicates the moment of adding the stimulant. The limiting factor in the monocyte calcium mobilization experiments was the number of cells available. For every patient (n = 21), two different stimuli were used: MIP-1α and fMLP. The number of cells available did not permit to perform replicates.

Statistical Analysis

Allele and genotype frequencies were calculated for the SNP rs1799987 A > G polymorphism and tested for Hardy–Weinberg equilibrium (HWE) in controls. Differences between cases and controls were analyzed by χ^2 test using contingency tables of genotype and allele frequencies. Hardy–Weinberg equilibrium (HWE), odds ratios, and confidence intervals (CIs) were calculated with an online tool, available at https://ihg.helmholtz-muenchen.de/ihg/snps.html. A p-value <0.05 was considered significant.

To compare the mean percentage of CCR5 expression on monocytes, we performed one-way analysis of variance (ANOVA). The comparison of expression levels of CCR5 on monocytes among and between the genotypes was tested with the

Kruskal–Wallis rank test and the Mann–Whitney-U rank test, respectively.
The occurrence of $[Ca^{2+}]_i$ in monocytes was tested with a χ^2 test using a contingency table. The comparison of MFI of $[Ca^{2+}]_i$ in monocytes among and between the genotypes was tested with the Kruskal–Wallis rank test and respectively with the Mann–Whitney-U rank test. A *p*-value < 0.05 was considered significant. Statistical analyses were performed using the Statistical Program for the Social Sciences SPSS, version 26 (SPSS, Inc., Chicago, IL, USA).

Results

Genotyping

Patient and control groups were in Hardy–Weinberg equilibrium ($p > 0.05$). The frequency of the G allele of rs1799987 was significantly higher in Löfgren's syndrome than in healthy controls ($p = 0.0015$, CI 1.22–2.32, OR 1.680) (Table 1). Furthermore, carriership of the G allele (GG + AG genotypes) was significantly increased in patients with Löfgren's syndrome with 80% of Löfgren's syndrome patients carrying the G allele versus 64% in controls ($p = 0.0028$; CI 1.31–3.88, OR 2.257).

Table 1. Allele and genotype frequencies of rs1799987 in controls and patients with Löfgren's syndrome.

***CCR5* rs1799987**	**Controls** (*n* = 257)	**Löfgren's syndrome** (*n* = 106) *
A (%)	299 (58%)	96 (45%)
G (%)	215 (42%)	116 (55%)
AA (%)	92 (36%)	21 (20%)
AG (%)	115 (45%)	54 (51%)
GG (%)	50 (19%)	31 (29%)

* Allele G was significantly increased in Löfgren's syndrome patients ($p = 0.0015$, confidence interval (CI) 1.22–2.32, odds ratio (OR) 1.680), and there was a significant increase in the number of patients with Löfgren's syndrome carrying the G allele (GG + AG genotypes; 80% in Löfgren's syndrome patients versus 64% in controls $p = 0.0028$, CI 1.31–3.88, OR 2.257).

CCR5 Expression on Peripheral Blood Monocytes

In patients with Löfgren's syndrome, overall, 30.0% ±16.6% of blood monocytes expressed CCR5. We investigated whether the percentage of monocytes expressing CCR5 was influenced by the presence of the G allele of rs1799987. There was no significant difference in the percentage of monocytes expressing CCR5 between the different genotypes ($p = 0.094$). However, a significantly higher percentage of $CCR5^+$ monocytes was seen in patients with the GG genotype 41.06 (±20.80) versus AA+AG 24.53 (±11.32) (*p*-value = 0.028, Figure 2a).

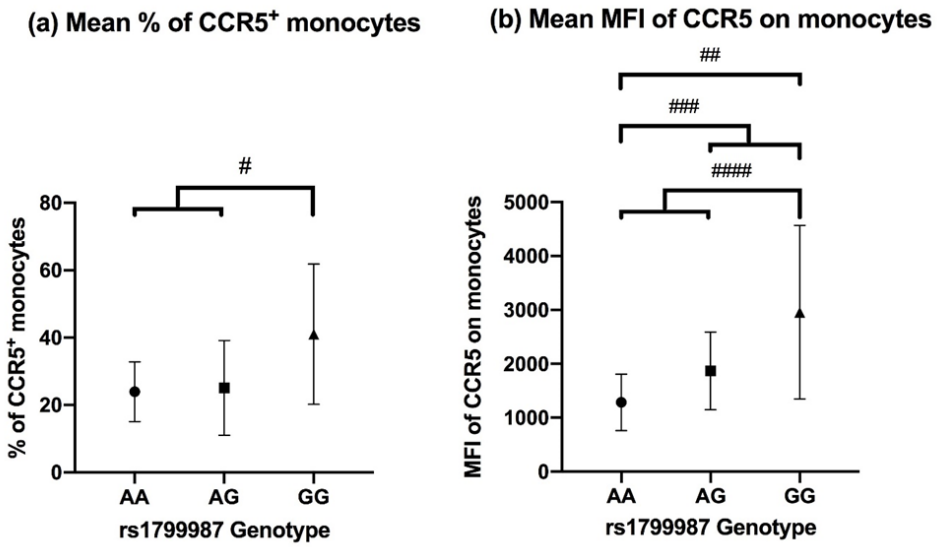

Figure 2. (**a**) Mean % of CCR5 positive (CCR5⁺) monocytes in 21 patients with the different genotypes for SNP rs1799987.
Each patient was analyzed once. Comparison of AA vs. AG vs. GG (p = 0.094) was not significant, but comparison of GG vs. AA + AG showed a significant difference (p = 0.028) [#]. (**b**) Mean MFI of CCR5 on monocytes in 21 patients with the different genotypes for rs1799987. Comparing AA vs. GG (p = 0.026) [##], AA vs. AG + GG (p = 0.038) [###], AA + AG vs. GG (p = 0.012) [####].

We also analyzed the median fluorescence intensity (MFI) of CCR5 on the monocytes. There was a significant difference (p = 0.030) in MFI between the genotypes. Post-hoc analysis with pairwise comparisons showed for patients with the GG genotype significantly higher MFI compared with patients with the AA genotype (p = 0.026, after Bonferroni correction). Investigating the G allele showed similar results with patients having a higher MFI, patients with AG + GG versus AA genotypes showed significant higher MFI 2324.97 (±1382.38) versus 1283.65 (±523.47), p = 0.038 (Figure 2b).

Further analysis of the monocyte subsets (classical, intermediate, and non-classical populations) did not show significant differences in CCR5 expression between the genotypes (data not shown).

CCR5 expression on lymphocytes was low and, in a number of cases, even undetectable (data not shown).

CCR5 Induced Calcium Mobilization Response in Monocytes

We performed the calcium mobilization assay in 21 patients. For the assay, we used the chemotactic peptide fMLP, which is known to induce changes in intracellular Ca ($[Ca^{2+}]_i$), as positive control.
Figure 1 illustrates the changes in $[Ca^{2+}]_i$ in monocytes after stimulation with fMLP and MIP-1α, a ligand for the CCR5 receptor.

In Figure 1, the first interval shows the baseline $[Ca^{2+}]_i$ level. After addition of fMLP and MIP-1α, a rise in $[Ca^{2+}]_i$ was observed in monocytes.

Kinetic Analysis of Changes in $[Ca^{2+}]_i$ after Stimulation

Stimulation of the monocytes with fMLP showed in all the 21 patients an immediate rise in $[Ca^{2+}]_i$, as illustrated by an elevated MFI, which persisted over the entire observation period of 200 s.

After stimulation of the monocytes with MIP-1α, 11 patients showed a rise in $[Ca^{2+}]_i$, similar to the figure presented earlier (Figure 1). Almost all patients who showed a rise in calcium influx upon stimulation with MIP-1α had the A allele (10 out of 11 patients) and mostly the AA genotype; this was significantly different from the patients who did not show any reaction, who mostly had the GG genotype ($\chi^2 = 7.3$, $p = 0.027$) (Table 2).

Table 2. $[Ca^{2+}]_i$ upon stimulation with MIP-1α in patients with Löfgren's syndrome according to rs1799987 genotypes.

Stimulation with MIP-1α		
Genotype	**Increase in $[Ca^{2+}]_i$**	**No increase in $[Ca^{2+}]_i$**
AA	6	1
AG	4	3
GG	1	6
Total	11	10

* Significantly more patients with the AA genotype showed a calcium influx reaction compared with patients with the GG genotype ($\chi^2 = 7.3$, $p = 0.027$).

Quantification of Changes in $[Ca2^{+}]_i$ after Stimulation

The median fluorescence intensity (MFI) of the calcium influx, measured after stimulation of the monocytes with MIP-1α, did not show any significant difference between the different genotypes for the rs1799987. Measuring the difference in calcium influx after stimulation with MIP-1α with Formula 1 showed a lower $[Ca^{2+}]_i$ in patients with the GG genotype for rs1799987, but this was not significant, $p = 0.11$ (Table 3).

Calculating the magnitude of the $[Ca^{2+}]_i$ to MIP-1α relative to the fMLP response, applying Formula 2, the Kruskal–Wallis test showed a significant difference (p = 0.035) in $[Ca^{2+}]_i$ between the different genotypes. Post-hoc analysis showed a significantly lower $[Ca^{2+}]_i$ in patients with the GG genotype for rs1799987 (p = 0.042 after Bonferroni correction). Patients with the GG genotype compared with patients with the AA + AG genotype showed a significant lower $[Ca^{2+}]_i$, p = 0.010.

Table 3. $[Ca^{2+}]_i$ measurements calculated upon stimulation with MIP-1α in patients with Löfgren's syndrome according to rs1799987 genotypes.

Genotype	MIP-1α *	MIP-1α/fMLP **
AA	23.25	0.38
AG	16.35	0.24
GG	5.35	0.07

* Stimulation with MIP-1α, as calculated with Formula 1 in Material and Methods (M and M) to calculate the net effect of the stimulant. ** Stimulation with MIP-1α relative to fMLP, as calculated with Formula 2 in M and M to reduce inter-patient differences in monocyte potency.

Discussion

In this study, we demonstrated that the G allele of SNP rs1799987 predisposes to Löfgren's syndrome, influences CCR5 expression on monocytes, and decreases the functional response of the CCR5 receptor. Our data support our hypothesis that variation in *CCR5* genetics and function contributes to a modified inflammatory response, which could explain the relatively benign course of sarcoidosis disease in patients with Löfgren's syndrome.

Associations between polymorphisms of the *CCR5* gene and sarcoidosis were described earlier. Spagnolo et al. found an association between a specific haplotype (HHC), which includes rs1799987 A > G, and parenchymal involvement in patients with sarcoidosis. They did not find an association with susceptibility for sarcoidosis;[11] however they excluded patients with Löfgren's syndrome. In a study with Löfgren's syndrome patients from Germany, two marker alleles in the *CCR5* promoter region, other than rs1799987 A > G, but part of the HHC haplotype, were associated with Löfgren's syndrome, in particular with female patients.[12] Interestingly, in patients with beryllium disease, which is a similar granulomatous disease as sarcoidosis, but with a known trigger, associations between worsening pulmonary function over time and *CCR5* gene polymorphisms were found. These gene polymorphisms were represented in the HHC haplotype.[18] Furthermore, associations between different inflammatory diseases and *CCR5*

haplotypes or gene polymorphisms represented in the known *CCR5* haplotypes are described. *CCR5* haplotypes HHE and HHG*2 are associated with susceptibility to SLE.[19] Two *CCR5* gene polymorphisms (rs1799987 and rs10577983) are associated with radiographic severity of rheumatoid arthritis.[20] For the CCR5Δ32 deletion, an association with susceptibility and disease severity was established with primary sclerosing cholangitis. No association with the CCR5Δ32 deletion was found in patients with ulcerative colitis and Crohn's disease.[21]

In the present study, we chose to analyze only rs1799987 and no other SNP's part of the *CCR5* haplotype, because the G allele of this SNP is part of haplotype HHC for which associations with sarcoidosis were found and, in HIV, the G allele was intensively analyzed, shown to slow HIV progression, independent of other polymorphisms, like the CCR5Δ32 deletion.

Löfgren's syndrome is a characteristically Western and Northern European manifestation of sarcoidosis, more commonly seen in the Netherlands and Sweden.[13] According to gnomAD, the European population has a rs1799987 G allele frequency of 0.4327, which is similar to what we found in our cohort, and our cohort completely consists of Western Europeans.

In the present study, carriers of the G allele showed a higher expression of CCR5 on monocytes, which is in contrast to earlier reports, which showed increased CCR5 expression on the cell surface when carrying the A allele of rs1799987.[9,22] The difference in cell types studied might be an explanation for the discrepancy in cell surface expression of CCR5. In the present study, CCR5 expression on monocytes was determined, while in previous reports, CCR5 expression on lymphocytes was addressed, where even T-cell subsets revealed different CCR5 expression.[22-24] It could be that this SNP does not affect the surface receptor expression on its own; for that, Shieh et al.[22] showed that there was no difference in surface expression of CCR5 on different cell types with the different genotypes of the rs1799987. However, individuals with the A allele for rs1799987 who also possessed the homozygous wild type of *pCCR5-59653C* showed a higher surface expression of CCR5 on different $CD4^+$ cells. Another study showed that individuals with the rs1799987 A > G genotype showed lower CCR5 expression on stored peripheral blood $CD4^+$ T cells and $CD14^+$ monocytes, only when they were also heterozygous for the CCR5Δ32 deletion (Δ32/wt).[25]

Furthermore, the CCR5Δ32 deletion variant (Δ32/Δ32), which is not part of the HHC haplotype, results in a truncated protein that fails to reach the cell surface. However, variations in gene expression among Δ32/wt and wt/wt subjects have been described,

suggesting other factors (e.g., other *CCR5* polymorphisms) contributing to CCR5 expression.[26] Altogether, data show that the effect of *CCR5* promoter polymorphisms on CCR5 expression may be cell type-specific and affected by other polymorphisms.

Another possible contributing aspect is the intracellular storage of CCR5 and its export to the plasma membrane after interacting with membrane associated or cytoplasmic proteins. In T-lymphocytes, the CCR5–CD4 interaction enhanced CCR5 transport to the plasma membrane.[14]

In the present study, we showed that Löfgren's syndrome patients with the G allele of the SNP rs1799987 had an impaired intracellular calcium influx, showing a dysfunctional chemokine–chemokine receptor interaction. One would not expect an effect of rs1799987 on the functionality of CCR5 given that this SNP is located in the promoter region. It could be that there are additional polymorphisms in the *CCR5* gene with strong linkage with rs1799987, which may have an impact on functionality, either in ligand binding and/or signaling function.
The expression and function of CCR5 is associated with differentiation of monocytes into macrophages as well as with phagocytosis and chemotaxis.[27] All these functions are crucial for regulation of inflammation and the formation and/or persistence of granuloma. We have shown previously that CCR5 is expressed at high levels on intermediate monocytes,[17] suggestive for a role of this chemokine receptor in monocyte differentiation. We assessed the impact of rs1799987, but, maybe because of the low number of evaluable patients per group, did not find significant differences in the intermediate monocytes.

Several studies[28-30] have shown a relation between chemokine signature and sarcoidosis. Higher CCR5 expression in BAL fluid (BALF) in sarcoidosis patients, regardless of sarcoidosis stage, and in Löfgren's syndrome patients have been shown.[31] Significantly higher protein levels or mRNA expression of chemokine C-C motif chemokine ligand 5 (CCL5) were found in the BALF of sarcoidosis patients compared with controls.[3,32] Palchevsky et al.[29] showed in lung biopsies from sarcoidosis patients that different chemokines (CCL2, CCL5) and chemokine receptors (CCR2, CCR5) were found in different cell types creating the sarcoid lung granulomas, regardless of the radiologic stage of the sarcoidosis and whether or not alveolitis was present. Chemokines and chemokine receptors were reported to play a role in recruiting mononuclear cells that form and expand the granulomas during the earlier phases of pulmonary sarcoidosis.[29]

After stimulation with chemoreceptors produced by APC (antigen presenting cell), CCR5 is recruited to the immunological synapse where it functions as a T cell

costimulatory molecule by improving and prolonging the T cell–APC interaction.[33] A dysfunctional CCR5 could lead to a less stable T cell–APC interaction and, thereby, a shorter duration of the T cell–APC interaction, which in turn could lead to a less prolonged inflammatory state and an unstable granuloma formation in sarcoidosis. The associations found between polymorphisms in *CCR5*, *CCR2*,[34] and sarcoidosis, including the present study, and the expression of the chemokines in the sarcoidosis granuloma suggest that genetic variants that cause decreased or dysfunctional chemokine receptors could lead to the formation of less stable granuloma, which in turn could lead to less prolonged disease, such as the Löfgren's syndrome phenotype of sarcoidosis. It would be very interesting and important to replicate these findings in cells derived from patients with chronic sarcoidosis. Translating these findings to the clinical practice with potential treatment options should be a further interest of future studies. A case report about Maraviroc, which is a CCR5 inhibitor and used in HIV treatment,[6] was recently published showing a resolution of sarcoidosis symptoms in an HIV-infected patient.[35]

It is clear that CCR5 plays an important role in T cell function and that this depends on chemokines and cytokines in the environment at sites of infection and inflammation.[36] Chemokine receptors, like other GPCRs, function through calcium channels, which is of importance for the further functioning of the cell in the process.[37]

For the calcium mobilization analyses, we used MIP-1α, a CCR5 ligand. The chemokine MIP-1α is a chemoattractant for $CD8^+$ T cells, which also is shown to be produced by $CD8^+$ T cell lymphocytes, and hence associated with a Th2 immune response.[28,29]

Sarcoidosis patients with advanced stages (stage II and III) have a higher concentration of MIP-1α in BALF compared with controls.[40] Furthermore, a significant correlation between higher MIP-1α concentration and $CD8^+$ T cell lymphocytes was observed in sarcoidosis patients with advanced stages of disease.[40] There seems to be a correlation between higher local MIP-1α concentrations and fibrotic lung changes, given the higher MIP-1α levels in progressive sarcoidosis and pulmonary fibrosis.[41] The expression of MIP-1α in interstitial fibroblasts found in biopsies obtained from patients with sarcoidosis and IPF emphasizes this.[42] In Japanese sarcoidosis patients, the plasma MIP-1α concentrations showed correlation with the course of disease, showing a decline in MIP-1α concentration in patients with spontaneous recovery.[43] In our group of patients with the G allele for rs1799987, the calcium mobilization response in monocytes following ligation of CCR5 with MIP-1α was impaired. This could be interpreted as dampening of otherwise inflammatory signaling towards Th2 dominated inflammation, or even directing the inflammation

more towards a Th1-type inflammation and inducing a more adequate Th1 response, resulting in a benign course. This allele and, thereby, the dysfunctional CCR5 could be important in conducting the T cell inflammation.

The observations made in the present study may fit into a more complex mechanism contributing to the favorable prognosis of Löfgren's syndrome. Löfgren's syndrome is known for its good prognosis in about 90% of the patients, which is characterized by remission of the inflammation within 2 years, even without treatment with immunosuppressive drugs.

Our data show that the G allele of SNP rs1799987 is overrepresented in patients with Löfgren's syndrome, and that this allele associates with quantitative and qualitative changes in CCR5, potentially dampening the inflammatory response. Additional research is needed to further decipher the role of CCR5 expression and function in sarcoidosis before targeted treatment approaches may be considered.

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The Exhale

The Clearing: The negative space of the lungs—the Hu loop—opens up and softens.

The Batin (Hidden) swelling of the hilar lymph nodes slowly subsides. The silent, invisible pressure deep within the chest lifts. If the onset was a restricted breath holding the body's struggle, this phase is the long, quiet exhale—releasing the trapped tension and restoring a natural, unobstructed rhythm to the lungs.

Chapter 8

Summary and general discussion

In the present thesis we aimed to contribute to the knowledge regarding genetic factors predisposing to Löfgren's syndrome, and to discover associations with clinical phenotypes and protein functionality. Furthermore, comparisons were made between patients with Löfgren's syndrome and patients without Löfgren's syndrome for a deeper understanding of differences between these two types of sarcoidosis. Besides assessment of genetic predisposition for Löfgren's syndrome, an important focus is the relationship between genetic background and manifestations of symptoms and prognosis by a long-term follow-up in a large Dutch cohort of patients with Löfgren's syndrome.

In this chapter, results of the genetic associations with Löfgren's syndrome are summarized and discussed, where HLA (*HLA-DRB1*03* and *HLA-DRB1*15*) and polymorphisms in *ANXA11*, *MIF* and *CCR5* are addressed respectively. For several genetic variants the functional consequences at the cellular level or the putative effect on clinical manifestations and disease prognosis will be discussed. Finally, the discussion will elaborate on practical implications of the findings and provide suggestions for future research.

Summary of main findings

In **Chapter 2**, we provided a comprehensive overview of disease pathogenesis, genetic background, clinical aspects, diagnosis, as well as patient management.

In **Chapter 3**, we validated the association of tag-SNPs for *HLA-DRB1*03* and *HLA-DRB1*15*. Furthermore, we showed that *HLA-DRB1*03* positive patients had a relatively lower lymphocyte percentage, a higher $CD4^+/CD8^+$ ratio and lower $CD103^+CD4^+/CD4^+$ ratio.

In **Chapter 4**, we showed that 94% of all patients with Löfgren's syndrome had resolving disease after a median follow-up time of 10.8 years. *HLA-DRB1*03* was associated with resolving disease (100%) with only 9% disease relapse. On the contrary, resolving disease was only seen in 74% with 16% disease relapse in *HLA-DRB1*03* negative patients. Supplemental data in this chapter show the evolution of treatment regimens over the years and that patients with Löfgren's syndrome can have fatigue years after diagnosis.

In **Chapter 5**, we performed a case-control study, and showed a decreased T allele of the *ANXA11* SNP rs1094550 in patients with sarcoidosis compared to controls. We showed that this protective effect was independent of sarcoidosis phenotype, i.e. similar associations were present in Löfgren's syndrome and chronic (non-Löfgren's

syndrome) sarcoidosis patients. Secondly, we performed a meta-analysis of 8 studies (including our case-control study), which confirmed the association of the T allele of rs1049550 with a reduced risk of developing sarcoidosis, regardless of phenotype.

In **Chapter 6**, we investigated the association of the *MIF* -173C allele with Löfgren's syndrome and found that the *MIF* -173C allele was not associated with overall susceptibility to Löfgren's syndrome, but was associated with the presence of erythema nodosum (EN) in patients with Löfgren's syndrome. Specifically, patients with Löfgren's syndrome with only EN showed a significantly higher frequency of this C allele compared to patients with only arthritis. This indicates that the C variant may contribute to, or be linked with the development of EN.

In **Chapter 7**, first we demonstrated that the rs1799987 G allele in *CCR5* gene was significantly higher in patients with Löfgren's syndrome compared to controls. Second, patients with the GG genotype showed a significantly higher percentage of CCR5+ monocytes in peripheral blood and a higher expression of CCR5 on monocytes, measured by median fluorescence intensity (MFI), compared to patients with the AA genotype. And third, following stimulation with MIP-1α, a significantly lower number of patients with the GG genotype showed a calcium influx reaction compared to patients with the AA genotype.

GENERAL DISCUSSION

Sarcoidosis is a systemic inflammatory disorder of unknown cause, characterized by the formation of noncaseating epithelioid cell granulomas and a heterogeneous clinical course.[1] A well-defined phenotype of sarcoidosis is Löfgren's syndrome, which manifests as acute onset, bilateral hilar lymphadenopathy, erythema nodosum, and/or bilateral ankle arthritis or periarticular inflammation.[2] Löfgren's syndrome is most commonly seen in Northern and Western Europe and is strongly associated with the presence of *HLA-DRB1*03*.[3] Patients with Löfgren's syndrome usually have a very good prognosis; up to 95% of patients who are *HLA-DRB1*03* positive experience resolving disease within 2 years.[4]

HLA

The first genetic findings discussed are those related to the *HLA-DRB1* gene. In accordance to previous literature it is clear that *HLA-DRB1*03* is not only strongly associated with development of Löfgren's syndrome but also with a good prognosis.[4] Furthermore, in Löfgren's syndrome *HLA-DRB1*15* has been associated with chronic/non-resolving disease.[4,5] Hence, typing of both DRB1-variants can be useful in risk stratification of patients with Löfgren's syndrome. Until now, HLA-DR typing is a time-consuming and expensive process. As we were only interested in *DRB1*03* and **15* typing, we developed a simple tagging assay (**Chapter 3**).
We first validated the assay and the association of tag-SNPs for *HLA-DRB1*03* and *HLA-DRB1*15* with Löfgren's syndrome. Next, associations between these SNPs and the degree of lymphocytosis and cellular phenotypes in bronchoalveolar lavage fluid (BALF) were determined. Finally, the long-term follow-up data of patients with Löfgren's syndrome were analyzed.

*Association of tag-SNPs for HLA-DRB1*03 and HLA-DRB1*15*

The tag-SNPs, rs2040410 and rs313538 for *HLA-DRB1*03* and *HLA-DRB1*15*, respectively, have each been used before independently in other diseases, such as diabetes mellitus and multiple sclerosis.[6,7] Our study was the first to validate a genetic tag for HLA typing in patients with sarcoidosis. We confirmed the representation of the A allele of rs2040410 with *HLA-DRB1*03* and the A allele of rs3135388 with *HLA-DRB1*15*.[8]

Lymphocytosis and cell phenotypes in bronchoalveolar lavage fluid

Second, we aimed to understand if the HLA-DRB1 genotype correlated with cellular variation in the bronchoalveolar lavage fluid (BALF). BAL in sarcoidosis is usually performed at diagnosis and when disease is active, and cell content is known to vary widely between patients. However, a lymphocytosis with an

increased ratio of $CD4^+/CD8^+$ and a decreased ratio of $CD103^+CD4^+/CD4^+$ is usually found in sarcoidosis and Löfgren's syndrome.[9] Our study showed that in all patients no difference in lymphocyte percentage was seen between *HLA-DRB1*03* positive and negative patients. However, *HLA-DRB1*03* positive patients showed a higher $CD4^+/8^+$ ratio and a lower $CD103^+/CD4^+/CD4^+$ ratio compared to *HLA-DRB1*03* negative patients. Previous studies show mixed results, with two studies[10,11] showing no difference in lymphocyte percentage between *HLA-DRB1*03* positive and negative patients, while other studies[12-14] show reduced lymphocyte percentages for *HLA-DRB1*03* positive patients compared to *HLA-DRB1*03* negative patients. All previous studies, except one,[10] showed a higher $CD4^+/8^+$ ratio in *HLA-DRB1*03* positive patients compared to *HLA-DRB1*03* negative patients. All the previous studies also included sarcoidosis patients with Löfgren's syndrome but were done with small patient cohorts. None of the previous studies made a distinction between patients with or without Löfgren's syndrome. In our cohort, patients with Löfgren's syndrome who were *HLA-DRB1*03* positive showed lower lymphocyte percentage compared to *HLA-DRB1*03* negative patients. Furthermore, *HLA-DRB1*15* was not investigated in the previous studies, which is why the effect of carriage of *HLA-DRB1*15* in *HLA-DRB1*03* patients had not been fully clarified. Remarkably, our data showed that *HLA-DRB1*03*$^+$*/15*$^+$ patients have significantly lower lymphocytes compared to *HLA-DRB1*03*$^-$*/15*$^+$ patients, which might suggest that the influence of *HLA-DRB1*03* is dominant over the influence of *HLA-DRB1*15*. This dominance of *HLA-DRB1*03* was previously proposed in studies of Grunewald[5] and Planck.[15] Furthermore, a recent study showed multiple SNPs in the extended HLA region, a region with a complex linkage disequilibrium (LD) pattern, to be associated with Löfgren's syndrome.[16] It is known that *HLA-DRB1*03* is part of the so-called "8.1 ancestral haplotype". Taken together, these data suggest that other HLA alleles could also play an important role in Löfgren's syndrome next to *HLA-DRB1*03*.

Another interesting finding in this study was that patients with *HLA-DRB1*03*$^+$*/15*$^-$, known to have a good prognosis, demonstrated a decreased $CD103^+CD4^+/CD4^+$ ratio, compared to patients with *HLA-DRB1*03*$^-$*/15*$^+$ and **03*$^-$*/15*$^-$. CD103 is especially known for its role in homing and retention of lymphocytes in peripheral tissues.[17] While sarcoidosis is characterized by a decreased ratio, these results show that patients with the lowest ratio are more likely to have Löfgren's syndrome which is associated with a good prognosis. Our results support earlier studies[9,18] showing that sarcoidosis patients with radiological Scadding stage I (including some Löfgren's syndrome patients) had lower $CD103^+CD4^+$ cells compared to sarcoidosis patients with more advanced radiological Scadding stage III/IV. Which suggests a relation between $CD103^+CD4^+$ cells and the extent of tissue involvement in the

lung parenchyma. Additionally, the lymphocyte subsets seem critical in determining whether inflammation can be resolved or progresses to chronic disease. As in our cohort, but also demonstrated in other studies, a higher $CD4^+/8^+$ ratio is seen in BALF of *HLA-DRB1*03* positive patients. Furthermore, distinct cytokine production patterns are observed in BALF from *HLA-DRB1*03* positive and *HLA-DRB1*03* negative individuals,[10] with the latter exhibiting a predominantly Th1-mediated immune response. A recent study showed higher Th17.1 cells in BALF of patients with Löfgren's syndrome, with higher levels of Th17 cytokines, such as IL-17A and IL-10 and lower levels of IFN-γ compared to patients with non-Löfgren's syndrome sarcoidosis.[19]

In this light it is interesting to mention the work of Grunewald et al.[13] They showed that in *HLA-DRB1*03* positive patients with pulmonary sarcoidosis, a higher proportion of $V\alpha2.3^+V\beta22^+CD4^+$ T cells is detected in BALF compared to *HLA-DRB1*03* negative patients. These T cells preferentially accumulated in the BALF while remaining at baseline levels in regional lymph nodes and peripheral blood. Moreover, several studies showed that a greater abundance of $V\alpha2.3^+$ T cells in the BALF of patients with pulmonary sarcoidosis correlated with a more favorable prognosis.[20,21] Previous studies established that *HLA-DRB1*03* positive sarcoidosis patients generally have a good prognosis.[4,22] This raises the question whether the favorable outcome is directly related to the HLA molecule itself, the binding of a specific antigen to it, its interaction or link with other genetic variants, or its association with $V\alpha2.3^+V\beta22^+CD4^+$ T cells that may facilitate more effective antigen clearance.[23]
To answer this question, several studies have been conducted searching for the antigen in Löfgren's syndrome. One of the potential antigens studied was the cytoskeletal protein vimentin, an autoantigen. Vimentin was found earlier to accumulate in BALF and to stimulate peripheral blood cells of *HLA-DRB1*03* positive sarcoidosis patients with Löfgren's syndrome and non-Löfgren's syndrome.[24,25] A subsequent study of the same group showed accumulation of $V\alpha2.3^+V\beta22^+CD4^+$ T cells in the lungs of *HLA-DRB1*03* positive sarcoidosis patients. Further molecular modeling pointed towards specific features of the *HLA-DRB1*03* molecule and its interaction with the $V\alpha2.3^+V\beta22^+CD4^+$ T cells, providing insights into the properties of this complex for accommodation of a potential antigenic peptide derived from the protein vimentin.[13]

In contrast, Greaves et al.[26] identified a different potential antigen, a peptide derived from Aspergillus nidulans. The peptide was recognized by specific T cell receptors (cells derived from patients with Löfgren's syndrome, and mainly expressing Vα2.3 and Vβ22), and stimulated these $CD4^+$ T cells, by inducing a higher IFN-γ and IL-2 secretion from BAL cells in *HLA-DRB1*03* positive Löfgren's syndrome patients compared to *HLA-DRB1*03* negative Löfgren's syndrome patients. In addition,

an increased serum IgG antibody response for Aspergillus nidulans was seen in blood of *HLA-DRB1*03* positive Löfgren's syndrome patients. Furthermore, other correlations were found between sarcoidosis and microbes, such as *Mycobacterium* and *Cutibacterium* species, of which genomic material was detected at higher frequencies in samples from patients with sarcoidosis.[27,28] In a Dutch cohort, presence of P. acnes in granulomas was especially seen in patients with chronic disease requiring treatment.[29]

These findings lead to the discussion whether sarcoidosis might be an autoimmune disease. A recent study showed that in patients with sarcoidosis several inflammatory proteins were elevated in plasma many years before sarcoidosis onset, similar to other immune-related diseases, indicating a predisposition to develop sarcoidosis and/or susceptibility for a pathogenic response to triggers of disease.[30] Subgroup analysis between 115 patients with non-Löfgren's syndrome and 37 patients with Löfgren's syndrome, showed no elevation of inflammatory proteins before onset in the Löfgren's syndrome group.[30] These results suggests that Löfgren's syndrome develops differently compared to non-Löfgren's syndrome. In addition, these findings suggest that Löfgren's syndrome is a different disease entity. The absence of elevated inflammatory proteins in a pre-clinical phase in combination with the acute presentation, could indicate that Löfgren's syndrome is an acute illness which only and immediately develops after encountering the antigen(s) without a preceding phase. This could mean that the antigen most likely originates from outside the body, making an autoimmune disease less likely. Although the strong association of Löfgren's syndrome with specific HLA types and the immune response against the autoantigen vimentin support an autoimmune mechanism, the acute and seasonal pattern of disease onset, along with the typically self-limiting course of Löfgren's syndrome, argues against a classical autoimmune etiology. In addition, examples of transient autoimmunity following infections[31,32] align with previous observations that strong anti-vimentin T-cell responses were absent in patients after recovery.[25]

Taken together, and also considering the different cytokine profiles in Löfgren's syndrome compared to non-Löfgren's syndrome,[33] this raises the following question: is Löfgren's syndrome a separate granulomatous disease entity, with or without an autoimmune origin? Further research is needed to elucidate the pathogenesis of Löfgren's syndrome. Prediagnostic differences, the acute and increased inflammatory response, and the effects of putative antigens, for example autoantigens, as vimentin, need to be studied in both Löfgren's syndrome and non-Löfgren's syndrome patients with sarcoidosis.

Longterm follow-up and prognosis

In our cohort (**chapter 4**) we evaluated long-term outcome of patients with Löfgren's syndrome in relation to *HLA-DRB1*03* and *HLA-DRB1*15* via tag SNP capturing. This was a unique part of the research, not only gathering data of patients who are still in follow-up clinically, but also requesting former patients with Löfgren's syndrome to return to the clinic for a one day visit and completing questionnaires. First, we confirmed the good prognosis of Löfgren's syndrome patients in general, by showing that 94% of all the Löfgren's syndrome patients had resolving disease after a median follow-up time of 10.8 years. Next, we analyzed our data dividing the patients in two groups: *HLA-DRB1*03* positive and *HLA-DRB1*03* negative patients.

HLA-DRB1*03 correlates with a good prognosis

In our cohort, all *HLA-DRB1*03* positive patients showed resolving disease after initial diagnosis. However, after a median period of 7.0 years, disease relapse was seen in 9% of the *HLA-DRB1*03* positive patients. This is higher than the relapse rate of 4%, detected by an earlier study that followed patients with Löfgren's syndrome for at least 2 years, but authors did not describe the maximum follow-up period or time to relapse.[4] In the same study with 205 *HLA-DRB1*03* positive patients with Löfgren's syndrome they detected 1% of patients with non-resolving disease.[4]

HLA DRB1*03 negative patients are at increased risk for chronic and relapsing disease

In the *HLA-DRB1*03* negative patients we showed that both non-resolving disease and relapse rate were more frequent during long-term follow-up then in *HLA-DRB1*03* positive patients. In comparison with one mid-term report[4] we found an increased relapse rate (16% vs 0%) and a decreased rate of non-resolving disease (26% vs 49%).

In the *HLA-DRB1*03* negative patients, 50% of the patients with non-resolving disease and 67% of the patients with relapsing disease were carrier of the *HLA-DRB1*15* allele.

From these studies we learn that patients with Löfgren's syndrome without *HLA-DRB1*03* are at an increased risk for chronic and relapsing disease, particularly those carrying the *HLA-DRB1*15* allele. The differences in reported rates between the earlier report by Grunewald[4] and our cohort may be largely due to the relatively small group of *HLA-DRB1*03* negative patients analyzed in both studies as well as possible differences in follow-up time. As discussed above *HLA-DRB1*03* appears to be the dominant allele, therefore the effect of *HLA-DRB1*15* is predominantly observed in *HLA-DRB1*03* negative patients. Other explanations may involve differences in methodology, including follow-up time and inclusion bias.

When combining reports in literature and our long-term follow-up, we can now conclude that 99-100% of carriers of *HLA-DRB1*03* will develop resolving disease. A meaningful observation that can be used for a personalized medicine approach for future patients with Löfgren's syndrome.

Comprehensive HLA-typing involves multi-steps which make the approach complex, time-intensive and costly. However, determining these tag SNPs is much cheaper and can be performed in more centers than HLA typing. We validated the representation of tag-SNPs for *HLA-DRB1*03* and *HLA-DRB1*15* in our Dutch cohort. For clinical implementation, the next step should involve a prospective study of tag-based *HLA-DRB1*03* and **15* typing in new patients diagnosed with Löfgren's syndrome and randomize patients into having follow-up or no follow-up visits at the outpatient clinics. This could ultimately lead to fewer outpatient clinic visits and shorter follow-up in the hospital, particularly in *HLA-DRB1*03* positive patients with Löfgren's syndrome, for whom follow-up may even be unnecessary. Future research is also needed to determine the role of *HLA-DRB1*03* in other phenotypes of sarcoidosis, especially regarding the prognosis at diagnosis of non-Löfgren's syndrome patients with sarcoidosis.
In conclusion, our data indicate that the *HLA-DRB1* genotype is a strong predictor of not only the evolution of disease but also the risk of relapse after disease resolution, and the clinical phenotype associated with relapse. Given the fact that *HLA-DRB1* tagging is straightforward and relatively inexpensive, it could be implemented in clinical practice to provide valuable information regarding both short- and long-term prognoses of patients with Löfgren's syndrome.

ANXA11 is associated with sarcoidosis, regardless of phenotype

In addition to HLA, we investigated the *ANXA11* gene, the association of which with sarcoidosis was first described in a German cohort.[34] The association of *ANXA11* with sarcoidosis is unique, because no associations have been found between *ANXA11* and other immune modulated disease, even though genetic commonalities are found between sarcoidosis and other immune modulated disease.[35]

In **Chapter 5,** we studied the association of *ANXA11* gene, particularly the SNP rs1049550, with Löfgren's syndrome. First, we performed a case-control study, which showed a decreased T allele frequency in sarcoidosis compared to controls, demonstrating a protective effect for the T allele regarding development of disease. We showed that this protective effect was independent of sarcoidosis phenotype, i.e. similar associations were present in Löfgren's syndrome and chronic (non-Löfgren's syndrome) sarcoidosis patients. Secondly, we performed a meta-analysis of 8 studies (including our case-control study), which confirmed the association of the T allele of

rs1049550 with a reduced risk of developing sarcoidosis, regardless of phenotype. The protective effect of *ANXA11* rs1049550, regardless of phenotype of disease, was additionally recently confirmed in a study with West-Slavonic sarcoidosis patients.[36]

In the meta-analysis we encountered variations in how disease stages (e.g., Scadding staging, chronic vs non-chronic etc.) and phenotypes (Löfgren's syndrome vs. non-Löfgren's syndrome, resolving vs. chronic) were defined and classified across studies. This resulted in heterogeneity and complicated the comparisons. Furthermore, there were only 2 studies[37,38] which included Löfgren's syndrome patients, both with low patient numbers. These findings underline the importance of research of genetic associations in a large cohort of patients with Löfgren's syndrome. Our own case-cohort study described the largest cohort of patients with Löfgren's syndrome, a chronic sarcoidosis cohort and a control cohort.

We confirmed the association between rs1049550 and susceptibility for sarcoidosis, including Löfgren's syndrome in both our own cohort as well as in the meta-analysis. With that, it is the first allele that predisposes to all types of sarcoidosis, an important observation, that confirms presence of identical pathways involved in disease in Löfgren's syndrome and non-Löfgren's syndrome sarcoidosis. However, the functional mechanisms by which this variant influences disease susceptibility is unknown. It is suggested that the amino-acid change caused by rs1049550 results in a dysfunctional Annexin A11,[37,39] which can influence cell processes,[40,41] by altering cell trafficking and apoptosis, which in turn can influence granuloma formation and maintenance.[42] But further research is needed to elucidate the functional effects of rs1049550 on protein function and cell processes and in different cell types, such as macrophages and monocytes, which are important in granuloma formation in sarcoidosis.

Furthermore, Levin et al.[43] found a significant SNP–SNP interaction between rs1049550 and rs9268839, a HLA SNP in an intergenic region with unknown function. This may indicate an interplay between HLA molecules, by presenting antigens, and *ANXA11* by influencing the inflammatory response. Unfortunately, we were unable to investigate the interaction between *ANXA11* rs1049550 and HLA. Due to a known strong association between *HLA-DRB1*03* and Löfgren's syndrome, it would be very interesting to investigate a possible interaction between *ANXA11* rs1049550 and *HLA-DRB1*03* in Löfgren's syndrome. However, to sufficiently enable such research more *HLA-DRB1*03* negative patients would be needed.

In conclusion, the T allele frequency of *ANXA11* rs1049550 is associated with susceptibility for sarcoidosis in general rather than with specific phenotypic forms.

Most importantly, *ANXA11* was not found to be associated with other immune mediated inflammatory disease, such as type I diabetes mellitus.[44] The group of immune mediated disease, including autoimmune diseases, clusters in families[45] and shared genes involved in disease susceptibility, including *HLA*-DRB1. However, the absence of a general association of these diseases with *ANXA11* suggests a unique pathway in all forms of sarcoidosis. Further research about the functional consequences of the *ANXA11* SNPs and its role in disease pathogenesis may therefore shed light on sarcoidosis defining pathogenic features. This is highly needed for a better understanding of the disease and may provide targets for sarcoidosis specific therapy.

MIF is associated with EN in Löfgren's syndrome

MIF has been associated with EN in sarcoidosis patients,[46] therefore to better understand the phenotype we investigated the association of MIF with Löfgren's syndrome.

In **Chapter 6**, the association of the macrophage migration inhibitory factor (*MIF*) -173C allele with susceptibility to Löfgren's syndrome and its clinical manifestations was investigated. MIF is relatively well studied and directly implied to play a role in sarcoidosis due to its immunomodulatory effect. We found that *MIF* -173C allele was not associated with overall susceptibility to Löfgren's syndrome, but it was associated with the presence of erythema nodosum (EN) in patients with Löfgren's syndrome. Specifically, patients with Löfgren's syndrome with only EN showed a significantly higher frequency of this C allele compared to patients with only arthritis, indicating that the C variant may contribute to, or be linked with, the development of EN. This association suggests that this polymorphism could serve as a biomarker for specific clinical manifestations. The results imply that the *MIF* -173C allele plays a role in the immune response pathway that leads to the manifestation of EN, possibly through increased MIF expression affecting the inflammatory process involved in skin reactions.

A recent study confirmed our results that there was no association between *MIF* -173C allele and susceptibility to sarcoidosis.[47] The same study also showed that there was no association with the severity of pulmonary sarcoidosis.[47] However, recent studies showed that MIF may play a modulatory role in immune activation in sarcoidosis,[48,49] while also having an effect on the phenotype of sarcoidosis. Elahi et al.[48] showed no difference in serum MIF concentration between sarcoidosis patients and healthy controls. But they identified two groups of sarcoidosis patients with different clinical and cytokine profiles. The first group had low MIF, IFN-γ and IL-10 serum concentrations and prominent extrapulmonary involvement. The second

group had high MIF, IFN-γ and IL-10 serum concentration. In this second group, more patients had skin involvement, but the type of skin involvement, specifically whether it concerned EN was not mentioned. Unfortunately, the *MIF* -173C allele was not analyzed in the study. It would be interesting to know whether there was a difference in the occurrence of *MIF* -173C between these two groups. *MIF* -173C allele is associated with increased MIF production, in vivo and in vitro.[50] Logically one would expect an increased frequency of *MIF* -173C in the second group and perhaps increased presence of the EN phenotype. Vice versa, in our study of the MIF allele, MIF expression levels in patient serum samples, were not measured. For a better understanding of the link between the *MIF* -173C allele and disease phenotypes this should be performed in future studies.

In contrast to *ANXA11*, associations of *MIF* -173C have been made with susceptibility, but also for the phenotype (severity or chronicity) for other systemic inflammatory diseases, such as rheumatoid arthritis[51] and juvenile idiopathic arthritis.[52] It has been demonstrated that MIF is a regulator of inflammatory and innate immune response. MIF is constitutively expressed by a broad variety of cells, like macrophages and monocytes and preformed MIF is released after exposure to microbial products and pro-inflammatory cytokines.[53] Once released, MIF functions as a regulator of innate and adaptive immune responses. Other features of MIF include its capacity to counterregulate the immunosuppressive effects of glucocorticoids on immune cells and to sustain pro-inflammatory functions by inhibiting p53-dependent apoptosis of macrophages.[54] Considering the pivotal role of MIF in orchestrating both innate and adaptive immune response, future therapeutic strategies modulating MIF activity might offer new treatment options for patients with inflammatory and autoimmune diseases, but also for sarcoidosis. In vitro studies with small molecule inhibitors and peptide inhibitors showed promising results.[55] An early phase trial with the anti-MIF monoclonal antibody imalumab, showed that it was well-tolerated by patients.[56] Further research with anti MIF therapy is needed.

Future research should focus on several key areas to build upon the findings of this study. First, larger and more diverse cohorts are needed to validate the association between the *MIF* -173C allele and erythema nodosum. This may enhance the generalizability and robustness of the findings. Second, further research is needed to determine a possible role for MIF in non-Löfgren's syndrome form of sarcoidosis, specifying organ involvement and type of manifestation. Third, functional studies are essential to establish a clear mechanistic link between the polymorphism, MIF expression, and clinical manifestation. Such studies may clarify how, why and when the C allele leads to increased MIF production in sarcoidosis patients. A next level of functional studies, likely requiring model systems, is needed to determine how

MIF influences granuloma formation, and other inflammatory disease processes, including EN associated immunecomplexes. In conclusion, *MIF -173C* allele was associated with erythema nodosum in Löfgren's syndrome patients, and not with susceptibility for Löfgren's syndrome.

CCR5 is associated with Löfgren's syndrome and have functional effects in cell-processes

The *CCR5* gene was previously associated with phenotypes of sarcoidosis and not with susceptibility for sarcoidosis.[57] In **chapter 7**, the association of *CCR5* rs1799987 with susceptibility to Löfgren's syndrome is evaluated. We demonstrated that the G allele of rs1799987 was significantly higher in patients with Löfgren's syndrome compared to controls. Next, the effect of this SNP on CCR5 expression and function by measuring calcium influx reaction to simulation was determined. Patients with the GG genotype showed a significantly higher percentage of $CCR5^+$ monocytes in peripheral blood and a higher expression of CCR5 on monocytes, measured by median fluorescence intensity (MFI), compared to patients with the AA genotype. Interestingly, the increased CCR5 expression on monocytes was not present in an earlier study done with HIV patients.[58] A possible explanation for this may be the study of different cell types or differences in underlying genetic profile. And third, following stimulation with MIP-1α, a significantly lower number of patients with the GG genotype showed a calcium influx reaction compared to patients with the AA genotype. The *CCR5* SNP rs1799987 G allele appears to modulate the immune response by increasing receptor availability but decreasing receptor activity, which, after the initial acute and intense inflammatory presentation, may lead to a tempered inflammatory response that characterizes the typically benign course of Löfgren's syndrome.
CCR5 has been shown to be present in granulomas seen in lymph node biopsies from sarcoidosis patients.[59] Studies showed that CCR5 functions as a T cell costimulatory molecule by improving and prolonging the T cell-APC interaction.[60] A dysfunctional CCR5 can interfere in this T cell-APC interaction, leading to a less stable interaction and thereby affecting the duration of the inflammatory state and an unstable granuloma formation in sarcoidosis.

While we did examine CCR5 expression and calcium signaling, further research is needed to explore ligand binding affinity, downstream signaling pathways, and effects on cellular migration and granuloma formation in vivo. CCR5 could be a potential treatment option in sarcoidosis patients, as a case report about the drug Maraviroc, which is a CCR5 inhibitor and used in HIV treatment,[61] was published showing a resolution of sarcoidosis symptoms in an HIV-infected patient.[62] In order to translate these findings to clinical practice, associations of genetic influences on

CCR5 expression and function as well as interaction with potential treatment options should be the focus of future research.

In conclusion, we demonstrated that the G allele of SNP rs1799987 predisposes individuals to Löfgren's syndrome, influences CCR5 expression on monocytes, and decreases the functional response of the CCR5 receptor.

Therapy

Following a diagnosis of sarcoidosis, evaluation of disease extent and severity is essential to determine the necessity of therapeutic intervention. The need for treatment depends on the risk of organ failure, mortality, or substantial impairment in quality of life. Due to the heterogeneous nature of sarcoidosis and the potential for spontaneous remission in many patients, treatment decisions remain complex. Current expert consensus supports the use of oral glucocorticoids as the first-line therapy. Recent evidence indicates no significant difference in clinical outcomes between oral glucocorticoids and methotrexate in patients with pulmonary sarcoidosis.[63] In cases where second-line agents, either alone or in combination with glucocorticoids, are ineffective or poorly tolerated, third-line treatment with biologic agents may be considered.
Patients with Löfgren's syndrome usually have a benign course with a high rate of spontaneous remission and many patients recover completely without long-term complications.

The fact that our cohort of patients with Löfgren's syndrome was collected over a long period also gave us insights into different treatment regimens over the years. These data are presented in the supplemental information included in **chapter 4**. Most patients with Löfgren's syndrome diagnosed before 1990 received corticosteroid treatment and some patients diagnosed before 1980 even received anti-tuberculosis treatment and were admitted to the hospital for longer periods. In later years some patients were treated with disease-modifying antirheumatic drugs (DMARD) and even with TNF-α blockers. We currently know that most patients with Löfgren's syndrome do not require systemic therapy and management is usually supportive. Fatigue is one of the complaints that, just as in non-Löfgren's syndrome sarcoidosis patients, also occurs regularly in patients with Löfgren's syndrome, even years after diagnosis. Interestingly, in our cohort significantly more *HLA-DRB1*03* negative patients suffered from fatigue after a long follow-up period. Recently, in patients with chronic fatigue syndrome associations between HLA were shown.[64] Furthermore, fatigue could be an expression of an ongoing low-level inflammatory process, given that less *HLA-DRB1*03* negative patients have resolving disease. Further prospective research is needed to demonstrate the association between

HLA and fatigue in sarcoidosis and what the influence of treatment will be.

The genomic human leukocyte antigen (HLA) region is central regarding antigen presentation and contains the strongest genetic link with Löfgren's syndrome. *HLA-DRB1*03* is strongly associated with Löfgren's syndrome and the good prognosis of Löfgren's syndrome. HLA-DRB1 is involved in many inflammatory diseases influencing both susceptibility and prognosis.[65-67]

Other genes, such as *ANXA11*, *MIF*, and *CCR5*, have emerged as notable candidates in sarcoidosis research due to their potential roles in disease mechanisms and phenotype-specific associations. Of particular interest is *ANXA11*, which harbors common genetic variants uniquely associated with sarcoidosis, including specific clinical manifestations such as Löfgren's syndrome. Targeting *ANXA11* may offer a means to modulate sarcoidosis-specific molecular pathways. In a therapeutic context, modulation of CCR5 is anticipated to influence the clinical course of both Löfgren's syndrome and sarcoidosis more broadly. Similarly, targeting MIF may alter the immune response by disrupting its immunomodulatory functions, potentially leading to the resolution of organ involvement or even complete disease remission. These genetically associated targets provide promising avenues for personalized treatment strategies in sarcoidosis.

GENERAL CONCLUSION

Löfgren's syndrome is a well-defined phenotype of sarcoidosis with a strong association with *HLA-DRB1*03* and a good prognosis. Our results show that even with Löfgren's syndrome, a well-established and defined phenotype, there is a complex and heterogeneous interplay between specific antigens, genetics and immune related processes. The network of interactions may be more extensive than previously thought and much more research is needed to elucidate this network accurately.

While we demonstrated differences between patients with Löfgren's syndrome and non-Löfgren's syndrome sarcoidosis, such as different cell types in BALF, we also showed similarities, such as the protective role of *ANXA11* in sarcoidosis, regardless of phenotype. We showed that a genetic variant of the *CCR5* gene is associated with susceptibility for Löfgren's syndrome and with quantitative and qualitative changes in CCR5. Furthermore, we have shown that an easy to perform test, HLA-DRB1 tagging with SNPs, can be used in practice in the management of patients with Löfgren's syndrome.

Overall, future research should aim to integrate genetic, molecular, and clinical data to develop comprehensive models of sarcoidosis pathogenesis and personalized medicine approaches. Because Löfgren's syndrome is so well described and framed, it is a good anchor for further research into the pathobiology of sarcoidosis. Unraveling the cause and the subsequent immunological mechanism in Löfgren's syndrome may provide us with more knowledge about sarcoidosis, in general, and prerequisites for resolving disease specifically. The key to unraveling the mystery of sarcoidosis may lie in Löfgren's syndrome.

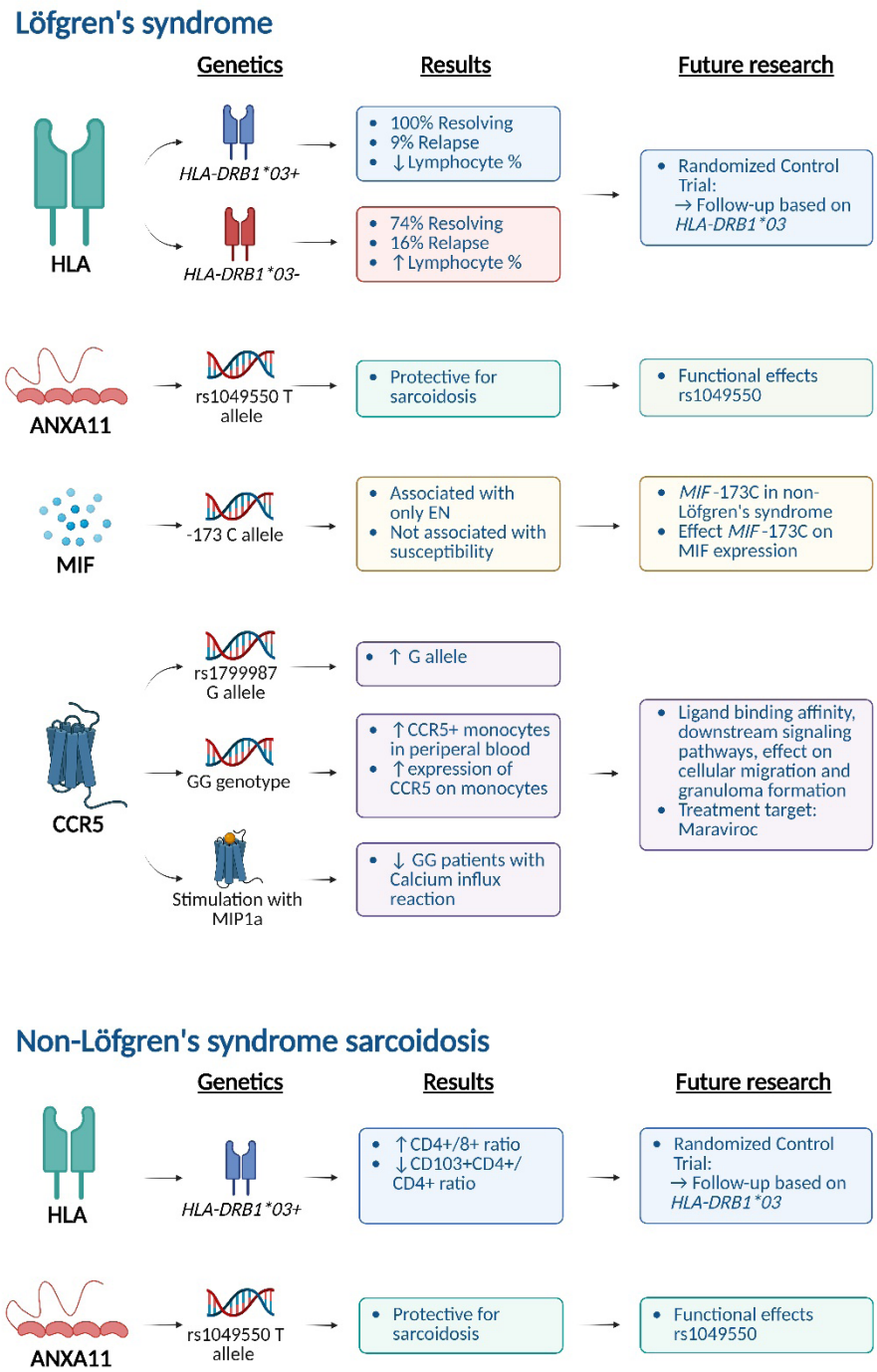

Figure 1. Schematic overview of the results of this thesis and suggestions for future research. EN = Erythema nodosum

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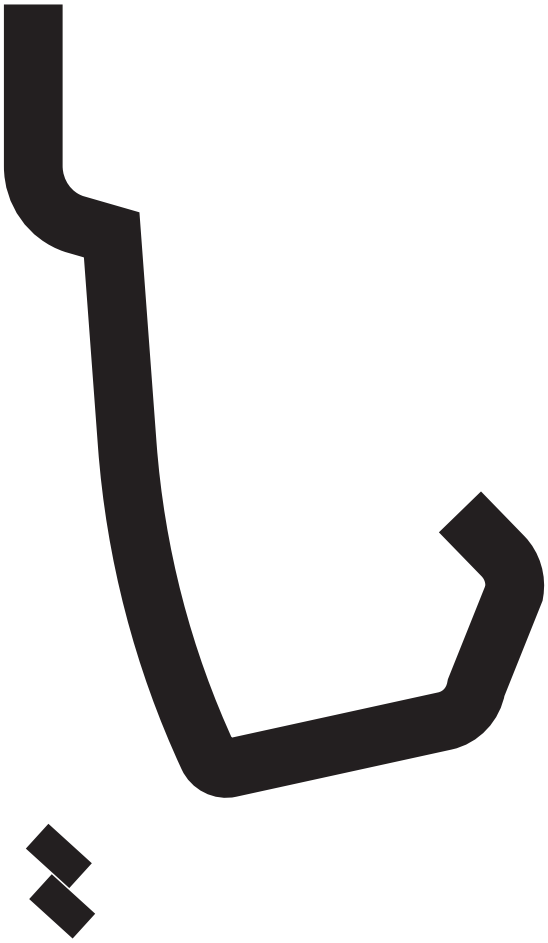


The Silence

The Release: The initial hook of the Ya (O!) transforms from a sudden gasp of pain into a grounded posture of resilience.

The clavicle and spine no longer bear the heavy, unseen burden of the unknown. The mental weight of the illness has been processed. The psyche, having traversed the peak of the syndrome, finds peace. The original "cry" dissolves into silence, leaving behind a body and spirit unified by the experience of healing.

Chapter 9

Appendix

Nederlandse samenvatting

Sarcoïdose is een systemische ontstekingsziekte met een onbekende oorzaak, gekenmerkt door de vorming van niet-verkazende granulomen en een heterogeen klinisch verloop. Het syndroom van Löfgren is een goed gedefinieerd, duidelijk fenotype van sarcoïdose.
Dit proefschrift richt zich op het syndroom van Löfgren, in het bijzonder op genetische factoren die vatbaarheid voor de ziekte predisponeren en factoren die samenhangen met de manifestatie van de symptomen.

Het syndroom van Löfgren

Het syndroom van Löfgren werd voor het eerst beschreven in 1946 door Sven Löfgren, een Zweedse longarts en kenmerkt zich door een acuut begin met koorts, bilaterale hilaire lymfadenopathie (BHL) op een thoraxfoto, erythema nodosum (EN) en/of bilaterale enkelartritis.
Het syndroom van Löfgren en “niet-Löfgren syndroom” sarcoïdose delen verschillende gemeenschappelijke kenmerken, zoals de vorming van niet-verkazende granulomen in de aangedane organen en een verhoogde $CD4^+/CD8^+$ T-celratio in de bronchoalveolaire lavagevloeistof (BALF). Het belangrijkste verschil tussen de twee types sarcoïdose is echter dat het fenotype van het syndroom van Löfgren een acute en meer homogene presentatie heeft, terwijl niet-Löfgren syndroom sarcoïdose een sluipend begin vertoont met een heterogene presentatie. De prognose van patiënten met het syndroom van Löfgren is zeer goed vergeleken met patiënten met niet-Löfgren syndroom sarcoïdose, vooral bij degenen die *HLA-DRB1*03* positief zijn; van hen herstelt 95% binnen 2 jaar.

Diagnose en incidentie

De diagnose van het syndroom van Löfgren wordt gesteld op basis van klinische en radiologische bevindingen. Patiënten hebben een acuut begin van de ziekte met bilaterale hilaire lymfadenopathie (BHL) op de röntgenfoto. Bij presentatie kunnen patiënten ook erythema nodosum (EN), artritis, of beide hebben. De combinatie van een acuut begin, BHL, EN en bilaterale enkelartritis heeft een hoge sensitiviteit en specificiteit voor de diagnose, respectievelijk tot 93 en 99%. Histopathologische bevestiging is niet vereist voor diagnostische doeleinden bij patiënten met typische klinische kenmerken van het syndroom van Löfgren. Erythema nodosum komt aanzienlijk vaker voor bij vrouwelijke patiënten, terwijl artritis vaker voorkomt bij mannelijke patiënten.
De wereldwijde incidentie van het syndroom van Löfgren varieert sterk; het beslaat tot de helft van de sarcoïdosepatiënten in Scandinavische landen, Nederland en

Spanje, terwijl het veel minder vaak voorkomt in het Verenigd Koninkrijk, de VS en Azië, waar minder dan 1% van de sarcoïdosepatiënten zich presenteert met het syndroom van Löfgren.

Genetica

De etiopathogenese van sarcoïdose blijft grotendeels onbekend, hoewel meerdere rapporten hebben gewezen op genetische erfelijkheid, infectieuze overdracht en gedeelde blootstelling aan omgevingsfactoren als hoofdoorzaken. De hypothese kan worden geformuleerd dat genetisch gepredisponeerde patiënten die worden blootgesteld aan bepaalde triggers uit de omgeving, waarschijnlijk de ziekte ontwikkelen. Er zijn studies die wijzen op een seizoensgebonden clustering van het syndroom van Löfgren. Een verhoogde incidentie in de lente is gerapporteerd op zowel het noordelijk als het zuidelijk halfrond, wat duidt op een gemeenschappelijke trigger in de omgeving.

Human Leukocyte Antigen (HLA)-regio

De immunologische kenmerken van sarcoïdose hebben geleid tot uitgebreid onderzoek naar de regio van het humaan leukocytenantigeen (HLA) op chromosoom 6. Deze regio speelt een centrale rol bij antigeenpresentatie, waarbij diverse allelen correleren met zowel de susceptibiliteit als het klinisch verloop van de aandoening. Specifiek voor het syndroom van Löfgren is een significante associatie aangetoond met het *HLA-DRB1*03* allel. Dit genotype is niet uitsluitend gerelateerd aan de ziekteontwikkeling, maar fungeert tevens als een onafhankelijke voorspeller voor een gunstige prognose, gekenmerkt door remissie binnen een termijn van twee jaar. Bovendien is bij het syndroom van Löfgren *HLA-DRB1*15* geassocieerd met een chronisch of niet-herstellend ziekteverloop. Daarom kan typering van beide DRB1-varianten nuttig zijn bij de risicostratificatie van patiënten met het syndroom van Löfgren.

*Associatie van tag-SNPs met HLA-DRB1*03 en HLA-DRB1*15*

Tot nu toe is *HLA*-typering een tijdrovend en duur proces geweest. Omdat we alleen geïnteresseerd waren in de typering van *HLA-DRB1*03* en *15, hebben we een eenvoudige ‘tagging assay’ ontwikkeld, waarbij tag-SNPs zijn gebruikt. In **hoofdstuk 3** hebben we deze tag SNPs gevalideerd met *HLA-DRB1*03* en **15* bij patiënten met het syndroom van Löfgren. We bevestigden de representatie van het A-allel van rs2040410 met *HLA-DRB1*03* en het A-allel van rs3135388 met *HLA-DRB1*15*.

Lymfocytose en celtypen in bronchoalveolair lavage

Verder hebben we in dit hoofdstuk ook gekeken naar de cellulaire variatie in de bronchoalveolaire lavagevloeistof (BAL-vloeistof) en of er een samenhang was

met de *HLA-DRB1* genotype. Het is bekend dat de cellulaire samenstelling in de BAL-vloeistof kan variëren tussen de fenotypen van sarcoidose. Meestal wordt er bij sarcoidose en het syndroom van Löfgren een verhoogd aantal lymfocyten (lymfocytose) gevonden met een verhoogd $CD4^+/CD8^+$ ratio en een lagere $CD103^+/CD4^+/CD4^+$ ratio. Wij toonden aan dat wanneer alle patiënten samen (Löfgren en niet-Löfgren) werden beoordeeld er geen verschillen in de lymfocytenpercentage werd gezien tussen *HLA-DRB1*03* positieve en negatieve patiënten. De *HLA-DRB1*03* positieve patiënten vertoonden echter wel een hogere $CD4^+/CD8^+$ ratio en een lagere $CD103^+/CD4^+/CD4^+$ ratio vergeleken met *HLA-DRB1*03* negatieve patiënten.
De patiënten met het syndroom van Löfgren die *HLA-DRB1*03* positief waren vertoonden een lager lymfocytenpercentage vergeleken met *HLA-DRB1*03* negatieve patiënten. Dit was onafhankelijk van de aan of afwezigheid van *HLA-DRB1*15*, wat suggereert dat de invloed van *HLA-DRB1*03* dominant is over de invloed van *HLA-DRB1*15*.
Een andere interessante bevinding in onze studie was dat patiënten met *HLA-DRB1*03*$^+$/15$^-$ (bekend om hun goede prognose) een verlaagde $CD103^+CD4^+/CD4^+$-ratio vertoonden vergeleken met patiënten met *HLA-DRB1*03*$^-$*/15*$^+$ en *03*$^-$*/15*$^-$. CD103 staat vooral bekend om zijn rol bij het nestelen en vasthouden van lymfocyten in perifere weefsels. Hoewel sarcoïdose wordt gekenmerkt door een verlaagde ratio, laten deze resultaten zien dat patiënten met de laagste ratio een grotere kans hebben op het syndroom van Löfgren, wat geassocieerd is met een goede prognose.

Lange termijn follow-up en prognose

In **hoofdstuk 4** hebben we de lange termijn uitkomsten van patiënten met het syndroom van Löfgren geëvalueerd in relatie tot *HLA-DRB1*03* en *HLA-DRB1*15* via 'tag SNP capturing', zoals beschreven in **hoofdstuk 3**. Dit was een uniek deel van het onderzoek, waarbij niet alleen gegevens werden verzameld van patiënten die nog onder controle waren, maar waarbij ook voormalige patiënten werden gevraagd terug te komen naar de kliniek voor een eendaags bezoek en het invullen van vragenlijsten. Ten eerste bevestigden we de goede prognose van patiënten met het syndroom van Löfgren in het algemeen, door aan te tonen dat 94% van alle patiënten hersteld was na een mediane follow-up tijd van 10,8 jaar. Vervolgens analyseerden we onze gegevens door de patiënten in twee groepen te verdelen: *HLA-DRB1*03* positieve en negatieve patiënten.

HLA-DRB1*03 correleert met een goede prognose

In ons cohort vertoonden alle *HLA-DRB1*03* positieve patiënten herstel na de initiële diagnose. Echter, na een mediane periode van 7,0 jaar werd bij 9% van deze patiënten een recidief (relapse) gezien. Dit is hoger dan het recidief percentage van 4% dat in een eerdere studie werd getoond.

HLA-DRB1*03 negatieve patiënten hebben een verhoogd risico op chronische en terugkerende ziekte

Bij de *HLA-DRB1*03* negatieve patiënten toonden we aan dat zowel een chronisch ziektebeloop als een recidief vaker voorkwamen tijdens de lang termijn follow-up dan bij positieve patiënten. Van de *HLA-DRB1*03* negatieve patiënten was een groot deel van degenen met een chronisch verloop of recidief ook drager van het *HLA-DRB1*15* allel. Uit onze studie leren we dat patiënten met het syndroom van Löfgren zonder *HLA-DRB1*03* een verhoogd risico lopen op een chronische en terugkerende ziekte, vooral degenen die het *HLA-DRB1*15* allel dragen. Omdat *HLA-DRB1*03* het dominante allel lijkt te zijn, wordt het effect van *HLA-DRB1*15* voornamelijk waargenomen bij *HLA-DRB1*03* negatieve patiënten.

Concluderend geven onze gegevens aan dat het *HLA-DRB1* genotype een sterke voorspeller is van niet alleen het beloop van de ziekte, maar ook het risico op recidief na herstel. Gezien het feit dat HLA-DRB1 tagging eenvoudig en relatief goedkoop is, zou het in de klinische praktijk kunnen worden geïmplementeerd om waardevolle informatie te verstrekken over zowel de korte- als langetermijnprognose van patiënten met het syndroom van Löfgren.

Single Nucleotide Polymorphisms (SNP)

Genetische associaties tussen sarcoïdose, het syndroom van Löfgren en niet-Löfgren syndroom sarcoïdose patiënten, beperken zich niet alleen tot de HLA-regio. Verschillende single nucleotide polymorphisms (SNPs) in niet-HLA-regio's blijken geassocieerd te zijn met een verhoogd ziekterisico voor sarcoïdose en het fenotype. Een SNP is een variatie van een enkel nucleotide op een specifieke genomische positie die voorkomt bij ten minste 1% van de populatie en staat voor algemene genetische diversiteit. In contrast hiermee is een mutatie elke verandering in de DNA-sequentie, die zeldzaam, spontaan of pathogeen kan zijn, en waarvoor geen minimumfrequentie in de populatie vereist is.

ANXA11 is geassocieerd met sarcoïdose, ongeacht het fenotype.

De bekendste en meest gerepliceerde genetische associatie bij sarcoïdose wordt gevonden in het gen voor annexine A11 (*ANXA11*) op de locus rs1049550, voor het eerst ontdekt in een Duits cohort van sarcoïdosepatiënten. Van *ANXA11* is bekend dat het betrokken is bij calciumsignalering, de celcyclus, vesikeltransport en apoptose. Een verandering in *ANXA11* zou kunnen resulteren in een disfunctioneel Annexine A11 dat celprocessen beïnvloedt, zoals celtransport, wat op zijn beurt de vorming en het behoud van granulomen bij sarcoïdosepatiënten kan beïnvloeden.

In **hoofdstuk 5** hebben we de associatie van het *ANXA11* gen, in het bijzonder

de SNP rs1049550, met het syndroom van Löfgren bestudeerd. Eerst voerden we een patiënt-controleonderzoek uit, dat een verlaagde T allelfrequentie liet zien bij sarcoïdose patiënten vergeleken met gezonde controle patiënten, wat wijst op een beschermend effect van het T allel voor de ontwikkeling van de ziekte. We toonden aan dat dit beschermende effect onafhankelijk was van het sarcoïdose fenotype; met andere woorden, vergelijkbare associaties waren aanwezig bij patiënten met het syndroom van Löfgren en patiënten met chronische sarcoïdose (niet-Löfgren syndroom). Ten tweede voerden we een meta-analyse uit van 8 studies (inclusief ons eigen onderzoek), die de associatie van het T allel van rs1049550 met een verminderd risico op het ontwikkelen van sarcoïdose bevestigde, ongeacht het fenotype.

In de meta-analyse stuitten we op variaties in de manier waarop ziektestadia (bijv. Scadding-stadia, chronisch vs. niet-chronisch, etc.) en fenotypes (syndroom van Löfgren vs. niet-Löfgren, herstellend vs. chronisch) werden gedefinieerd en geclassificeerd in de verschillende studies. Dit leidde tot heterogeniteit en bemoeilijkte de vergelijkingen. Bovendien waren er slechts 2 studies die patiënten met het syndroom van Löfgren includeerden, beide met lage patiënten aantallen. Deze bevindingen onderstrepen het belang van onderzoek naar genetische associaties in een groot cohort van patiënten met het syndroom van Löfgren. Onze eigen studie beschreef het grootste cohort van patiënten met het syndroom van Löfgren, een chronisch sarcoïdose-cohort en een controle-cohort.

We bevestigden de associatie tussen rs1049550 en de vatbaarheid voor sarcoïdose, inclusief het syndroom van Löfgren, in zowel ons eigen cohort als in de meta-analyse. Daarmee is dit het eerste allel dat predisponeert voor alle typen sarcoïdose; een belangrijke waarneming die de aanwezigheid bevestigt van identieke processen die betrokken zijn bij de ziekte in zowel het syndroom van Löfgren als niet-Löfgren syndroom sarcoïdose. De functionele mechanismen waarmee deze variant de vatbaarheid voor de ziekte beïnvloedt, zijn echter onbekend. Er wordt gesuggereerd dat de aminozuurverandering veroorzaakt door rs1049550 resulteert in een disfunctioneel Annexine A11, wat celprocessen kan beïnvloeden, zoals verandering van transport-processen in de cel en de apoptose (geprogrammeerde celdood), wat op zijn beurt de vorming en het behoud van granulomen kan beïnvloeden. Maar verder onderzoek is nodig om de functionele effecten van rs1049550 op de eiwitfunctie en celprocessen op te helderen, ook in verschillende celtypen zoals macrofagen en monocyten, die belangrijk zijn bij granuloomvorming bij sarcoïdose.

MIF is geassocieerd met EN bij het syndroom van Löfgren

Macrophage migration inhibitory factor (*MIF*) is een immunoregulerend cytokine

dat een rol speelt bij de activering en migratie van T-cellen en macrofagen. MIF is betrokken bij antigeenspecifieke immuunresponsen. De interactie tussen antigeenpresenterende cellen en T-cellen, die leidt tot T-celactivering en cytokineproductie, speelt een sleutelrol in de immunopathogenese van sarcoïdose door de vorming en het behoud van granulomen aan te sturen.

Er zijn associaties gevonden tussen een genetische variatie in *MIF* (het -173C allel, dat geassocieerd is met een verhoogde MIF-productie) en chronische ontstekings- en auto-immuunziekten, zoals sarcoïdose en systemische sclerose. Bij sarcoïdosepatiënten met erythema nodosum (EN) bleek de frequentie van het *MIF -173C* allel aanzienlijk hoger te zijn dan bij patiënten met EN door andere oorzaken of controles, wat wijst op een rol voor MIF in de klinische presentatie van sarcoïdose.

In **hoofdstuk 6** hebben we de associatie van het macrophage migration inhibitory factor (*MIF*) -173C allel met de vatbaarheid voor het syndroom van Löfgren en de klinische manifestaties ervan onderzocht. We vonden dat het *MIF* -173C allel niet geassocieerd was met de algemene vatbaarheid voor het syndroom van Löfgren, maar wel met de aanwezigheid van EN bij patiënten met het syndroom van Löfgren. Specifiek vertoonden patiënten met het syndroom van Löfgren met alleen EN een significant hogere frequentie van dit C allel vergeleken met patiënten met alleen artritis, wat aangeeft dat de C-variant kan bijdragen aan, of gekoppeld kan zijn aan, de ontwikkeling van EN. Deze associatie suggereert dat dit polymorfisme zou kunnen dienen als een biomarker voor specifieke klinische manifestaties. De resultaten impliceren dat het *MIF* -173C allel een rol speelt in het immuunresponstraject dat leidt tot de manifestatie van EN, mogelijk door een verhoogde MIF-expressie die het ontstekingsproces bij huidreacties beïnvloedt.

CCR5 is geassocieerd met het syndroom van Löfgren en heeft functionele effecten op celprocessen

De CC-chemokinereceptor 5 (CCR5) speelt een cruciale rol in de T-celfunctie, en van polymorfismen in het *CCR5* coderende gen is bekend dat ze de expressie ervan beïnvloeden. Hoewel genetische studies naar *CCR5*-varianten geen associatie met vatbaarheid voor sarcoïdose hebben aangetoond, is een *CCR5*-haplotype gekoppeld aan aanhoudende longbetrokkenheid bij Nederlandse en Britse sarcoïdosepatiënten. Daarnaast zijn bij het syndroom van Löfgren polymorfismen in de *CCR5* promotor geassocieerd met vrouwelijke patiënten.

In **hoofdstuk 7** hebben we de associatie van *CCR5* rs1799987 met de vatbaarheid voor het syndroom van Löfgren geëvalueerd. We toonden aan dat het G allel van rs1799987 significant vaker voorkwam bij patiënten met het syndroom van Löfgren vergeleken met controle patienten. Vervolgens werd het effect van deze SNP op de

CCR5 expressie en -functie bepaald door de calcium-influxreactie na stimulatie te meten. Patiënten met het GG genotype vertoonden een significant hoger percentage $CCR5^+$ monocyten in het perifere bloed en een hogere expressie van CCR5 op monocyten (gemeten via de mediane fluorescentie-intensiteit, MFI) vergeleken met patiënten met het AA genotype. Interessant genoeg werd de verhoogde CCR5-expressie op monocyten niet geobserveerd in een eerdere studie bij HIV-patiënten. Een mogelijke verklaring hiervoor kan het onderzoek naar verschillende celtypen zijn of verschillen in het onderliggende genetische profiel. Ten derde vertoonde in onze studie een significant lager aantal patiënten met het GG genotype een calcium-influxreactie na stimulatie met MIP-1α vergeleken met patiënten met het AA genotype. Het G allel van de *CCR5* SNP rs1799987 lijkt de immuunrespons te moduleren door de beschikbaarheid van receptoren te vergroten, maar de activiteit van de receptoren te verlagen. Dit kan, na de initiële acute en intense ontstekingspresentatie, leiden tot een getemperde ontstekingsreactie die kenmerkend is voor het doorgaans goedaardige verloop van het syndroom van Löfgren.

Hoewel we de CCR5 expressie en calciumsignalering hebben onderzocht, is er meer onderzoek nodig naar de bindingsaffiniteit van liganden, signaleringsroutes in de cel en de effecten op celmigratie en granuloomvorming in vivo. CCR5 zou een potentiële behandelingsoptie kunnen zijn; er is een casus gepubliceerd over het medicijn Maraviroc (een CCR5 remmer gebruikt bij HIV), waarbij de sarcoïdose symptomen bij een HIV-geïnfecteerde patiënt verdwenen. Om deze bevindingen naar de klinische praktijk te vertalen, zou toekomstig onderzoek zich moeten richten op de associaties van genetische invloeden op de CCR5-expressie en -functie, evenals de interactie met potentiële behandelopties.

Therapie

Na de diagnose sarcoïdose is evaluatie van de uitgebreidheid en ernst van de ziekte essentieel om de noodzaak van therapeutisch ingrijpen te bepalen. De behoefte aan behandeling hangt af van het risico op orgaanfalen, sterfte of aanzienlijke verslechtering van de kwaliteit van leven. Vanwege de heterogene aard van sarcoïdose en de kans op spontane remissie bij veel patiënten, blijven beslissingen over de behandeling complex. De huidige consensus onder experts ondersteunt het gebruik van orale glucocorticoïden als eerstelijnsbehandeling. Recent bewijs wijst uit dat er geen significant verschil is in klinische uitkomsten tussen orale glucocorticoïden en methotrexaat bij patiënten met pulmonale sarcoïdose. In gevallen waarin tweedelijnsmiddelen (alleen of in combinatie) niet effectief zijn of slecht worden verdragen, kan een derdelijnsbehandeling met biologicals worden overwogen.
Patiënten met het syndroom van Löfgren hebben meestal een goedaardig verloop

met een hoge mate van spontane remissie; veel patiënten herstellen volledig zonder langetermijn complicaties. Het feit dat ons cohort over een lange periode is verzameld, gaf ons ook inzicht in de verschillende behandelregimes door de jaren heen. De meeste patiënten die vóór 1990 de diagnose kregen, ontvingen corticosteroïden, en sommige patiënten van vóór 1980 kregen zelfs anti-tuberculosebehandeling en werden langdurig in het ziekenhuis opgenomen. In latere jaren werden sommige patiënten behandeld met DMARD's en zelfs met TNFα-remmers (biologicals). Tegenwoordig weten we dat de meeste patiënten met het syndroom van Löfgren geen systemische therapie nodig hebben en dat het beleid meestal ondersteunend is. Vermoeidheid is een van de klachten die, net als bij andere vormen van sarcoïdose, regelmatig voorkomt bij patiënten met het syndroom van Löfgren, zelfs jaren na de diagnose. Opvallend is dat in ons cohort significant meer *HLA-DRB1*03* negatieve patiënten last hadden van vermoeidheid na een lange follow-up periode. Recentelijk zijn er bij patiënten met het chronisch vermoeidheidssyndroom associaties met HLA aangetoond. Bovendien zou vermoeidheid een uiting kunnen zijn van een aanhoudend laaggradig ontstekingsproces, aangezien minder *HLA-DRB1*03* negatieve patiënten volledig herstellen. Verder prospectief onderzoek is nodig om het verband tussen HLA en vermoeidheid bij sarcoïdose aan te tonen en wat de invloed van behandeling daarop is.

Het genomische humaan leukocytenantigeen (HLA)-gebied staat centraal bij antigeenpresentatie en bevat de sterkste genetische link met het syndroom van Löfgren. *HLA-DRB1*03* is sterk geassocieerd met zowel de ziekte als de goede prognose. HLA-DRB1 is betrokken bij veel ontstekingsziekten en beïnvloedt zowel de vatbaarheid als de prognose.
Andere genen, zoals *ANXA11*, *MIF* en *CCR5*, zijn naar voren gekomen als opvallende kandidaten in sarcoïdose-onderzoek vanwege hun potentiële rol in ziektemechanismen en fenotype-specifieke associaties. Bijzonder interessant is *ANXA11*, dat genetische varianten bevat die uniek geassocieerd zijn met sarcoïdose, inclusief specifieke uitingen zoals het syndroom van Löfgren. Het therapeutisch beinvloeden van *ANXA11* zou een manier kunnen bieden om sarcoïdose-specifieke moleculaire paden te moduleren.

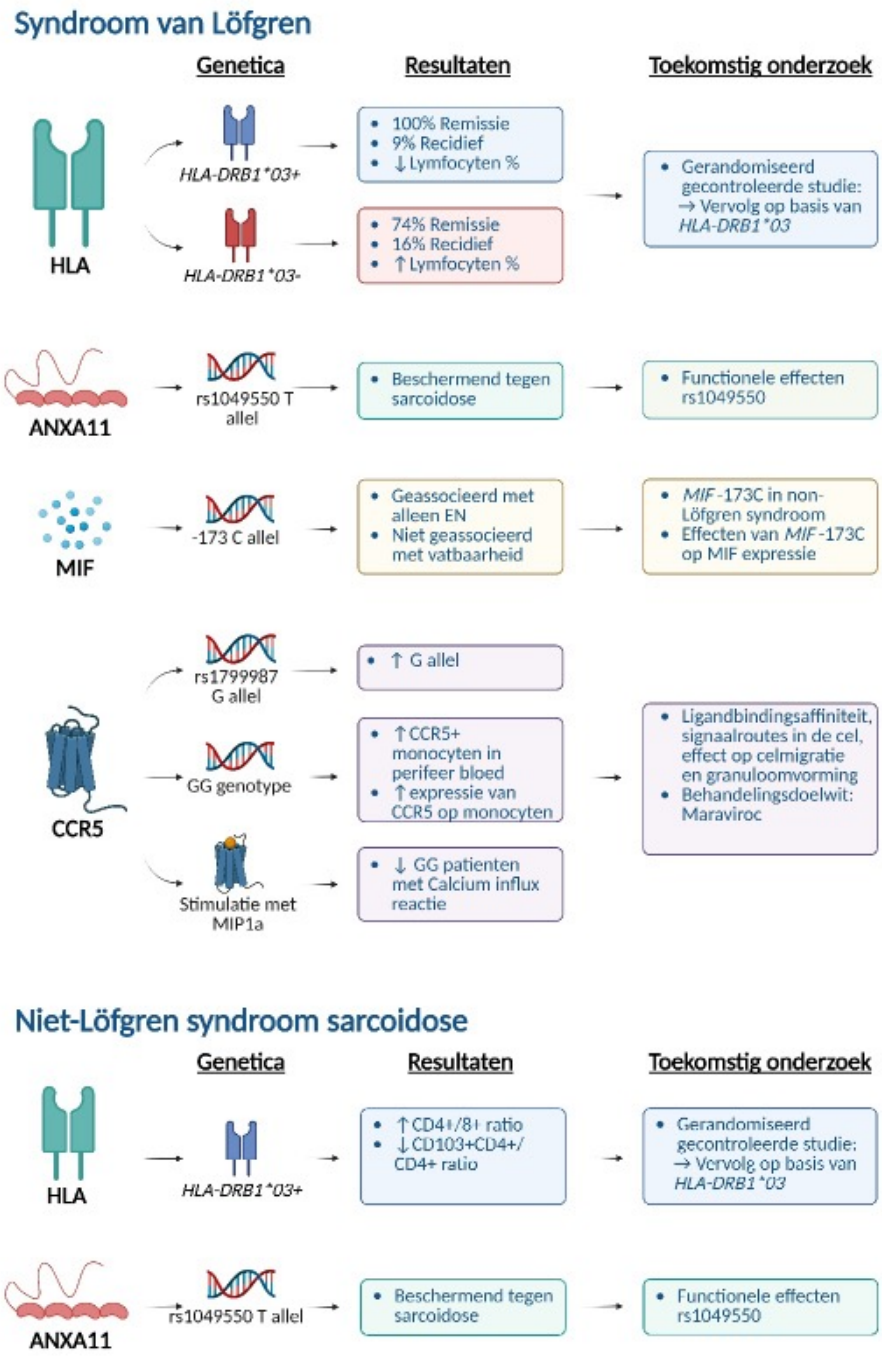
In een therapeutische context wordt verwacht dat modulatie van CCR5 het klinische verloop van zowel het syndroom van Löfgren als sarcoïdose in bredere zin zal beïnvloeden. Op vergelijkbare wijze zou het targeten van MIF de immuunrespons kunnen veranderen door de immunomodulerende functies ervan te verstoren, wat mogelijk kan leiden tot het herstel van orgaanbetrokkenheid of zelfs volledige remissie van de ziekte. Deze genetisch geassocieerde targets bieden veelbelovende wegen voor gepersonaliseerde behandelstrategieën bij sarcoïdose.

Algemene conclusie

Het syndroom van Löfgren is een goed gedefinieerd fenotype van sarcoïdose met een sterke associatie met *HLA-DRB1*03* en een goede prognose. Onze resultaten laten zien dat er zelfs bij het syndroom van Löfgren sprake is van een complexe en heterogene wisselwerking tussen specifieke antigenen, genetica en immuungerelateerde processen. Het netwerk van interacties is mogelijk uitgebreider dan voorheen gedacht en er is veel meer onderzoek nodig om dit netwerk nauwkeurig in kaart te brengen.

Hoewel we verschillen hebben aangetoond tussen patiënten met en zonder het syndroom van Löfgren, zoals verschillende celtypen in de BAL-vloeistof, toonden we ook overeenkomsten aan, zoals de beschermende rol van *ANXA11* bij sarcoïdose, ongeacht het fenotype. We lieten zien dat een genetische variant van het *CCR5* gen geassocieerd is met de vatbaarheid voor het syndroom van Löfgren en met kwantitatieve en kwalitatieve veranderingen in CCR5. Bovendien hebben we aangetoond dat een eenvoudig uit te voeren test, HLA-DRB1 tagging met SNP's, in de praktijk kan worden gebruikt bij de behandeling van patiënten met het syndroom van Löfgren.

Al met al moet toekomstig onderzoek gericht zijn op het integreren van genetische, moleculaire en klinische gegevens om uitgebreide modellen van de pathogenese van sarcoïdose en benaderingen voor gepersonaliseerde geneeskunde te ontwikkelen. Omdat het syndroom van Löfgren zo goed beschreven en afgebakend is, vormt het een goed ankerpunt voor verder onderzoek naar de pathobiologie van sarcoïdose. Het ontrafelen van de oorzaak en het daaropvolgende immunologische mechanisme bij het syndroom van Löfgren kan ons meer kennis verschaffen over sarcoïdose in het algemeen, en specifiek over de voorwaarden voor het genezen van de ziekte. De sleutel tot het ontrafelen van het mysterie van sarcoïdose ligt mogelijk in het syndroom van Löfgren.



Figuur 1. Schematisch overzicht van de resultaten van dit proefschrift en suggesties voor toekomstig onderzoek. EN = Erythema nodosum

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2011 ERS conference, Amsterdam, Netherlands: Oral Presentation: ***'Polymorphisms in CCR5 confer susceptibility to Löfgren's syndrome and may regulate the immune response'***

Poster presentations

2023 ATS conference, Washington DC, USA: Thematic Poster: L***öfgren's Syndrome: Prognostically as good as we think?***

2022 ERS conference, Barcelona, Spain: Thematic Poster: *ANXA11* ***rs1049550 Associates with Löfgren's Syndrome and Chronic Sarcoidosis Patients***.

2014 WASOGBAL conference, Izmir, Turkiye: Thematic Poster: ***'Polymorphisms in CCR5 confer susceptibility to Löfgren's syndrome and may regulate the immune response'***

2013 WASOG conference, Paris, France: Thematic Poster: ***'Genetic analysis of ethnicity of sarcoidosis patients in the Netherlands'***

2013 ERS conference, Barcelona, Spain: Thematic Poster: '***Genetic analysis of ethnicity of sarcoidosis patients in the Netherlands'***

2009 ERS conference, Vienna, Austria: E-communication: ; ***Macrophage migration inhibitory factor (MIF) -173 polymorphism is associated with clinical phenotypes of Löfgren's syndrome'***

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Ümüş ik kan het niet te vaak zeggen: 사랑해 (Saranghae)

Curriculum Vitae

Bekir Karakaya was born on March 8th, 1982, in Utrecht. He graduated from Dr. F.H. de Bruijne Lyceum in Utrecht in 2000. After not being selected for the medical program, he pursued studies in biology, completing the propaedeutic year, and later studied Biomedical Sciences for a year. On his third attempt, he was accepted into medical school at the University Medical Center Utrecht in 2002. During his final year, he completed an internship in the Department of Pulmonary Medicine at St. Antonius Hospital. Subsequently, he applied for a residency and was hired under the supervision of Prof. J.M.M. van den Bosch.

In 2008, he joined the Interstitial Lung Disease Center of Excellence as a researcher and initiated his PhD project under the guidance of Prof. Dr. J.M.M. van den Bosch and later Prof. Dr. J.C. Grutters.

In 2009, he began his residency training and, in June 2017, officially became a pulmonologist. Following this, he started working as a fellow at St. Antonius Hospital Nieuwegein, with a focus on interstitial lung disease (ILD) and pulmonary vascular disease. After completing a two-year fellowship, he continued as a pulmonologist at the same hospital, specializing in ILD. Throughout his training in pulmonary medicine and after becoming a pulmonologist, he remained dedicated to advancing his PhD trajectory.