gene polymorphisms in fibrotic sarcoidosis

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gene polymorphisms in
FIBROTIC SARCOIDOSIS

Genpolymorfismen in fibrotische sarcoïdose
(met een samenvatting in het Nederlands)

proefschrift

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For Aaldert H. Kruit (1920-1972) and Emery A. Kruit (2006-)

Content

chapter ONE

1

general INTRODUCTION

1. SARCOIDOSIS

The term sarcoidosis, also known as Morbus Besnier-Boeck, was coined by the Norwegian dermatologist Ceasar Boeck in 1899 to describe the clinical features of this granulomatous disorder. The word "sarcoidosis" is derived from the Greek word "σαρκοδεσ," meaning "fleshy," and the Greek suffix "οσισ," meaning "condition." The "fleshy condition" refers to the skin lesions that often appear on various parts of the body. These lesions are non-caseating granulomas, a hallmark of sarcoidosis. They can be present in virtually any organ but are most often found in the lungs (1).

The descriptive definition of sarcoidosis, as reported at the World Congress in Kyoto in 1991 (2) and updated since the proclaimed definition back in 1979 (3), reads as follows: "Sarcoidosis is a multisystem disorder of unknown cause. It commonly affects young and middle-aged adults and frequently presents with bilateral hilar lymphadenopathy, pulmonary infiltration, ocular and skin lesions. Other organs may also be involved. The diagnosis is established when clinicoradiological findings are supported by histological evidence of noncaseating epithelioid cell granulomas. Granulomas of known causes and local sarcoid reactions must be excluded. Frequently observed immunologic features are depression of cutaneous delayed-type hypersensitivity and increased CD4/CD8 ratio at the site of involvement. Circulating immune complexes along with the signs of B-cell hyperactivity may also be detectable. The course and prognosis may correlate with the mode of the onset and the extent of the disease. An acute onset with erythema nodosum or asymptomatic bilateral hilar lymphadenopathy usually heralds a self-limiting course, whereas an insidious onset, especially with multiple extra-pulmonary lesions, may be followed by relentless progressive fibrosis of the lungs and other organs."

1.1 Sarcoidosis: a granulomatous disorder

In general, granulomatous disorders are classified according to 1) infection (M. tuberculosis, Schistosoma), 2) chemical exposure (beryllium, silica), 3) immunologic disorders (sarcoidosis, Crohn's disease, histiocytosis X, allergic granulomatosis), 4) neoplasia (malignant nasal granuloma, lymphoma) and 5) hypersensitivity pneumonitis (Farmer's lung, Bird fancier's lung). Each disease is characterized by an immunologic response that causes the formation of

granulomas. Granulomas are organized structures of inflammatory and non-inflammatory cells. The structures may appear similar irrespective of the causative agent, in which case the clinical features will aid in defining the exact type of granulomatous disease. In other cases, granulomas will have distinct histological features. For instance, M. tuberculosis and M. lepra both cause caseation within the core of the granuloma, whilst the sarcoid granuloma, at least in most cases, does not. The progression and outcome vary between the different granulomatous disorders. The evolution towards fibrosis can be found in virtually all types of granulomas, but is almost invariably present in granulomas caused by worm cysts such as those of the Schistosoma mansoni (4). On the other hand, sarcoid granulomas as well as many other granulomatous disorders may either resolve without sustaining visible damage to the tissue, or progress towards permanent scarring.

1.2 Clinical features

Sarcoidosis commonly presents with symptoms such as fever, malaise, anorexia or weight loss. These symptoms may vary from being mild to severe. Many patients have respiratory symptoms, including cough, dyspnea and retrosternal chest discomfort. Alternatively, sarcoidosis can be occasionally discovered in a completely asymptomatic individual. Although sarcoidosis is often acute and self-limiting, in many individuals it becomes chronic, flaring up occasionally over a period of many years (5). Failure to regress within the first 2 years following onset is believed to be predictive for developing a chronic and persistent course (6). Although the majority of sarcoidosis patients undergo spontaneous remission, approximately 10 percent of the sarcoidosis cases develop parenchymal fibrosis (7). Fibrosis of the lung parenchyma is associated with poor pulmonary function and a bleak prognosis with increased morbidity and mortality (8).

The classification of pulmonary sarcoidosis is based on roentgenological staging according to the Scadding criteria (1). Chest radiographs are classified by stages: 0, normal chest radiographic findings; I, bilateral hilar adenopathy with normal lung parenchyma; II, bilateral hilar adenopathy with pulmonary infiltrates; III, pulmonary infiltrates without hilar adenopathy; IV, pulmonary fibrosis/fibrocystic parenchymal changes. However, the use of advanced radiographic techniques such as high resolution computer tomography (HRCT) scans are superior in discerning subtle changes and details that can be

overlooked using standard chest X-rays. Where standard chest radiography would classify fibrosis as one stage, namely stage IV, HRCT scans define three main patterns of fibrosis: bronchial distortion, honeycomb patterns and a linear pattern which all correlate closely with pulmonary function impairment (9) (Figure 1).

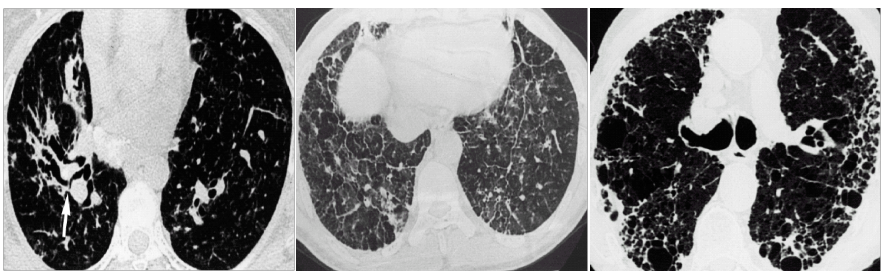
The disease pattern of sarcoidosis varies greatly and is dependent on the type of organs involved as well as the severity and/or duration of inflammation. Extrapulmonary manifestations of sarcoidosis may vary from benign to severe symptoms. Acute sarcoidosis or Löfgren's syndrome is often accompanied by a harmless skin condition called erythema nodosum while neuro- or myocardial sarcoidosis can pose a serious threat to life (10-12). Uveitis, an ophthalmic complication of sarcoidosis often seen in Asians, causes an unfavorable outcome in 15% of the cases, and the risk of developing blindness, regardless of ethnicity, is also present (13, 14). Other more or less common complications are also seen, including involvement of extrathoracic lymph nodes, skin lesions other than erythema nodosum, abnormalities of calcium metabolism, and involvement of the liver, phalangeal bones and bone marrow (1, 15, 16).

1.3 Epidemiology

Sarcoidosis is most common in young adults between the ages of 20 and 40, although a number of studies have suggested a second peak after 50 years, especially for females (17-19). A population-based study of incidence and survival in adults with sarcoidosis in the United States of America reported incidence rates of 5.9 per 100,000 person-years for men and 6.3 per 100,000 person-years for women (17). A European epidemiological study has reported incidence rates of 16.5 for men and 21.7 per 100,000 person-years for women. The accumulated lifetime risk was found to be higher in females (1.3%) than males (1.0%) (18).

The incidence, but also the clinical phenotypes of sarcoidosis, vary between countries and different racial groups. For instance, Scandinavian countries and the US African-American population in the United States show the highest incidence rates (20). In African-Americans, the incidence rate is 3.8-fold higher than in Caucasians, conferring an overall lifetime risk of 2.4% versus 0.85% (21). Moreover, sarcoidosis patients of African descent have more severe clinical manifestations and poorer outcomes compared to Caucasians

(15). The progression towards pulmonary fibrosis is more frequently seen in Caucasians and Blacks than in Japanese (1). On the other hand, Japanese sarcoidosis patients are more often complicated with uveitis as well as cardiac and skin involvement (22). The overall mortality is 1–5% and is caused by myocardial involvement in Japanese and respiratory failure in Caucasians (1).



A **B** **C**

Figure 1 Three main HRCT patterns of pulmonary fibrosis.
Bronchial distortion (A), linear pattern (B), and honeycombing (C) of the lungs in three different patients with fibrotic pulmonary sarcoidosis.

1.4 Pathogenesis in the lung

A major endeavor in sarcoidosis research to date has been the attempt to elucidate the origin of granuloma formation in sarcoidosis. Since the first clinical description of sarcoidosis over a century ago, research efforts have contributed significantly to the understanding of the pathways involved in granuloma formation, yet the cause has not been identified.

The analogy of sarcoid granulomas with other granulomas such as tuberculosis, hypersensitivity pneumonitis and other forms of granulomatosis has lead to the assumption that sarcoidosis is an exuberant immune response to a putative causative agent. The general paradigm of immune granuloma formation suggests a specific, T-cell-mediated response to an antigenic agent that has been processed by macrophages and has then been presented to antigen-specific T lymphocytes. The activated T cells and macrophages subsequently direct the accumulation and differentiation of mononuclear phagocytes in the local microenvironment (7, 23). The tapestry of

immunologic responses that follow the exposure to a putative antigen and the subsequent formation of a granuloma is based on data from patients and analogies with experimental immune responses (24). The granulomatous formation and evolution has been suggested to occur in three important series of events:

I. Disease onset

The antigen-presenting cells (APC) engulf, process and present the antigen to naïve CD4+ T-helper lymphocytes (Th-0 cells). The processed antigen, nestled in the groove of the MHC class II molecules present on the APC/dendritic cell, binds with the Th-0 cell receptor (TCR) in association with co-stimulatory molecules (B7 with CD28, CD40 with CD40 ligand). This results in activation and polarization of Th-1 helper cells (25).

II. Granuloma formation

At the core of the granuloma are the macrophage-derived epithelioid cells and giant multinucleated cells, surrounded by a rim of lymphocytes and fibroblasts. Additional cells that are found within or around the perimeter of the granuloma include fibroblast, mast cells, and various other types of cells.

The formation of the granuloma is thought to ensue following continuous presentation of antigens by lung macrophages to Th-1 cells. This cascade involves the expression and actions of a myriad of cytokines and chemokines and the recruitment, migration and differentiation of inflammatory cells. Most data attribute the Th-1 driven response to cytokines IFN-γ, TNF-α, IL-12 and IL-18 (26-29). Other mediators include IL1β, and IL-6, which both amplify and maintain granuloma formation (30). In addition, chemokines such as MIP-1, MIP-3, MCP-1, RANTES and others are also found in sarcoid granulomas and are believed to further propel the formation of the granuloma through the recruitment of monocytes and T-cells to the sarcoid lesion (31).

III. Resolution or perpetuation of the granuloma (the Th-1/Th-2 paradigm)

Somewhere along the route may lay the junction at which the granuloma formation either becomes chronic - in some cases culminating in irreversible scarring - or regresses virtually without sustaining permanent damage to the affected tissue. The contrasting disease patterns of sarcoidosis, i.e. acute/self-remitting versus chronic/end-stage disease, has lead to the idea that differential immune responses may be the foundation for this dichotomy of disease

patterns (figure 2). As detailed above, sarcoidosis is initiated and propagated by the innate Th-1 type immune response. A switch from the Th-1 to the adaptive Th-2 type response is considered beneficial in terms of eliminating the antigen more effectively (32). Ironically, a switch from a Th-1 to Th-2 response is believed to occur in sarcoidosis patients who develop a chronic type disease which subsequently may activate the fibrotic pathway (26, 33). A Th-2 response is also the driving force behind the formation of fibrosis in many diseases such as schistosomiasis (4) and idiopathic pulmonary fibrosis (IPF), a disease leading to progressive fibrotic destruction of the lung tissue (34). It has also been speculated that a concurrent Th-1 and Th-2 response during the onset of sarcoidosis may predestine a chronic disease progression. This is supported by an experimental model of tuberculosis. In this model, when Th-2 primed cells are superimposed upon a pre-existing Th-1 response, the cells involved in the inflammation site are sensitized by cytokines resulting in exacerbation of the inflammation (35). In tuberculosis, the actions of TNF-α strongly depend on the presence of Th-1 and Th-2 primed cells. In a mixed Th-1/Th-2 response, TNF-α exacerbates tissue damage while in a 'pure' Th1 response it does not (36). Thus, it seems that failure of the Th-2 to eradicate the causative antigen will propel a fibrogenic cascade. Th-1 cells mediate the immune response through the excretion of key cytokines IFN-γ and IL-2 while Th-2 cells include antibody production and the excretion of IL-4, IL-5 and IL-10. The Th-1 and Th-2 responses are designed to rid the infected tissue of the antigen and, in case of failing to do so, subsequently form a clean perimeter of fibrous tissue around the granuloma and to mediate regression of the granuloma by initiation of a controlled tissue repair and remodeling.

One of the latest additions to the line of key factors in fibrotic responses in tissue injury is the CC chemokine ligand 18 (CCL18). This chemokine is produced by BAL-derived cells and has been found to be markedly increased in patients with pulmonary fibrosis (IPF, UIP and stage IV sarcoidosis). Apparently, CCL18 expression is synergistically activated by Th-2 cytokines IL-4 and IL-10 (figure 2). The results of this study are in support of the hypothesis that a Th-2 immune response, represented by alveolar macrophages, plays a crucial role in the perpetuation of fibroproliferative processes in diffuse parenchymal lung diseases (37).

Although the Th-1/Th-2 paradigm in fibrotic disorders is intriguing, a Th-2 immune response does not fully explain the occurrence of pulmonary fibrosis. For example, asthma does not typically involve progressive fibrosis but is

associated with elevated levels of Th-2 cytokines and pulmonary fibrosis in patients with progressive systemic sclerosis occurs in the absence of a Th-2 response. Since other cells beside T-helper lymphocytes express 'Th-2 cytokines', it is more accurate to define certain diseases in terms of cytokine profiles than the predominant T- helper subset. Mast cells, epithelioid cells, fibroblasts and monocytes are all able to express 'Th-2 cytokines' such as IL-4, IL-5, IL-9 and IL-10 (38-41).

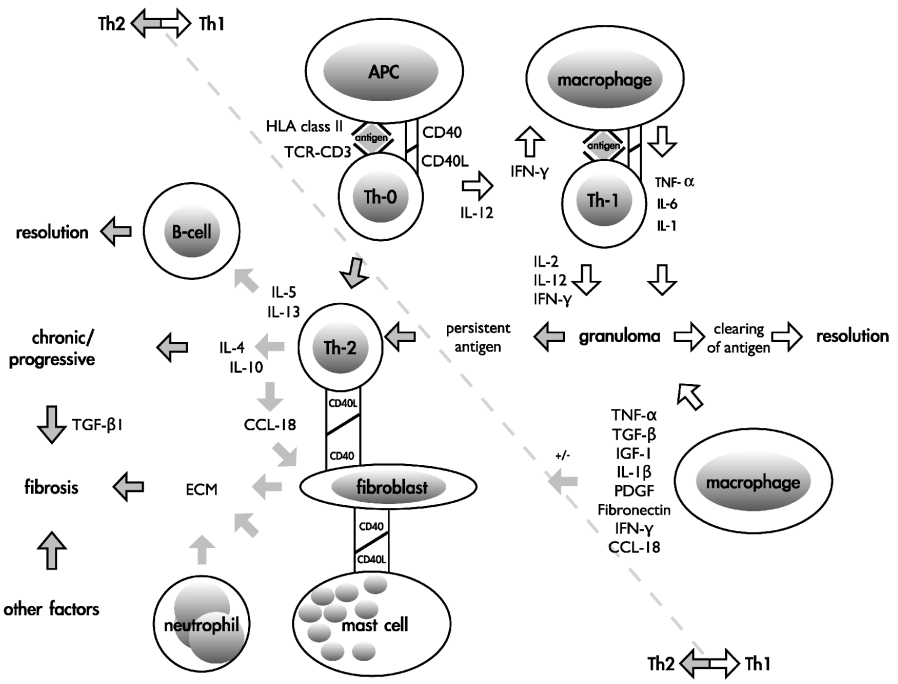


Figure 2 The Th-1/Th-2 paradigm in the development of fibrosis of the sarcoid granuloma.

The failure to eliminate the antigen through the initial Th-1 immune-response may cause a switch to a Th-2 response. The Th-2 associated cytokines may either lead to resolution of the granuloma or fuel the progression with fibrotic development. The meticulously orchestrated actions of interleukins and other factors, the recruitment of cells and the interactions between numerous cell types are all key in the fate of the granuloma.

Some reports have established a connection between cytokine expression profiles and the prognosis of sarcoidosis. For instance, the outcome of sarcoidosis is predicted by the inverse levels of TNF-α, a pro-inflammatory cytokine and TGF-β1, an anti- inflammatory cytokine. Low TNF-α and high TGF-β1 in broncho-alveolar lavage fluid (BALF) are indicative of resolution of sarcoidosis whilst high TNF-α and low TGF-β1 levels predict chronic progression (42-44). In the case of sarcoidosis, the question as to when and how the granuloma progresses into a destructive fibroproliferative disease remains suggestive at best. The fate of the granulomatous tissue may be attributed to a myriad of cytokines which plays a crucial role in inflammation as well as in tissue repair and remodeling. Disturbances in the carefully orchestrated interactions between cells, cytokines and other factors may lead to an exuberant fibrotic response in the sarcoid granuloma.

2. PULMONARY FIBROSIS

Fibrosis is exaggerated scarring of tissue and is often defined as wound healing that has gone awry. Wound healing takes place in response to tissue damage inflicted by inflammation, noxious stimuli, and other (unknown) causes. Under normal circumstances, when the causative agent of the inflammation has been eradicated by the immune system, the wound is repaired by the formation of connective tissue which replaces the destroyed or damaged cells of the injured tissue. Basically, four components are involved in this process: 1) angiogenesis, which involves the proteolytic degradation of the parent vessel basement membrane, the formation of capillary sprouts, proliferation and migration of endothelial cells, and recruitment and proliferation of pericytes and smooth muscles cells, 2) migration and proliferation of fibroblasts which are mediated by growth factors derived from platelets, endothelial cells and inflammatory cells such as macrophages, 3) deposition of ECM by fibroblasts containing mostly collagens type I and III but also a variety of other connective tissue components, and 4) maturation and reorganization of fibrous tissue (scar tissue remodeling) in order to restore tissue function (45).

Fibrosis is characterized by a disproportionate increase of extracellular matrix (ECM) proteins (46). As will be elaborated on in paragraph 2.2, the exuberant ECM production is believed to originate from an imbalance between cytokines which play a role in tissue healing and remodeling. Fibrosis can occur in virtually any organ and the mechanisms underlying tissue injury with fibrotic development may all share a common pathway.

Granulomas feature in many interstitial lung diseases which culminate in fibrosis, although not all interstitial lung diseases with fibrosis are preceded by granulomas. Cases of interstitial lung diseases which are not hallmarked by granulomas include drug-induced fibrosis (e.g. bleomycin, nitrofurantoin), interstitial pneumonias (desquamative, lymphocytic), systemic collagen vascular disease, radiation-induced fibrosis and nonspecific interstitial pneumonia/fibrosis (47). These fibrotic entities show considerable variation in the patterns of scarring. Most interstitial lung diseases (granulomatous or not) show three types of histological patterns of fibrosis: 1) intraluminal buds that protrude into the alveoli and/or distal bronchioles, 2) obliterative changes, in which loose connective tissue masses destroy the lumens of alveoli, alveolar ducts or distal bronchioles and 3) mural incorporation of intraluminal

connective tissue masses, which fuse with alveolar, alveolar ductal, or bronchiolar structures (48). These three patterns have common morphologic features suggesting that, regardless of their severity, they result from a common pathogenetic mechanism, i.e. the accumulation of ECM and destruction of tissue structure (48).

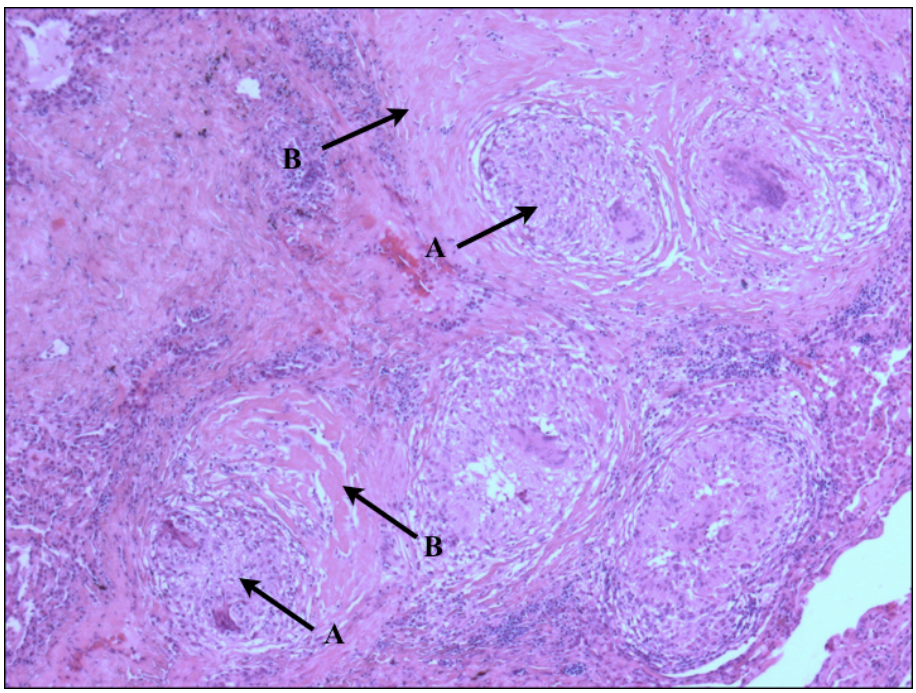


Figure 3 Histochemical staining of a lung tissue biopsy from a sarcoidosis patient who succumbed to respiratory failure due to advanced pulmonary fibrosis.
The sarcoid granulomas show inflammatory cells (A) and the intrusion of excessive fibrous cell mass (B).

As seen in approximately 10 percent of patients with sarcoidosis, the granulomatous inflammation becomes chronic with concomitant wound healing of the inflamed tissue which ultimately leads to the formation of scar tissue, i.e. fibrosis (7) (figure 3). As with other granulomatous disorders, the pattern of fibrosis in sarcoidosis may either be restriced to the granuloma or

exhibit a more diffuse pattern. The latter type of fibrosis may result from granulomatous activities of cells that are not necessarily organized in a granuloma. The precipitating factor may either be diffusely present throughout the lung or the responding cells may not be able to assemble into organized structures (49). The spatial and temporal events in pulmonary fibrosis are dictated by inflammatory cells and their cytokines as well as by other components which promote the production of ECM.

2.1 Cell types in granulomas involved in pulmonary fibrosis

In sarcoid granulomas, fibrosis may occur as proliferating fibroblasts move from the perimeter towards the center, replacing the granulomatous tissue with fibrous mass. These fibrotic changes are even sometimes seen in early granulomas (50, 51). A more diffuse, progressive fibrosis may develop subsequently when the surrounding tissue has sustained significantly more damage (49). Different cell types are known or believed to play functional roles in the events that take place in the onset and/or evolution of fibrosis. Interactions between cells through the signaling of cytokines and growth factors as well as direct communication between fibroblasts and adjacent cell types are essential to the propagation of fibrosis.

Macrophages

Fibrosis is controlled by a variety of inflammatory cells, but mostly by macrophages and the vast array of cytokines they elaborate which directly stimulate fibroblasts to proliferate and deposit ECM. As detailed earlier, the macrophage is the driving force behind the Th-1 response in sarcoidosis and plays a pivotal role in the development and progression of the granuloma through the macrophage-derived cytokines and growth factors.

Neutrophils

Aside from the hallmark cell types that typify the sarcoid granuloma, neutrophils are also often increased in number in the BALF of patients with advanced radiographic stages (III/IV) (52, 53), yet the exact role of neutrophils in fibrosis is poorly defined. It has been suggested that the chemotactic activity for monocytes and lymphocytes of neutrophil-derived serine proteases may play a role in switching acute inflammation to chronic inflammation which may precede fibrosis (54). In addition, neutrophils excrete cathepsin G which

is able to convert angiotensin I into angiotensin II (55), a potent growth factor for pulmonary fibroblasts (56). Contrasting features of neutrophils in fibrosis are based on the finding that these cells express collagenases (MMP-2 and MMP-9) in BALF of IPF patients (57). The ability of neutrophils to produce pro- (cathepsin) as well as anti-fibrotic (MMPs) factors illustrate the complexity of these cells in relation to sarcoidosis with pulmonary fibrosis.

Eosinophils

Another cell type associated with pulmonary fibrosis is the eosinophilic granulocyte. The classic role of eosinophils pertains to the immune response to a parasitic infection. Eosinophils take part in the formation of a fibrous capsule around the unsuccessfully eradicated worm cyst. Their granules contain pro-fibrotic cytokines such as IL-1β, IL-6, IL-8, TNF-α and TGF-β1. This fact has led researchers to believe that eosinophils might have a substantial involvement in fibrotic diseases. A study by Hao et al. showed, however, that eosinophils were not indispensable in bleomycin-induced pulmonary fibrosis in mice (58). Moreover, not all eosinophilic pneumonias in which excessive numbers of eosinophils are infiltrating the lung parenchyma will necessarily culminate in fibrosis (59). Yet, in patients with IPF or sarcoidosis, the increased number of eosinophils has been found to correlate with a worse prognosis of both diseases (60, 61). It seems therefore feasible that eosinophils may not actually cause fibrosis, but rather fuel the preexisting fibrotic changes in injured tissue.

Mast cells

The role of mast cells in the development of pulmonary fibrosis of different origins has been acknowledged for some time. Mast cells are reportedly increased in advanced radiographic stages of sarcoidosis and are also found to correlate with impaired lung function and increased levels of fibrosis markers (62). In fact, the number of mast cells has been found to be indicative of the extent to which fibrosis occurs in various pulmonary disorders (63). Direct cell-cell interactions between the CD40 receptor on mast cells and the CD40 ligand (CD40L) on T-cells and fibroblasts have further defined the implication of mast cells in pulmonary fibrosis (64). Mast cells are a major source for IL-4, IL-5, IL-6, IL-10, latent TGF-β1, TIMP-1, MMPs and basic fibroblast growth factor (bFGF). The number of bFGF-containing mast cells is found to be increased in pulmonary fibrotic disorders, including sarcoidosis (65). Mast cell

granules also constitute serine proteases that directly or indirectly stimulate fibroblast proliferation. Two of these enzymes, chymase and tryptase, are used to determine two sets of mast cell phenotypes: chymase/tryptase-positive and tryptase-positive (chymase-negative) mast cells (66, 67). These phenotypically different cells are each presumed to have distinct roles in different pathological conditions. Recently, Edwards et al. found that mast cells expressing chymase (as well as other proteins) in lung biopsies of sarcoidosis patients were localized at the sites where tissue remodeling was prominent (51). The role of chymase in fibrotic disorders is addressed in detail below.

2.2 Pro- and anti-fibrotic factors in pulmonary fibrosis

As mentioned earlier in this chapter, the occurrence of fibrosis as a response to tissue injury is likely due to the imbalance between factors that either promote or counter the accumulation of ECM. Over the last decade, considerable evidence has emerged suggesting that cytokines are important stimuli to ECM deposition in pulmonary fibrosis (68). Our knowledge of the actions of cytokines involved in the fibrotic response of the lung has been gained from animal studies. Agents such as irradiation, bleomycin, cyclophosphamide, and silica are administered to animals in order to inflict lung injury with subsequent inflammatory and fibrotic responses.

A vast number of cytokines are implicated in pulmonary fibrosis, of which most have been investigated in the inflammatory response and disease evolution in sarcoidosis. Of the array of cytokines, TNF-β, TGF-β1, interleukins (e.g. IL1β, IL-2, IL-13, IL-18), platelet derived growth factor (PDGF), and endothelin (ET)-1 are classified as having pro-fibrotic properties, while IFN-γ and prostaglandin E_2 (PGE_2) are known to exert anti-fibrotic actions in pulmonary fibrosis (69-72). To complicate matters more, some cytokines, such as TGF-β3, are known to exert both pro- and antifibrotic actions, which appear to manifest in the context of expression levels of other cytokines (73).

There is also a significant number of factors of which the biochemical properties are known in context of normal physiology. Yet these factors' functions become oblique when placed in the context of pathogenesis where they are found to be highly expressed at the site of inflammation or injury. Such factors may be part of an endogenous, physiological environment that is present in normal tissue. Upon tissue injury, this physiological system may be

utilized to trigger events that lead to tissue repair and remodeling. The renin-angiotensin-aldosterone system (RAAS), for instance, is also believed to play a central role in the development of fibrosis (74). In particular, the profibrotic properties of angiotensin II (Ang II) is receiving due attention.

Cytokines

In sarcoidosis, TNF-α can act as a strong inducer of pulmonary fibrosis (49). Armstrong et al. found that in stage II/III sarcoidosis patients, the TNF receptor (TNF-R) was elevated as compared to patients with stage I sarcoidosis. The authors concluded that soluble TNF-R may sequester bioactive TNF-α and consequently hamper the chronic development of sarcoidosis (75). Anti-TNF-α is in fact a promising therapeutic regimen for the treatment of sarcoidosis (76). TGF-β1 is probably the most extensively investigated cytokine in relation to fibrosis. Its actions are vast and they vary depending on the disease. It can stimulate fibroblast proliferation through an autocrine pathway leading to subsequent expression of collagens and other ECM components (77-79) and it is also capable of inhibiting MMPs (80, 81). Exuberant expression of TGF-β1 can lead to progressive fibrosis which culminates in organ dysfunction (79).

TGF-β1 has both pro- and anti-inflammatory as well as pro- and anti-fibrotic properties, depending on expression levels and the type of cell that produces it (79, 82, 83). The complexity of actions exerted by TGF-β1 is illustrated by the notion that high expression of TGF-β1 in sarcoidosis patients is associated with a favorable prognosis (42, 43), while lung function is decreased in sarcoidosis patients who exhibit elevated TGF-β1 levels (84). In granulomas, TGF-β1 is mainly produced by monocytes and macrophages, eosinophils, mast cells, neutrophils and platelets, although most of these cell types produce only the latent (inactive) form. Latent TGF-β is bound to the so-called latency-associated protein (LAP). Disassociation of LAP from TGF-β is activated by several enzymes, including IL-13 (83) and chymase (85). Consequently, active TGF-β is rendered available for binding to its receptors TGFβ-receptors II (except TGF-β2) and III, leading to various downstream effects.

The link between inflammatory responses in granulomas and the evolution toward fibrosis was supported by a study by Roman et al (50). In their study, experimental murine granulomas and human sarcoid granulomas showed similar spatial patterns of ECM deposition distribution, even in early granuloma formation. TGF-β1 was found to be highly expressed by macrophages and

giant cells and on fibroblasts in fibrotic areas.
More recently, factors that were previously known for their potent pro-fibrotic characteristics have shown to exert milder or transient fibrotic actions. TGF-β3 is the most intriguing in this regard (figure 4). *In vitro* studies have demonstrated that all three TGF-β isoforms are potent growth factors for primary lung fibroblast proliferation with subsequent deposition of ECM (81). However, *in vivo* studies have shown that exogenous administration of TGF-β3 is able to reduce scarring in cutaneous wounds by downregulating TGF-β1-induced ECM deposition (86). Furthermore, TGF-β1-driven fibrosis in injured rat lungs is also mitigated by TGF-β3 administration while TGF-β3 administration in the absence of elevated TGF-β1 results in a more transient, non-persistent type of fibrosis (73). These studies suggest that a balance between the expression patterns of TGF-β1 and TGF-β3 isoforms is important in preventing the deleterious effects of prevailing expression of TGF-β1.
Similar co-dependent behavior of cytokines which control the outcome of injured tissue repair was recently found for connective tissue growth factor (CTGF) and TGF-β1. CTGF alone induced transient fibrosis in rat lungs, while TGF-β1 was needed to sustain permanent fibrosis (87). CTGF is abundantly present in the lungs and its expression levels are found to increase with advanced radiographic stages (III and IV) in pulmonary sarcoidosis (52).

Matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP)
ECM turnover is mediated through a balance of synthesis and degradation through the action of matrix metalloproteinases and their inhibitors, TIMPs. Degradation of ECM is accomplished by MMPs, which are collagenases involved in the process of tissue remodeling. MMP-8 and MMP-9 activity is elevated in IPF and chronic sarcoidosis patients (88). MMP-8 is the major contributor to the collagenase activity in the airways of patients with idiopathic pulmonary fibrosis and sarcoidosis and may initiate collagen destruction and remodeling which are associated with the development of pulmonary fibrosis (88). Furthermore, MMP-1 expressed by mast cells has also been found at the site of tissue remodeling in pulmonary sarcoidosis (51).
The recent discovery of a collagen receptor, discoidin domain receptor 1 (DDR1), showed that the deterioration of pulmonary sarcoidosis was accompanied by a DDR1 subtype-dependent upregulation of MMP-9 and MCP-1 in CD14-positive BALF cells (89). Components that act directly on the

accumulation of ECM include the tissue inhibitor of metalloproteinases (TIMP)-1 through TIMP-4. TIMPs are stromal factors with multiple functions which favor the accumulation of ECM by antagonizing the actions of MMPs (90). An immunohistochemical study by Gonzales and colleagues revealed that the cells in sarcoid granulomas contain an abundance of MMPs and a paucity of TIMPs (91). The observation that TIMPs are down-regulated implies that these enzymes are not the cause of fibrosis, but rather respond to the pro-fibrotic milieu by allowing MMPs to prevail during matrix degradation.

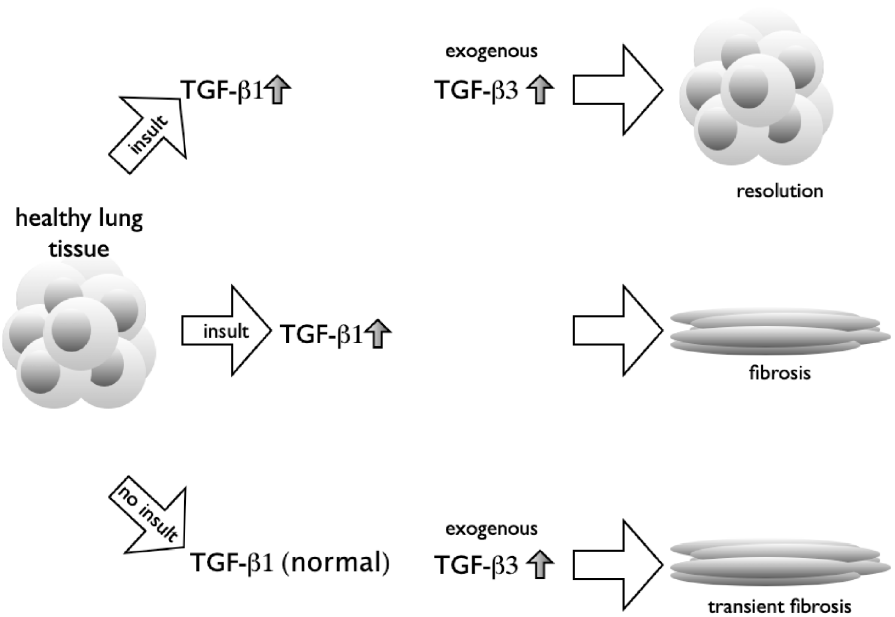


Figure 4 Differential transforming growth factor (TGF)-β1 and β3 levels in the fibrotic response to lung tissue injury.
Elevated TGF-β1 as a result of tissue injury causes progressive fibrosis. The concurrent presence of elevated TGF-β3 thwarts the progression towards fibrosis, while TGF-β3 administration to healthy tissue (normal TGF-β1 levels) will cause transient, non-sustainable fibrosis.

The renin-angiotensin-aldosterone system (RAAS) in tissue injury and fibrosis

Tissue injury caused by inflammation or other causes triggers inflammatory cell recruitment, the production of cytokines, growth factors, chemokines and enzymes. These components all serve the purpose of healing the tissue with all means necessary. Their biochemical and immunologic properties are commonly investigated contextually, i.e. by the merit of each of these properties in inflammation/injury, healing, remodeling and fibrosis.

Interest in the pathogenesis of sarcoidosis in relation to the RAAS is undoubtedly inspired by the aberrantly elevated circulating ACE levels, a hallmark of this diffuse lung disease. Disease activity of sarcoidosis is monitored by circulating ACE activity and although it serves as a reasonably reliable marker (92, 93), the exact role of ACE in disease progression still remains elusive. The elevated ACE expression originates from the active granulomas which are present in the sarcoidosis-affected organs. The total mass of active granulomas seems to correlate with circulating ACE levels, i.e. radiological stages II/III correspond with the highest ACE levels (94).

Ang II is found within sarcoid granulomas suggesting that ACE is functionally active in the local inflammation (95). One of the first studies to address the role of Ang II in granulomas was performed in an experimental model using a mouse infected with schistosomiasis (96). The lesions in the liver were found to contain components of the entire RAAS. Subsequent studies revealed that granuloma macrophages, but not lymphocytes, release Ang II (97).

Evidence suggests that Ang II is not only key in the systemic RAAS, but that locally formed Ang II is a crucial element in the events that take place in tissue injury and remodeling, thus in a locally acting RAAS.

Systemic RAAS

The RAAS is a system of enzymes and hormones that regulate blood pressure and electrolyte and fluid homeostasis in mammals. As one of the body's humoral cardiovascular mechanisms, the RAAS plays a major role in the regulation of sodium, potassium and water balance, vascular tone and sympathetic nervous system activity. Renin is synthesized and stored in the juxtaglomerular cells of the kidney and is released in response to decreases in renal perfusion pressure and decreases in sodium and chloride concentrations in the kidney, as well as to the presence of catecholamines and Ang II. Renin catalyzes the concentration of angiotensinogen from the liver to Ang I. Angiotensin-converting enzyme (ACE) is mostly present on the surface

membrane of the vascular endothelial cells of the lungs. ACE cleaves off two amino acids from Ang I to form the octapeptide Ang II. The actions of Ang II include, but are not limited to, vasoconstriction and the release of aldosterone from the adrenal cortex. Aldosterone acts upon the kidneys to increase sodium and fluid retention and it enhances vascular permeability and smooth muscle contraction, all of which are actions to raise blood pressure (98).

Local RAAS

The local formation of Ang II exerts a variety of cytokinetic effects under normal or pathological conditions (99-102). For instance, Ang II has the ability to stimulate fibroblast proliferation in the human lungs (56). Ang II acts on the G protein-coupled receptors called type 1 and 2 (AGTR1 and AGTR2) which are present in organs such as the heart (103, 104), kidneys (105) and lungs (106). The AGTR1 type is known for its more deleterious effects upon binding of its ligand, namely vasoconstriction and cardiovascular hypertrophy. The AGTR2 receptor is believed to regulate opposing effects although the complete picture of this process remains to be elucidated (107, 108).

The discovery of other Ang II-forming enzymes, especially those that seem to act in a more local manner has yielded great insights into the mechanisms and growth factors involved in tissue repair and remodeling following inflammation, ischemia or noxious stimuli. The mast cells and neutrophils that are present in granulomas are also able to convert exogenous Ang I to Ang II by mast cell chymase and neutrophil cathepsin G, respectively (55).

Chymase, a serine proteinase, is predominantly stored in the cytoplasmic secretory granules in mast cells and is released following mast cell activation (109) (figure 5). Chymase positive mast cells are present in a number of organs including the heart, skin and lung (110-112). Expression of chymase is, however, not limited to mast cells but is also found in mesangial cells and vascular smooth muscle cells in the kidney (113). In the human heart, chymase is localized in the cardiac interstitium, endothelial cells and some mesenchymal interstitial cells (114). The affinity of chymase for its substrate Ang I is much higher than that of ACE and this has led to the assumption that chymase is the main enzyme, at least locally, for Ang II formation (115). In fact, chymase is found to be the major enzyme for Ang II formation in the human lung (116). Direct involvement of chymase in the matrix collagen accumulation has also been described, with equivocal reports that either support a fibrogenic or anti-fibrogenic character of chymase. For instance,

mast cell chymase is able to cleave type 1 procollagen to collagen fibrils *in vitro*, suggesting a fibrogenic role for chymase in the formation of matrix collagen depositions (117). By contrast, chymase causes degradation of the collagen matrix by activating latent matrix-metalloproteinase (MMP-1) (118-120) and by inactivating TIMP-1 (121), suggesting anti-fibrogenic rather than fibrogenic properties. Pharmacological inhibition of chymase has been proven to successfully abrogate the development of fibrosis in animal models (67, 122) which indicates a pro- rather than antifibrotic nature of chymase.

Factors that provide a putative feedback mechanism for Ang II formation include a novel homologue of ACE, namely ACE2. ACE2 is a membrane-associated and secreted enzyme which is abundantly present on the surface of lung alveolar epithelial cells (123). ACE2 shares a 42% homology with ACE and the genomic structure of ACE2 indicates that both genes have evolved from a common ancestor (124). In vitro studies have shown that ACE2, unlike ACE, is able to convert Ang I to Ang 1-9 but unable to cleave bradykinin (124). Whereas Ang II is a potent vasoconstrictor, Ang 1-9 has no known effects other than being a substrate for ACE, resulting in Ang 1-7, a putative vasodilator (125). Thus, ACE2 seems to counter the formation of Ang II by ACE, as confirmed by studies using ACE2 knockout mice (126). Although no studies to date have addressed the possible role of ACE2 in sarcoidosis, the observed Ang II modulation by ACE2 in injured sites of other disease models which are similar to the systemic model (126) suggests an analog role for ACE2 in compensating the pro-fibrotic effects of Ang II formation by ACE or other Ang I-converting enzymes (figure 4). In fact, recombinant ACE2 administration has been shown to markedly mitigate severe acute lung injury in mice (108).

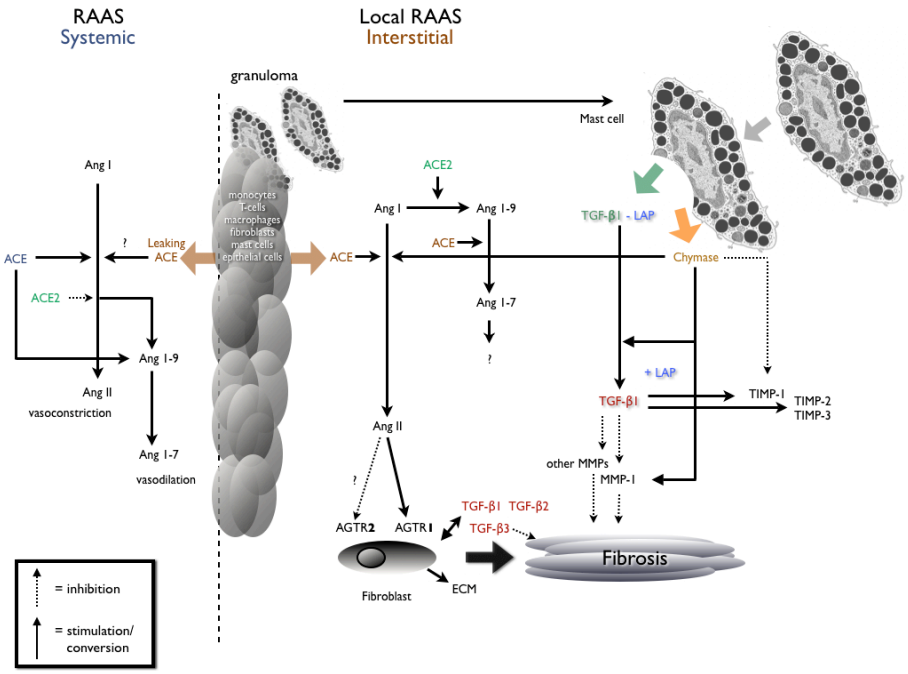


Figure 5 The putative role of the Renin-Angiotensin-Aldosterone System (RAAS) in the sarcoid granuloma and fibrosis.

Angiotensin II (Ang II) is generated by both chymase and ACE, originating from the granuloma. Ang II binds to the AGTR1 receptor which leads to fibroblast proliferation through the autocrine actions of TGF-β1 with subsequent extracellular matrix (ECM) deposition. Chymase may concomitantly inhibit tissue inhibitors of MMP-1 (TIMP-1) and activate MMP-1 to promote ECM degradation. ACE2 negates the formation and potentially deleterious effects of Ang II. Unlike the presence of local ACE, ACE2 and chymase, the systemic actions of these enzymes are separate from their local actions and may not have significant bearing on the fibrotic changes in the sarcoid granuloma.

2.3 Prognostic markers for sarcoidosis with fibrotic evolution

Many reported studies have assessed a wide range of clinical, radiologic, and serologic/biochemical sarcoidosis disease markers (23, 127). Despite these efforts, not one marker has shown its utility when used as a single measure for disease activity or prognosis. Chest radiography and respiratory function tests are still the best indices of lung dysfunction and failure, despite their inability to predict the course or outcome of sarcoidosis. These tests still remain the standards against which novel putative disease markers are assessed.

Classic biochemical and serologic markers

As mentioned earlier, the measurement of ACE levels in blood has not been shown to have any prognostic value for the course of sarcoidosis, let alone being predictive for the development of pulmonary fibrosis. It appears that ACE, for lack of better alternatives, is still the most commonly used disease activity marker in clinical practice, despite its low specificity. Serum lysozyme, neopterin, beta$_2$-microglobulin, and adenosine deaminase, to list a few, have not been able to show superiority to ACE as markers for sarcoidosis disease activity nor have any of these markers proven their merit in predicting the course of the disease or pulmonary fibrosis (128-134).

Serum procollagen III peptide (S-PCP-III) has been shown to correlate well with disease progression and to be predictive for the development of fibrotic sarcoidosis (135) as do collagenase levels in BALF (136). While the latter marker has a better specificity, the application of marker measurements in BALF is not practical since performing a regular lavage during disease follow-up would be cumbersome.

Cytokines

Novel candidates for sarcoidosis disease markers that continue to receive considerable interest include the soluble IL-2 receptor (sIL-2R) (43, 137), receptors for TNF-α TNF-RI (the 55-kD receptor) and TNF-RII (the 75-kD receptor) (138), IL-12 in serum (139) and in BALF (140), and IL-8 (141). Most of these studies have delivered convincing or promising results in terms of improved sensitivity and specificity and have shown the merits of these markers for predicting the course of sarcoidosis.

Krebs von den lungen-6 (KL-6)

KL-6, a mucin excreted by type II cells in the lungs, has been evaluated as a novel marker for diffuse lung diseases. KL-6 is found to be increased in progressive fibrotic disorders of the lung including IPF (142) and pulmonary fibrosis in scleroderma (143). Besides being a marker for pulmonary fibrosis, an active role for KL-6 in the pathogenesis of fibrosis has been suggested. KL-6 is chemotactic for fibroblasts which indicates a possible active role in the development of fibrosis (144). Attempts to utilize KL-6 as a useful disease activity marker for sarcoidosis have also been reported and have shown promising results in terms of monitoring disease activity (145, 146) as well as predicting pulmonary disease progression (147), however, KL-6 is not sufficiently specific to qualify as a diagnostic tool (147).

Clara cell protein 10 (CC10)

CC10 (CC16, uteroglobulin), a low-molecular-weight protein of 10 kDa, is secreted in large amounts into the lumen of the respiratory tract by nonciliated bronchiolar Clara cells (148). CC10 has potent immuno-suppressive properties and is believed to be an important down-regulator of airway inflammation (149). CC10 levels are found to be increased in serum, but not in BALF in sarcoidosis, meaning that CC10 levels in serum are not a reflection of augmented expression but are rather due to an increased air-blood barrier permeability (150). Moreover, serum CC10 levels have been correlated positively with radiologic progression (147, 150) and spontaneous regression of sarcoidosis (151).

Surfactant protein (SP)-A and SP-D

Both SP-A and SP-D, excreted by type II pneumocytes, have also shown their merits in predicting the course of pulmonary fibrosis. Greene at al. found that serum SP-A and SP-D levels were significantly elevated in patients with IPF and systemic sclerosis and that SP-D correlated with radiographic abnormalities in patients with IPF (152). In addition, the authors found that both serum SP-A and SP-D levels were highly predictive of survival in patients with IPF (152). The same study reported that a correlation was only found between SP-D and sarcoidosis patients with parenchymal involvement, while no details were provided regarding sarcoidosis patients with pulmonary fibrosis (152).

YKL-40

YKL-40 (human cartilage glycoprotein 39), a 40 kD glycoprotein produced by e.g. inflammatory cells (153, 154), has been investigated as a new marker for cancer. Elevated serum YKL-40 has been found in all cancers studied to date including lung cancer (155).

More recently, YKL-40 was shown to be a promising candidate for a marker for sarcoidosis (156). Not only has this protein proven to be a valuable tool for monitoring disease activity, but YKL-40 levels were also associated with a decreased lung diffusing capacity (Dlco), suggesting that YKL-40 correlated with the presence of fibrosis.

It is, however, unlikely that merely one sarcoidosis activity or prognostic marker will suffice in assessing the patient's disease status and outcome. A reliable tool for monitoring sarcoidosis will likely comprise multiple markers that will each complement a complete body of clinical information.

2.4 Therapeutic interventions for sarcoidosis with pulmonary fibrosis

The common regimen for the treatment of sarcoidosis is the use of corticosteroids. Although the short-term efficacy is generally successful, the long-term effects of corticosteroids have not been proven (157). Other steroid-sparing agents have been evaluated as an option for treatment of patients with chronic sarcoidosis. These include cytotoxic drugs, antifungal compounds, interleukin-inhibiting drugs, and folate antagonists (158). Each agent has proven to be more or less efficacious depending on the sarcoidosis phenotype (159).

New insight into the roles of cytokines and other factors important to the formation and evolution of the granulomas have led to speculation that new treatment strategies will specifically target these cytokines and factors. TNF-α is currently receiving due attention as a new target in the treatment of sarcoidosis (76).

It has been acknowledged that Ang II may be a potential target for the treatment of fibrotic development upon tissue damage. In fact, several reports have provided evidence for deploying pharmaceutical inhibitors of ACE (160, 161) and chymase (122), as well as AGTR1 blockers (162-164) in thwarting the development of fibrosis in injured tissue. A publication in Nature by Imai et al.

supports the notion that therapeutic interventions targeting RAAS components may be efficacious in hindering the progression of pulmonary injury (108). The authors found that recombinant ACE2 administration can mitigate the severity of severe acute lung failure in mice (108).

Recently, a retrospective study conducted at the Mayo Clinic failed to demonstrate the efficacy of ACE-inhibitor therapy in IPF patients in terms of survival (165). These results seemed to reject the implication of Ang II in the pathogenesis of IPF.

One may propose that ACE inhibition might abrogate the effects of Ang II-mediated fibrosis in stage IV sarcoidosis patients, but ACE levels can reach a magnitude of the normal values without showing any correlation with fibrosis. This lack of Ang II-mediated pro-fibrotic effects may be explained by the negligible influence of circulating ACE and by the fact that local rather than systemic levels of Ang II contribute to an unfavorable progression towards fibrosis.

3. GENETIC ASPECTS OF SARCOIDOSIS

The observation that disease incidence is linked to race, ethnicity and clusters in families led early investigators to hypothesize that there might be a genetic predisposition to sarcoidosis (166). Some years later, an elaborate study named ACCESS (A Case-Control Etiology Study of Sarcoidosis) provided clear evidence for increased risk of contracting sarcoidosis among first- and second-degree relatives of sarcoidosis cases compared with relatives of matched control subjects (167).
Sarcoidosis is likely to be a genetically complex disease that involves a combination of genetic loci (polygenic) conferring disease predilection or phenotypic variation of disease manifestation.

3.1 Approaching the genetic aspects of diseases

There are a number of approaches that can be taken to evaluate the genetic basis for a given disease. In the case of sarcoidosis, linkage analysis in families affected with sarcoidosis and association analysis of genetic polymorphisms such as 'single nucleotide polymorphisms' (SNP) are popular means of establishing the genetic compound of sarcoidosis disease susceptibility. The association is performed in a case-control setting with unrelated case subjects compared to unrelated control subjects. Significant differences in allele or genotype frequencies between cases and controls are taken as evidence for involvement of an allele in disease susceptibility. Alternatively, allele or genotype frequencies may also be compared between phenotypically different subsets within the patient group and thus provide information on the disease-modifying properties of the polymorphic site.
SNPs are defined as 'DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered.' Each individual has many single nucleotide polymorphisms that together create a unique DNA pattern for that person. SNPs are generally considered to be a form of point mutation that has been evolutionarily successful enough to recur in a significant proportion of a species' population. A SNP differs from a (point) mutation by its frequency observed in a given population. This frequency has been set arbitrarily at 1%. SNPs can influence the expression of the gene depending on the location in which they are found. A SNP located in an exon

may cause a codon change that consequently may cause an amino acid substitution, while SNPs in the intron/exon boundary may potentially influence intron splicing. Promoter (5′ region) polymorphisms can change the consensus sites of transcription factor binding which may result in either diminished or enhanced gene expression (168). Finally, the 3′ untranslated region may contain sequences that regulate translation efficiency, mRNA stability, and polyadenylation signals (169). The presence of SNPs in this region may thus alter these regulatory properties.

A variation on the approach to investigate the direct functional effects of polymorphisms on a disease is the utilization of SNPs that may exist in strong linkage with a putative functional SNP. With this approach, the association between the SNP and disease can be observed vicariously through the functional SNP.

SNPs that show significant linkage disequilibrium with each other are grouped to form haplotypes (a contraction of the phrase "haploid genotype"). The identification of a few alleles of a haplotype block unambiguously identifies all other polymorphic sites in this region. Haplotypes have a great advantage over individual SNP evaluation, in particular when multiple disease susceptibility alleles are present with the haplotype block (170). The disadvantage of this approach is the significant reduction of the number of subjects who carry a specific haplotype that can be analyzed. Consequently, the statistical power needed to identify any association will be reduced.

3.2 Polymorphisms in sarcoidosis

Genetic studies on sarcoidosis have demonstrated that specific gene polymorphisms are involved in both susceptibility to and phenotypic determination of the disease (171). These polymorphisms include genes governing antigen presentation such as human leukocyte antigen (HLA) genes for the major histocompatibility complex (MHC), cytokines such as TNF-α, lymphotoxin-α, IL-1α, and chemokines (172-176). In addition, recent reports indicate an important contribution of T-lymphocyte co-stimulatory genes such as BTNL2 to sarcoidosis susceptibility (177).

A study revealing the first link between a cytokine gene polymorphism and the prognosis of sarcoidosis was established in a population of Japanese sarcoidosis patients (178). The TNFB*1 allele variant of the TNF-β gene was identified as a marker for prolonged clinical course in patients with

sarcoidosis. Subsequent studies have shown that the DQB1*0201 allele is strongly protective against severe sarcoidosis in British and Dutch Caucasians, in which it is strongly associated with stage I disease, whereas DQB1*0602 tended to have opposite effects (179). The same study found a clear association between the *0201 allele and Löfgren's syndrome which indicated that carriage of this allele reduced the risk of disease progression (179). The power of haplotype construction is illustrated by a case-control study by Spagnolo et al. in which the C-C chemokine receptor 2 (CCR2) was analyzed in a group of Dutch sarcoidosis patients. One specific haplotype was found to be strongly associated with the propensity to contract Löfgren's syndrome (176). A following study using sarcoidosis patients, their kin and control subjects, could not confirm the findings by Spagnolo et al., although the authors suggested that CCR2 is in strong linkage with a putative gene that may contribute to disease susceptibility (180).
In summary, these associations are illustrative of the approaches used in genetic studies on sarcoidosis and how investigations can yield direct (causative SNP) or indirect (in strong linkage with the functional SNP) evidence for the involvement of polymorphisms in the susceptibility or disease phenotypes of sarcoidosis.

3.3 Polymorphisms involved in the pathogenesis of sarcoidosis and other fibrotic diseases

Gene polymorphisms that warrant a predictive value to either disease susceptibility, severity, outcome or manifestation have contributed significantly to the understanding of the underlying pathogenesis of any given disease, including fibrotic disorders. A significant number of studies have focused on polymorphisms present in the genes of key factors involved in fibrotic diseases of different etiologies. Genes encoding for components which are believed or known to play a role in the pathogenesis of sarcoidosis have been subjected to scrutiny in various types of fibrotic disorders. These genetic association studies may be used as a lead when looking for the genetic predisposition for developing pulmonary fibrosis in sarcoidosis. A general overview of the polymorphisms in genes of cytokines, growth factors, RAAS components and extracellular matrix components which have been described in fibrotic diseases and sarcoidosis are described below.

Transforming growth factor (TGF)-β family

The TGF-β1 gene is one of the most extensively evaluated genes in the context of fibrosis. As this gene contains a number of functional SNPs that influence the expression levels, the likelihood of identifying the influence of genetically controlled TGF-β1 levels on the disease susceptibility or progression of potentially fibrotic diseases is assumed to be relatively high. Two well-known SNPs in the TGF-β1 gene are those present in exon 1. They both result in amino acid changes in codon 10 (Leu -> Pro) and codon 25 (Arg -> Pro) and they are both associated with inter-individual variation in TGF-β1 production (181). These, as well as other polymorphisms of TGF-β1, have been shown to confer an increased risk to e.g. the rate or severity of hepatic (182, 183) and pulmonary (184-186) fibrosis. Studies on gene polymorphisms of the TGF-β family in sarcoidosis, however, are scant. SNPs in the TGF-β1 gene present in codon 10 and codon 25 have been evaluated for implication in sarcoidosis disease progression in Japanese (187) and Caucasians (188). However, no associations were found between polymorphisms and disease progression, susceptibility or severity in either study.

Isoforms TGF-β2 and TGF-β3 gene polymorphisms have also been identified and tested for associations with various (fibrotic) disorders. For instance, polymorphisms in both TGF-β2 and TGF-β3 genes were found to be associated with cutaneous fibrosis in systemic scleroderma (189).

Interleukins (IL)

Because interleukins exert key signaling actions between cell types in the process of inflammation, interleukin gene polymorphisms have been prime candidates for studies on such fibrotic diseases as IPF (IL-10) (172), (IL-1α, β) (172), (IL-6) (190), (IL-12) (191); sarcoidosis (IL-6) (192), (IL-1α, β) (172), (IL-10) (188); cryptogenic fibrosing alveolitis (IL-8) (193); liver fibrosis (IL-10) (194, 195); chronic (fibrotic) pancreatitis (IL-10) (196); and systemic sclerosis (IL-10) (197), (IL-8) (193). The expression levels of most of these cytokines are influenced by the polymorphisms present in the encoding genes. The IL-10 gene, for instance, has highly polymorphic sites that cause either higher (198) or lower (199) baseline expression. The results of the aforementioned studies in terms of finding associations between polymorphisms and the propensity to develop fibrosis vary widely, with some pointing towards a relationship between functional polymorphisms and either pace and severity of fibrotic

development, while others could not find any association. Although IL-1β (200) and IL-10 (188) SNPs do not seem to have any bearing on sarcoidosis disease phenotypes or outcome, the IL-6 –174 C/T promoter polymorphism was found to be associated with sarcoidosis patients who progressed towards pulmonary fibrosis (192).

Tumor necrosis factor (TNF)-α

Promoter gene polymorphisms in the TNF-α gene cause variation in expression levels (201, 202). TNF-α is regarded as a pro-fibrotic cytokine (203) and high expression levels may thus be unfavorable for the progression of sarcoidosis and other fibrotic diseases.

TNF-α gene variants are associated with the severity of pulmonary cystic fibrosis (204, 205) and have been found to attribute to the risk to developing IPF (206) and fibrosing alveolitis (207). Although it would make sense that polymorphism-attributed elevated TNF-α expression might fuel a fibrotic response following inflammation, ample studies have been unable to link high expression of TNF-α to fibrosis. For instance, hepatic periportal fibrosis (PPF), a major pathological consequence of infections with Schistosoma, is unrelated to TNF-α genetic variants (208).

In a study on sarcoidosis, haplotyping of the TNF gene revealed that haplotype 4 was increased in both Dutch and UK sarcoid patients (173). In addition, haplotype 2 was present at a higher frequency in patients suffering from Löfgren's syndrome, while haplotype 4 was lower compared to other sarcoidosis patients (173). TNF-α gene polymorphisms have, however, not been found to be associated with pulmonary fibrosis in sarcoidosis patients.

Interferon (IFN)-γ

The IFN-γ gene also has a number of SNPs that result in varying expression levels. Although IFN-γ is a key cytokine in the Th-1 driven response as seen in sarcoidosis, very few studies have addressed the possible influence of IFN-γ gene variants on the onset or development of sarcoidosis. Only one study by Akahoshi and colleagues revealed a positive association between polymorphisms in the IFN-γ gene that are known to influence expression and sarcoidosis disease susceptibility (209). Others have acknowledged the putative implications of the IFN-γ SNPs in relation to fibrosis, since genetically determined higher expression of IFN-γ, a potent suppressor of the fibrotic

response, might be beneficial to patients who are in danger of developing fibrosis upon tissue injury. Indeed, the IFN-γ +2109 A/G polymorphism is associated with a higher risk for developing periportal fibrosis (PPF), whereas the IFN-γ +3810 G/A polymorphism is associated with less PPF (210). In IPF patients, however, the 5644 G/A SNP does not seem to contribute to disease susceptibility (190).

Matrix metalloproteinases (MMP) and their inhibitors (TIMP)

Gene polymorphisms in the genes of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) make interesting candidates for the genetic influence on ECM accumulation, which hallmarks the development of fibrosis. Functional studies have revealed that some of the sequence variants in the MMP genes affect MMP gene expression. These include the MMP-1 gene (1G/2G, a run of 1 or 2 guanines), the MMP-9 gene (–1562 C/T) and the MMP-3 (5A/6A, 5 or 6 adenosines) polymorphism (211-213). MMP-1, MMP-3, and MMP-9 gene polymorphisms have been found to be associated with the progression of chronic liver disease in Japanese (214).

In sarcoidosis, the MMP-1 1G variant tended to be more prevalent in patients with ocular involvement as well as in patients with more than three organs involved (215).

The tissue inhibitors of MMPs, TIMP-1, TIMP-2 and TIMP-3 are also gaining interest in genetic studies. Polymorphisms at position –418 and +853 in the TIMP-2 gene are both associated with chronic obstructive pulmonary disorder (COPD), another disease in which lung matrix changes are key in the pathogenesis (216). Recently, the promoter variant of TIMP-3 was investigated in Mexican pigeon breeder's disease (PBD) and IPF to find a possible association between the –915 A/G or –1296 T/C polymorphisms and developing pulmonary fibrosis (217). Although no such associations were observed, haplotyping revealed an association between the two polymorphisms and the susceptibility to developing PBD and lymphocytosis in this group, suggesting that TIMP-3 plays a role in inflammation rather than the previously supposed fibrotic response in PBD (217). Furthermore, a genetic study employing microsatellite markers revealed a possible role for TIMP-1 in genetic susceptibility to systemic sclerosis in determining the degree of cutaneous fibrosis (189).

The RAAS

Genetic polymorphisms have been described in a number of RAAS components including Angiotensin-converting enzyme (ACE), ACE2, chymase, angiotensin receptors (AGTR)1 and AGTR2, bradykinin receptors and renin. Table 1 summarizes the genetic studies of RAAS component polymorphisms in fibrotic or fibrosis-related diseases.

Angiotensin-converting enzyme (ACE)

Cambien et al. were the first to indicate the genetic influence on ACE levels by revealing a major quantitative trait locus (QTL) responsible for nearly half of the variance in serum ACE levels (218) which was later identified as a polymorphism in the gene encoding for ACE. This polymorphism is caused by an insertion of a 287 bp fragment within the intron 16 of the ACE gene and is present in a high frequency in various populations studied (219). An absence of the inserted fragment is referred to as a deletion and the two alleles of this polymorphism are designated as I (insertion) and D (deletion). Carriers of homozygous D (DD) or I (II) express the highest and lowest ACE levels, respectively, with intermediate ACE levels for heterozygous I/D individuals (220).

With the discovery of the ACE I/D polymorphism and its strong influence on the expression levels of ACE, attempts have since been made to resolve the clinical relevance of genotype-dependent influence on susceptibility or progression of diseases in which ACE is or may be implicated. Ample studies have searched for the relationship between the ACE I/D polymorphism and either susceptibility for, or disease phenotypes of, sarcoidosis. The results were mostly inconsistent between the studies and it seems that the influence of ACE polymorphisms depend on the number and the race of the patients studied (221). For instance, in African-Americans, the II genotype is associated with a progression towards higher stages of chest X-rays while in Finnish patients it is the DD variant that leads to a worsening of pulmonary disease (221, 222). In a UK cohort, no associations were found between ACE I/D variants and either sarcoidosis susceptibility or severity (223).

The role of ACE I/D in fibrotic diseases is uncertain, as shown by varying results of association studies which focused on different diseased organs (table 1). Circulating Ang II levels do not appear to be higher in people with the ACE DD genotype which implies that increased ACE activity, at least in blood, does not contribute to increased Ang II formation (224). On the other hand, local

Ang II generation in the internal mammary artery is enhanced in individuals carrying the D allele (225) which suggests that genetically altered ACE expression may be more likely to enhance Ang II formation locally rather than systemically. Hence, higher Ang II formation may precipitate the development of fibrosis in cases of injurious stimuli to tissue.

Angiotensin receptor type (AGTR)1 and AGTR2

The C variant of the AGTR1 receptor polymorphism 1166 C/T causes a differential response to Ang II (226). For this reason, many association studies have searched for the influence of the AGTR1 1166 C/T polymorphism in various diseases including coronary atherosclerosis (227) and myocardial infarction (228). Two studies have reported an actual contribution of this polymorphism to the progression of nephropathy (with fibrosis) (229, 230) [(231). Genetic variation studies of the X-chromosome-linked AGTR2 gene are scant and since the exact role of AGTR2 is still suggestive (107), the interpretation of association studies are precarious. Nevertheless, a Japanese study showed that the 4599 C/A polymorphism was associated with hypertension in women, and the A allele of the –1332 C/A promoter polymorphism was found to contribute to developing coronary artery disease in Caucasian males (232). AGTR2 polymorphisms in relation to fibrotic diseases have not been found (231) or have yet to be reported.

In Japanese sarcoidosis patients, an epistatic interaction was observed between the ACE I/D and AGTR1 1166 C/T polymorphism. Namely, the ACE D allele showed less ACE activity than the ACE I allele in the patients who also carried the 1166 C allele, while the opposite was found in patients with the 1166 A allele (233). It appeared that, rather than a synergistic effect on ACE levels, an inverse effect was found. This finding also serves as a clear illustration of how gene polymorphisms of the RAAS components interact with each other and lead to phenotypical changes.

Angiotensin-converting enzyme 2 (ACE2)

Polymorphisms in the ACE2 gene have been evaluated in patients with essential hypertension and disease outcome in SARS, but have not revealed any support for a genetic basis of ACE2 in either disease (234, 235). Although no studies to date have yet addressed the genetic variation of the ACE2 gene in context of fibrosis, one publication has reported an interesting association between ACE2 haplotypes and the presence as well as the extent of left

ventricular hypertrophy in men (236). The authors of this study suggested that genetic variants of ACE2 may alter the process of cardiac remodelling in an Ang II-mediated process. With the increasing number of reports now supporting the importance of ACE2 in tissue injury and repair (237, 238) and the genetic associations between the ACE2 gene and injury of the heart (236), it will not be long before genetic studies on fibrotic disorders will supplement the list of RAAS components with the ACE2 gene.

Chymase (CMA1)

Polymorphisms in the chymase gene (CMA1) have been investigated in several diseases that involve the detrimental effects of Ang II formation. For instance, a polymorphism at position -1897 (designated CMA/B) is associated with higher coronary artery bypass graft degeneration (239) and hypertrophic cardiomyopathy (240). Functional polymorphisms in CMA1 leading to diminished expression of chymase have not been shown to have any bearing on blood pressure (241). Ono et al. performed an extensive study with thirteen SNPs in CMA1 of which two were loss-of-function mutations and comparison of hypertensive and normotensive patients did not show differences between genotype frequency distributions (241). As suggested by this study, as well as evidenced by biochemical studies that chymase is easily inhibited by protease inhibitors in serum (242), the systemic actions of chymase are believed to be negligible.

Angiotensinogen (ATG)

The most extensively investigated functional polymorphism in the ATG gene is the methionine to threonine substitution in codon 235 (M235T). Hypertension has been a particularly popular choice for association studies in relation to the M235T polymorphism (243). The outcomes of these studies vary widely which is likely due to the different racial backgrounds between the study groups (244).

A study in New Zealanders showed that the 235T variant independently contributed to a 2-fold increase to developing coronary heart disease (245).

Evidence that ATG polymorphisms can influence the outcome of tissue injury and remodeling has been provided by Wang and coworkers (246). They showed that hypertensive dialysis patients with the 235TT genotype had significantly more left ventricular hypertrophy (LVH) of the heart compared to patients with genotypes 235 MM and/or 235MT (246). Yet in Japanese

patients, the extent of LVH following myocardial infarction is not associated with any M235T genotype (247). Possibly, the relevance of the M235T polymorphism in the response to tissue injury may depend on the preceding injury and the racial or ethnic background of the patient. Recently, a study in African-Americans showed that the rare allele of the promoter polymorphism (-6 A/G) was protective for developing chronic kidney disease (248). Although these studies strongly support the influence of genetic variation of the ATG gene on fibrotic development in the heart and the kidney's, studies on genetic polymorphisms in the ATG gene in relation to pulmonary tissue injury have yet to be published.

Table 1 RAAS polymorphisms and fibrotic development in disease.

Disease	Gene	Poly-morphism	Association	Ethnicity (Country)	Reference
Kidney					
IgA nephropathy	ACE ATG AGTR1	I/D M235T 1166 A/C	no no no	Asian (Taiwan)	(230)
diabetic nephropathy	ACE	I/D	no	Asian (Japan)	(249)
type 2 diabetic nephropathy	ACE	I/D	yes	Asian (China)	(250)
type 1 diabetic nephropathy	ACE ATG AGTR1	I/D M235T 1166 A/C	yes yes yes	Caucasian (Denmark)	(229)
renal scarring	ACE ATG AGTR1 AGTR2	I/D M235T 1166 A/C 3123 A/C	no no no no	Caucasian (Spain)	(231)
chronic kidney disease	ATG AGTR1	1166 A/C G(-6)A	yes yes	African-American (US)	(248)
Heart					
myocardial fibrosis	ACE	I/D	no	Asian (Japan)	(251)
left ventricular hypertrophy (renal failure)	ACE ATG	I/D M235T	no yes	Asian (China)	(246)
left ventricular hypertrophy (myocardial infarction)	ACE ATG	I/D M235T	yes no	Asian (Japan)	(247)
right ventricular hypertrophy (COPD)	ACE ATG	I/D M235T	yes no	Caucasian (Netherlands)	(252)
collagen I synthesis and QT dispersion in essential hypertension	ACE	I/D	yes	Asian (Japan)	(253)
tachycardia cardiomyopathy	ACE	I/D	yes	Caucasian (US)	(254)
myocardial fibrosis in rats	ACE	Microsatellite	yes	N/A (animal)	(255)
rheumatic heart disease	ACE	I/D	yes	Asian (Taiwan)	(256)

Table 1 Continued.

Disease	Gene	Polymorphism	Association	Ethnicity (Country)	reference
Liver					
disease progression in chronic hepatitis C	ATG	M235T	yes	Causians (Australia)	(257)
liver fibrosis	ATG	M235T	yes	Caucasian (Australia)	(258)
liver fibrosis in chronic HCV infection	ATG ACE AGTR1	M235T I/D 1166 A/C	no no no	Caucasian (UK)	(259)
Lung					
pulmonary fibrosis	ACE	I/D	yes	Caucasian (US)	(260)
sarcoidosis pulmonary disease severity	ACE	I/D	no	Caucasian (UK)	(223)
portal hypertension in CF patients	ACE	I/D	yes	Caucasian (UK)	(261)
increased risk of death or bronchopulmonary dysplasia	ACE	I/D	no	Caucasian (US)	(262)
acute respiratory distress syndrome	ACE	I/D	yes	Caucasian (UK) Asian (China)	(263) (264)
noninfectious pulmonary dysfunction following allogeneic stem cell transplant	ACE	I/D	yes	Asian (Japan)	(265)
Other					
chronic allograft dysfunction	ATG	M235T	yes	Caucasian (Turkey)	(266)
chronic pancreatitis	ACE	I/D	no	Caucasian (US)	(267)
systemic sclerosis	ACE	I/D	yes	Caucasian (Italy)	(268)

ACE = angiotensin-converting enzyme, ATG = angiotensinogen, AGTR = angiotensin receptor, N/A = not applicable.

3.4 Polymorphisms in sarcoidosis disease markers

As discussed in paragraph 2.3, the availability of markers for sarcoidosis which are able to predict the outcome or monitor disease activity are limited. Moreover, markers that are currently being used or have been evaluated as possible marker candidates are not yet reliable enough. To complicate matters further, some proteins used as disease markers may be influenced by functional polymorphisms and consequently skew the interpretation of disease marker levels. As mentioned earlier, gene polymorphisms can influence the production of the gene product in various manners. For instance, a promoter polymorphism may disrupt a regulatory consensus sequence and result in a diminished or enhanced expression of the gene. As illustrated by TGF-β1, polymorphisms in exons may cause an amino-acid substitution which may also have significant bearing on expression levels (181). The polymorphic site that is associated with altered expression levels may not necessarily be the QTL itself, as illustrated by the ACE insertion/deletion (I/D) polymorphism. The I/D is positioned in an intron with no functional consequences but it is rather in strong linkage with a putative QTL that actually causes the variation in expression levels in blood. The ACE I/D polymorphism and its strong association with ACE level variation has led to reconsideration of the standard use of reference intervals which are based on a general population. Reported ACE I/D genotype-dependent ACE levels have shown its usefulness in improving the interpretation of ACE levels in a given individual (269, 270) and it was pointed out that the highly variable I/D allele frequencies between races are bound to change the overall range for ACE levels for a particular population (271).

Expression of SP-D, another marker for sarcoidosis, is also influenced by the presence of gene polymorphisms. One of the polymorphisms in the gene encoding SP-D (SFTPD) causes a methionine (Met) for a threonine (Thr) substitution in codon 11. Leth-Larsen et al. subsequently reported that individuals with the genotype SFTPD Thr11Thr have significantly lower SP-D serum levels than individuals with the SFTPD Met11Met genotype (272). In a similar study, Heidinger and colleagues found six SNPs in the 5' untranslated region, the coding region and the 3' region of the SFTPD gene from which they deduced haplotypes. One of these SFTPD haplotypes was found to be associated with reduced SP-D levels (273).

Another sarcoidosis disease marker that is genotypically controlled is CC16. The A38G polymorphism is associated with altered CC16 levels in both

healthy individuals and in sarcoidosis patients (274). Laing et al. demonstrated that the CC16 38AA genotype is associated with reduced plasma CC16 levels in asthma patients as well as in healthy control subjects (275). Data on the CC16 A38G polymorphism in relation to the clinical progression of sarcoidosis has been conflicting (274, 276) but deserves further investigation to conclude whether CC16 expression variation may influence the course of sarcoidosis.

This thesis will show that the ongoing discovery of polymorphisms which influence levels of disease markers will lead to an amended take on the interpretation of normal or deviating disease marker levels in a given individual and it will provide a renewed perspective on previously published evaluations of disease markers.

4. AIM OF THE THESIS

There is ample evidence to support the contribution of single nucleotide polymorphisms (SNPs) to the different disease phenotypes of many diseases. In the case of sarcoidosis, most genetic studies have focused mainly on disease susceptibility in case-control studies, although an increasing number of reports now indicate the disease-modifying properties of SNPs on sarcoidosis. What has been lacking in most of these studies, however, is the focus on the subset of patients who develop the life-threatening complication of pulmonary sarcoidosis, namely pulmonary fibrosis.

In addition to some key cytokines that are known to be involved in the mechanisms of tissue injury, remodeling and fibrosis, a central role for angiotensin II (Ang II) in response to an injurious stimulus continues to gain more interest. Accumulating evidence strongly suggests the involvement of the organ-localized renin-angiotensin-aldosterone system, or local RAAS, in the evolution of fibrosis of numerous organs including the lung. Genetic variation in the genes that encode fibrosis-related cytokines and RAAS components may contribute to the propensity to develop pulmonary fibrosis in sarcoidosis patients.

The influence of SNPs on sarcoidosis disease parameters is presented in this thesis. Genetic polymorphisms may serve as a predictive tool by providing the likelihood for an individual to contract a disease or to develop a particular phenotype of the disease. This is denoted as differences in allele-, genotype- or haplotype frequencies between patients and healthy controls or between subgroups within the cohort. The effects of a polymorphism on sarcoidosis phenotypes may appear as changes of such parameters as radiography, lung function or serum levels of the disease marker.

5. SCOPE AND OUTLINE OF THE THESIS

Chapter 2 describes the results of four SNPs and five constructed haplotypes in the chymase gene (*CMA1*) in Dutch and Japanese sarcoidosis patients and their racially matched controls. Radiographic evolution in Dutch sarcoidosis patients as well as clinical manifestations in Japanese patients is evaluated for association with the *CMA1* polymorphisms.

In **Chapter 3,** seven intron SNPs in ACE2 are investigated in Dutch sarcoidosis patients. The frequency distributions of the ACE2 genotypes and constructed haplotypes are compared between patients with different pulmonary disease phenotypes. Patients suffering from either acute, with no parenchymal involvement (including a substantial number of patients with Löfgren's syndrome), or chronic type disease with parenchymal involvement (including fibrotic end-stage), according to radiographic evolution over a 4-year follow-up period are analyzed.

Chapter 4 presents the results of SNPs in TGF-β isoforms 1, 2 and 3 and the propensity to develop pulmonary fibrosis in Dutch sarcoidosis patients with a 4-year follow-up period. Patients with different pulmonary disease phenotypes (according to radiographic evolution and lung function parameters) are compared in terms of SNP and haplotype frequency distributions.

Chapter 5 discusses the importance of adjusting serum ACE levels for the ACE I/D genotype. Genotype-specific ACE levels were established using a large group of healthy individuals. The added value of this method is illustrated by a retrospective comparison of ACE levels in patients with and without the consideration of the ACE I/D genotype.

Chapter 6 demonstrates the influence of a functional SNP in the MUC1 gene on KL-6 values in patients with interstitial lung diseases and healthy controls. Genotype-specific reference values are established and applied to KL-6 values in patients with sarcoidosis.

Chapter 7 evaluates a novel serum marker, YKL-40, for sarcoidosis and the presence of pulmonary fibrosis. The gene encoding this protein (CHI3L1) is screened for single nucleotide polymorphisms which may influence the serum YKL-40 levels in healthy controls and in sarcoidosis patients.

Chapter 8 summarizes and provides concluding remarks on the thesis.

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chapter TWO

2

Chymase Gene (*CMA1*) Polymorphisms
IN DUTCH AND JAPANESE SARCOIDOSIS PATIENTS

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Abstract

Chymase is released from mast cells following activation. Evidence suggests that chymase plays an important role in tissue injury and remodeling of the lungs, heart and skin.

We postulated that chymase gene *(CMA1)* polymorphisms are associated with pulmonary fibrosis in Dutch and with cardiac and skin involvement in Japanese sarcoidosis patients.

Dutch (n=153) and Japanese (n=122) sarcoidosis patients with controls (Dutch, n=309; Japanese, n =111) were studied. Pulmonary involvement in Dutch patients as well as clinical manifestations in Japanese patients was evaluated for association with five *CMA1* polymorphisms.

The *CMA1* polymorphisms were not associated with disease susceptibility in either population, or with radiographic evolution in the Dutch or with cardiac or skin involvement in the Japanese patients. The -526 T allele was associated with a lower IVC in Dutch patients.

The *CMA1* polymorphisms studied do not contribute to disease susceptibility in Japanese or Dutch sarcoidosis patients. *CMA1* polymorphisms do not influence radiographic evolution in Dutch sarcoidosis patients, nor do they predispose to cardiac or skin involvement in Japanese patients. However, the association between *CMA1* –526 C/T and iVC in the Dutch patients suggests that chymase may modify the functional outcome of pulmonary sarcoidosis.

Introduction

Sarcoidosis is a systemic disease of unknown cause and is characterized by the presence of noncaseating granulomas in one or multiple organs (1). The involvement of the Renin-Angiotensin-Aldosterone system (RAAS) in the pathophysiology of sarcoidosis, in which angiotensin-converting enzyme (ACE) is responsible for the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) (2), has been extensively investigated, but its relevance remains elusive (3).

More recently, the existence of a *local* RAAS has been confirmed or suggested in a number of organs, including the lung, heart, skin and even the eye. In these organs, the local formation of Ang II has the ability to stimulate proliferation of fibroblasts (4-8). Expression of Ang II receptors on macrophages in bronchoalveolar lavage fluid (BALF) of sarcoidosis patients is found to correlate with disease activity, suggesting Ang II-mediated responses in sarcoidosis (9). Moreover, the presence of Ang II within the sarcoid granuloma supports the implication of local Ang II in the formation or perpetuation of the granulomatous inflammation (10).

Chymase, a serine proteinase, is also capable of forming Ang II. Chymase is predominantly stored in the cytoplasmic secretory granules of mast cells and is released following mast cell activation (11). Mast cells release a vast array of cytokines and enzymes known to mediate the inflammatory events (12). Chymase-positive mast cells are present in a number of organs, including the heart, skin and lung (13-15).

The affinity of chymase for its substrate Ang I is much higher than that of ACE and this has led to the assumption that chymase is the main enzyme, at least locally, for Ang II formation (16, 17). Chymase also activates transforming growth factor-β1 (TGF-β1), a key factor in tissue injury and repair (18-20).

Chymase has been reported to be upregulated in a number of diseases that have the potential to eventuate in fibrosis, such as diabetic nephropathy (21), ischemic heart disease (22, 23) and pulmonary fibrosis (24). In a subset of sarcoidosis patients, the disease progresses insidiously towards the development of pulmonary fibrosis. This is more frequently seen in Caucasians and Blacks than in Japanese (25). Instead, Japanese sarcoidosis patients are more often complicated with cardiac and skin involvement (26). While the involvement of mast cells in sarcoidosis has been suggested for quite some time (27), only one study to date has addressed the role of mast cell chymase in sarcoidosis (28). The authors found that chymase-positive mast cells were present in non-parenchymal fibrous areas of cutaneous lesions in sarcoidosis

and that chymase-positive mast cell subtypes showed a positive correlation with the presence of systemic (eye and lung) manifestations in Japanese sarcoidosis patients. Patients with sarcoidosis limited to the skin, on the other hand, showed mostly tryptase-positive (chymase negative) mast cells in the skin lesions (28), suggesting that the recruitment of chymase-positive mast cells might play a role in the severity of sarcoidosis.

Chymase may contribute to an unfavorable course of sarcoidosis by increasing local Ang II and concomitant TGF-β1 upregulation, which may ultimately lead to irreversible scarring of the lungs. In addition, the high incidence of cardiac sarcoidosis (26) often leading to fibrotic changes (29) and the reported presence of chymase-positive mast cells in sarcoid skin lesions in Japanese patients (28) may be related to chymase-mediated tissue repair and fibrosis of the heart and skin.

The different patterns by which sarcoidosis is manifested are believed to have a genetic basis (30). The occurrence of fibrosis in sarcoidosis, as often seen in Caucasians and Blacks, but rarely in Japanese (25), may possibly be found in the genetics that underlie the mechanism of tissue repair and remodeling. Single nucleotide polymorphisms (SNPs) of genes encoding for proteins that are known to be involved in tissue injury and repair have been associated with fibrotic events in pulmonary diseases (31-34). Polymorphisms of the chymase gene (*CMA1*) have been investigated in several diseases that involve the detrimental effects of Ang II formation. For instance, a polymorphism at position -1897 (designated CMA/B) is associated with higher coronary artery bypass graft degeneration (35) and hypertrophic cardiomyopathy (36).

Studies on sarcoidosis in relation to *CMA1* genotypes, however, have not yet been performed.

We hypothesized that chymase may be involved in sarcoidosis and that differential *CMA1* genotypes would reveal an association with fibrosis in a group of Dutch Caucasian sarcoidosis patients. We also investigated whether cardiac involvement and sarcoid skin lesions in Japanese sarcoidosis patients were related to *CMA1* polymorphisms.

For this study, the biallelic polymorphisms evaluated included: -1897 (C/T); –526 (C/T); -153 (C/T), all of which are located in the promoter site; 1628 (A/G), present in the intron/exon boundary as previously described (36) and a polymorphism at position 2821 (C/T), which is present in the 3′ untranslated region that may contain sequences that regulate translation efficiency, mRNA stability, and polyadenylation signals (37).

Subjects and Methods

Patients and controls

One hundred and fifty-three unrelated Dutch Caucasian sarcoidosis patients (88 males/65 females; mean age at diagnosis ± standard deviation (years): 36.6 ± 10.5, range: 17 – 71) were included in this retrospective study. The diagnosis of sarcoidosis was established when clinical findings were supported by histologic evidence and after exclusion of other known causes of granulomatosis. Histologic proof was obtained by either bronchial mucosa biopsy, mediastinal biopsy, cervical lymph node biopsy, peripheral lung biopsy, open lung biopsy, VATS (video-assisted thoracoscopic surgery) lung biopsy, skin/muscle biopsy, or liver biopsy. In 46 patients, the diagnosis was made without biopsy proof because these patients presented with the classic symptoms of Löfgren's syndrome with fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy. Verbal and written consent was obtained from all patients and authorization was given by the Ethics Committee of the Sint Antonius Hospital, Nieuwegein (region Utrecht), The Netherlands.

One hundred and twenty-two Japanese sarcoidosis patients (37 males/85 females; mean age at diagnosis ± SD (years): 44.6 ± 14.1, range: 10 - 70) were recruited from the Central Clinic in Kyoto, Japan and included in this retrospective study. All patients were native Japanese and unrelated to each other. The diagnosis of sarcoidosis was established when clinical and radiological findings were supported by histological evidence of noncaseating epithelioid cell granulomas, after exclusion of other known causes of granulomatosis. Biopsies were obtained using identical techniques as those described for the Dutch patients. Verbal and written patient consent for genetic analysis was obtained prior to phlebotomy and authorization was given by the Ethics Committees of the clinics.

Chest radiographs were assessed to determine disease severity using standard radiographic staging for sarcoidosis. Chest radiographs were classified according to the Scadding criteria: 0, normal chest radiographic findings; I, bilateral hilar adenopathy with normal lung parenchyma; II, bilateral hilar adenopathy with pulmonary infiltrates; III, pulmonary infiltrates without hilar adenopathy; IV, pulmonary fibrosis/fibrocystic parenchymal changes (25).

Presentation chest radiographic data were available for 149 Dutch and 121 Japanese patients. The distribution of chest radiographic stages, at presentation is shown in table 1. Chest radiographs, taken at 2 and 4 years after initial presentation, were available for Dutch patients only (n = 107). These patients

included 4 patients with radiographic stage IV who had been diagnosed outside of this hospital prior to referral. Of these patients, radiographic data at presentation were unavailable. Radiographic evolution over a 4-year follow-up period (presentation, 2 and 4 years) was categorized as follows: A (normalization or improvement towards stage I), B (persistent stage II/III or progression in that direction), and C (stable stage IV or progressive towards this stage) (31). Patients who had been diagnosed with Löfgren's syndrome at presentation (n = 46) were considered as a distinct group with radiographic evolution not exceeding stage I.

All patients were assessed for extrapulmonary disease (table 2), *e.g.* ophthalmic, cardiac, neurological involvement, according to the ATS/ERS/WASOG statement on sarcoidosis during follow-up (25).

DNA was obtained from 309 (111 males/198 females; mean age ± SD (years): 39.2 ± 10.9, range: 18 - 68) unrelated, ostensibly healthy employees of the St. Antonius Hospital. By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases. The Japanese control population comprised 111 (65 males/46 females; mean age ± SD (years): 38.2 ± 12.2, range: 19 - 75) healthy, unrelated, native Japanese, mainly gathered from the Kinki area such as Kyoto, Osaka, and Shiga.

Table 1 Chest X-ray at presentation in Japanese and Dutch sarcoidosis patients.

Stage	Japanese (n = 122)		Dutch (n = 153)	
	n	%	n	%
0	1	0.8	1	0.6
I	61	50.0	89	58.2
II/III	57	46.8	56	36.6
IV	2	1.6	3	2.0
unknown	1	0.8	4	2.6

Table 2 Extrapulmonary disease at presentation in Japanese and Dutch sarcoidosis patients.

	Japanese (n = 122)		Dutch (n = 153)	
	n	%	n	%
Uveitis	94	77	11	7.4
Skin	40	32.8	11	7.4
Cardiac	32	26.2	6	4.0
Neuro	25	20.5	2	1.3
Muscle	14	11.5	0	0
Joints	8	6.6	33	22.1
Erythema Nodosum	0	0	50[b]	33.6
Löfgren's syndrome	0	0	46	30.9
Unknown	0	0	4	-

Pulmonary function testing.

Dutch patients had performed lung function tests at presentation, and 2 and 4 years following diagnosis. Vital (inspiratory) capacity (iVC), forced expiratory volume in 1 second (FEV_1) and diffusing capacity of the lung for carbon monoxide (D_{LCO}) were used to assess the presence of lung function impairment at presentation and follow-up of disease. iVC and FEV_1 were calculated from volumes in liters and adjusted to BTPS (body temperature, ambient pressure, saturated with water vapor) in accordance with the ERS recommendations and expressed as percent predicted values (38).

Lung function data of the Japanese patients were unavailable.

Genetic analysis

Five biallelic *CMA1* single nucleotide polymorphisms: -1897 (C/T); - 526 (C/T); -153 (C/T) [promoter region]; 1628 (A/G) [intron/exon boundary]; 2821 (C/T) [untranslated region], were determined using sequence-specific primers (SSPs) -polymerase chain reaction (PCR).

The sequences of SNP-specific primers with their complementary consensus primers are shown in table 3 and the PCR conditions were as previously described (39). The final reaction concentrations used were 7.6 ng/µl.

CMA1 haplotypes were determined using Phase version 2 (Mac OS X). This program uses an advanced algorithm based on a method by Stephens et al. (40, 41).

Table 3 Primer sequences for the identification of 54 biallelic single nucleotide polymorphisms of CMA1.

polymorphism	dbSNP accession[a]	GenBank accession	specific primers for SNP	consensus primers
-1897 (C/T)	rs1800875	NT 026437	5′-CCT CAG CCA GGC AGG TG **G/A** (reverse)	5′- GAA GAG AAT CCG GAG CTG GA (forward)
-526 (C/T)	rs1956923	NT 026437	5′-CCA CTC CAT TCC ACT ACA C **G/A** (reverse)	5′- AGA GCA GAA AGG GAC CTT GA (forward)
-153 (C/T)	rs5244	NC 000014.4	5′- GGT GAT TCT AGG GGA ACT TC **C/T** (forward)	5′- GT TTC AGG AGC TGA TAC TGC (reverse)
1628 (A/G)	rs5248	NC 000014.4	5′-AA GGG TGA CTG TTA TAG ACC TG **T/C** (reverse)	5′- TCA GGG CCA CTT CCT CTA TG (forward)
2821 (C/T)	rsrs5250	NT 026437	5′- GAT ACT TCT GGA GGC TTA GG **G/A** (reverse)	5′- CCT CTG ACC ATC CAT TCC AG (forward)

[a] The SNP loci were identified using accession numbers according to the SNP database at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp

Statistical analysis

Statistical analysis was performed using chi-square contingency table analysis with the appropriate number of degrees of freedom (df) (SPSS for Windows; SPSS Inc., Chicago, IL). Fisher's exact test was used if expected cell frequencies were lower than five. Adjustment for multiple tests was made using the formula $p_c = p \times n$, where p_c is the corrected value, p the uncorrected value and n the number of tests performed (Bonferroni method). Genotype frequencies were tested for Hardy-Weinberg equilibrium. Univariate analysis was performed to assess the influence the *CMA1* genotypes and haplotypes on lung function parameters at presentation, and 2 and 4 years following presentation. One-way repeated-measures ANOVA was used to chart the changes of lung function parameters over a 4-year follow-up period (presentation and 4 years), followed by analyses of two-way interactions between grouping variables as described in the results paragraph. All values of lung function parameters were normally distributed.

A value of $p < 0.05$ was considered significant.

Results

Single nucleotide polymorphisms

Table 4 summarizes the results of *CMA1* genotype and allele carrier frequencies in Dutch and Japanese sarcoidosis patients and healthy controls. No deviation from Hardy-Weinberg equilibrium was observed for any of the groups studied.

In the Dutch population, no differences were found for genotype, allele or allele carrier frequencies between healthy controls and sarcoidosis patients.

In the Japanese population, the C allele of polymorphism at position -153 was found to be slightly more prevalent in the control group (carrier frequency: 46.8%) than in patients (32.0%) ($p = 0.02$; OR = 1.7) and was reflected by a lower TT (53.3% vs. 68.0%) and higher CT (41.4% vs. 27.0%) genotype frequency in the control group. In addition, the carrier frequency of the G variant of allele 1628, an intron SNP in close proximity of exon 3, was lower in the patient group compared to controls (32.0% vs. 44.1%; $p = 0.03$ (OR = 1.3)). After correcting the calculated probabilities for the number of investigated SNPs ($p \times 4 = p_c$), both results were rendered not significant ($p_c = 0.08$ and $p_c = 0.12$, respectively). The -1897 (C/T) and -526 (C/T) promoter polymorphisms were found to be in complete linkage with each other in the Japanese, as reported by Ono et al. (42) as well as in the Dutch subjects. The latter polymorphism was arbitrarily chosen for further analysis.

Haplotype construction

SNPs that show significant linkage disequilibrium with each other are grouped to form haplotypes. In this study, we established five major haplotypes with four polymorphic sites in the *CMA1* gene. Japanese and Dutch patients and healthy controls were assessed for haplotype distributions and comparisons were made between patients and controls as well as between the Japanese and the Dutch (table 5). There was no difference in haplotype frequency distribution between the Dutch controls and sarcoidosis patients. In the Japanese population, however, a slightly decreased carrier frequency of *CMA1* haplotype 4 was observed in patients (30.3%) compared to the control group (44.1%); $p = 0.04$; $p_c = 0.2$; df = 4.

The most striking differences observed for haplotype distributions between the Japanese and Dutch, both for patient-patient and control-control comparisons, were found for haplotype 3 and 4. Namely, haplotype 3 carriage was extremely rare in Japanese controls (2.7%), whereas it was found in 23.9% of

the Dutch control population ($p < 0.0001$; $p_c = 0.0005$; df = 1). Conversely, haplotype 4 carriage was more common in Japanese controls (44.1%) than in the Dutch controls (13.9%) ($p < 0.0001$; $p_c = 0.0005$; df = 1).

Extrapulmonary disease phenotypes

Examination of genotype frequencies in relation to cardiac and skin involvement in the Japanese sarcoidosis patients did not reveal any association (data not shown). However, haplotype 4 carriage in sarcoid uveitis patients (27.7%) vs. control subjects (44.1%) and sarcoid patients without uveitis (39.3%) suggested a slight protective effect against uveitis in sarcoidosis ($p_{trend} = 0.05$; $p_c = 0.25$; df = 2). The significance was increased, yet not statistically significant following the correction of the p-value, when haplotype 4 carriage was compared between sarcoid patients with uveitis and controls alone ($p = 0.02$; $p_c = 0.1$; OR = 2.1 [95% confidence interval (CI) 1.1 - 3.7]). In the Dutch population, 7.4% of the sarcoidosis patients were complicated by uveitis (table 2), but haplotype 4 carriage in this group (18.2%) did not appear to be significantly different from Dutch healthy controls (13.9%) ($p > 0.1$). However, the low number of uveitis patients (n = 11) in the Dutch group as well as the low frequency of haplotype 4 carriage in the total sarcoidosis population (13.8%) made a reliable comparison difficult due to insufficient statistical power.

Evaluation of all other extrapulmonary manifestations of sarcoidosis in Dutch and Japanese patients did not reveal any association with the *CMA1* polymorphisms studied (data not shown).

Table 4 Genotype and allele carrier frequencies of the *CMA1* polymorphisms in Dutch and Japanese sarcoidosis patients and healthy controls.

Polymorphism position		Dutch patients (n=153)	Dutch controls (n=309)	Japanese patients (n=122)	Japanese controls (n=111)	
-1897 (promoter)	CC	82 (53.6)	160 (51.8)	48 (39.3)	42 (37.8)	
	CT	60 (39.2)	130 (42.0)	51 (41.8)	53 (47.8)	
	TT	11 (7.2)	19 (6.2)	23 (18.9)	16 (14.4)	
	C	142 (92.8)	290 (93.8)	99 (81.1)	95 (85.6)	
	T	71 (46.4)	149 (48.2)	74 (60.6)	69 (62.2)	
-526 (promoter)	CC	82 (53.6)	160 (51.8)	48 (39.3)	42 (37.8)	
	CT	60 (39.2)	130 (42.0)	51 (41.8)	53 (47.8)	
	TT	11 (7.2)	19 (6.2)	23 (18.9)	16 (14.4)	
	C	142 (92.8)	290 (93.8)	99 (81.1)	95 (85.6)	
	T	71 (46.4)	149 (48.2)	74 (60.6)	69 (62.2)	
-153 (promoter)	TT	101 (66.0)	200 (64.7)	83 (68.0)	59 (53.3)	
	CT	48 (31.4)	99 (32.0)	33 (27.0)	46 (41.4)	
	CC	4 (2.6)	10 (3.3)	6 (5.0)	6 (5.4)	
	T	149 (97.4)	299 (96.8)	116 (95.1)	105 (94.6)	[a] $p = 0.02$; $p_c = 0.08$ (OR
	C	52 (34.0)	109 (35.3)	39 (32.0)[a]	52 (46.8)[a]	= 1.7); df = 1
1628 (intron/exon)	AA	131 (85.6)	264 (85.4)	83 (68.0)	62 (55.8)	
	AG	22 (14.4)	44 (14.3)	34 (27.9)	43 (38.7)	
	GG	0	1 (0.3)	5 (4.1)	6 (5.4)	
	A	153 (100)	308 (99.7)	117 (96.0)	105 (94.6)	[b] $p = 0.03$; $p_c = 0.12$
	G	22 (14.4)	45 (14.6)	39 (32.0)[b]	49 (44.1)[b]	(OR = 1.3); df = 1
2821 (untranslated)	CC	121 (79.1)	235 (76.1)	120 (98.4)	108 (97.3)	
	CT	31 (20.3)	73 (23.6)	2 (1.6)	3 (2.7)	
	TT	1 (0.6)	1 (0.3)	0	0	
	C	152 (99.3)	308 (99.7)	122 (100)	111 (100)	
	T	32 (20.9)	74 (24.0)	2 (1.6)	3 (2.7)	

Data are given as absolute numbers and percentages (%) in parentheses.
Probability (p), odds ratio (OR) and degrees of freedom (df) are provided for initial results (before Bonferroni correction).

Table 5 Haplotype (carrier) frequencies of CMA1 in Dutch and Japanese sarcoidosis patients and healthy controls.

						Dutch patients (n=153)	**Dutch controls (n=309)**	**Japanese patients (n=122)**	**Japanese controls (n=111)**
Haplotype	**position**					Haplotype (carrier) frequency	Haplotype (carrier) frequency	Haplotype (carrier) frequency	Haplotype (carrier) frequency
	-1897	-526	-153	1628	2821				
1	C	C	T	A	C	52.7 (75.9)	53.2 (79.3)	41.0 (64.8)	35.5 (55.0)
2	T	T	T	A	C	27.7 (47.0)	27.3 (48.2)	39.8 (60.6)	38.3 (62.2)
3	C	C	C	A	T	12.2 (22.9)	12.1 (23.9)	0.8 (1.6)	1.4 (2.7)
4	C	C	C	G	C	6.8 (13.8)	7.1 (13.9)	17.2 (30.3)[a]	24.8 (44.1)[a]
5	C	C	T	G	C	0.6 (1.2)	0.3 (0.6)	1.2 (2.5)	0 (0)

Data are given as frequencies with carrier frequencies in parentheses. Probability (p) and degrees of freedom (df) are given for significant results only. [a] Japanese controls vs. patients: $p = 0.04$; $p_{corr} = 0.2$, degrees of freedom (df) = 4
Japanese controls vs. Dutch controls: $p < 0.0001$; $p_{corr} < 0.0005$, df = 4
Japanese patients vs. Dutch patients: $p < 0.002$; $p_{corr} < 0.01$, df = 4

Radiographic staging and evolution

Genotype, allele, and haplotype (carrier) frequencies in Dutch patients that had either presented with stage IV or progressed from lower stages (over a 4 year follow-up period) (n = 22) were compared with the frequencies of those that had a normal (0) chest x-ray, stage I or stages II/III. For this purpose, the patient population was categorized according to radiographic evolution: A (normalization or improvement towards stage I), B (persistent stage II/III or progression in that direction), and C (stable stage IV or progressive towards this stage). Patients who had been diagnosed with Löfgren's syndrome at presentation were considered as a distinct group with radiographic evolution not exceeding stage I.

Table 6 shows data of allele carriage frequencies in Dutch patients, grouped according to radiographic evolution and Löfgren's syndrome.

Table 6 CMA1 allele carrier frequencies in 107 Dutch sarcoidosis patients in relation to radiographic evolution during 4-year follow-up period.

polymorphism	radiographic evolution[a]			
	A (n = 49)	B (n = 36)	C (n = 22)	Löfgren's [b] (n = 46)
-526 C/T				
C	44 (89.7)	35 (97.2)	21 (95.5)	44 (95.6)
T	25 (51.0)	20 (55.6)	12 (54.5)	19 (41.3)
-153 C/T				
T	48 (98.0)	36 (100)	21 (95.4)	45 (97.8)
C	15 (30.6)	12 (33.3)	6 (27.3)	16 (34.8)
1628 A/G				
A	49 (100)	35 (97.2)	21 (95.5)	46 (100)
G	5 (10.2)	8 (22.2)	2 (9.1)	7 (15.2)
2821 C/T				
C	49 (100)	36 (100)	21 (95.5)	46 (100)
T	11 (22.4)	5 (13.9)	5 (22.7)	10 (21.7)

Data are given as absolute numbers and percentages (%) in parentheses.
[a] The patient population was categorized according to radiographic evolution:
A (normalization or improvement towards stage I);
B (persistent stage II/III or progression in that direction),;
C (stable stage IV or progressive towards this stage).
[b] Patients with Löfgren's syndrome were regarded as a distinct group with radiographic evolution not exceeding stage I.

No association was found for *CMA1* genotype or allele (carrier) frequencies with radiographic evolution or Löfgren's syndrome. None of the constructed haplotypes revealed an association with radiographic evolution (data not shown). Radiographic staging at any given time point (presentation, 2 and 4 years) also failed to show any association with *CMA1* genotype and haplotype distributions (data not shown). Furthermore, the sarcoidosis patients were grouped according to improvement, stable disease or worsening of pulmonary sarcoidosis based on radiographic data. These categories did not show any association with genetic variants of *CMA1* (data not shown).

For the Japanese patient group, only two stage IV patients were present in the total population, which did not allow us to perform an analysis of fibrotic patients with genotype distributions with any degree of precision. All other

radiographic stages (only available at time of presentation) did not show any association with any *CMA1* haplotype or single locus polymorphism (data not shown).

Lung function parameters in Dutch sarcoidosis patients

Univariate analysis was performed to assess the influence of *CMA1* genotypes and haplotypes on lung function parameters measured at the time of presentation, 2 and 4 years. Other variables in the analysis included radiographic staging, smoking and received treatment. Neither of the investigated *CMA1* SNPs or constructed haplotypes showed to have significant bearing on lung function at any time point (data not shown).

Complete serial lung function data of iVC (%) and FEV_{-1} over a 4-year follow-up period were available for 95 patients (group A (n = 36), B (n = 31), C (n = 13) and Löfgren's (n = 15)). Serial Dl*co* data were available for 50 patients (group A (n = 18), B (n = 17), C (n = 6) and Löfgren's (n = 9). One-way repeated-measures ANOVA analysis was performed to chart the changes in iVC, FEV_{-1} and DL*co* over a 4-year follow-up period and was controlled for radiographic evolution, smoking, and received treatment (Table 7). Radiographic evolution was strongly associated with iVC, FEV_{-1} and Dl*co*, such that group C had an overall lower lung function than group A, B or Löfgren's ($p < 0.0001$ for all) (Table 7). None of the lung function parameters were significantly altered by smoking history or received treatment (data not shown). The analysis further showed that the –526 C/T genotype of the *CMA1* gene was strongly associated with a reduced iVC in patients who had been followed-up for 4-years ($p = 0.000002$; $p_c = 0.000008$; degrees of freedom (df) = 2). iVC appeared to be lower with an increase of the T allele in the genotype with the following pattern (genotype (estimated marginal mean iVC (%) ± SEM [95% CI])): CC (94.2 ± 2.3 [89.6 – 98.8]), CT (85.9 ± 2.7 [80.5 - 91.4]) and TT (77.9 ± 4.2 [69.6 - 86.1]) (figure 1). Consequently, carriage of haplotype 2, which includes the –526 T allele, caused a lower iVC in sarcoidosis patients in a similar manner (94.2 ± 2.4 [89.6 – 98.9] vs. 84.5 ± 2.7 [79.2 - 89.8], $p = 0.000002$ ($p_c = 0.000008$)).

Sarcoidosis patients with parenchymal involvement (radiographic evolution group B and C) or without (group A and Löfgren's syndrome) both had a lower iVC in presence of the –526 T allele. In patients without parenchymal involvement this pattern was as follows: (genotype (estimated marginal mean iVC (%) ± SEM [95% CI])) CC (103.0 ± 2.8 [97.4 - 108.6]), CT (93.3 ± 3.4

[86.5 – 100.1]), TT (88.9 ± 4.4 [80.1 - 97.8]), p = 0.00008, p_c = 0.0003. Patients with parenchymal involvement showed a similar genotype-specific trend with a lower baseline iVC value: CC (86.1 ± 4.2 [77.9 - 94.5]), CT (79.2 ± 4.7 [69.7 - 88.7]), TT (62.0 ± 9.0 [44.0 - 80.0]), p = 0.00009, p_c = 0.0004.
Haplotype 2 carriage reduced the iVC values in a similar fashion in patients with (77.7 ± 4.8 [68.2 – 87.3] vs. 86.3 ± 4.3 [77.7 – 95.0], p = 0.01, p_c = 0.04) and without (92.1 ± 3.2 [85.7 – 98.6] vs. 102.9 ± 2.8 [97.4 – 108.5], p = 0.00008, p_c = 0.0003) parenchymal involvement.
Neither any of the investigated SNPs nor any of the constructed haplotypes were found to alter the course of any of the lung function parameters over the 4-year follow-up period (data not shown).

Discussion

We were able to construct five *CMA1* gene haplotypes using four biallelic single nucleotide polymorphisms (SNPs) in sarcoidosis patients and healthy controls from two racially different populations. We discovered that the distribution and frequencies of these haplotypes were different between the two populations studied. Since the manifestation of sarcoidosis can differ vastly between populations of different ethnicity (43), single nucleotide polymorphism (SNP) frequency distributions of disease modifying genes or genes that predispose to the disease may vary in function of this phenomenon.
CMA1 genotype distributions in Dutch patients did not deviate from the healthy Dutch controls.
Recently, Ono et al. (42) described a total of thirteen single nucleotide polymorphisms in *CMA1* in a Japanese population consisting of normotensive and hypertensive individuals. All but one SNP from our study were covered in Ono's study with comparable allele frequency data between the Japanese controls. Two additional SNPs are mutations resulting in a premature stop codon (785 C/A in exon 2) and atypical splicing (881 G/C at the junction between exon 2 and intron 2) which both disable enzyme activity. None of these or any other SNPs were found to influence blood pressure in their study groups. As observed by Ono et al., the low allele frequency of either polymorphism (0.62% of 785 A and 0.78% of the 881 C variant) with an expected homozygozity of 1 in 26,000 and 1 in 16,000, respectively, would not have sufficed to perform association studies with the sarcoidosis cohorts used in our study.

This study revealed no association between any *CMA1* genotype and fibrotic development in Dutch sarcoidosis patients in terms of radiographic evolution or staging. Pulmonary fibrotic sarcoidosis is by no means homogeneous; based on CT-scans, three main categories exist, each with a corresponding severity and progression of fibrosis (44). Although this study failed to show an association between *CMA1* polymorphisms and susceptibility to fibrotic disease, *CMA1* variants may influence the severity and progression of fibrosis within a group of stage IV sarcoidosis patients.

Table 7 Characteristics of sarcoidosis patients used for the 4-year follow-up assessment of lung function parameters.

Gender (males/females)		61/34		
Age at presentation				
Mean ± SD (years)		36.6 ± 11.0		
Range (years)		20 - 71		
Smoking history (yes/no/unknown)[a]		37/56/2		
Treatment (yes/no)[b]		62/33		
Lung function (%-predicted) (mean ± SEM)		**iVC**	**FEV_1**	**DL*co***
Radiographic evolution[c]	A	99.8 ± 11.9 (n = 36)	95.1 ± 14.2 (n = 36)	91.4 ± 15.7 (n = 18)
	B	93.8 ± 14.8 (n = 31)	84.8 ± 16.4 (n = 31)	83.2 ± 18.6 (n = 17)
	C	79.4 ± 17.6[d] (n = 13)	61.6 ± 24.4[e] (n = 13)	61.5 ± 15.0[f] (n = 6)
	Löfgren's	103.6 ± 12.6 (n = 15)	93.1 ± 15.9 (n = 15)	92.7 ± 15.2 (n = 9)

Gender, number of cases with smoking history, and received treatment are given as absolute numbers (n). Age (years) and iVC (% predicted) are given as mean ± standard error (SEM) and range. iVC (% predicted) according to radiographic evolution, was calculated as the mean ± SEM from 2 repeated measures per individual (2 x n) over a 4-year follow-up period (presentation and 4 years). [a] A cut-off period of 5 years following cessation of smoking was considered as negative ('no') smoking history. [b] Individuals who were treated for at least 3 months during the 4-year follow-up period, were considered as 'yes' for treatment. [c] The patient population was categorized according to radiographic evolution: A (normalization or improvement towards stage I), B (persistent stage II/III or progression in that direction), C (stable stage IV or progressive towards this stage) and Löfgren's (stage I and normalization).

[d] Mean iVC of group C vs. A, B or Löfgren's ($p < 0.001$)

[e] Mean FEV_1 of group C vs. A, B or Löfgren's ($p < 0.001$)

[f] Mean DL*co* of group C vs. A, B or Löfgren's ($p < 0.001$)

Indeed, studies on the gene encoding for TGF-β1, a key factor in fibrogenesis, have shown that polymorphisms can have some bearing on the fibrotic phenotypes in liver and lung fibrosis (32, 33, 45). A substantially larger group of fibrotic sarcoidosis patients is needed to allow for similar association studies with *CMA1* polymorphisms.

Interestingly, a strong association between the *CMA1* –526 C/T polymorphism and iVC, a parameter for pulmonary restriction, was observed. The effect of the T allele on the iVCs of all patients, acting independent of radiographic evolution, evokes ample propositions for explaining the observed phenomenon. It may be that chymase is involved in tissue remodeling in sarcoidosis at a very early stage of the disease. In fact, ECM turn-over and tissue remodeling have been found to be present within the active granulomas of even the early phase of the sarcoid granuloma (46, 47). The increase of ECM in granulomas may be transient and the affected tissue may not necessarily sustain permanent scarring. The machinery of collagen expression and ECM turn-over, partly regulated by chymase (48), may consequently lead to subtle changes in the rigidity of the tissue that may not be discerned by chest x-ray. This may underlie the commonly observed disparity between radiographic staging and lung function data in sarcoidosis (49). The absence of any association between –526 C/T and diffusing capacity (Dl*co*) indicates that the integrity of the alveolar surface area was not compromised and thus suggests against substantial scarring of the alveolar walls.

The position of the promoter polymorphism (-526) was found to have a 87% match with a consensus for factor cAMP-responsive element binding protein (http://motif.genome.jp). Replacement of the C with a T allele negates this consensus, which suggests that the –526 C/T polymorphism may influence the regulation of *CMA1* expression. Since the -526 T substitution is in complete linkage with a T substitution of the -1897 polymorphism, we also tested whether the sequence of the promoter region at position -1897 might show a modification of a regulatory consensus. It showed that the C to T substitution at position -1897 might introduce a transcription factor E47 (87% match) or a possible USF (ubiquitous transcription factor upstream stimulatory factor) binding site (85% match). We cannot however infer from our studies which SNP has an actual functional effect that leads to the observed change of lung function parameter iVC. Moreover, whether the *CMA1* –526 T or the *CMA1* -1897 T allele either diminishes or augments *CMA1* gene expression may only be established when expression studies are performed. The -1897 C/T

polymorphism has also revealed associations with hypertrophic cardiomyopathy (36) and, more recently, with atopy (50). It seems therefore that this polymorphic site is able to influence disease phenotypes at different levels for reasons that have yet to be elucidated.

The influence of *CMA1* polymorphisms could not be evaluated in the Japanese patients due to lack of lung function data and information on radiographic evolution. However, such evaluations were not the rationale for studying the Japanese patient groups since the progression towards pulmonary fibrosis in this cohort was observed in two cases only.

None of the investigated SNPs or haplotypes were found to be associated with cardiac or skin involvement in Japanese patients. Since many patients with cardiac sarcoidosis show no clinical symptoms, the diagnosis is often missed (51, 52). However, patients who are diagnosed with cardiac sarcoidosis, like those in this study, are consequently selected for more advanced cardiac involvement (52). Therefore, genetic variation of *CMA1* would profoundly affect tissue remodeling of the sarcoid heart, the cardiac sarcoidosis group in this study would have sufficed to exemplify associations between cardiac sarcoidosis and *CMA1* genetic variation.

Conclusion

This is the first study to describe *CMA1* genetic variation in sarcoidosis patients and healthy controls of two different ethnicities. No associations were found between any *CMA1* genotype and fibrotic development in Dutch sarcoidosis patients or with cardiac or skin involvement in Japanese sarcoidosis patients. The observed association between the CMA1 –526 C/T polymorphism and iVC suggests that chymase influences the functional outcome of pulmonary sarcoidosis. Additional studies using larger groups of sarcoidosis patients with different clinical phenotypes are recommended to confirm our findings.

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chapter THREE

3

Angiotensin-Converting Enzyme 2 (ACE2) Haplotypes are Associated with PULMONARY DISEASE PHENOTYPES IN SARCOIDOSIS PATIENTS

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Abstract

Angiotensin II (Ang II) formation by angiotensin-converting enzyme (ACE) or other enzymes has shown to exhibit profibrotic properties in a variety of fibrotic diseases. A homologue of ACE called ACE2 has been shown to counteract the formation of Ang II. Genetic variation in the components involved in Ang II formation may underlie the progression of pulmonary sarcoidosis.

Seven ACE2 SNPs, located on the X-chromosome, were investigated using SSP-PCR and haplotypes were constructed. Gender-matched analyses of sarcoidosis patients (80 males/64 females) and controls (110 males/218 females) were performed to correlate disease susceptibility and pulmonary disease phenotypes with ACE2 genotypes and haplotypes.

ACE2 SNPs or haplotypes were not associated with susceptibility for sarcoidosis. Haplotype 4 was only present in sarcoid males without parenchymal involvement (frequency: 0.19) and absent in males with parenchymal involvement ($p=0.006$; $p_{corr.}=0.05$; degrees of freedom (df)=1; OR=0). No significant difference was observed between haplotype 4 frequencies in females with (0.08) or without (0.13) parenchymal involvement ($p=0.5$). Although not significant after correction, analysis of the patient group with fibrosis showed that males with haplotype 5 (0.27) were predominant over those with haplotype 5 of the groups without fibrosis (0.03); $p=0.01$; $p_c=0.08$; df=1; OR=11.4. Females with fibrosis vs. no fibrosis revealed no difference between haplotype 5 frequencies: 0.05 vs. 0.03; $p=0.37$; $p_c=1$; df=1.

These results suggest that ACE2 might be involved in the progression of pulmonary sarcoidosis which may depend on gender. Subsequent studies using larger groups are needed to confirm these findings.

Introduction

Sarcoidosis is a systemic disease of unknown cause which is characterized by the presence of noncaseating granulomas in one or multiple organs (1). The disease is often accompanied by elevated angiotensin-converting enzyme (ACE) levels (2).

ACE is a key enzyme in the renin-angiotensin-aldosterone system (RAAS) and has the ability to form angiotensin II (Ang II), a potent vasoconstrictor (3). The implications of ACE and other components of the RAAS in the pathophysiology of sarcoidosis have not yet been clarified (4). In addition to the systemic RAAS, a local RAAS is also known to exist in a number of organs, including the lung (5). In this system, Ang II is formed locally and is known to exert fibrogenic (6-11) as well as angiogenesis-inducing (12-14) actions under pathological conditions.

The complexity of the RAAS, either acting locally or systemically, is also illustrated by the recent discovery of a homologue of ACE, dubbed ACE2 (15, 16). ACE2 is, like ACE, a membrane-associated and secreted enzyme that is expressed predominantly by, but not limited to, endothelial cells (16). ACE2 is present in many tissues, including the lung, but is less ubiquitous than ACE with the highest expression in renal, cardiovascular and gastrointestinal tissue (17). In vitro studies have shown that ACE2, unlike ACE, is able to convert Ang I to Ang 1-9 (nine amino acids) but unable to cleave bradykinin (15). Whereas Ang II is a potent vasoconstrictor, Ang 1-9 has no known effects other than being a substrate for ACE, resulting in Ang 1-7 (seven amino acids), a putative vasodilator (18). Thus, ACE2 seems to counter the formation of Ang II by ACE, as confirmed by studies using ACE2 knockout mice (19) (see figure 1).

ACE2 has also been identified as a receptor for the SARS (severe acute respiratory syndrome) and has been mapped to a quantitative trait locus (QTL) for hypertension on the X-chromosome (19, 20).

An increasing number of reports now address the role of ACE2 in tissue injury and repair (21, 22). ACE2 studies in relation to sarcoidosis, however, have not yet been reported. Hypothetically, when ACE levels are elevated, as often seen with sarcoidosis, ACE2 could mitigate the potentially deleterious effects of increased Ang II formation. The presence of ACE2 in the lung parenchyma (17) may therefore be crucial in the events involving the pulmonary RAAS in the pathophysiology of sarcoidosis.

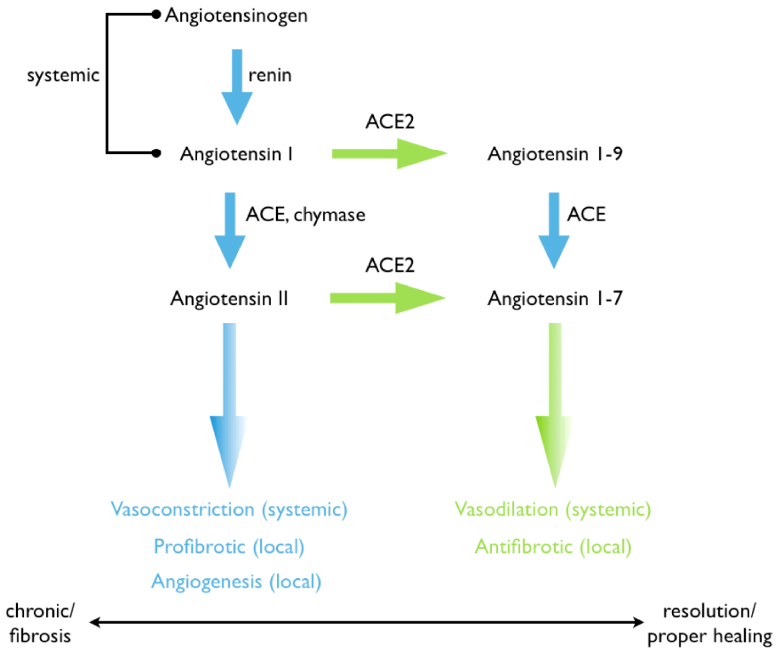

Figure 1 The counteractive systemic and local actions between ACE, chymase and ACE2 in formation of Angiotensin II (Ang II) and other biologically active and inactive peptides, based on published work (15, 16, 45). In this model, the balance between Ang II formation and hydrolysis mediated by ACE2 is central in the outcome of the injured tissue.

Association studies on polymorphisms in genes that encode for components of the RAAS such as ACE have been amply investigated in sarcoidosis. However, the associations between ACE gene polymorphisms and disease susceptibility or severity vary between the studies published (23-25). These inconsistent findings are likely due to additional factors that modulate the activity of ACE against the background of ethnic origin of the studied groups.

In this study, we evaluated seven polymorphisms spanning virtually the total distance of the ACE2 gene. We investigated whether ACE2 genotypes and haplotypes would reveal an association between either sarcoidosis susceptibility or pulmonary disease phenotypes in a group of Dutch Caucasian sarcoidosis patients. The cohort was stratified as patients suffering from either acute, with no parenchymal involvement (including a substantial number of patients with Löfgren's syndrome), or chronic type disease with parenchymal involvement (including fibrotic end-stage), according to radiographic evolution over a 4-year follow-up period.

Subjects and Methods

Patients

One hundred and forty-four unrelated Dutch Caucasian sarcoid patients (80 males/64 females; age at diagnosis ± standard deviation (years): 36.2 ± 10.5, range: 17 – 71) were included in this study. The diagnosis of sarcoidosis was established when clinical findings were supported by histological evidence and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis (26). In 44 (16 males/28 females) patients, the diagnosis was made without biopsy proof because these patients presented with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy.

The medical ethical committee of the St. Antonius Hospital approved the study conducted and all subjects gave formal written consent.

Controls

Venous blood samples were obtained from 328 (118 males/210 females; age (years): 39.0 ± 11.4, range: 18 - 68) unrelated, Dutch Caucasian employees of the St. Antonius Hospital in The Netherlands. By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases.

Evaluation of extrapulmonary disease

All patients were assessed for extrapulmonary disease, in agreement with the consensus provided by the ATS/ERS/WASOG statement on sarcoidosis (26).

Chest radiography

Chest radiographs were assessed to determine disease severity using standard radiographic staging for sarcoidosis, classified according to the Scadding criteria: 0, normal chest radiographic findings; I, bilateral hilar adenopathy with normal lung parenchyma; II, bilateral hilar adenopathy with parenchymal involvement; III, parenchymal involvement without hilar adenopathy; IV, pulmonary fibrosis/fibrocystic parenchymal involvement (26).

Radiographic evolution over a 4-year follow-up period (presentation, 2 and 4 years) was available for 95 patients (60 males/35 females) and was categorized as follows: A (normalization or improvement towards stage I), B (persistent stage II/III or progression in that direction), and C (stable stage IV or progressive

towards this stage) (27). An additional 5 patients (4 males/1 female) with radiographic stage IV had been diagnosed outside of this hospital prior to referral. Of these patients, radiographic data at presentation were unavailable. Patients who had been diagnosed with Löfgren's syndrome at presentation (16 males/28 females) were considered as a distinct group with radiographic evolution not exceeding stage I. Patient characteristics are summarized in table 1.

Pulmonary function tests.
Spirometric tests were performed at presentation and upon 2 years, and 4 years following diagnosis. Forced (inspiratory) vital capacity (FVC), forced expiratory volume in 1 second (FEV_1) and carbon monoxide diffusing lung capacity (D*lco*) were used to assess the presence of lung function impairment at presentation and follow-up of disease. All lung function parameters are expressed as percent predicted values. FVC and FEV_1 were calculated from volumes in liters and adjusted to BTPS (body temperature, ambient pressure, saturated with water vapor) in accordance with the ERS recommendations (28).

Sequence-Specific Primers and Polymerase Chain Reaction
A total of 7 biallelic ACE2 single nucleotide polymorphisms: 971 G/A [intron 1]; 1298 C/T [intron 1]; 3581 C/T [intron 1]; 8686 G/A [intron 3/exon 3 boundary]; 14169 T/C [intron 6]; 21010 C/T [intron 8] and 36425 G/A [intron 18], were determined using sequence-specific primers (SSPs) and polymerase chain reaction (PCR). The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in table 2. The PCR conditions were as previously described (29).

Table 1 Characteristics of 144 Dutch sarcoidosis patients used for 4-year follow-up evaluation.

Gender (males/females)	80/64
Age at presentation (years)	
males (mean ± SD) / range	34.2 ± 9.3 / 17-67
females (mean ± SD) / range	39.4 ± 11.4 / 20-71
Smoking history (yes/no/unknown)[a]	
males	36/36/8
females	19/42/3
Treatment (yes/no/unknown)[b]	
males	52/23/5
females	29/33/2
Radiographic evolution (males/females)[c]	
A	27/18
B	22/9
C	15/9
Löfgren's	16/28
Lung function parameters[d]	
Males	
FVC (mean ± SD) (% predicted) (n = 48)	93.4 ± 13.8
DL*co* (mean ± SD) (% predicted) (n = 23)	86.8 ± 16.9
FEV_1 (mean ± SD) (% predicted) (n = 50)	86.0 ± 21.4
females	
FVC (mean ± SD) (% predicted) (n = 31)	99.4 ± 15.2
DL*co* (mean ± SD) (% predicted) (n = 18)	80.6 ± 17.8
FEV_1 (mean ± SD) (% predicted) (n = 35)	86.4 ± 16.6

Gender, number of cases with smoking history, and treatment are given as absolute numbers (n). Age (years) is given as mean ± standard deviation (SD) and range. [a] A cut-off period of 5 years following cessation of smoking was considered as negative ('no') smoking history. [b] Individuals who were treated for at least 3 months during the 4-year follow-up period, were considered as 'yes' for treatment. [c] A (normalization or improvement towards stage I), B (persistent stage II/III or progression in that direction), C (stable stage IV or progressive towards this stage) and Löfgren's (stage I and normalization). [d] Mean ± SD of lung function parameters was calculated from 3 repeated measures per individual over a 4-year follow-up period (presentation, 2 years and 4 years).

Haplotype determination

ACE2 haplotypes were determined using Phase, version 2 (Mac OS X). This program uses an advanced algorithm based on a statistical method by Stephens et al. (30, 31). Samples with rare haplotypes (in our total cohort occurring less than 4 times) were re-genotyped to rule out erroneous typing (32). No mistyping was observed for any of the repeated samples.

Table 2 Primer sequences for the identification of 7 biallelic single nucleotide polymorphisms of ACE2.

Polymorphism	dbSNP accession[a]	Primer	Consensus	final conc. (ng/μl)	product size (bp)
971 (G/A)	rs1978124	5′-TGC TGA TGT AGA AGT GTG GAG A G/A (forward)	5′- TGG GCC TCA TGC TCT CTC T (reverse)	7.6	248
1298 (C/T)	rs4646120	5′- CCT CCT GGG CTC AAA TGA T C/T (forward)	5′- CAG GAA TAG GTT CAG AAA TGG G (reverse)	7.6	267
3581 (C/T)	rs4646127	5′- CGC AGT GAG CCA TGA TC G/A (reverse)	5′- GCA GAC CAA ACT CCT CTT CC (forward)	7.6	392
8686 (G/A)	rs2285666	5′- GCT TAT TAC TTG AAC CAG GTA G/A (forward)	5′- TGC TTG CCG ACC TCA GAT CT (reverse)	7.6	475
14169 (T/C)	rs1514279	5′-TAT TGC ACC AGG TAC TAT GCT A/G (reverse)	5′-AGA AAG GAC CTG TGG AGG CT (forward)	7.6	345
21010 (C/T)	rs4646152	5′- GTT ACA ACT ACT CAG CTC TGC C C/T (forward)	5′-GAT AGT GGG AGG GCA TGG TA (reverse)	7.6	398
36425 (G/A)	rs233574	5′-CTA GGT GAC AGA GCA AGA CTC C/T (reverse)	5′-TCC CCA GTG CTA CCT CCA AAT (forward)	7.6	425

[a] The SNP loci were identified using accession numbers according to the SNP database at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp (accessed September 2004).

Statistical analysis

Statistical analysis was performed using chi-square contingency table analysis with the appropriate number of degrees of freedom (df) (SPSS for Windows; SPSS Inc., Chicago, IL). Fisher's exact test was used if expected cell frequencies

were lower than 5. Adjustment for multiple tests was made using the formula $p_c = p \times n$, where p_c is the corrected value, p the uncorrected value and n the number of SNPs or haplotypes determined (Bonferroni method). Genotype frequencies were tested for Hardy-Weinberg equilibrium. One-way ANOVA, controlled for smoking and radiographic evolution, was used to assess the influence of ACE2 genotypes and haplotypes on lung function parameter changes at presentation, 2-years and 4-years following presentation). All values of lung function parameters were log transformed.
Statistical significance was denoted by a value of $p < 0.05$ for all tests performed.

Results

Allele and genotype frequencies.
Because the ACE2 gene is located on the X-chromosome, the Hardy-Weinberg (HW) equilibrium was only calculated for the female subjects in the patient group and controls. No deviation from the HW equilibrium was found for the females of either group.
The allele and genotype frequencies of seven SNPs are listed in table III. The sarcoidosis and control groups were subdivided into males and females for comparing the X-chromosome-located alleles between patients and controls. No differences were found between healthy controls and sarcoidosis patients of either gender with respect to allele and genotype distributions (table 3).

Haplotype construction and frequencies.
The construction of haplotypes based on 7 SNPs produced a total of 13 haplotypes of which 5 were most common, 3 were rare and 6 occurred only once or twice in the total population. Haplotype frequencies in male and female sarcoidosis patients and controls are summarized in table 4. The frequency of haplotype 2 was lower in sarcoid males (0.12) than in healthy controls (0.23), but was not statistically significant, $p = 0.06$. Haplotype 6 was found in sarcoid males (0.08), but not in healthy controls. After correcting the p-value, the observed difference lost its significance ($p = 0.02$, $p_c = 0.16$). Haplotype frequencies between sarcoid and healthy females were not different between sarcoidosis patients and healthy controls (table 4).

Table 3 Allele and genotype frequencies in Dutch Caucasian male and female sarcoidosis patients and controls.

polymorphism	allele and genotype	Sarcoidosis patients		Controls	
		Males[a] n = 80	Females n = 64	Males[a] n = 118	Females n = 210
971	G	0.62 (50) [b]	0.49 (63) [b]	0.58 (68) [b]	0.50 (210) [b]
	A	0.38 (30)	0.51 (65)	0.42 (50)	0.50 (210)
	GG	-	0.26 (34)	-	0.23 (96)
	AG	-	0.47 (60)	-	0.53 (224)
	AA	-	0.27 (34)	-	0.24 (100)
1298	C	0.62 (50)	0.49 (63)	0.60 (71)	0.50 (210)
	T	0.38 (30)	0.51 (65)	0.40 (47)	0.50 (210)
	CC	-	0.26 (34)	-	0.23 (96)
	CT	-	0.47 (60)	-	0.53 (224)
	TT	-	0.27 (34)	-	0.24 (100)
3581	C	0.77 (62)	0.63 (81)	0.69 (81)	0.65 (273)
	T	0.23 (18)	0.37 (47)	0.31 (37)	0.35 (147)
	CC	-	0.40 (51)	-	0.41 (172)
	CT	-	0.46 (59)	-	0.48 (202)
	TT	-	0.14 18)	-	0.11 (46)
8686	G	0.71 (57)	0.81 (104)	0.75 (88)	0.81 (340)
	A	0.29 (23)	0.19 (24)	0.25 (30)	0.19 (80)
	GG	-	0.63 (80)	-	0.65 (272)
	AG	-	0.36 (46)	-	0.32 (136)
	AA	-	0.01 (2)	-	0.03 (12)
14169	T	0.76 (61)	0.63 (80)	0.67 (79)	0.64 (268)
	C	0.24 (19)	0.37 (48)	0.33 (39)	0.36 (152)
	TT	-	0.40 (51)	-	0.41 (172)
	CT	-	0.46 (59)	-	0.46 (193)
	CC	-	0.14 (18)	-	0.13 (55)
21010	C	0.76 (61)	0.63 (80)	0.68 (80)	0.64 (268)
	T	0.24 (19)	0.37 (48)	0.32 (38)	0.36 (152)
	CC	-	0.40 (51)	-	0.41 (172)
	CT	-	0.46 (59)	-	0.46 (193)
	TT	-	0.14 (18)	-	0.13 (55)
36425	G	0.81 (65)	0.65 (83)	0.74 (87)	0.68 (286)
	A	0.19 (15)	0.35 (45)	0.26 (31)	0.32 (134)
	GG	-	0.45 (58)	-	0.46 (194)
	AG	-	0.41 (52)	-	0.44 (184)
	AA	-	0.14 (18)	-	0.10 (42)

[a] Because males are hemizygous for ACE2, genotype frequencies in males are omitted as they are not representative. [b] Frequencies are calculated from the number of alleles (n). Absolute numbers of alleles are in parentheses.

Parenchymal involvement and fibrosis in pulmonary sarcoidosis

Male and female patients were grouped according to disease with and without parenchymal involvement, including those with Löfgren's syndrome, as assessed by radiographic evolution over a 4-year follow-up period. By taking this dichotomous approach, a gender-matched analysis could be performed while retaining a substantial number of subjects. The results are summarized in table 5.

Haplotype 4 was only present in sarcoid males (frequency = 0.19) without parenchymal involvement and absent in men with parenchymal involvement; $p = 0.006$; $p_c = 0.05$; degrees of freedom (df) = 1; OR = 0. No significant difference was observed between haplotype 4 frequencies in females with (0.08) or without parenchymal involvement (0.13), $p = 0.5$. The haplotype 4 frequency in male controls (0.10) was not significantly different from males either with (0) or without (0.19) parenchymal involvement ($p > 0.1$ for either comparison). Females also failed to show a significant difference between haplotype 4 frequencies in controls (0.14) and sarcoidosis patients with (0.08) or without (0.13) parenchymal involvement ($p > 0.1$ for either comparison). Received treatment did not confound the observed effects of haplotype 4 carriage for either gender.

Analysis of the patient group with fibrosis (group C) showed that males with haplotype 5 (0.27) were predominant over those with haplotype 5 of groups A, B and Löfgren's combined (0.03); $p = 0.01$; $p_c = 0.08$; df = 1; OR = 11.4 (table 5). Females of group C vs. groups A, B and Löfgren's revealed no difference between haplotype 5 frequencies: 0.05 vs. 0.03; $p = 0.37$; $p_c = 1$; df = 1 (table not shown). No further associations were found between radiographic evolution and ACE2 genotype or allele frequencies (data not shown).

Lung function parameters for ACE2 genotypes and haplotypes

We also evaluated the possibility of ACE2 genotypes or haplotypes influence on lung function parameters FVC, FEV_1 and D*lco*. One-way ANOVA analysis was performed to chart the influence of ACE2 genotypes and haplotypes on lung function at the time of presentation, at 2 and 4 years after presentation. This univariate analysis was controlled for radiographic evolution, smoking, gender and treatment.

Neither any of the investigated SNPs nor any of the haplotypes was associated with FVC, FEV_1 or D*lco* (data not shown).

Table 4 ACE2 haplotype frequency distributions in male and female sarcoidosis patients and healthy controls.

haplo type	SNP position							sarcoidosis patients		controls	
	971	1298	3581	8686	14169	21010	36425	Males n = 80	Females n = 64	Males n = 118	Females n = 210
1	G	C	C	G	T	C	G	0.44 (35) [a]	0.33 (42) [a]	0.35 (41) [a]	0.31 (129) [a]
2	A	T	T	G	C	T	A	0.12 (10) [c]	0.32 (41)	0.23 (27) [c]	0.28 (117)
3	G	C	C	A	T	C	G	0.16 (13)	0.16 (21)	0.18 (22)	0.13 (57)
4	A	T	C	G	T	C	G	0.10 (8)	0.12 (14)	0.10 (12)	0.14 (61)
5	A	T	T	G	C	T	G	0.06 (5)	0.02 (3)	0.07 (8)	0.02 (8)
6	A	T	C	A	T	C	G	0.08 (6) [d]	0.03 (4)	0 [d]	0.05 (21)
7	G	C	C	A	T	C	A	0.03 (2)	0	0.02 (2)	0.03 (13)
8-13[b]	-	-	-	-	-	-	-	0.01 (1)	0.02 (3)	0.05 (6)	0.04 (16)

[a] Frequencies are calculated from the number of alleles (n). Absolute numbers of alleles are in parentheses.
[b] Each haplotype occurred no more than twice in each group.
[c] $p = 0.06$, odds ratio (OR) = 2.3, degrees of freedom (df) = 1
[d] $p = 0.02$, $P_{corrected} = 0.16$, OR = 0, df = 1

Discussion

In this study, we were able to construct haplotypes using 7 biallelic single nucleotide polymorphisms (SNPs) from the gene coding for ACE2 in a group of clinically well-defined unrelated Dutch Caucasian sarcoidosis patients and controls. Association analyses revealed gender-dependent effects of haplotypes 4 and 5 on radiographic evolution. The observed protective effect of haplotype 4 against progression of parenchymal involvement in males may provide a clue about the implication of ACE2 in the development of granulomas in the lung parenchyma. In addition, the preponderance of haplotype 5 presence in males with fibrotic disease (stage IV) may suggest a role of ACE2 in the development of chronic disease with subsequent fibrosis in

sarcoidosis. Consequent to the rare occurrence of these haplotypes in the total population and the further reduction of the cohort by the gender-based division, the remaining number of patients used for comparison were low. Although the results described here may indicate the implication of genetic variation of ACE2 on pulmonary phenotypes in sarcoidosis, our data should be interpreted with caution until a study with a larger number of subjects can confirm our findings.

Table 5 Parenchymal involvement and ACE2 haplotype frequencies in male and female sarcoidosis patients.

haplotype	**parenchymal involvement (n = 144)**				**controls (n = 328)**	
	no		yes			
	Males (n = 43)	Females (n = 46)	Males (n = 37)	Females (n = 18)	Males (n = 118)	Females (n = 210)
1	0.49 (21) [a]	0.32 (30) [a]	0.38 (14) [a]	0.33 (12) [a]	0.35 (41) [a]	0.31 (129) [a]
2	0.12 (5)	0.32 (29)	0.12 (4)	0.33 (12)	0.23 (27)	0.28 (116)
3	0.14 (6)	0.16 (15)	0.22 (8)	0.17 (6)	0.18 (21)	0.15 (62)
4	0.19 (8) [b]	0.13 (12) [c]	0 [b, d]	0.08 (3) [c, e]	0.10 (12) [d]	0.14 (57) [e]
5	0.02 (1) [f]	0.03 (3)	0.14 (5) [f]	0.03 (1)	0.07 (8)	0.05 (20)
6	0.04 (2)	0.01 (1)	0.09 (3)	0.03 (1)	0	0.02 (7)
7	0	0	0.03 (1)	0	0.02 (3)	0.03 (12)
8-13	0	0.02 (2)	0.06 (2)	0.03 (1)	0.05 (6)	0.04 (17)

[a] Frequencies are calculated from the number of alleles (n). Absolute numbers are in parentheses. The development of parenchymal involvement was based on radiographic evolution over a 4-year follow-up period: patients who had presented with or had progressed towards radiographic stages II and higher were considered as 'yes' for parenchymal involvement; patients with Löfgren's syndrome and those who had normalized stage 0 or resolved to radiographic stage I were considered as 'no' for parenchymal involvement.

[b] parenchymal vs. non-parenchymal involvement: $p = 0.006$; $p_{corr.} = 0.05$; OR = 0

[c] parenchymal vs. non-parenchymal involvement: $p = 0.5$

[d] controls vs. parenchymal involvement: $p = 0.24$

[e] controls vs. parenchymal involvement: $p = 0.31$

[f] Four out of 5 male patients with haplotype 5 had pulmonary fibrosis (radiographic evolution, group C): group C vs. group A, B and Löfgren's, $p = 0.01$; $p_c = 0.08$; OR = 11.4

The observation that haplotype-associated differences were found only among men may suggest that predisposing genetic factors causing parenchymal damage in sarcoidosis are dependent on gender. In our cohort, males were

more likely to develop sarcoidosis with parenchymal involvement than females (males: 47% vs. females: 27%; OR = 2.3; p = 0.02). To our knowledge, such a difference has not been described in epidemiological studies on sarcoidosis (26, 33, 34). The ACE2 gene is located on the X-chromosome and the effects of ACE2 expression may act in function of the different genetic backgrounds between males and females. In this regard, whether the observed protective effect of haplotype 4 in males rather than females in our cohort has any relation to the increased propensity of men to exhibit parenchymal involvement cannot be concluded from this study. In order to establish whether the observed protective effect of haplotype 4 to parenchymal involvement in males is indeed gender-specific or based on the fact that no other haplotype can be concomitantly present (one X-chromosome) that could negate the protective effects, female patients who are homozygous for haplotype 4 are needed. In such case, a comparison could be made between heterozygous and homozygous females with respect to parenchymal involvement. Similarly, females homozygous for haplotype 5 would allow us to further explore the preliminary results of the effects of haplotype 5 on pulmonary fibrosis in males.

In agreement with earlier reports, a difference between FVC, a parameter for restrictive impairment, was clearly seen between patients with or without parenchymal involvement (90% vs. 100%, respectively; $p < 0.000002$) (35, 36). Our analysis revealed no influence of SNPs or haplotypes on FVC of the groups with different radiographic evolution. Thus, modulation of lung function is not likely to be influenced by differential ACE2 genotypes or haplotypes.

Ang II is mitogenic for fibroblasts (6) and is also found to enhance angiogenesis in vivo (12-14), of which the latter has been found in organs affected by sarcoidosis (37, 38). It has been shown that in case of injured heart (21) and kidney (22), the down-regulation of Ang II by ACE2 may co-determine the outcome of tissue inflammation, repair and ensuing fibrosis. ACE2 is abundantly present on the surface of lung alveolar epithelial cells (39). Thus, modulation of Ang II expression by ACE2 may be key in the perpetuation of sarcoidosis, exemplified by parenchymal damage and development of fibrosis.

The associations between radiographic evolution and ACE2 haplotypes, but not between individual SNPs, reiterates the pertinence of using the haplotype-construction approach for association studies. Using haplotypes to capture diversity within the gene of interest has substantial power to detect associations

with other, as yet unidentified, common variants within the same gene (40-42). Polymorphisms in the ACE2 gene have been evaluated in patients with essential hypertension and disease outcome in SARS, but have not revealed any support for a genetic basis of ACE2 in either disease (43, 44). Our study included two SNPs used in the aforementioned studies, namely at position 971 (intron 1) and position 8686 (intron 3). Neither the hypertension study nor the SARS study employed haplotyping of the ACE2 gene to search for associations. It is therefore possible that these associations exist but have yet to be uncovered.

How ACE2 coded by either haplotype 4 or 5 exert differential actions on their substrate cannot be deduced from this study. In terms of the model described in this paper, however, it is feasible to suggest that ACE2 haplotypes 4 and 5 result in contrasting phenotypic characters of their gene product.

On a critical note, as with all genetic association studies, considerations such as population size and bias should be taken seriously. To fully rely on the association found with ACE2 haplotypes 4 in relation to parenchymal involvement and haplotype 5 with the presence of fibrosis should therefore be repeated with a substantially larger group of patients with various degrees of pulmonary sarcoidosis.

Conclusion

In conclusion, this first study to address genetic variation of ACE2 in relation to sarcoidosis has yielded intriguing data which indicate that ACE2 haplotypes are associated with the course of the disease. Protein studies are needed to delineate the role of ACE2 in sarcoidosis.

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chapter FOUR

Transforming growth factor-β gene polymorphisms in SARCOIDOSIS PATIENTS WITH AND WITHOUT FIBROSIS

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Abstract

About 25% of patients with chronic sarcoidosis develop pulmonary fibrosis. Transforming growth factor (TGF)-β1 plays a central role in fibrosis and accruing reports address the implication of TGF-β2 and β3 in this process.

We determined whether single nucleotide polymorphisms (SNPs) in the TGF-β1, β2 and β3 genes might contribute to pulmonary fibrosis in sarcoidosis patients. Five SNPs per TGF-β gene were investigated.

Patients with either acute/self-remitting (n=50) and Löfgren's syndrome (n=46) or chronic disease without (n=34) and with fibrosis (n=24) assessed over a 4-year follow-up period were compared. The controls comprised 315 individuals.

Polymorphism frequencies were not discordant between the patients and controls. The TGF-β3 4875 A allele was significantly higher in fibrotic patients (carrier frequency: 0.29) than in patients with acute/self-remitting (0.06) and chronic (0.03) sarcoidosis combined, $p_{corrected}$ =0.01, OR=7.9. The TGF-β3 17369 C allele carrier frequency was significantly higher in fibrotic patients (0.29) compared to acute/self-remitting (0.08) and chronic (0.06) patients combined, p_c = 0.05, OR = 5.1.

Although not significant after correction, the TGF-β3 15101 G allele carrier frequency was lower in fibrotic patients (0.79) compared to acute/self-remitting (0.94) and chronic (1.00) patients combined, p=0.02, p_c=0.1, OR=0.15. The TGF-β2 59941 G allele was more abundant in fibrotic patients (0.62) compared to patients with acute/self-remitting (0.41) and chronic sarcoidosis combined (0.28), p = 0.04, p_c = 0.2, OR = 2.9. TGF-β1 gene polymorphisms were not associated with fibrosis.

This study is the first to suggest the implication of genetic variation of TGF-β3 in the predilection to develop pulmonary fibrosis in sarcoidosis patients.

Introduction

Sarcoidosis is a systemic disease of unknown cause, which is characterized by the presence of noncaseating granulomas in one or multiple organs.(1) In approximately 90 percent of patients with sarcoidosis, the disease is manifested as pulmonary granulomas (2). Although parenchymal abnormalities often resolve spontaneously, about 20-25% of cases will ultimately develop pulmonary fibrosis (3). Pulmonary fibrosis is marked by a disproportionate increase in extracellular matrix (ECM) deposition, produced by proliferating fibroblasts that reside in the lungs (4, 5). Isoform 1 of the TGF-β family has been extensively scrutinized in fibrotic diseases such as sarcoidosis with radiographic stage IV (6, 7) and idiopathic pulmonary fibrosis (8, 9).

In contrast to their relative, isoforms TGF-β2 and TGF-β3 have yet to receive due attention. In the lung, the expression pattern of TGF-β3 suggests that an imbalance between TGF-β1 and TGF-β3 is key in the development of fibrosis upon tissue injury (10-12).

Polymorphisms in the genes encoding for all three TGF-β isoforms have been identified and linked to variations of protein expression or functionality. SNPs in the TGF-β1 gene, present in codon 10 (Leu10Pro) and codon 25 (Arg25Pro) are both associated with interindividual variation in TGF-β1 production (13). These, as well as other polymorphisms of TGF-β1, have been shown to confer an increased risk to e.g., pulmonary fibrosis (14, 15).

Both TGF-β2 and TGF-β3 gene polymorphisms, identified with microsatellite markers, have been associated with cutaneous fibrosis in systemic scleroderma (16). A genetic basis for the pathogenesis of this fibrotic disease is thus strongly supported.

In sarcoidosis, studies on gene polymorphisms of the TGF-β family are scant. Both Leu10Pro and Arg25Pro polymorphisms in the TGF-β1 gene have been evaluated for implication in sarcoidosis disease progression in Japanese(17) and Caucasians (18). However, no associations were found between polymorphisms and disease progression, susceptibility or severity in either study.

We hypothesize that TGF-β plays an important role in the development of pulmonary fibrosis in sarcoidosis and that genetic variation of TGF-β1, β2 and β3 underlies the propensity to develop fibrosis in patients affected with sarcoidosis. Therefore, we sought to determine whether SNPs and constructed

haplotypes of either isoform TGF-β1, 2 or 3 might be differentially distributed in sarcoidosis patients who develop fibrosis compared to those who do not, as assessed by radiographic evolution over a 4-year follow-up period.

Subjects and Methods

Subjects

One hundred and forty-five unrelated Dutch Caucasian sarcoidosis patients (88 males/66 females; mean age ± SD/range at diagnosis: 36.5 ± 10.4/17 – 71), diagnosed between 1965 and 1999 were included in this study. The diagnosis of sarcoidosis was established when clinical findings were supported by histological evidence and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis.(19) In 46 patients, the diagnosis was made without biopsy proof because these patients presented with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy.

The medical ethical committee of the St. Antonius Hospital approved the study conducted and all subjects gave formal written consent.

Chest radiographs were assessed in consensus by chest physicians specialized in diffuse lung diseases to determine disease severity using standard radiographic staging for sarcoidosis, classified according to the Scadding criteria (19). Radiographic data were available of 101 sarcoidosis patients. One patient had presented with stage 0 (normal), 44 patients had presented with stage I, 28 patients with stage II, 25 with stage III, and 3 patients with stage IV. Radiographic evolution over a 4-year follow-up period (presentation, 2 and 4 years) was categorized as follows: acute/self-remitting (normalization or improvement towards stage I) (n = 50), chronic (persistent stage II/III or progression towards this stage) (n = 34), and fibrosis (stable stage IV or progressive towards this stage) (n = 24). Of the last group, 7 patients with radiographic stage IV had been diagnosed outside of this hospital prior to referral. High resolution computed tomography (HRCT) was available for 16 patients with stage IV sarcoidosis at 0-6 years following presentation. Of these patients, three main patterns of fibrosis (linear pattern, bronchial distortion and honeycombing) were semiquantified, according to a classification established by Abehsera et al (20). Namely, 11 patients had

minimal to moderate linear patterns of whom 6 had concomitant moderate to severe bronchial distortion; 4 patients had both severe linear patterns and severe bronchial distortion; 1 patient had severe honeycombing, a severe linear pattern and severe bronchial distortion.
Forty-six patients had been diagnosed with Löfgren's syndrome (radiographic stage I at presentation). Of this group, 45 patients had received radiographic follow-up. These patients had normalized chest radiography after 2 or 4 years following presentation, except for 1 patient, who remained at radiographic stage I after 4 years. The diagnosis, rather than radiographic follow-up, was regarded as a category. Due to limitation of available sample material, 4 patients of the acute/self-remitting group, 3 of the chronic group and 2 patients with Löfgren's syndrome could not be genotyped for TGF-β2.
The control group comprised 315 (118 males/197 females; age (years): 39.0 ± 11.4, range: 18 - 68) healthy, unrelated, Dutch Caucasian employees of the St. Antonius Hospital in The Netherlands.

Genotyping
Biallelic single nucleotide polymorphisms were determined using sequence-specific primers (SSPs) and polymerase chain reaction (PCR).
The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in Table 1. The PCR conditions were as previously described (21). The final primer concentrations used were 7.6 ng/μl except for SNP rs1800472 and rs8179181, which were both 3.8 ng/μl. To control for erroneous genotyping, previously TGF-β genotyped samples were inserted blindly to the person performing the assay. No discrepancies were found between the genotypes of the analyzed and re-analyzed samples.
In addition, all patients were genotyped for the TGF-β1 28 T/C polymorphism using the LightCycler (LC) system (Roche Diagnostics, Basel, Switzerland) as previously described (22). The LightCycler and SSP-PCR methods for determination of the 28 T/C polymorphism revealed no discrepancies.

Pulmonary function tests
Pulmonary function tests were performed at presentation and upon 2 years, and 4 years following diagnosis. Vital capacity (inspiratory) (iVC), forced expiratory volume in 1 second (FEV_1) and carbon monoxide diffusing lung capacity (D*lco*) were used to assess the presence of lung function impairment

at presentation and follow-up of disease. All lung function parameters are expressed as percent predicted values. IVC and FEV_1 were calculated from volumes in liters and adjusted to BTPS (body temperature, ambient pressure, saturated with water vapor) in accordance with the ERS recommendations (23).

Statistical analysis

Statistical analyses of SNP and haplotype frequency distributions were performed using chi-square contingency table analysis with the appropriate number of degrees of freedom (df) (SPSS for Windows; SPSS Inc., Chicago, IL). Fisher's exact test was used if expected cell frequencies were lower than 5. Adjustment for multiple tests was made by multiplying the p-value by the number of SNPs or haplotypes of each gene (Bonferroni method). Genotype frequencies were tested for Hardy-Weinberg equilibrium. Haplotypes were determined using Phase, version 2 (Mac OS X) (24, 25).

Statistical power was calculated with an online tool (case-control for discrete traits test), available at http://pngu.mgh.harvard.edu/~purcell/gpc/.

Multivariate analysis, controlled for radiographic evolution, smoking, and treatment, was used to assess the influence of TGF-β genotypes and haplotypes on lung function parameter changes over a 4-year follow-up period. A cut-off period of 5 years following cessation of smoking was considered as negative ('no') smoking history. Individuals who were treated with corticosteroids for at least 3 months during the 4-year follow-up period, were considered as 'yes' for treatment. No other treatment regimens had been used in the study group. All values of lung function parameters were log transformed.

Statistical significance was denoted by a value of $p < 0.05$ for all tests performed.

Results

The distributions of allele carrier and genotype frequencies of TGF-β1, TGF-β2 and TGF-β3 in the sarcoidosis patient group and healthy controls did not deviate from Hardy-Weinberg equilibrium.

Polymorphism frequencies were not discordant between the total patient population and controls (data not shown).

Polymorphism frequencies did not differ between patients with or without received treatment, between smoking history or between different radiographic stages at presentation (data not shown). The C allele carrier frequency of the

TGF-β1 28 T/C polymorphism was lower in Löfgren's patients (0.52) compared to controls (0.69), which was ascribed to an increased TT (0.48) and decreased CT (0.35) genotype frequency compared to the controls ((TT: 0.31 and CT: 0.51), p = 0.05; p_c = 0.25; degrees of freedom (df) = 2). Patients with fibrosis showed a preponderance of the G allele (carrier frequency: 0.62) of the TGF-β2 59941 A/G polymorphism compared to patients with acute/self-remitting (0.41) and chronic sarcoidosis (0.28) combined, p = 0.04, p_c = 0.2, odds ratio (OR) [95% confidence interval (CI)] = 2.9 [1.1 - 7.4] (Figure 1). Furthermore, controls had a TGF-β2 59941 A/G genotype distribution, AG (0.33) and AA (0.64), that was different from that of patients with fibrosis, AG (0.58) and AA (0.38), p = 0.03, p_c = 0.15, df = 2.

Table 2 summarizes the results of TGF-β3 gene polymorphisms in sarcoidosis patients with different radiographic evolutions and controls. The presence of the A allele of the TGF-β3 4875 G/A polymorphism was significantly higher in patients with fibrosis compared to patients with acute/self-remitting and chronic sarcoidosis combined, with carrier frequencies of 0.29 vs. 0.06 and 0.03 respectively, p = 0.002, p_c = 0.01, OR = 7.9 [2.1 - 30.9] (see also Figure 1).

The TGF-β3 4875 G/A frequency distributions were not different between controls and patients with fibrosis, p = 0.13, df = 2.

For TGF-β3 SNP 15101 G/A, more AA (0.21) homozygous and less GG (0.50) homozygous individuals were observed in the fibrosis group compared to acute/self-remitting (AA (0.06), GG (0.60)) and chronic (AA (0), GG (0.59)) patients combined, p = 0.017, p_c = 0.09, df = 2. Moreover, the carrier frequency of the G allele of SNP 15101 G/A was lower in fibrosis patients (0.79) compared to patients with acute/self-remitting (0.94) and chronic (1.00) sarcoidosis combined, p = 0.02, p_c = 0.1, OR = 0.15 [1.6 - 32.3] (see also Figure 1). Compared to controls, the G allele carrier frequency in patients with fibrosis was also lower (0.79 vs. 0.94, p = 0.016, p_c = 0.08, OR = 0.23 [0.08 – 0.71].

Finally, the C allele of the TGF-β3 17369 T/C SNP was found to be significantly more abundant in sarcoidosis patients with fibrosis (carrier frequency: 0.29) compared to acute/self-remitting (0.08) and chronic (0.06) disease combined, p = 0.01, p_c = 0.05, OR = 5.1 [1.6 - 17.7] (see also Figure 1).

Haplotypes were constructed for all TGF-β genes from 5 polymorphic loci in each gene. The genotypes assigned to seven TGF-β1 and thirteen TGF-β2

haplotypes (data not shown). Six haplotypes were found for the TGF-β3 gene (Table 3). The resulting haplotypes and their frequency distributions were evaluated in patients with classified radiographic evolutions and controls. All haplotype frequencies of TGF-β1, β2 and β3 were equally distributed between sarcoidosis patients and controls, and between patient with different radiographic evolutions, treatment status or smoking history (data not shown). Only TGF-β3 haplotype 4 correlated with fibrosis in sarcoidosis patients (Table 3). This haplotype is different from the others by the 4875 G to A substitution and thus showed a similar frequency distribution as the individual 4875 G/A SNP.

We also evaluated the possibility of TGF-β genotypes or haplotypes influence on lung function parameters IVC, FEV_1 and D*lco*. Multivariate analysis was performed to chart the influence of TGF-β genotypes and haplotypes on lung function at the time of presentation, at 2 and 4 years after presentation. This analysis was controlled for radiographic evolution, smoking, and treatment. Table 4 summarizes the effects of different variables on lung function parameters of sarcoidosis patients over a 4-year follow-up period. TGF-β3 haplotype 4 is used to illustrate the analysis of all TGF-β polymorphisms and its potential effect on lung function. All lung function parameters were significantly decreased in patients with fibrosis compared to those without, according to radiography, $p < 0.0001$ for all (Table 4). In addition, smoking had a slight, yet significant effect on D*lco* but not on other lung function parameters, $p = 0.01$ (Table 4).

Neither any of the investigated SNPs nor any of the haplotypes was associated with IVC, FEV_1 or D*lco* values (data not shown).

Table 1 Primer sequences for the identification of biallelic single nucleotide polymorphisms in the TGF-β1, TGF-β2 and TGF-β3 genes.

Polymorphism	dbSNP*	Specific primer	Consensus primer†
TGF-β1			
-1347 C/T	rs1800469	5′- CCC TCC TGA CCC TTC CAT CC C/T	5′- CCT GAG CCC TCC AAG CTA AA (r)
28 T/C	rs1982073	5′- GCA GCG GTA GCA GCA GC A/G	5′- AGA CAC CCC CGG TCC AAG (f)
73 G/C	rs1800471	5′- GTG CTG ACG CCT GGC C G/C	5′-GGC TCC GGT TCT GCA CTC (r)
11089 C/T	rs1800472	5′-TTC CTG CTT CTC ATG GCC A C/T	5′- CCT GAG CCC TCC AAG CTA AA (r)
20743 C/T	rs8179181	5′- CAG GCA GGA GAG ACG C A/G	5′- CGT GGA TGG TCA TCT GGC T (f)
TGF-β2			
23267 G/T	rs4625350	5′- CTT TGA ACA GGA AGA CTT TAC AC G/T	5′- TAG TTT GGT AGT CAT CAA GCA GC (r)
36158 C/T	rs2799086	5′- CCT TCA AAT GCC ACT TCT ACT T C/T	5′- GGC CCT AGG ACT GGA TTT ATG (r)
41537 C/G	rs2796814	5′- GGG ATC AAC TGG AGT TTT TA AAA C/G	5′- TCC TTT CCT TGC TTG TCC CT (r)
59941 A/G	rs1891467	5′-AAA TTT CAC CAG GGA GCT GA CAT TTT A/G	5′- TCT ATG TGC AGA AAC CGC TAG T (r)
69153 C/T	rs2796813	5′- CTA ATG AGT TCC TGT GAG GAG AA C/T	5′- CAA GAT GTT AAG GGC TGC AGT (r)
TGF-β3			
-614 C/T	n/a	5′- AGC TAA AGG TGG GGG CA A/G	5′- GGC TTT TCC TGT GCC TGT TTA (f)
4875 G/A	rs3917165	5′- CTT TGC TTA TGT TTT GCC CC A/G	5′- CCC AGG TCT TCT GAA CAT ACC (r)
15101 G/A	rs3917187	5′- CCA GGG CCT CTG CTC CTA T/C	5′- TTG CCT CCA AGT AGG TAG GTG (f)
17369 T/C	rs3917200	5′-TCC AAG GGC TCT GCT CTC C/T	5′-TCT GTT GAG TGT GGC TTG G (r)
17682 A/G	rs3917201	5′- TCA ACA GAG GGT CCC TGA T A/G	5′- CTA GCT CCA ACT TTC CGT TCA (r)

* The SNP loci were identified using accession numbers according to the SNP database at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp † r = reverse primer; f = forward

Table 2 TGF-β3 allele carrier and genotype frequencies in sarcoidosis patients, patients with Löfgren's syndrome and healthy controls.

Polymorphism	RADIOGRAPHIC EVOLUTION				
	acute/self-remitting (n = 50)	chronic (n = 34)	fibrosis (n = 24)	Löfgren's (n = 46)	controls (n = 315)
-614 C/T					
allele C	50 (1.0)	34 (1.0)	24 (1.0)	46 (1.0)	315(1.0)
T	7 (0.14)	6 (0.18)	0	4 (0.09)	50 (0.16)
genotype CC	43 (0.86)	28 (0.82)	24 (1.0)	42 (0.91)	265 (0.84)
CT	7 (0.14)	6 (0.18)	0	4 (0.09)	50 (0.16)
TT	0	0	0	0	0
4875 G/A					
allele G	50 (1.0)	34 (1.0)	24 (1.0)	46 (1.0)	315 (1.0)
A	3 (0.06)	1 (0.03)	7 (0.29)*	5 (0.11)	45 (0.14)
genotype GG	47 (0.94)	33 (0.97)	17 (0.71)	41 (0.89)	271 (0.86)
AG	3 (0.06)	1 (0.03)	7 (0.29)	5 (0.11)	44 (0.14)
AA	0	0	0	0	1 (0.003)
15101 G/A					
allele G	47 (0.94)	34 (1.0)	19 (0.79) †,‡	41 (0.89)	296 (0.94)
A	20 (0.40)	14 (0.41)	12 (0.51)	21 (0.46)	139 (0.45)
genotype GG	30 (0.60)	20 (0.59)	12 (0.50)	25 (0.54)	176 (0.56)
AG	17 (0.34)	14 (0.41)	7 (0.29)	16 (0.35)	123 (0.39)
AA	3 (0.06)	0	5 (0.21) §,‖	5 (0.11)	16 (0.05)
17369 T/C					
allele T	50 (1.0)	34 (1.0)	24 (1.0)	46 (1.0)	312 (0.99)
C	4 (0.08)	2 (0.06)	7 (0.29) **	6 (0.13)	53 (0.17)
genotype TT	46 (0.92)	32 (0.94)	17 (0.71)	40 (0.87)	262 (0.83)
CT	4 (0.08)	2 (0.06)	7 (0.29)	6 (0.13)	50 (0.16)
CC	0	0	0	0	3 (0.01)
17682 A/G					
allele A	47 (0.94)	34 (1.0)	20 (0.83)	42 (0.91)	297 (0.94)
G	18 (0.36)	14 (0.41)	12 (0.50)	21 (0.46)	140 (0.44)
genotype AA	32 (0.64)	20 (0.59)	12 (0.50)	25 (0.54)	176 (0.56)
AG	15 (0.30)	14 (0.41)	8 (0.33)	16 (0.35)	121 (0.38)
GG	3 (0.06)	0	4 (0.17)	5 (0.11)	18 (0.06)

Data are provided as absolute numbers with genotype and allele carrier frequencies in parentheses.

* fibrosis vs. 'acute/self-remitting+chronic': p = 0.002, p_c = 0.01, odds ratio (OR) = 7.9 (df = 1)

† fibrosis vs. 'acute/self-remitting+chronic': p = 0.02, p_c = 0.10, OR = 0.15 (df = 1)

‡ fibrosis vs. controls: p = 0.016, p_c = 0.08, OR = 0.23 (df = 1)

§ fibrosis vs. 'acute/self-remitting+chronic': p = 0.02, p_c = 0.10 (df = 2)

‖ fibrosis vs. controls: p = 0.017, p_c = 0.08, (df = 2)

** fibrosis vs. 'acute/self-remitting+chronic': p = 0.01, p_c = 0.05, OR = 5.1 (df = 1)

Table 3 TGF-β3 haplotype carrier frequencies in sarcoidosis patients with different radiographic evolutions over a 4-year follow-up period, patients with Löfgren's syndrome and healthy controls.

haplotype	-614 C/T (promoter)	4875 G/A (intron)	15101 G/A (intron/exon)	17369 T/C (intron/exon)	17682 A/G (intron)	RADIOGRAPHIC EVOLUTION				
						acute/self-remitting (n = 50)	chronic (n = 34)	fibrosis (n = 24)	Löfgren's (n = 46)	controls (n = 315)
1	C	G	G	T	A	46 (0.92)	33 (0.97)	19 (0.79)	39 (0.85)	277 (0.88)
2	C	G	A	T	G	13 (0.26)	12 (0.36)	7 (0.29)	16 (0.35)	95 (0.30)
3	T	G	G	T	A	7 (0.14)	6 (0.18)	0	5 (0.11)	50 (0.16)
4	C	A	A	C	G	3 (0.06)	1 (0.03)	7 (0.29)*	5 (0.11)	44 (0.14)
5	C	G	A	C	G	1 (0.02)	1 (0.03)	0	1 (0.02)	9 (0.03)
6	C	G	A	T	A	2 (0.04)	0	1 (0.04)	0	3 (0.01)

Data represent absolute numbers with haplotype carrier frequencies in parentheses.
* fibrosis vs. acute/self-remitting+chronic: $p = 0.002$, $p_c = 0.01$, odds ratio (OR) = 7.9 (degrees of freedom (df) = 1).

Discussion

In this study, sarcoidosis patients with pulmonary fibrosis revealed differential allele distributions of SNP loci in the TGF-β3 gene compared to sarcoidosis patients without pulmonary fibrosis, assessed over a 4-year follow-up period. Figure 1 shows the most noticeable results for the SNP allele carrier frequencies in sarcoidosis patients with and without pulmonary fibrosis. TGF-β3 SNP 4875 G/A and 17369 T/C were the only polymorphisms that retained a p-value below 0.05 after the Bonferroni correction was applied, suggesting a genetic influence of TGF-β3 on the propensity to develop pulmonary fibrosis in patients with sarcoidosis. TGF-β1, β2 or β3 gene polymorphisms do not appear to contribute to sarcoidosis disease susceptibility as evidenced by the similar polymorphism frequency distributions between patients and healthy controls.

Functional polymorphisms with moderate to high heterozygosity have not yet been mapped for TGF-β2. One TGF-β2 SNP, present in exon1, is known to result in an Arginine to Histidine amino acid substitution in codon 91 (26). Our patient group was also genotyped for this polymorphism, but revealed no variation (data not shown).

The TGF-β2 59941 A/G gene polymorphism located within intron 2 showed a substantial increase of the G allele in the fibrotic patients compared to non-fibrotic sarcoidosis patients that was significant before correction ($p = 0.04$). This SNP deserves future investigations in association studies with additional patients with sarcoidosis or other fibrotic disorders. Previous reports have shown the genetic influence of TGF-β2 and TGF-β3 on fibrotic diseases such as scleroderma.(16) Our present study shows that genetic variation of TGF-β3 may contribute to the development of pulmonary fibrosis in sarcoidosis patients, at least according to radiographic data. With the exception of the TGF-β3 17282 A/G polymorphism (27), none of the TGF-β3 SNPs described in our study have been addressed in other association studies. Thus, speculations or conclusions pertaining to any phenotypic changes that may be caused by the 4875 G/A and/or 17369 T/C polymorphisms should be approached with care until functional data becomes available. Kim et al. found a strong association between the SNP locus 17282 A/G and cleft palate syndrome in Koreans (27). This polymorphism was not associated with pulmonary fibrosis in our sarcoidosis group, which suggests that either this polymorphism has differential influences on different disease types or that it acts in function of racial background.

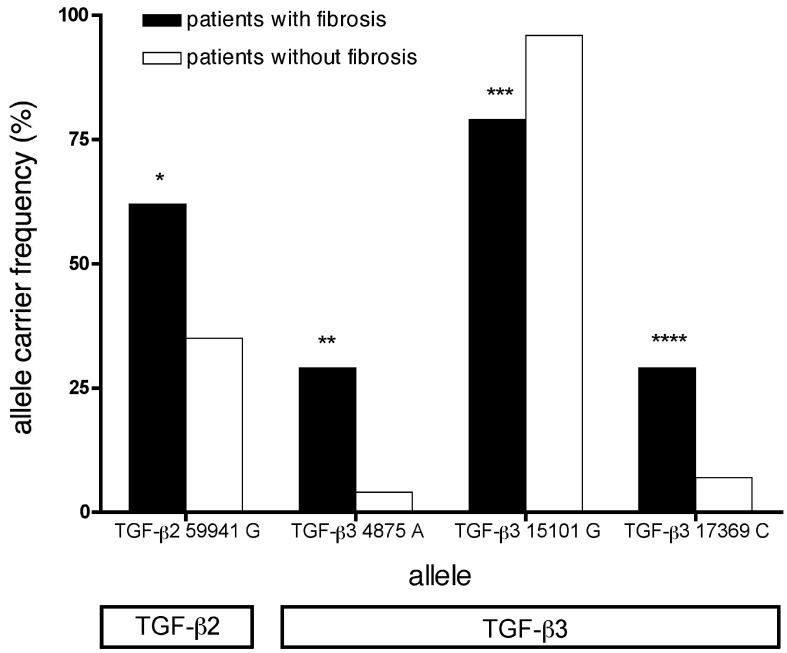

Figure 1 Allele carrier frequencies of SNPs in the TGF-β2 and TGF-β3 genes which were different between sarcoidosis patients with and without fibrosis, based on radiographic evolution over a 4-year follow-up period. p = 0.04, p_c = 0.2, ** p = 0.002, p_c = 0.01, *** p = 0.02, p_c = 0.1, **** p = 0.01 , p_c = 0.05

The observation that neither any of the fibrosis-associated SNPs nor haplotype 4 had any significant influence on lung function was not unexpected. It has been known that lung function does not always clearly correlate with radiographic staging (28). Although the fibrotic group as a whole had a significantly lower lung function compared to the non-fibrotic groups, a subset of patients within a single category, i.e. fibrosis may have normal iVC and DL*co* values as the pattern of pulmonary fibrosis may vary (20). As a consequence, the distribution of the polymorphism frequency in the fibrotic group compared to the non-fibrotic group according to radiography may still poorly correlate with lung function parameters that are commonly associated with fibrosis.

The polymorphisms in the gene encoding TGF-β1 did not reveal associations as seen with TGF-β2 and TGF-β3. All but one SNP (20743 C/T, located in the intron/exon boundary) are known to influence the expression levels of TGF-β1.

(13, 29). Based on available literature describing the influence of individual SNPs on variation of expression levels, TGF-β1 haplotypes that were constructed in this study would each have a net effect on the expression levels. None of the TGF-β1 haplotypes could be ascribed to the different phenotypes of sarcoidosis. Thus, and in accordance with previous reports (17, 18), the absence of associations between haplotypes and disease phenotype implies that genetically controlled levels of TGF-β1 neither predict the outcome of, nor contribute to the susceptibility to sarcoidosis. On a critical note, the lack of associations found between the genetic variation of TGF-β1 and the development of fibrosis may not yet be conclusive. The statistical power was estimated to be 41% with a relative risk of 2, which may not have sufficed to identify the impact of TGF-β1 polymorphisms on the development of pulmonary fibrosis. A substantially larger group (n = 62 to achieve 80% power) of sarcoidosis patients with pulmonary fibrosis is needed to confirm the negative findings.

It is speculated that the balance between all three TGF-β isoforms may determine the nature of healing. Isoforms 1 and 2 of the TGF-β family are generally described as having a profibrotic nature, whilst TGF-β3 shows a more diverse character which may either sustain or resolve the progression of fibrosis (30, 31). The latter is also supported by the observation that the exogenous addition of TGF-β3 can mitigate the deleterious effects of increased TGF-β1 and thus prevent fibrosis in the injured lung (32). The outcome of sarcoidosis may be predestined by the genetics that control the immune response and the subsequent repair and remodeling of the injured tissue. Just as in those described in TGF-β1, functional polymorphisms in TGF-β3 which may exist in strong linkage with the fibrosis-associated SNPs described in this study, may lead to decreased expression of TGF-β3. Consequently, this may cause a shift towards TGF-β1 and possibly TGF-β2 expression which may be unfavorable to the outcome of sarcoidosis. Such speculations can however only be confirmed by functional studies.

The results described in this present studies indicate that genetic variation of TGF-β3 is associated with pulmonary fibrosis that could be discerned by chest X-rays. Available high resolution CT data were able to confirm fibrotic changes present as three main patterns of fibrosis, according to Abehsera et al. (20).

Although an association between these different HRCT patterns and TGF-β3 polymorphisms could not be identified at this point due to a limited sample size, future studies using a larger group of fibrotic patients may lead to additional associations between TGF-β polymorphisms and different fibrotic patterns. Five patients who had presented with stage II and 9 patients who had presented with stage III progressed towards stage IV 4 years later. A recent study by Akira et al. showed that HRCT-scans failed to discriminate between ground-glass opacities that had either disappeared or evolved into honeycombing of the lung after 7.4 (mean) years (33). The associations between TGF-β3 gene variations and fibrosis would not have been found had we not followed up on these patients. Thus, a follow-up period of a significant number of years proves to be imperative in order to establish a category of patients with apparent pulmonary fibrosis for genetic association studies.

Table 4 TGF-β3 haplotype 4 carriage in sarcoidosis patients and its effect on lung function over a 4-year follow-up period.

Radiographic evolution	**iVC (%)**	**FEV_1 (%)**	**D*lco* (%)**
acute/self-remitting	95.9 ± 5.9 (n = 37)	94.1 ± 4.9 (n = 37)	101.0 ± 4.7 (n = 20)
chronic	93.3 ± 5.8 (n = 30)	87.7 ± 4.7 (n = 30)	93.9 ± 4.3 (n = 15)
fibrosis	74.7 ± 6.3 (n = 14)*	66.6 ± 5.2 (n = 14) †	63.0 ± 5.4 (n = 7) ‡
Löfgren's syndrome	101.1 ± 5.8 (n = 15)	91.2 ± 5.3 (n = 14)	92.7 ± 5.2 (n = 9)
Smoking history			
yes	91.5 ± 5.0 (n = 36)	84.1 ± 5.0 (n = 36)	94.9 ± 4.4 (n = 20) §
no	92.7 ± 4.8 (n = 59)	85.0 ± 4.5 (n = 59)	86.6 ± 4.1 (n = 31)
Treatment			
yes	91.6 ± 6.0 (n = 61)	79.2 ± 4.4 (n = 61)	91.4 ± 4.1 (n = 24)
no	90.2 ± 6.4 (n = 29)	82.3 ± 4.9 (n = 29)	83.0 ± 4.6 (n = 22)
unknown	91.3 ± 6.3 (n = 5)	87.6± 6.8 (n = 5)	97.8 ± 6.0 (n =5)
Follow-up			
presentation	89.6 ± 5.8 (n = 95)	81.1 ± 4.6 (n = 95)	91.5 ± 4.2 (n = 51)
4 years	92.5 ± 5.6 (n = 95)	84.9 ± 4.6 (n = 95)	89.9 ± 4.2 (n = 51)
TGF-β3 haplotype 4			
present	91.6 ± 6.0 (n = 11)	89.6 ± 5.8 (n = 11)	85.4 ± 2.1 (n = 6)
absent	91.6 ± 6.0 (n = 84)	92.5 ± 5.6 (n = 84)	87.5 ± 4.3 (n = 45)

Lung function values are provided as percent predicted (%) with standard error (SEM) in parentheses and number of subjects (n).

* fibrosis vs. acute/self-remitting (with or without Löfgren's) + chronic: $p < 0.0001$

† fibrosis vs. acute/self-remitting (with or without Löfgren's) + chronic: $p < 0.0001$

‡ fibrosis vs. acute/self-remitting (with or without Löfgren's) + chronic: $p < 0.0001$

§ smokers vs. non-smokers: $p = 0.01$

Conclusion

Although efforts have been made to find associations between TGF-β1 polymorphisms and sarcoidosis, the results of this study are the first to suggest the implication of genetic variation of TGF-β3 in the predilection to develop pulmonary fibrosis in patients affected with sarcoidosis. Additional studies in other sarcoidosis populations, preferentially in different ethnic groups, are recommended to confirm our findings.

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chapter FIVE

5

ACE I/D-corrected Z-scores to identify normal and elevated ACE activity in sarcoidosis

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Abstract

The value of elevated serum angiotensin-converting enzyme (ACE) activity in the diagnosis and follow-up in sarcoidosis is a matter of ongoing debate. This may be at least related to the insertion (I)/deletion (D) polymorphism in the ACE gene (ACE I/D). ACE activity is influenced by the ACE I/D polymorphism. As a consequence, the use of one reference interval instead of three genotype-specific reference intervals for ACE activity may lead to a less precise interpretation of ACE activity.

In order to assess whether determination of ACE activity indeed requires the ACE I/D genotype to be taken into account, we established ACE I/D-corrected reference intervals in healthy, Caucasian volunteers (n=200). In addition, ACE activities in ACE I/D genotyped patients suspected of or having sarcoidosis (n=129) were expressed as the Z-score related to ACE I/D-corrected reference intervals.

Comparison of the Z-score with ACE activity in which ACE I/D is ignored rendered 8.5% misclassification of 'elevated' versus 'normal' ACE or vice versa.

Our data demonstrate a convenient way to circumvent the use of three reference intervals by introducing a Z-score for ACE activity. It also illustrates the need to re-investigating the possible clinical value of serum ACE activity in sarcoidosis by considering ACE I/D.

Introduction

For at least twenty-five years, angiotensin-converting enzyme (ACE) (peptidyl-dipeptidase A, EC 3.4.15.1) measurements in blood are commonly requested for diagnosis and follow-up in patients with sarcoidosis. Sarcoidosis is a granulomatous disease with unknown etiology in which serum ACE activity is found to be elevated in approximately 60% of the patients (1). However, given the gross inter-individual variation of ACE levels and the observations that population-based reference intervals for ACE have been acknowledged as being deceptive in terms of identifying abnormal values, the added value of ACE levels in the diagnosis and follow-up of sarcoidosis is still subject to debate (2, 3). Part of this discussion may find its cause in insufficient regard for the contribution of the most extensively studied ACE gene polymorphism, namely the ACE I/D polymorphism, into account. Cambien et al. were the first to indicate the genetic influence on ACE levels by revealing a major quantitative trait locus (QTL) responsible for nearly half of the variance in serum ACE levels. This was later identified as a polymorphism in the gene encoding for ACE (4). The polymorphism is caused by an insertion of a 287 bp fragment within intron 16 and it is commonly present in various populations (5). An absence of the inserted fragment is referred to as a deletion and the two alleles of this polymorphism are denoted I (insertion) and D (deletion). Homozygous carriers of D (DD) or I (II) express the highest and lowest ACE levels, respectively, with intermediate ACE levels for heterozygous ID individuals (6).

As reported in other populations, the approach of using ACE I/D-corrected reference intervals of ACE activity may improve the interpretation of ACE levels during follow-up and diagnosis (7-9). 'Elevated' versus 'normal' ACE activity which is not based on ACE activity in ACE I/D-characterized reference populations may introduce a less precise interpretation of ACE activity in a patient suspected of sarcoidosis who presents for the first time. As a consequence, ACE activity may be underestimated in II individuals and overestimated in DD individuals. On the one hand, the introduction of three reference intervals for ACE activity, i.e. II, ID and DD, seems a rather simple approach to prevent this under- or overestimation. On the other hand, three reference intervals for one analyte, i.e. ACE, raises questions about how to report laboratory results in the (near) future, since an increasing number of reference intervals of analytes based on genetic polymorphisms can be expected.

In order to study the relevance of the ACE I/D polymorphism in the interpretation of ACE activity in patients suspected of sarcoidosis, we first established ACE I/D-corrected reference intervals in Dutch, white Caucasian, healthy volunteers. Secondly, we introduced the Z-score approach to report ACE activity based on ACE I/D polymorphism. The Z-score, or standard score, is a dimensionless quantity derived by subtracting the population mean from an individual (raw) score and then dividing the difference by the population standard deviation. The quantity *Z* represents the distance between the raw score and the population mean in units of the standard deviation. Using the Z-score, the ACE activity in patients (raw score) is reported as the deviation from the mean ACE activity in the ACE I/D-corrected reference population. This approach may not only contribute to renewed gathering and evaluation of data with respect to the role of ACE activity in the diagnosis and follow-up of sarcoidosis but may also be a pragmatic way to meet the physician's future need of reporting analyte activity based on genotype-corrected reference intervals.

Subjects and Methods

Healthy volunteers and patients

Healthy employees of the St. Antonius Hospital volunteered for venapunction. Relevant information about medication, ethnicity and hereditary diseases was obtained by having the volunteers complete a questionnaire. Non-Caucasian subjects were omitted from further analysis in order to maintain genetic homogeneity of the population. Subjects who were treated with medication known to affect the renin-angiotensin system, either by directly inhibiting ACE or angiotensin II type 1 receptor blocking, were also excluded from our studies to prevent pharmacologically altered ACE activity. The remaining total of 200 subjects, used for evaluation and analyses, comprised of 100 women (mean age ± SD [range] (years): 39 ±12.4 [18-67] and 100 men (42 ± 10.4 [19-68]. The medical ethical committee of this hospital approved the study conducted and all subjects gave formal written consent.

One hundred and twenty-nine ACE measurements from patients suspected of sarcoidosis or reactivation of previously diagnosed sarcoidosis were retrospectively evaluated for ACE levels and genotyped for the ACE I/D polymorphism. The group that was diagnosed with sarcoidosis included 94 Dutch Caucasian patients (53 men/41 women). The diagnosis of sarcoidosis

was established when clinical findings were supported by histological evidence and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis (10). In 9 patients, the diagnosis was made without biopsy proof because these patients presented with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy. Fifteen patients with sarcoidosis had been diagnosed outside of this hospital prior to referral. The age at which the initial diagnosis was made was unknown. Five patients had presented at our clinic for the first time. The mean age ± SD [range] at the time the diagnosis was made was 38.0 ± 12.6 [20-81] years. The remaining 89 sarcoidosis patients had been clinically monitored for 10 (median); 1–37 (range) years, after which time ACE activity was being measured. Chest radiographs were assessed in consensus by chest physicians specialized in diffuse lung diseases to determine disease severity using standard radiographic staging for sarcoidosis, classified according to the Scadding criteria (10). Over the median follow-up period of 10 years at which ACE was measured, 51 patients normalized or improved towards stage I), 21 patients had persistent stage II/III or progression towards this stage, and 12 patients developed fibrosis (stage IV or progressive towards this stage). The patients who had been diagnosed with Löfgren's syndrome (radiographic stage I at presentation). (n = 9) had received radiographic follow-up of 4 (median); 1–27 (range) years. These patients had normalized chest radiography in less than 2 years following presentation. At the time ACE activity was measured, 54 sarcoidosis patients were receiving treatment with corticosteroids and 1 patient with methotrexate. Forty-six patients were cigarette smokers, while the smoking status of 30 patients was unknown.

The group that was suspected of sarcoidosis included 23 men and 12 women (smokers/non-smokers/unknown: 10/23/2). None of these patients were taking medication that directly interferes with the renin-angiotensin-aldosterone system such as ACE-inhibitors or angiotensin II receptor antagonists. Upon histological evaluation, sarcoidosis could be excluded in 28 patients. The mean age ± SD [range] (years) at the time ACE was measured was 53.1 ± 13.4 [26–84]).

Genotyping

Genomic DNA of volunteers and patients was isolated from EDTA blood using the MagNA Pure LC DNA Isolation kit I (MagNA Pure; Roche Diagnostics).

ACE I/D polymorphisms were determined by real-time PCR using fluorescent hybridization probes and a LightCycler (Roche Diagnostics) as described earlier with some slight modifications (5, 10, 11). Briefly, the reaction volume was 20 µl, containing 1 µl of DNA (40 – 80 ng), 0.2 µM forward primer and 0.8 µM reversed primer reported by Rigat et al. (5), 2 µl of 10 x reaction buffer (LightCycler DNA master hybridization probes, Roche Diagnostics), 1.6 µl of 25 mM $MgCl_2$ stock solution and 0.1 µM of each of the probes. The detection probes were the same as described by Somogyvári et al. (10). The PCR conditions were as follows: denaturation at 95 °C for 60 s, followed by 50 cycles denaturation (95 °C for 10 s), annealing (first 10 cycles: 67 °C for 20 s, followed by 0.5 °C stepwise decrease per cycle to 61 °C) and extension (72 °C for 30 s). Melting curve analysis consisted of heating to 95 °C for 5 s, 45 °C for 60 s, followed by an increase of the temperature to 75 °C at 0.2 °C/s. To exclude mistyping of I/D heterozygotes as D/D homozygotes, a second PCR reaction was performed under the same conditions except for using the primer pair described earlier (10, 11). Verification of the real-time PCR results with those of electrophoresis and using SSP-PCR revealed no mistyping.

Determination of ACE activity

Quantification of ACE activity was measured in lithium heparin plasma using the Bühlmann ACE kinetic test, according to previously described methods (Bühlmann Laboratories AG, Switzerland (12, 13)). The manufacturer's reference interval is 12-68 U/l.

Statistical analyses

The statistical evaluation of our data was performed using SPSS 11 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism v. 4 (Graphpad Software, Inc., San Diego, CA, USA) software packages.

χ^2 test was used for categorical variables and Student's *t* test or ANOVA, followed by Tukey's post-test for multiple comparisons between groups, was used for analysis of continuous variables that were normally distributed.

Variance analysis was performed and the effect size was calculated using the omega squared approach (14). This equation describes the portion of variance

explained by the ACE I/D genotype and other factors. Fixed factors were gender and the ACE I/D genotype. Age was included as a covariate.
Genotype frequencies were tested for Hardy-Weinberg equilibrium. P values lower than 0.05 were considered statistically significant.
Z-score was calculated as ($ACE_{patient}$ – mean $ACE_{reference\ group}$)/$SD_{reference\ group}$, where mean $ACE_{reference\ group}$ and $SD_{reference\ group}$ are calculated from the ACE values measured in either the II, ID or DD reference group. If *z* is less than -1.96 below the mean or z is greater than + 1.96 above the mean, the ACE level value is considered deviant from the reference interval used.

Results

ACE activity and ACE/ I/D genotypes in healthy volunteers
ACE activity and reference intervals categorized to ACE I/D genotype were determined in healthy volunteers (table 1). Significant differences in ACE activity (U/l) between subjects grouped according to ACE I/D were observed as follows: II (25.9), ID (38.1) and DD (53.1) (table 1; ANOVA $p < 0.0001$). The DD group exhibited ACE levels that reached twice the value of that of the II group whereas intermediate values were observed in the ID group. ACE I/D-corrected ACE values grouped according to sex showed a similar trend (ANOVA $p < 0.0001$, data not shown). A moderate yet significant difference between ACE levels (mean ± SD, range (U/l) in healthy males (42.5 ± 16.5, 11.8 – 84.1) and females (36.2 ± 13.5, 12.9 – 79.0) ($p < 0.001$) was observed (table not shown). Variance analysis showed that 34.0% of the variance in ACE activity was attributed to the ACE I/D genotype.
In order to confirm the ACE I/D genotype-dependent ACE activity observed in plasma, ACE activity was also measured in sera of the healthy population using the Fujirebio test (Fujirebio Inc., Tokyo, Japan, cat. no. FU 116) (16, 17). The results between plasma and serum were identical in terms of ACE I/D genotype-dependent ACE activity (data not shown).
I/D allele frequencies in healthy volunteers were 48.2% for I and 51.8% for D whereas ACE I/D genotype frequencies for II, ID and DD were 21.5%, 53.5% and 25%, respectively (table 2). No deviation from the Hardy-Weinberg equilibrium was found for the ACE genotypes in the three populations studied. No significant differences were found for allele, allele carrier or genotype frequencies of ACE I/D between males and females in healthy volunteers (table 2).

Table 1 ACE I/D-corrected reference intervals for ACE.

n		**200**	
ACE genotype	**II**	**ID**	**DD**
n	43	107	50
mean ACE (SD)	25.9 (8.7)	38.1 (12.2)	53.1 (14.9)
calculated reference interval	9 - 43	14 – 62	24 – 82
ANOVA		$p < 0.0001$	
multiple comparisons		$p < 0.001$ for II vs. ID; ID vs. DD and II vs. DD	

Mean ACE and reference intervals (+ 1.96 SD and -1.96 SD reference limits) are shown for each ACE genotype. One-way ANOVA, followed by Tukey's post-test was performed for genotype-corrected ACE levels comparisons.

Z-scores of patients

In order to demonstrate the consequences of ACE I/D-corrected reference intervals for the classification of ACE in 'elevated' versus 'normal' ACE activity, 129 ACE measurements in patients suspected of sarcoidosis were calculated and interpreted in two ways: ACE activity while disregarding ACE I/D polymorphisms and ACE activity while taking ACE I/D-corrected reference intervals into account. In the second approach, ACE activity is reported as the Z-score related to the three ACE I/D-corrected reference intervals. Using both approaches, 11 out of 129 measurements (8.5%) were shown to lead to a different interpretation, i.e. from 'elevated' to 'normal' or vice versa (table 3).

Discussion

Discordant classification of ACE activity in 11 out of 129 (8.5%) ACE measurements in patients suspected of having (reactivated) sarcoidosis clearly demonstrates the consequences of the ACE I/D polymorphism on the interpretation of ACE activity. All elevated ACE levels changing from 'elevated' to 'normal' were from DD individuals, of whom two patients had histiocytosis X and extrinsic allergic alveolitis, respectively. Normal ACE levels changing to 'elevated' were from II individuals. ACE measurements in these individuals, which are used to illustrate the importance of ACE I/D genotyping clearly show the imprecise interpretation that can occur if the knowledge of genetic influence on ACE levels is ignored.

Compared to females in the control group, males had slightly higher mean ACE activity, which was not due to differences in ACE I/D genotype distributions between the two genders. Although some studies have reported serum ACE activity to be gender-independent (15, 16), other studies indicate that, for reasons unknown, males have a modestly higher ACE activity compared to females (17, 18). However, integration of gender in the Z-score calculation did not change the percentage of patients who were imprecisely marked as having normal or elevated ACE activity before genotype correction. Therefore, the gender consideration in addition to the ACE I/D genotype may not be critical when reporting ACE values as Z-scores.

Table 2 ACE I/D genotype distribution in Dutch Caucasians.

group	**Total**			**Males**			**Females**		
n	200			100			100		
Genotype frequency	II	ID	DD	II	ID	DD	II	ID	DD
n	43	107	50	20	54	26	23	53	24
(%)	(21.5)	(53.5)	(25.0)	(20.0)	(54.0)	(26.0)	(23.0)	(53.0)	(24.0)
Allele carrier frequency	I	D		I	D		I	D	
n	150	157		74	80		76	77	
(%)	(75.0)	(78.5)		(74.0)	(80.0)		(76.0)	(77.0)	
Allele frequency	I	D		I	D		I	D	
n	193	207		94	106		99	101	
(%)	(48.2)	(51.8)		(47.0)	(53.0)		(0.49)	(51.0)	
χ^2-square (allele carrier, carrier and genotype frequencies)	$p > 0.05$ (not significant)								

Data are shown as number of individuals (n) in each group, subdivided according to gender. Genotype, allele carrier and allele frequencies are shown as number of individuals (n) and percentage (%). Chi-square test was performed to compare allele and genotype distributions between genders.

The purpose of this study is not to contribute to the clinical validation of ACE. The patients merely serve to demonstrate the importance of applying genotype-corrected reference intervals for ACE. Moreover, we regard the utilization of ACE I/D-corrected reference intervals for ACE activity as a prerequisite for renewed research to the relevance of measuring ACE in sarcoidosis. For instance, previous studies evaluating alternative markers for sarcoidosis disease

activity such as soluble interleukin (IL) receptor-2 (22), procollagen 1 and 2 (23), IL-12 (24), IL-8 (25) and, more recent,YKL-40 (26), have traditionally been benchmarked against ACE, without correcting the latter for its genotype. The re-evaluation of these markers by comparing its performance as a possible marker for sarcoidosis disease activity with ACE I/D genotype-corrected Z scores, may shed a new light on previous findings. The Z-score approach may also offer its value in prospective studies or re-evaluation of studies in which ACE activity has been measured in the context of other respiratory diseases including pneumonia (27), asthma (28), tuberculosis (29, 30), and chronic obstructive pulmonary disorder (COPD) (29).

Additional factors that significantly influence the normal levels of an analyte in a given individual necessitates the use of reference values that are corrected for those factors. The inconvenience of adopting a multitude of reference intervals specific for genotype and possibly other factors can be avoided by utilizing the Z-score approach. This way, and as demonstrated in this study, reporting a laboratory parameter as a Z-score is a pragmatic way of anticipating the increasing number of reference intervals as a result of genetic polymorphisms that lead to differential analyte concentrations. Moreover, changes in pre-analytical procedures or analytical methods do not necessarily lead to a change of values and the Z-score may thus prevent the introduction of new reference intervals in the clinic.

Only one effort to establish genotype-corrected ACE reference values has been reported for white Caucasians (8). This study was aimed at the improvement of the identification of sarcoidosis patients when ACE genotypes were considered. The authors suggested that more intricate studies should be undertaken to establish a genotype-corrected reference interval for any particular population. A more elaborate study was undertaken using Japanese healthy subjects and the obtained reference interval depending on ACE I/D genotypes also made it clear that for II genotype individuals in particular, a seemingly moderate ACE elevation could lead to overlooking the activity of sarcoidosis and that healthy DD genotype individuals could easily be identified as having abnormal (elevated) ACE levels (7). Similar to what we have shown here, both authors clearly illustrated the importance of genotype for reference values by reporting an immense increase of sensitivity compared to the conventional reference interval.

In our study, variance analysis showed that the ACE I/D genotype accounted for 34.0% of variation of ACE levels in the control population. This was in

accordance with previous reports which focused on I/D polymorphisms and additional putative quantitative trait loci (QTL's) (6, 31-33).
As pointed out by Csaszar et al. (34), who repeated the study by Sharma et al. in a Hungarian population, genotype-corrected reference values for ACE should be established locally. A suggested standard reference interval, as provided by the manufacturers of ACE measuring assays, will not apply, given the fact that I/D allele frequencies can differ vastly between ethnicities. Indeed, compared to Caucasians (32, 35, 36), and confirmed by our data, which show that the D and I allele distributions are roughly the same (52% and 48%, respectively.), the I allele in Japanese is more abundant than the D allele and thus attributable to the lowering of the overall reference range for ACE levels in Japanese.

Table 3 ACE measurements leading to discordant classification between two approaches for reporting ACE activity.

Measurement	ACE (U/l)	ACE genotype	Z-score 1[Δ]	Z-score 2[∇]	Change[§]	disease[¶]
1	45	II	0.36	2.20	elevated	stage II/III sarcoidosis
2	62	II	1.47	4.15	elevated	stage II/III sarcoidosis
3	63	ID	1.53	2.04	elevated	stage II/III sarcoidosis
4	65	ID	1.66	2.20	elevated	stage II/III sarcoidosis
5	65	II	1.66	4.49	elevated	stage 0/I sarcoidosis
6	54	II	0.95	3.23	elevated	stage 0/I sarcoidosis
7	70	ID	1.96	2.61	elevated	stage II/III sarcoidosis
8	75	DD	2.31	1.47	normalized	stage II/III sarcoidosis
9	75	DD	2.31	1.47	normalized	histiocytosis X
10	80	DD	2.64	1.81	normalized	stage 0/I sarcoidosis
11	70	DD	1.99	1.13	normalized	EAA[†]

Z-scores greater than 1.96 are regarded as elevated ACE levels.
[Δ] Z-score 1: based on conventional* reference interval (*conventional reference interval: 9 – 70 U/l (calculated as -1.96 and +1.96 standard deviation in our healthy population))
[∇] Z-score 2: based on ACE I/D-corrected reference intervals
[§] ACE activity based on ACE I/D-corrected reference interval relative to conventional reference interval.
[¶] stage 0/I sarcoidosis = normalized or improved towards radiographic stage I; stage II/III sarcoidosis = persistent stage II/III or progression towards this stage.
[†] EAA = extrinsic allergic alveolitis.

Conclusion

ACE I/D-corrected ACE activity in a Dutch Caucasian population has shown that a relatively simple genetic profile can provide a more precise reference interval. Reporting laboratory results in a way in which reference intervals are incorporated may be a simple and generally applicable approach for dealing with the increasing number of genotype-corrected reference intervals for other analytes.

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chapter SIX

6

The mucin-1 568 adenosine to guanine polymorphism influences SERUM KREBS VON DEN LUNGEN-6 LEVELS

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Abstract

Krebs von den Lungen (KL)-6 offers a new perspective as a disease marker in pulmonary diseases. The aim of this study was to analyze whether serum KL-6 levels are dependent on the functional adenosine to guanine mucin-1 gene polymorphism at nucleotide position 568 in a well-characterized white population. Polymorphisms were determined in 327 healthy, white Caucasian individuals and 74 sarcoidosis patients, using a polymerase chain reaction-sequence-specific primers assay. The serum KL-6 levels were measured by ELISA. Significant differences between serum KL-6 levels of healthy subjects who were grouped according to MUC1 568 genotype were observed ($p < 0.0001$) (mean ± SEM; 95%CI): AA (195.2 ± 9.9 U/ml; 95%CI: 175.7–214.8 U/ml), AG (246.0 ± 8.6 U/ml; 95%CI: 229.0–263.1 U/ml) and GG (302.6 ± 11.8 U/ml; 95%CI: 279.3–326.0 U/ml). In the sarcoidosis patients, the results were: (mean ± SD; 95% CI): AA (550.1 ± 411.7; 95% CI: 380.2-720.1), AG (716.3 ± 452.4; 95% CI: 547.4-885.2), GG (1151.0 ± 1122; 95% CI: 610.1-1692.0), $p = 0.02$. Comparison of the KL-6 levels in which the 568 genotype was ignored rendered six out of 74 (7.5%) misclassifications of 'elevated' versus 'normal' KL-6 levels or vice versa In conclusion, the mucin-1 568 A to G polymorphism may be of interest for diagnostic purposes as our study delivered *in vivo* evidence that it contributes to inter-individual variations in KL-6 levels.

Introduction

Many acute and chronic lung disorders with variable degrees of pulmonary inflammation and fibrosis are collectively referred to as interstitial lung diseases (ILD)s. In order to evaluate the activity and monitor the course of the ILD several methods such as chest roentgenogram, pulmonary function testing, gallium-67 lung scan, and bronchoalveolar lavage are available. The lung epithelium-specific protein Krebs von den Lungen-6 (KL-6) offers a new perspective as disease marker in ILDs (1). Serum KL-6 is elevated in a majority of ILD patients and normal in patients with bacterial pneumonia or in healthy subjects (2). KL-6 levels depend on the number of regenerating type II epithelial cells and the integrity of the alveolar-capillary membrane (3, 4). Since KL-6 is chemotactic for human fibroblasts, this protein may also play a functional role in fibrosis (5). Serum KL-6 levels predict outcome in idiopathic pulmonary fibrosis (IPF), acute respiratory distress syndrome and sarcoidosis (6-9).

The KL-6 antibody recognizes a specific sugar chain on the mucin-1 (MUC1) protein (3). There are known variations in the length and structure of the MUC1 protein which result from two known polymorphisms. The variable number of tandem repeats (VNTR) polymorphism present within the coding region codes for a twenty amino acid motif, resulting in many different alleles that show a bimodal distribution, i.e. small and large (10). In addition, the MUC1 pre-mRNA uses either one of two neighboring splice acceptor sites for exon 2 resulting in a MUC1 protein difference of nine amino acids. The MUC1 splice site recognition is based on an adenosine (A) to guanine (G) single nucleotide polymorphism in exon 2 at nucleotide position 568 (11). Previously, it has been demonstrated that larger MUC1 proteins express more sugar chains on their surface compared to smaller proteins (12).

We hypothesized that the functional MUC1 polymorphisms might contribute to variance in serum KL-6 levels. For this purpose, we analyzed the MUC1 568 A/G polymorphism utilizing an easy-to-perform polymerase chain reaction-sequence-specific primers (PCR-SSP)s assay in a well characterized Dutch white population. The MUC1 568 genotypes were related to serum KL-6 levels in order to determine a possible gene-protein relationship.

Subjects and Methods

Healthy controls

Venous blood samples were obtained from 327 ostensibly healthy employees of the St Antonius Hospital; 210 women (39 ± 12.1 years) and 117 men (42 ± 10.4 years). By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases. The individuals were assessed with a complete history and physical examination. The overrepresentation of women who participated in this study is explained by the predominantly female workforce at this hospital. Fifty-five individuals (33 women and 22 men) smoked for at least five pack-years. Ethnicity of both parents was used as the criterion for assuming Dutch Caucasian ethnicity of the subject. Exclusion criteria included known pulmonary disease and non-Dutch Caucasian ethnicity. The medical ethical committee of this hospital approved the study conducted and all subjects gave formal written consent.

Patients

Seventy-four unrelated and randomly selected Dutch white patients with sarcoidosis (43 men, 31 women; mean age ± SD: 39 ± 11.2 years) were included in the study. In 49 patients, the diagnosis of sarcoidosis was established when clinical findings were supported by histologic evidence, and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis (13). One patient presented with radiographic stage 0, 20 patients presented with stage I, 11 patients presented with stage II, 14 patients presented with stage III, and 3 patients presented with stage IV disease. Twenty-five patients presented with the classic Löfgren's syndrome of fever, erythema nodosum, bilateral hilar lymphadenopathy, and joint symptoms. The diagnosis in these patients was made without biopsy proof.

Serum KL-6 measurements

KL-6 concentrations were measured by an ELISA technique using a specific KL-6 antibody kit (ED046; kindly provided by Eisai Co., Tokyo, Japan) as described previously (14). All samples were run in duplicate and mean values were used for analysis.

Sequence-specific primers and polymerase chain reaction

The biallelic MUC1 568 A/G single nucleotide polymorphism (exon 2; rs4072037; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp) was determined with PCR-SSPs. The reverse SSPs 5′-AGC TTG CAT GAC CAG AAC CC and 5′-AGC TTG CAT GAC CAG AAC CT were used in combination with the consensus forward primer 5′-CTA TGG GCA GAG AGA AGG AG, leading to expected PCR product sizes of 233 bp. The PCR conditions were as previously described (15).

Statistical analyses

The statistical evaluation of our data was performed using SPSS 11 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism version 4 (Graphpad Software, Inc., San Diego, CA, USA) software packages. χ^2 test was used for categorical variables and multivariate analysis of continuous variables that were normally distributed. The latter analysis was performed using a linear regression model, controlled for gender and smoking as fixed factors and age as a covariate, followed by a post-test for multiple comparisons between groups. Serum KL-6 levels were reported as estimated marginal mean ± SEM and 95% confidence intervals (CI) in U/ml (unless otherwise stated). The reference interval of serum KL-6 in each genotype group was calculated by the formula: mean ± 1.96 SD. Genotype frequencies were tested for Hardy-Weinberg equilibrium. Statistical significance was denoted by a value of $p < 0.05$ for all tests performed.

Results

MUC1 genotypes

The study population was in Hardy-Weinberg equilibrium for the MUC1 568 genotype distribution ($p = 0.44$). In healthy controls, the MUC1 568 allele frequency was 359 (54.9%) for A and 295 (45.1%) for G. The genotype frequency for AA, AG and GG was 102 (31.2%), 155 (47.4%) and 70 (21.4%), respectively. In sarcoidosis patients, the allele frequency was 80 (54.0%) for A and 68 (46.0%) for G. The genotype frequencies were 25 (33.8%) AA, 30 (40.5%) AG and 19 (25.7%) GG. No significant difference in MUC1 568 genotype or allele frequency distributions were found between sarcoidosis patients and controls.

Relationship between MUC1 568 A/G polymorphism and serum KL-6 levels
Healthy controls
The mean serum KL-6 ± SD in all healthy individuals was 238.7 ± 101.5 U/ml and the reference interval was 39.8 – 437.6 U/ml. Table 1 summarizes the serum KL-6 levels that were categorized according to MUC1 568 A/G genotype. Pronounced differences between serum KL-6 levels of subjects who were grouped according to MUC1 568 genotype (mean ± SEM; 95% CI) were observed: AA (195.2 ± 9.9 U/ml; 95% CI: 175.7 – 214.8 U/ml), AG (246.0 ± 8.6 U/ml; 95% CI: 229.0 – 263.1 U/ml) and GG (302.6 ± 11.8 U/ml; 95% CI: 279.3 – 326.0 U/ml). Serum KL-6 values were found to be significantly different when all three genotype-group serum levels were compared ($p < 0.0001$). Post-test analysis was performed for genotype-specific KL-6 levels comparisons ($p < 0.0001$ between all genotype comparisons). Males tended to have slightly higher KL-6 levels (mean ± SEM; 95% CI) (267.9 ± 8.6 U/ml; 95% CI: 251.0 – 284.8) than females (234.0 ± 6.7 U/ml; 95% CI: 220.8 – 247.2); $p = 0.019$). Smoking had no influence on KL-6 levels ($p = 0.8$). KL-6 levels were co-dependent on age ($p = 0.01$), although grouping the individuals in age brackets 10-19 did not reveal profound differences in KL-6 levels ((females: 186.9 ± 7.2 U/ml; male 224.9 U/ml ± 0 U/ml), 20-29 (females: 213.1 ± 10.0 U/ml; males: 214.2 ± 10.1 U/ml), 30-39 (females: 242.2 ± 9.0 U/ml; males: 256.5 ± 12.0 U/ml), 40-49 (females: 214.2 ± 9.4 U/ml; males: 251.1 ± 10.0 U/ml), 50-59 (females: 255.9 ± 10.3 U/ml; males: 271.9 ± 16.8 U/ml) and > 60 years (females: 208.2 ± 7.9 U/ml; males: 217.9 ± 12.9 U/ml)). Figure 1 illustrates the MUC1 genotype-grouped serum KL-6 levels.

Table 1 MUC1 genotype-specific serum KL-6 levels in healthy, white Caucasians.

MUC1 polymorphism	***568***		
genotype	***AA***	***AG***	***GG***
number of individuals	102	155	70
serum KL-6 levels (U/ml)	195.2 (9.9)	246.0 (8.6)	302.6 (11.8)
95% confidence interval (U/ml)	175.7 – 214.8	229.0 - 263.1	279.3 - 326.0
Normal range (U/ml)	29.2 – 355.4	67.6 – 413.4	71.1 – 533.7

SEM are shown in parentheses. Values were corrected for age, smoking, and gender.

Sarcoidosis patients

Figure 2 illustrates the influence of the 568 A/G polymorphism on KL-6 levels in sarcoidosis patients. When the KL-6 levels of the patients who were grouped according to the genotype, the results were (mean ± standard deviation (SD); 95% CI): AA (550.1 ± 411.7; 95% CI: 380.2 - 720.1), AG (716.3 ± 452.4; 95% CI: 547.4 - 885.2), GG (1151.0 ± 1122; 95% CI: 610.1 - 1692.0), p = 0.02.

KL-6 levels measured in sarcoidosis patients at presentation were also dependent on radiographic staging. Namely, patients who had presented with stages 0/I and Löfgren's syndrome had lower KL-6 levels (mean ± SEM; 95% CI) (548.9 ± 121.0 U/ml; 95% CI: 307.4 – 790.3 U/ml) than those with stages II, III and IV (1118.5 ± 133.1 U/ml; 95% CI: 852.9 – 1384.2 U/ml), p=0.0003. When the 568 A/G genotype influence on KL-6 levels in the patients were corrected for radiographic staging, gender, age and smoking, significant differences in KL-6 levels (mean ± SEM (U/ml); 95% CI) between patients grouped according to the MUC1 568 A/G genotype were observed as follows: AA (606.5 ± 136.5 U/ml; 95% CI: 334.0 – 879.0 U/ml), AG (670.5 ± 133.9 U/ml; 95% CI: 403.1 – 937.8 U/ml) and GG (1224.2 ± 165.3 U/ml; 95% CI: 894.2 – 1554.1 U/ml), p = 0.003, (Table 2). Smoking, gender or age did not show any significant influence on KL-6 levels (data not shown).

Table 2 MUC1 genotype-specific serum KL-6 levels in sarcoidosis patients.

MUC1 polymorphism	***568***		
genotype	***AA***	***AG***	***GG***
number of individuals	25	30	19
serum KL-6 levels (U/ml)	606.5 (136.5)	670.5 (133.9)	1224.2 (165.3)
95% confidence interval (U/ml)	334.0– 879.0	403.1 – 937.8	894.2 – 1554.1

SEM are shown in parentheses. Values were corrected for radiographic staging, age, smoking, and gender.

Comparison of the KL-6 levels in which the 568 genotype was ignored rendered six out of 74 (7.5%) misclassifications of 'elevated' versus 'normal' KL-6 levels or vice versa. Specifically, KL-6 levels of 568 AA patients (n = 3) changed from being elevated (> 437.6 U/ml, according to the reference interval: 39.8 – 437.6 U/ml) to normal (within to the AA genotype-specific

reference interval in table 1) and 568 GG genotyped patients (n = 3) changed from having normal (between 39.8 – 437.6 U/ml) to elevated (> upper reference limit of the GG genotype-specific reference interval in table 1) KL-6 levels (data not shown).

Discussion

This study is the first to describe a MUC1 genotype influence on KL-6. We demonstrated that the 568 A to G polymorphism contributes to inter-individual differences in serum KL-6 levels. Serum KL-6 levels were higher in those individuals carrying the 568 G allele with 568 A homozygotes having the lowest levels, 568 G homozygotes having the highest levels and heterozygotes having intermediate levels, which is compatible with a gene-dose effect. A similar MUC1/KL-6 gene-dose effect was observed in the sarcoidosis patients, despite the staggering 3-fold increase of the overall KL-6 levels compared to controls. In addition, and as observed previously (9), radiographic staging showing parenchymal involvement (stages II and higher) correlated with a significantly higher KL-6 levels compared to patients without parenchymal involvement (stages 0/I and Löfgren's patients). Discordant classification of KL-6 levels (normal vs. elevated) in 7.5% of the evaluated sarcoidosis patients clearly demonstrates the consequences of the 568 A/G polymorphism on the interpretation of KL-6 levels.

Ligtenberg and colleagues have demonstrated that the MUC1 gene polymorphism at nucleotide position 568 is functional (11). The choice of the MUC1 pre-mRNA splice acceptor site, resulting in a 27 bp difference between the two alleles, is determined by this polymorphic nucleotide. Furthermore, there is a strong linkage disequilibrium between the MUC1 568 A/G and VNTR polymorphism (11, 16). Pratt and colleagues reported that determination of the MUC1 568 A/G using PCR-SSPs is a much easier way to establish MUC1 haplotypes than determination of the VNTR itself (16). Most alleles containing a large number of tandem repeats have a G at nucleotide position 568 and splice to the upstream splice acceptor site, which results in a larger MUC1 protein. Conversely, most alleles containing a small number of repeats have an A at this position and splice to an acceptor site located 27 bp further downstream, which results in a smaller MUC1 protein (11, 16).

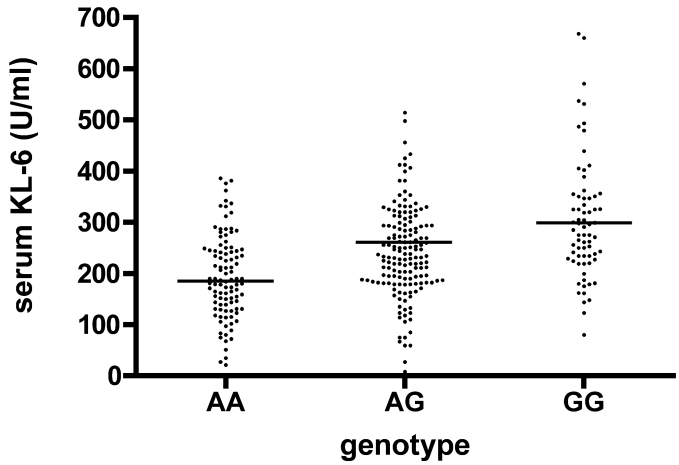

Figure 1 Scatterplot illustrating the association between MUC1 568 A/G genotype and serum KL-6 levels (U/ml) in 327 healthy whites. Horizontal bars in scatter plots represent estimated marginal mean for each group.

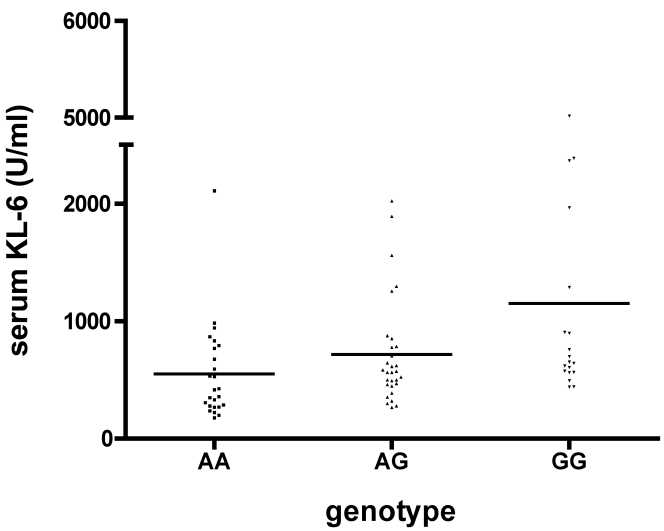

Figure 2 Scatterplot illustrating the association between MUC1 568 A/G genotype and serum KL-6 levels (U/ml) in 74 sarcoidosis patients. Horizontal bars in scatter plots represent mean for each group.

The tandem repeat unit is rich in serine and threonine. Following translation, the MUC1 protein undergoes extensive modification. Sugar chains are joined through O-glycosylation with serine and threonine residues of the protein backbone (17). Silverman and co-workers demonstrated that mucins with a high number of repeat units have more sugar chains compared to those with fewer tandem repeats (12). Since the monoclonal IgG_1 KL-6 antibody recognizes a sugar chain on the MUC1 protein and O-glycosylation of MUC1 is influenced by the primary sequence of peptide core (3), it is conceivable that the larger MUC1 protein encoded by the MUC1 568 G allele expresses more KL-6 on its surface than the smaller MUC1 protein encoded by the 568 A allele.

Serum KL-6 is a promising disease marker in ILDs (2). Serum KL-6 levels are elevated in a majority of patients with a number of ILDs, including IPF, hypersensitivity pneumonitis, interstitial pneumonitis associated with collagen vascular disease and sarcoidosis (2, 9). The clinical value of KL-6 measurements as diagnostic test is likely to benefit from genotyping for the MUC1 polymorphism at position 568. There may be additional SNPs in the MUC1 gene which were not identified and evaluated in this study, that could lead to differential functionality of the gene. Like the 568 A/G polymorphism, such quantitative trait loci may contribute to variance in serum KL-6 levels in addition to SNP 568 A/G. Further scrutiny of the coding and promoter regions of MUC1 is needed to map the MUC1 genetic variability and its phenotypic effect on KL-6 levels more completely.

Serum KL-6 is also a prognostic marker in various lung diseases. High serum KL-6 levels were found to predict a resistance to corticosteroid treatment in IPF patients (6). Kohno and colleagues estimated survival in IPF using the Kaplan-Meier method and they showed that patients with serum KL-6 levels above 1,000 U/ml have a significantly worse prognosis (7). In patients with acute respiratory distress syndrome, KL-6 levels were higher in non-survivors than survivors (8). Moreover, serum KL-6 levels tended to be associated with pulmonary disease outcome in sarcoidosis (9). Correcting serum KL-6 levels for the MUC1 haplotypes may increase its value as a prognostic marker.

Conclusion

In conclusion, our study is the first to deliver *in vivo* evidence that the MUC1 568 A to G polymorphism accounts for significant inter-individual variations in serum KL-6 levels.

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chapter SEVEN

7

A polymorphism in the CHI3L1 gene influences levels of YKL-40, A NOVEL SARCOIDOSIS MARKER

Submitted

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Abstract

YKL-40 has recently shown its potential as a marker for sarcoidosis.

This study aimed to assess whether YKL-40 may predict the course of sarcoidosis over a 4-year follow-up period and to investigate whether polymorphisms in the chitinase 3-like 1 (CHI3L1) gene might influence serum YKL-40 levels in sarcoidosis patients (n=63) and controls (n=333).

Patients had significantly higher (mean, 95%CI) serum YKL-40 levels (181.3 ng/ml, 50.7-648.1) compared to controls (36.6 ng/ml, reference interval: 11.9 – 110.0 ng/ml), $p < 0.0001$. Serum YKL-40 was elevated in 79% of the patients and was inversely correlated with DLco at presentation ($r^2 = -0.27$, $p=0.03$), but not after 2-4 years of follow-up ($r^2 = -0.16$, $p=0.27$).

Serum YKL-40 levels in controls were dependent on the CHI3L1 -329 G/A polymorphism (mean, 95%CI): GG (n=213) 48.3 ng/ml, 41.7-56.0; GA (n=101) 31.2 ng/ml, 26.6-36.3; AA (n=17) 17.8 ng/ml, 13.6-23.4, $p < 0.0001$. In patients, this effect was not observed.

YKL-40 may be used to exclude sarcoidosis, but it is unable to predict the course of the disease. The CHI3L1 -329 G/A polymorphism may be of interest for investigations involving YKL-40, as our study delivered evidence that it contributes to inter-individual variations in YKL-40 levels.

Introduction

Sarcoidosis is a systemic disease of unknown cause and is characterized by the presence of noncaseating granulomas in one or multiple organs (1). The majority of patients have pulmonary involvement and although most undergo spontaneous remission, about 10-15 percent of the cases progress insidiously towards pulmonary fibrosis (2). Fibrosis of the lung parenchyma is marked by excessive extra-cellular matrix (ECM) deposition which can lead to end-stage fibrosis with honeycombing of the lungs. This sequela is associated with loss of lung capacity and has a poor prognosis with high mortality (3).

It is challenging to find reliable sarcoidosis disease markers that are capable of predicting the course of the disease. The human cartilage glycoprotein-39, or YKL-40, has recently shown its potential merit as a marker for sarcoidosis (4). Beside the reported overall increase of serum YKL-40 levels in sarcoidosis patients compared to controls, pulmonary carbon monoxide diffusion (DLco) was found to be inversely correlated with serum YKL-40 levels (4). The authors of this study concluded that YKL-40 may serve as a marker for disease activity as well as fibrosis in sarcoidosis patients.

In addition to sarcoidosis, serum YKL-40 is also elevated in systemic sclerosis (5, 6), rheumatoid arthritis (7-9), liver fibrosis (10, 11) and inflammatory bowel disease (12). Moreover, elevated YKL-40 has been reported to predict shorter survival in cancer patients (13-18).

YKL-40 is a member of the chitinase family and is secreted by articular chondrocytes and synovial cells (19) as well as by disease-associated cells including cancer (20, 21) and inflammatory cells (4, 22, 23). Although its precise function has not yet been elucidated, the biological properties of YKL-40 suggest that it plays a role in tissue inflammation, remodeling and the development of fibrosis (24-26).

Single nucleotide polymorphisms (SNPs) of genes that are involved in the pathogenesis of sarcoidosis have shown to contribute to the vastly different patterns with which this disease can evolve (27). SNPs may alter the nature of a protein and is reflected by altered physiologic properties or changed expression levels. Functional polymorphisms present in proteins which are used as disease markers may complicate the interpretation of differential protein levels in both healthy and affected individuals. In the ACE gene, for instance, an insertion/deletion (I/D) polymorphism in intron 16 is known to exhibit gross differences of baseline expression levels based on the genotype of

the coding gene (28). As a consequence, the precision needed to differentiate between normal and deviating levels may be compromised (29).
The YKL-40 coding chitinase 3-like 1 (CHI3L1) gene also bears a large number of polymorphic sites, some of which are potentially functional. The aim of this study was to investigate whether SNPs in the CHI3L1 gene may influence serum YKL-40 levels in Dutch Caucasian sarcoidosis patients and healthy controls. The biallelic polymorphisms which we evaluated included two that are present in the promoter region, three in different intron/exon boundaries and one SNP in exon 5 that causes an amino acid substitution (Arg145Gly).
We also tested the hypothesis by Johansen et al. (4) that YKL-40 levels may predict the course of pulmonary sarcoidosis over a 4-year follow-up period, assessed by radiographic evolution and pulmonary function tests.

Subjects and Methods

Controls

Venous blood samples were obtained from 333 ostensibly healthy employees of the St Antonius Hospital; 214 women (39 ± 12.0 years) and 119 men (42 ± 10.2 years). By completing a questionnaire, relevant background information was provided by these volunteers which included medication, ethnicity and hereditary diseases. The overrepresentation of women who participated in this study is explained by the predominantly female workforce at this hospital. Fifty-five individuals (33 women and 22 men) smoked for at least five pack-years. Ethnicity of both parents was used as the criterion for assuming Dutch Caucasian ethnicity of the subject. Exclusion criteria included known pulmonary disease and non-Dutch Caucasian ethnicity. The medical ethical committee of this hospital approved the study conducted and all subjects gave formal written consent.

Patients

Seventy-five unrelated Dutch Caucasian sarcoidosis patients (44 males/31 females; age at diagnosis (years) ± SD/range: 39 ± 11.2 years/17 – 71) were included in this retrospective study. The diagnosis of sarcoidosis was established in 50 patients when clinical findings were supported by histological evidence and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis (30).

Chest radiographs were assessed to determine disease severity using standard radiographic staging for sarcoidosis, classified according to the Scadding criteria (30). One patient presented with radiographic stage 0, 20 patients presented with stage I, 11 patients presented with stage II, 15 patients presented with stage III, and 3 patients presented with stage IV disease. In 25 patients, the diagnosis was made without biopsy proof because these patients presented with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy.
Radiographic evolution over a 4-year follow-up period was categorized as follows: acute/self-remitting (normalization or improvement towards stage I) (n = 25), chronic (persistent stage II/III or progression towards this stage) (n = 16), and fibrosis (stable stage IV or progressive towards this stage) (n = 9). Patients who had been diagnosed with Löfgren's syndrome at presentation (n = 25) were regarded as a distinct group. Except for one, all patients with Löfgren's had received radiographic follow-up. These patients had normalized chest radiography after 2 or 4 years following presentation, except for 1 patient, who remained at radiographic stage I after 4 years.
Pulmonary function tests were performed at presentation and upon 2 years, and 4 years following diagnosis. Vital capacity (inspiratory) (VC), forced expiratory volume in 1 second (FEV_1) and carbon monoxide diffusing lung capacity (DLco) were used to assess the presence of lung function impairment at presentation and follow-up of disease. All lung function parameters are expressed as percent predicted values. VC and FEV_1 were calculated from volumes in liters and adjusted to BTPS (body temperature, ambient pressure, saturated with water vapor) in accordance with the ERS recommendations (31).
The medical ethical committee of the St. Antonius Hospital approved the study conducted and all subjects gave formal written consent.

Serum YKL-40 measurements
Serum YKL-40 was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Metra YKL-40, Quidel Corporation, San Diego, CA, USA), in accordance with the manufacturer's instructions (32). All samples were run in duplicate and mean values were used for analysis.

Genotyping
Biallelic single nucleotide polymorphisms were determined using sequence-specific primers (SSPs) and polymerase chain reaction (PCR).

The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in Table 1. The PCR conditions were as previously described (33). The final primer concentrations used were 7.6 ng/µl. DNA was available from 63 patients.

Table 1 Primer sequences for the identification of 6 biallelic single nucleotide polymorphisms of CHI3L1.

Polymorphism and location	dbSNP accession*	Primer	Consensus	product size (bp)
-329 (G/A) promoter	rs10399931	5′-CGG CTG AGT CAC ATC TCC G/A (forward)	5′-CAG GCC CTG TAC TTC CTT TA (reverse)	261
-247 (C/T) promoter	rs10399805	5′- AGA GGA GGG TTG AGA AAC CG/A (reverse)	5′-TGC TGA AGA TGC AAA GGT AGA G (forward)	220
48 (T/A) intron1/exon1	rs7515776	5′-ATC TGG GCT AGC CAA GGT TAA T/A (forward)	5′-AGA GAA ATC CAG GAT GAG ACC (reverse)	247
1219 (C/T) intron2/exon3	rs1538372	5′-CCT GAA GGA GAA GTC TGG G G/A (reverse)	5′- TAG CGT ATT CAT CCC CTG CA (forward)	265
2117 (C/G) intron4/exon4	rs2071579	5′- GAA CAT CCA TAC AGT GGA TG G/C (reverse)	5′- TAA CCC AGC CTC TCA CCC AA (forward)	264
2950 (G/A) exon 5	rs880633	5′- GGT GGT AAA ATG CTG TTT GTC TC C/T (reverse)	5′- GCA TGC TAC AGG GCT GAT TTT (forward)	171

*The SNP loci were identified using accession numbers according to the SNP database at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp (accessed July 2005).

Statistical analysis

The statistical evaluation of our data was performed using SPSS 11 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism v. 4 (Graphpad Software, Inc., San Diego, CA, USA) software packages. Statistical analyses of SNP and haplotype frequency distributions were performed using chi-square contingency table analysis with the appropriate number of degrees of freedom. Fisher's exact test was used if expected cell frequencies were lower than 5. Adjustment for multiple tests was made by multiplying the p-value by the number of SNPs being investigated (Bonferroni method). Genotype frequencies were tested for Hardy-Weinberg equilibrium. Haplotypes were determined using Phase, version 2 (Mac OS X) (34, 35).

YKL-40 values were ln-transformed before analysis, since these were ln-normal distributed; antilog geometric mean values are denoted as mean. Analysis of variance was performed and the portion of the serum YKL-40 level variance, explained by the CHI3L1 genotype and other factors, was estimated. Fixed factors in the ANOVA-model were gender, smoking history, and the CHI3L1 genotype. Age was included as a covariate. The ANOVA was followed by the appropriate post-testing for comparisons between groups.
Correlation between variables was assessed with Spearman's rho.
The sensitivity and the specificity were established using the receiver operating characteristic (ROC) curve by plotting the sensitivity against the reverse specificity (1 minus specificity) at each value.
Statistical significance was denoted by a value of $p < 0.05$ for all tests performed.

Results

Serum YKL-40 levels in controls

Two YKL-40 values were considered outliers (680.0 and 703.4 ng/ml) and were omitted from further analysis. Mean (median) serum YKL-40 in the remaining 331 controls was 36.6 (37.3) ng/ml. The calculated reference range (mean ± 1.96 x SD) was: 11.9 – 110.0 ng/ml.
Age correlated with YKL-40 levels ($r^2 = 0.23$, $p < 0.0001$) and explained 5% of the variation. Smoking history or gender did not significantly influence serum YKL-40 levels (data not shown).

Serum YKL-40 levels in patients

Sarcoidosis patients had significantly higher (mean (median), 95%CI) serum YKL-40 levels (181.3 (210.0) ng/ml, 50.7-648.1) compared to controls, $p < 0.0001$. Based on the reference interval of controls, 59 sarcoidosis patients (79%) showed serum YKL-40 levels that exceeded the upper reference limit.
A receiver operating characteristic curve revealed that at the value of 72.0 ng/ml, serum YKL-40 had a sensitivity of 90% and a specificity of 72%. The area under the curve was 0.953 (Figure 1). The positive and negative predicted values of YKL-40 were 50% and 98%, respectively.

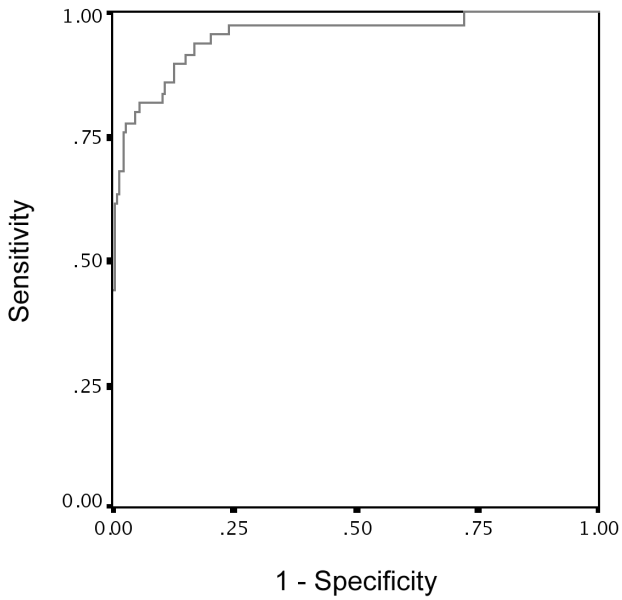

Figure 1 Receiver operating characteristic curve of serum YKL-40 with 331 healthy controls and 75 sarcoidosis patients.

YKL-40 levels and clinical parameters in sarcoidosis patients

Radiographic staging at presentation or radiographic evolution were not associated with YKL-40 levels (data not shown).

Serum YKL-40 showed an inverse correlation with DLco (%) at presentation (r^2 = -0.27, p = 0.03), but not with DLco measured at 2-4 years (r^2 = -0.16, p = 0.27). Sarcoidosis patients who had DLco levels below the mean (85%) had significantly higher mean serum YKL-40 levels than those with DLco values equal to or above the mean: 259.8 ng/ml, 95%CI: 200.3 – 317.3 vs. 170.7 ng/ml, 95%CI: 134.3 – 210.6, p = 0.004 (Figure 2).

Neither FEV_1 nor VC (both measured at presentation or after 2-4 years) correlated with serum YKL-40 levels (data not shown).

CHI3L1 single nucleotide polymorphisms

Table 2 summarizes the results of CHI3L1 genotype and allele carrier frequencies in the Dutch sarcoidosis patients and controls. No deviation from Hardy-Weinberg equilibrium was observed for any of the groups studied. No differences were found for genotype, allele or allele carrier frequencies between sarcoidosis patients and controls.

Four major haplotypes with 6 polymorphic sites in the *CHI3L1* gene were established (Table 3). There was no difference in haplotype frequencies between sarcoidosis patients and controls.

Association between genetic variants of CHI3L1 and serum YKL-40 levels in controls and sarcoidosis patients

Each SNP in the CHI3L1 gene was analyzed for association with serum YKL-40 levels. All 6 polymorphic sites were found to influence serum YKL-40 levels in a gene-dose dependent manner. Figure 3A illustrates the effect of SNP -329 G/A on serum YKL-40 levels in healthy controls. To test whether the variation in YKL-40 levels was influenced independently by the individual SNPs or by linkage disequilibrium between them, a multivariate analysis was performed. In this model, which was adapted from the method used by Zhu et al. (36), the SNP that explained most of the variation of serum YKL-40 levels was selected as a covariate to identify the influence of the remaining SNPs (analyzed separately). The -329 G/A polymorphism showed the highest variance: 23%, $p < 0.0001$).

Also, age was included as a covariate and gender and smoking history were included as fixed factors. The analysis revealed that the correction of serum YKL-40 levels by the -329 G/A polymorphism negated the influence of the other polymorphisms on serum YKL-40 levels (Table 4). The isolated effect of the -329 G/A SNP was also illustrated by determining haplotype-specific serum YKL-40 levels.

Table 2 CHI3L1 allele carrier and genotype frequencies in Dutch Caucasian sarcoidosis patients and controls.

polymorphism	allele and genotype	controls (n = 333)	patients (n = 63)
-329 (G/A)	G	0.94 (314)	0.97 (61)
	A	0.35 (118)	0.29 (18)
	GG	0.65 (215)	0.71 (45)
	AG	0.30 (101)	0.25 (16)
	AA	0.05 (17)	0.03 (2)
-247 (C/T)	C	0.98 (328)	0.98 (62)
	T	0.24 (79)	0.25 (16)
	CC	0.76 (254)	0.74 (47)
	CT	0.22 (74)	0.24 (15)
	TT	0.02 (5)	0.02 (1)
48 (T/A)	T	0.98 (328)	0.98 (62)
	A	0.24 (79)	0.25 (16)
	TT	0.76 (254)	0.74 (47)
	TA	0.22 (74)	0.24 (15)
	AA	0.02 (5)	0.02 (1)
1219 (C/T)	C	0.94 (314)	0.98 (62)
	T	0.47 (157)	0.44 (28)
	CC	0.53 (176)	0.56 (35)
	CT	0.41 (138)	0.43 (27)
	TT	0.06 (19)	0.02 (1)
2117 (C/G)	C	0.81 (269)	0.79 (50)
	G	0.70 (233)	0.62 (39)
	CC	0.30 (100)	0.38 (24)
	CG	0.51 (169)	0.41 (26)
	GG	0.19 (64)	0.20 (13)
2950 (G/A)	G	0.83 (277)	0.79 (50)
	A	0.69 (230)	0.62 (39)
	GG	0.31 (103)	0.38 (24)
	GA	0.52 (174)	0.41 (26)
	AA	0.17 (56)	0.20 (13)

When heterozygous carriers of either of the haplotypes in combination with haplotype 1 were compared with each other in terms of (mean) serum YKL-40 levels, the only significant differences were those observed between haplotypes that differed from the -329 allele: haplotype carrier 1+1 (43.8 ng/ml) vs. 1+2 (28.8 ng/ml), $p < 0.0001$; haplotype carrier 1+2 (28.8 ng/ml) vs. 1+3 (49.4 ng/ml), $p < 0.0001$; haplotype carrier 1+2 (28.8 ng/ml) vs. 1+4 (44.7 ng/ml), $p < 0.05$; haplotype carrier 1+2 (28.8 ng/ml) vs. 1+5 (60.3 ng/ml), $p < 0.01$; haplotype carrier 1+5 (60.3 ng/ml) vs. 1+6 (22.3 ng/ml), $p < 0.05$.

Table 3 CHI3L1 haplotype frequencies in sarcoidosis patients and controls.

haplotype	-329 (G/A)	-247 (C/T)	48 (T/A)	1219 (C/T)	2117 (C/G)	2950 (G/A)	frequency in controls (n = 333)	frequency in patients (n = 63)
1	G	C	T	C	C	G	0.53	0.58
2	A	C	T	T	G	A	0.17	0.13
3	G	T	A	C	G	A	0.12	0.13
4	G	C	T	T	G	A	0.09	0.10
5	G	C	T	C	G	A	0.02	0.03
6	A	C	T	C	C	G	0.02	0.01
7-10*	-	-	-	-	-	-	0.05	0.02

*One of each haplotype occurred no more than 3 times.

The -329 G/A polymorphism did not appear to significantly influence serum YKL-40 levels in sarcoidosis patients (Figure 3B) despite the trend towards lower mean values associated with the presence of the A allele: genotype AG (153.2 ng/ml) compared to GG (192.7 ng/ml), p = 0.16, nor did correcting the effect of the -329 G/A SNP for smoking, gender and age in a multivariate analysis reveal a genotype-dependent influence on serum YKL-40 levels (data not shown). Age correlated significantly with serum YKL-40 in a similar pattern as that which was observed in the control population ($r^2 = 0.24$, p = 0.04). No association was found between the remaining 5 polymorphisms and serum YKL-40 levels in sarcoidosis patients (data not shown).
The observed association between DLco and serum YKL-40 levels was not attributed to the -329 G/A genotype distribution between patients below and equal/above the mean DLco (85%) (data not shown).

Age and genotype-specific reference intervals
In order to calculate the expected serum YKL-40 levels of an individual that is corrected for age and the -329 G/A genotype, the following formulas were deduced from the multivariate analysis:

-329 GG genotype: ln (serum YKL-40 (ng/ml)) = 3.348 + 0.012 x age (years) ± 0.944
-329 AG genotype: ln (serum YKL-40 (ng/ml)) = 2.916 + 0.012 x age (years) ± 0.944
-329 AA genotype: ln (serum YKL-40 (ng/ml)) = 2.362 + 0.012 x age (years) ± 0.944

When the expected serum YKL-40 serum levels were calculated for the sarcoidosis patients, the number of patients with either elevated or normal serum YKL-40 levels remained unchanged.

Table 4 CHI3L1 gene polymorphisms and the influence on serum YKL-40 levels in controls.

Polymorphism	Genotype-specific YKL-40 levels (ng/ml) (mean, 95% CI)[a]	Differences between genotype-specific YKL-40 serum levels
-329 (G/A)	GG (n = 213) 48.3, 41.7-56.0 GA (n = 101) 31.2, 26.6-36.3 AA (n = 17) 17.8, 13.6-23.4	P < 0.00001
-247 (C/T)	CC (n = 254) 39.4, 34.2-45.5 CT (n = 72) 43.1, 36.4-51.0 TT (n = 5) 50.1, 32.0-78.4	P = 0.25[#]
48 (T/A)	TT (n = 252) 39.4, 34.1-45.4 TA (n = 72) 43.1, 36.4-51.2 AA (n = 7) 47.7, 31.6-72.1	P = 0.27[#]
1219 (C/T)	CC (n = 176) 40.5, 34.7-47.3 CT (n = 138) 40.0, 34.2-46.8 TT (n = 19) 42.2, 32.0-55.5	P = 0.07[#]
2117 (C/G)	CC (n = 100) 36.9, 31.2-43.6 CG (n = 168) 41.0, 35.5-47.5 GG (n = 63) 43.1, 35.9-51.8	P = 0.14[#]
2950 (G/A)	GG (n = 103) 39.5, 34.2-45.6 GA (n = 173) 42.4, 35.7-50.6 AA (n = 55) 49.2, 35.3-68.6	P = 0.43[#]

*Corrected for smoking history, gender, and age. [#]SNP -329 G/A was used as a covariate to determine the effects of the remaining 5 SNPs on serum YKL-40 levels. Smoking history, gender and age were also included as fixed factor or covariate. Mean YKL-40 and 95% CI were calculated from log-transformed data.

Discussion

This study supports the merits of serum YKL-40 as a disease marker for sarcoidosis, as previously shown by Johansen and co-workers (4). Serum YKL-40 levels were found to be elevated in 79% of the sarcoidosis patients. The strong negative predictive value (98%) indicates that YKL-40 in serum may

be useful as an exclusion criterion for sarcoidosis in suspected cases or in patients who are being monitored for disease activity. However, the low positive predictive value (50%) and the previously described use of YKL-40 as a marker for various other diseases (5-12) may limit the use of YKL-40 as a diagnostic marker for sarcoidosis.
Serum YKL-40 levels were found to increase with age, although the extent to which serum YKL-40 was elevated in sarcoidosis patients was too high to allow age to influence the accuracy of serum YKL-40 as a sarcoidosis marker.

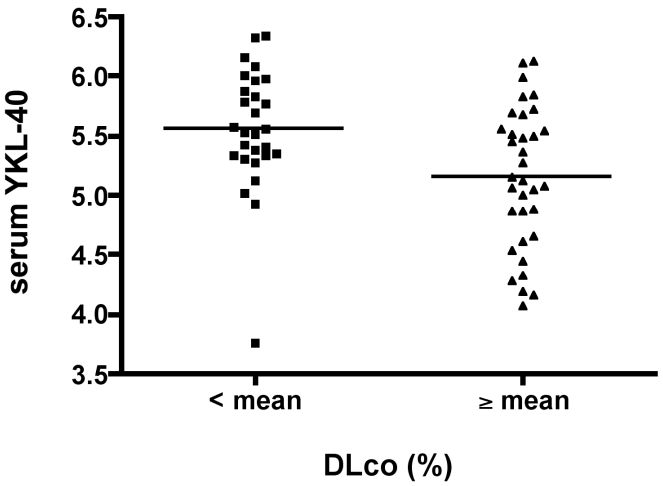

Figure 2 Scatter plot of serum YKL-40 levels (ng/ml) in sarcoidosis patients (n = 62) with diffusion capacity (DLco) below and equal/above the mean (85%). Horizontal bars in scatter represent mean values Y-axis represents log-transformed values. Student's t-test: p = 0.005.

The inverse correlation between serum YKL-40 levels and DLco seemed to suggest that YKL-40 is related to the development of pulmonary fibrosis. However, since the development of pulmonary fibrosis is usually irreversible (3), the lack of correlation between DLco measured at later time points during follow-up and serum YKL-40 levels measured at presentation does therefore suggest against the presence of fibrosis. Vascular involvement in pulmonary sarcoidosis is often seen (37) and is, like fibrosis, often reflected by a decreased DLco (38). The observed correlation between YKL-40 and DLco may therefore

be a result of granulomatous burden in the pulmonary vasculature rather than the presence of fibrosis. The relationship between elevated YKL-40 levels in serum and vascular involvement remains to be determined. Alternatively, sarcoidosis may involve fibrotic changes that are too small to be discerned on chest x-rays. Such disparities between staging and lung function parameters that indicate fibrosis have been reported (39, 40). It is therefore important to consider which parameter is the most suitable for establishing the presence, extent and progression of fibrosis of the lungs. If DLco does indeed reflect fibrotic changes, the absence of correlation between the pulmonary phenotypes according to radiography and YKL-40 is not necessarily incongruous. In our study group, only 3 sarcoidosis patients with radiographic stage IV at presentation also had YKL-40 data available. Consequently, the group size was too small to deliver useful data in terms of present fibrosis and changes in serum YKL-40 levels. Contrary to what Johansen et al. described (4), YKL-40 levels above the median were not associated with decreased DLco values. Instead, sarcoidosis patients with DLco values below the mean had higher YKL-40 levels. An observation similar to ours has been reported in systemic sclerosis patients with pulmonary fibrosis by chest x-ray. In this report, patients with decreased DLco showed higher serum YKL-40 levels in serum (5). Although our findings transpired from assessing a larger group of patients of whom DLco as well as YKL-40 data were available (n = 44 vs. n = 27), even larger groups should be used to resolve the inconsistent findings between Johansen's and our study.

This study is the first to describe a genotypic influence of the CHI3L1 -329 G/A polymorphism on serum YKL-40 levels in healthy individuals. This polymorphism was shown to explain as much as 23% of the variation in serum YKL-40 levels. In the sarcoidosis patients, the genotype-dependent effect of the -329 G/A polymorphism was not observed. Possibly, in a disease state, pathologic factors involved in sarcoidosis which contribute to an increase of YKL-40 levels may overrule the variance attributed to the -329 G/A polymorphism in CHL3L2. Just as what was argued for age, there may not be a diagnostic merit in correcting serum YKL-40 levels for the -329 G/A genotype.

The hypothesis as to whether the -329 G/A genotype might predispose to pulmonary fibrosis in our patient group was performed by comparing the -329 G/A genotype distribution between patients with stage IV and other stages as well as with normal and decreased DLco values. The lack of negative associations between DLco and the -329 G/A genotype in our patient group

does not however support a protective effect of the A allele on pulmonary fibrosis, although significantly more patients with complete lung function, radiographic data and genotypes are required to confirm our finding with more certainty. Using an online tool to check for functional motifs at position -329 did not reveal any changes in the motif found for the wild-type nucleotide (http://motif.genome.jp/). The actual position of the SNP is therefore unlikely to be the quantitative trait locus (QTL) itself. Instead, it may be in linkage with another, yet to be identified QTL. Like ACE, in which the I/D polymorphism explains as much as 50% of the variance of ACE activity through linkage with the actual QTL (28, 41), additional sequencing of the promoter region of the CHI3L1 gene may indicate a site that is responsible for an even higher variation of YKL-40 levels.

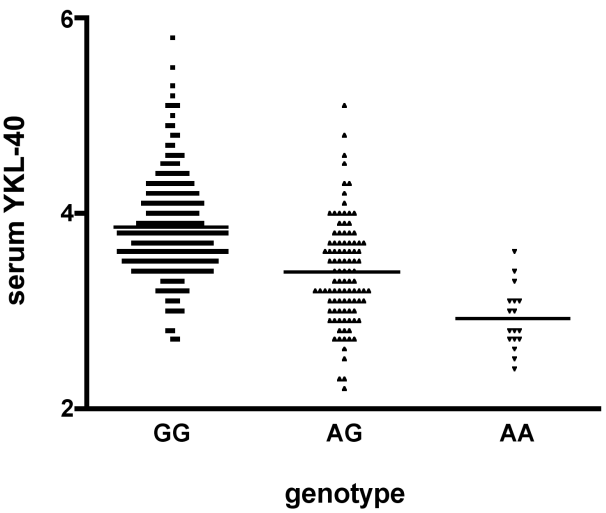

Figure 3A Scatterplot illustrating the association between the -329 G/A polymorphism in the CHI3L1 gene and serum YKL-40 levels in healthy controls (n = 331). Horizontal bars in scatters represent mean values. Y-axis represents log-transformed values. ANOVA: $p < 0.0001$.

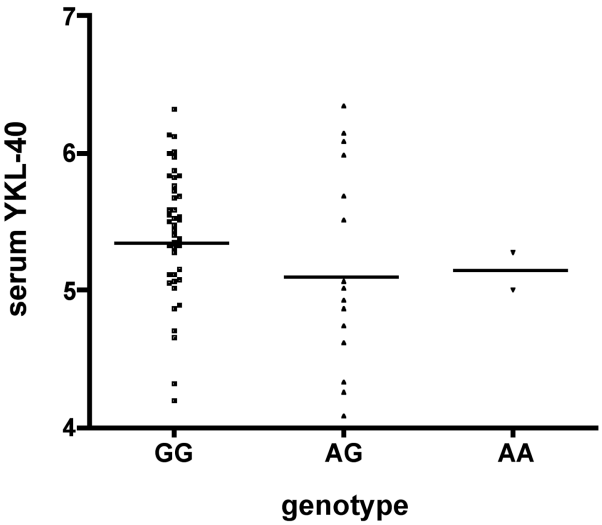

Figure 3B Scatterplot illustrating the association between the -329 G/A polymorphism in the CHI3L1 gene and serum YKL-40 levels in sarcoidosis patients (n = 63). Horizontal bars in scatters represent mean values. Y-axis represents log-transformed values. ANOVA: p = 0.29

Conclusion

This study shows that YKL-40 can be used as an exclusion marker for sarcoidosis with the stipulation that other diseases associated with elevated serum YKL-40 levels can be excluded upon assessment. Serum YKL-40 levels could not predict the course of pulmonary disease phenotypes according to radiographic evolution over a 4-year follow-up period. A considerably larger group of stage IV sarcoidosis patients and DLco data are needed to evaluate the potential of YKL-40 as a marker for pulmonary fibrosis. Furthermore, the CHI3L1 -329 G/A polymorphism may be of interest for investigations involving YKL-40, as our study delivered in vivo evidence that it contributes to inter-individual variations in YKL-40 levels.

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chapter EIGHT

8

Summary & CONCLUDING REMARKS

Summary

Although the majority of sarcoidosis patients undergo spontaneous remission, approximately 10 percent of the sarcoidosis cases develop parenchymal fibrosis. Accumulating fibrotic lesions may lead to end-stage sarcoidosis, catagorized radiographically as stage IV pulmonary sarcoidosis. Fibrosis of the lung parenchyma is associated with poor pulmonary function and a bleak prognosis with increased morbidity and mortality.

Fibrosis is characterized by a disproportionate increase in extracellular matrix (ECM) proteins. The exuberant ECM production is believed to originate from an imbalance between cytokines involved in tissue healing and remodeling. Additional factors which have shown to pricipitate the development of fibrosis are those of the renin-angiotensin system. In this system, angiotensin II exhibits a pivotal role in the process of tissue injury with ensuing fibrosis.

Sarcoidosis is likely to be a genetically complex disease that involves a combination of genetic loci (polygenic) conferring disease predilection or phenotypic variation of disease manifestation. Single nucleotide polymorphisms (SNP's) in genes that encode for components involved in tissue injury and repair may influence the risk of developing pulmonary fibrosis in sarcoidosis. The effects of genetic variation on sarcoidosis phenotypes may appear as changes of such parameters as radiography, lung function or serum levels of the disease marker.

The use of the classic sarcoidosis disease marker, ACE, has not unequivocally enabled the clinician to predict the course of sarcoidosis. Ang II levels in serum are apparently not influenced by circulating ACE, which likely makes the potentially detrimental effects of elevated ACE levels in circulation negligible. The role of Ang II in sarcoidosis with pulmonary fibrosis may still be of great relevance, even though it may be more locally restricted, i.e. in the granuloma. The involvement of locally formed Ang II by enzymes such as chymase in the granuloma may thus have a profound impact on the development of pulmonary fibrosis in sarcoidosis.

The genetic variation of the gene encoding for chymase (CMA1) in relation to pulmonary fibrosis in Dutch sarcoidosis patients and cardiac and skin involvement of Japanese sarcoidosis patients was investigated in **chapter 2**. No associations were found between CMA1 genotypes or haplotypes with respect to any of the investigated clinical phenotypes. Although the CMA1

genetic variation may not be associated with the propensity to develop pulmonary fibrosis, further characterization of stage IV (pulmonary fibrosis) patients by using HRCT may reveal an influence of CMA1 polymorphisms on the severity of fibrosis. A substantially larger group of fibrotic patients is needed to evaluate such a relationship. The association between CMA1 –526 C/T and vital capacity in sarcoidosis patients suggests that chymase may modify the functional outcome of pulmonary sarcoidosis. Chymase deserves further scrutiny in order to gain a better grasp on the exact mechanisms of fibrotic development in pulmonary sarcoidosis.

The hypothesis that genetic variation of ACE2 may influence the course of pulmonary sarcoidosis was investigated in **chapter 3**. ACE2 is believed to be an important factor that protects the deleterious effects of Ang II in tissue injury. ACE2 polymorphisms with deduced haplotypes were analyzed in patients suffering from either acute, with no parenchymal involvement (including a substantial number of patients with Löfgren's syndrome), or chronic type disease with parenchymal involvement (including fibrotic end-stage), according to radiographic evolution over a 4-year follow-up period (chapter 3). Analysis of the patient group showed that the frequency of haplotype 5 was higher in males with pulmonary fibrosis compared to those without. Haplotype 4 was only present in sarcoid males without parenchymal involvement and absent in men with parenchymal involvement. The observed differential associations between the genetic variation of ACE2 and parenchymal involvement and fibrosis in males suggest that ACE2 might play an important role in the progression of pulmonary sarcoidosis that is dependent on gender.

Chapter 4 presents the results of SNPs in TGF-β isoforms 1, 2 and 3 and the propensity to develop pulmonary fibrosis in sarcoidosis patients with a 4-year follow-up period. None of the five functional SNPs in the TGF-β1 gene were found to be associated with sarcoidosis with pulmonary fibrosis. Interestingly, a strong association was found between two SNPs in the TGF-β3 gene and pulmonary fibrosis. In addition, a borderline significant association was observed between a SNP in the TGF-β2 gene and pulmonary fibrosis. According to the available literature, the focus on (pulmonary) fibrosis in relation to genetics has been mainly reported for TGF-β1 gene polymorphisms.

The results described in chapter 5 may possibly prompt investigators to direct their interest towards the genetics of TGF-β isoforms 2 and 3. This will most likely add to the understanding of the mechanisms involved in fibrosis.

The aforementioned chapters describe the influence of genetic variation on different disease phenotypes of sarcoidosis. The choice of these particular genes provides insights into the pathways in which the encoded proteins are involved. When a relationship is found between gene polymorphisms and disease phenotypes, we assume that these polymorphisms change the behavior or levels of the protein, either directly or due to linkage. Consequently, the alteration of the protein may lead to differential behavior of the disease. When levels of serum proteins act in function of disease states, these proteins can aid in the diagnosis or tracking the course of the disease. These serum markers may also be influenced by gene polymorphisms. Depending on the genotype of the marker, serum levels may vary in healthy individuals. For most markers, including those used for sarcoidosis, the physiological reason for changing levels as a results of the disease is largely unknown. If a genetically influenced variation is observed for a marker, these findings may at least improve the precision of the interpretation of serum levels.

Despite repetitive calls from different reports to establish ACE I/D genotype-corrected reference intervals, subsequent studies on ACE I/D polymorphisms have only briefly touched on this subject before elaborating on associations with numerous diseases. Clinically useful reference intervals corrected for the ACE I/D genotypes were never given. ACE I/D-corrected reference intervals were established for Dutch, white Caucasian, healthy volunteers (**chapter 5**). In addition, ACE activities in patients suspect of or having sarcoidosis and genotyped for ACE I/D were expressed as a Z-score related to ACE I/D-corrected reference intervals. Comparison of the Z-score with ACE activity in which ACE I/D was ignored rendered an 8.5% misclassification of 'elevated' versus 'normal' ACE or vice versa. These data did not only demonstrate a convenient way to circumvent the use of three reference intervals by introducing a Z-score for ACE activity but also illustrated the need to re-investigate the clinical value of serum ACE activity in sarcoidosis by taking ACE I/D into account.

Chapter 6 demonstrates the influence of a functional SNP at position 568 in the MUC1 gene on serum KL-6 levels in patients with sarcoidosis and healthy controls. The A to G substitution was associated with increased serum KL-6 levels in a gene-dose dependent manner. Genotype-specific reference values were shown to improve the accuracy of using KL-6 values. As discussed in chapter 6, KL-6 has been suggested to have a profibrotic property. The functional polymorphism described in MUC1 may predispose a patient to a poorer outcome of a disease by promoting fibrosis. Such studies are worth conducting in diseases which appear to deteriorate with increasing KL-6 levels. If it can be established that KL-6 actually contributes to the progression of fibrosis, the MUC1 568 A/G genotype could pose a threat to affected individuals carrying the G allele. The identified association between the MUC1 568 polymorphism and serum KL-6 levels will likely prompt investigators to look for genetic associations between the 568 polymorphism and diseases in which KL-6 is of interest.

A similar genotype-dependence on serum levels was observed for a SNP in the YKL-40 encoding gene, CHI3L1 (**chapter 7**). The difference was, however, that instead of showing an association with higher serum levels, as seen for KL-6, individuals carrying the rare allele of the SNP had lower serum YKL-40 levels than those carrying the wild-type. Moreover, the consideration of genotypes in assessing YKL-40 levels as an indicator of sarcoidosis did not improve the accuracy with which patients were identified as being affected. This was likely caused by the observation that in affected individuals YKL-40 was elevated to such an extent that the -329 G/A genotype could not discriminate between elevated and normal YKL-40 levels. The actual position of the SNP is unlikely to be the quantitative trait locus (QTL) itself. Instead, it may be in linkage with another, yet to be identified QTL. Additional sequencing of the promoter region of the CHI3L1 gene may indicate a site that is responsible for an even higher variation of YKL-40 levels. Future studies on the CHI3L1 -329 G/A polymorphism may reveal a genetic component of the pathophysiologic relevance of YKL-40 in diseases such as sarcoidosis.

Concluding remarks

The results described in this thesis strongly support the influence of genetic variation on the disease parameters that are used to describe the phenotypes of pulmonary sarcoidosis. These parameters include disease marker levels in serum, evolution of chest X-ray, and lung function data. The results show that a number of SNPs can add to the propensity to develop pulmonary fibrosis as seen for TGF-β3 polymorphisms in relation to radiographic evolution, or that a combination of SNPs on one allele (haplotypes) may predict the odds of developing chronic sarcoidosis and fibrosis in males (chapter 3). The influence of gene polymorphisms on disease parameters is in essence an indirect measure of the changed functionality of a gene. This is illustrated by the association between genotypes (ACE2, TGF-β3) and radiographic evolution in which X-ray is used as a parameter, as well as between genotypes and lung function (CMA1). The choice of the parameter which would somehow quantitate the (fibrotic) manifestation of sarcoidosis is imperative in understanding the influence of gene polymorphisms. The following questions illustrate this point. Firstly, is the lower VC associated with a CMA1 variant an indication of fibrosis? Secondly, is the observed relationship between high levels of serum YKL-40 and lower Dlco at presentation only, a reflection of a transient type of fibrosis? And lastly, is radiographic evolution towards fibrosis really a homogenous category with a unique sequence of events, or is this category a collection of different types of scarring? In order to understand these observations, the meaning of what different parameters represent in terms of (patho) physiologic mechanisms needs to be pondered. As outlined in the general introduction and in chapter 2, fibrosis ranges from being transient and reversible to being progressive. These different types of fibrosis may result in changes of one parameter for pulmonary fibrosis, but not in the other. Thus, if we can establish what a parameter specifically indicates in the events of pulmonary fibrosis, the associations between genetic variation and disease parameters will improve our understanding of the genetic component in developing a certain disease phenotype of sarcoidosis. Intricate histologic descriptions of pulmonary fibrosis in lung biopsies may be needed to complement the radiologic and physiologic findings.

Contrary to the conclusions or speculations that emerge from comparing genotypes and changes in disease parameters, the polymorphisms of disease markers ACE, KL-6 and YKL-40 inform us directly about their influence on protein functionality. The identification or evaluation of polymorphisms in

genes that encode proteins which are or may be used as sarcoidosis disease markers clearly underline the relevance of considering gene polymorphisms in an attempt to identify normal and deviating levels of disease markers. Studies performed in the past which have unsuccessfully tried to link ACE, KL-6 or other marker levels to predict or monitor the course of any given disease may lead to alternative results upon the consideration of these marker's genotypes. The ability to better interpret marker levels in healthy and affected individuals is the first step in using disease markers to describe the progression or resolution of a disease. For the clinician, however, having to account for the genetic variation of a disease marker may be cumbersome. Means to circumvent the potentially expansive number of reference intervals that are based on e.g. gender, age, length, and gene polymorphisms can be accomplished by formulating a simple algorithm that accounts for any variable known to influence the measures of a marker. As illustrated in chapter 3, the Z-score offers a useful way to apply genetically-specific marker levels in practice.

In order to grasp the genetic influence on the vast and complex pathways which lead to pulmonary fibrosis in sarcoidosis, the expansion of useful fibrosis parameters are needed to be able to give the affected individual a proper risk assessment and a fitting treatment regimen.

Most genetic studies on fibrotic diseases agree on one important conclusion: there is no single gene that underlies the development of fibrosis. An undesirable consequence of this fact is that the combined influence of more than one gene and one or more environmental factors compromises the statistical power to isolate the effect of any single gene. In light of this principal, the genetic association studies on the RAAS components in relation to pulmonary fibrosis in sarcoidosis as presented in this thesis have an important limitation. The RAAS is a meticulously orchestrated system that allows for correction of imbalances between concentrations of RAAS components. As a result, a phenotypic change caused by a polymorphism in one RAAS component may be compensated by an up- or downregulation of a counter-acting component and may consequently negate the effects of the polymorphism being studied. Moreover, genetic heterogeneity in other RAAS components may mask any effect of the SNP being investigated. The epistatic interactions between SNPs in multiple RAAS genes may reveal stronger effects on the sarcoidosis phenotypes, i.e. the course of pulmonary sarcoidosis with fibrosis. In order to perform such association studies, a significantly higher

number of sarcoidosis patients are needed. Studying a substantially larger cohort of sarcoidosis patients with pulmonary fibrosis will enable an assessment of the effect of disease-modifying genes on the rate and/or severity of pulmonary fibrosis. The use of HRCT scans will enable a more detailed characterization of phenotypical differences between the types or extent of pulmonary fibrosis and may possibly reveal associations with polymorphisms in the genes described in this thesis. An important limitation is that there is no consensus to date with which HRCT findings can be categorized. In order to associate genetic variation with phenotypic categories, ordinal or dichotomous quantitative HRCT readings must be invented. With the ongoing gathering and recruitment of sarcoidosis patients with pulmonary fibrosis, the number of meticulously phenotyped subjects and new candidate genes will lead to a wealth of new insights into the genetic influence on the development of pulmonary fibrosis in sarcoidosis. Furthermore, the associations described in this thesis will hopefully prompt future investigations into the delineation of the functional roles of polymorphisms in genes that are involved in the pathogenesis of pulmonary fibrosis in sarcoidosis.

list of DEFINITIONS

Allele - Any one of a number of alternative forms of the same gene occupying a given locus (position) on a chromosome.

Chromosome - A large macromolecule which carries densely packed genetic information in biological cells.

Disease phenotype - A specific manifestation of a disorder or structure in a human in terms of clinical characteristics.

Epistasis - The control of a phenotype by two or more genes.

Exon - The coding DNA region in a gene.

Gene - The DNA that encodes the chemical structure of a protein.

Gene polymorphism - A DNA sequence variation, occurring when a single nucleotide in the genome is altered.

Genome - The whole hereditary information of an organism that is encoded in the DNA. This includes both the genes and the non-coding sequences.

Genotype - The specific genetic makeup (the specific genome) of an individual, usually in the form of DNA. It codes for the phenotype of that individual.

Haplotype - A set of single nucleotide polymorphisms (SNPs) found to be statistically associated on a single chromatid.

Heterozygous - An organism carrying two different versions of a gene on the two corresponding chromosomes.

Homozygous - An organism carrying identical versions of a gene on the two corresponding chromosomes.

Intron - The non-coding DNA region in a gene that is spliced out in the process of translation.

Linkage disequilibrium (LD) - The non-random association of alleles at two or more loci on a chromosome. It describes a situation in which some combinations of alleles or genetic markers occur more or less frequently in a population than would be expected from their distance.

Locus (plural: loci) - The position of a gene (or other significant sequence) on a chromosome. A locus can be occupied by any of the alleles of the gene.

Primer - A short DNA strand (or related molecule) that serves as a starting point for DNA replication.

Quantitative trait locus (QTL) - A region of DNA that is associated with a particular trait (e.g., biomarker levels). Though not necessarily genes themselves, QTLs are stretches of DNA that are closely linked to the genes that underlie the trait in question.

Reference interval - The range of values (usually 95% confidence limits) found in an apparently healthy population (i.e, not afflicted by an illness).

Nederlandse SAMENVATTING

Sarcoïdose

Sarcoïdose, ook wel bekend als de ziekte van Besnier-Boeck, heeft een onbekende oorzaak. De ziekte kenmerkt zich door de aanwezigheid van ontstekingshaarden in diverse organen, genaamd granulomen. Hoewel de meeste patiënten met sarcoïdose spontaan genezen, krijgt een klein deel van de patiënten te maken met longfibrose. Littekenweefsel neemt de plaats in van het granuloom en kan zich over de hele long uitbreiden. Fibrose wordt gekenmerkt door de opeenhoping van de zg. extracellulaire matrix, bestaande uit collagenen en andere structurele eiwitten. Deze eiwitten worden geproduceerd door fibroblasten die aanwezig zijn in de longen. Het ontstaan van deze onomkeerbare littekens van het longweefsel wordt waarschijnlijk veroorzaakt door een storing in de balans tussen bepaalde belangrijke eiwitten waardoor wondgenezing niet naar behoren verloopt.

Genetica van sarcoïdose

Hoewel omgevingsfactoren een belangrijke rol spelen, zijn er voldoende aanwijzingen dat sarcoïdose een genetisch component heeft. Er zijn namelijk grote verschillen gevonden in het voorkomen en de mate van ernst van sarcoïdose tussen bevolkingsgroepen. Zo hebben Amerikanen van Afrikaanse afkomst vaker een ernstiger ziekteverloop dan blanke Amerikanen en komt bij Japanse sarcoïdose patiënten veel vaker aantasting van het hart en de ogen voor. Tussen individuen van dezelfde bevolkingsgroep bestaan er ook kleine genetische verschillen. Door vergelijking van DNA is duidelijk geworden dat er vele kleine variaties, de zogenaamde single nucleotide polymorfismen (SNP's), aanwezig zijn. De schatting is dat er per gen ongeveer 100 SNP's kunnen voorkomen. Een aantal van deze SNP's kunnen de functie van het eiwit veranderen. Dit kan zich bijvoorbeeld uiten als een verhoging of verlaging van expressie, verandering van affiniteit voor een receptor, of een gestoorde functie ten gevolge van een veranderde structuur. Een individu kan, afhankelijk van de aanwezige SNP's, vatbaarder zijn voor een ziekte of de ziekte kan een ander klinisch berloop hebben. Indien een eiwit wordt gebruikt als merker voor een ziekte, zoals ACE in sarcoïdose, kunnen genetische variaties de hoeveelheid van de merker - zelfs in gezonde personen - significant beïnvloeden. Deze verschillen bemoeilijken dus de interpretatie van de waarden van een ziektemerker.

Longfibrose in sarcoïdose

In dit proefschrift worden de genen van bepaalde eiwitten onderzocht op genetische variatie en op het mogelijke verband met het ontstaan van longfibrose als gevolg van sarcoïdose.

Eiwitten die worden gemeten in het bloed van patiënten met sarcoïdose kunnen iets zeggen over de activiteit of over de prognose van de ziekte. Omdat sommige genetische variaties tot een hogere of juist lagere hoeveelheid van een eiwit kunnen leiden, in zowel zieke als gezonde personen, kan het onduidelijk zijn of een verhoging (of verlaging) van een merker door de ziekte of door de genetica wordt bepaald. In dit proefschrift worden reeds bestaande en nieuwe sarcoïdosemerkers geëvalueerd en wordt de invloed van de genetische variatie meegewogen in het bepalen van referentiewaarden (waarden in gezonde personen). Deze 'genotypenafhankelijke' referentiewaarden laten voor de meeste merkers zien dat verhoogde of normale waarden van de merker preciezer kunnen worden geïdentificeerd.

Hoofdstuk 2: Genetische verschillen in chymase (CMA1) in Japanse en Nederlandse sarcoïdosepatiënten

In dit hoofdstuk worden SNP's in het chymase gen onderzocht die wellicht een rol kunnen spelen in het krijgen van longfibrose als gevolg van sarcoïdose. Chymase is een enzym dat in het beschadigde hart, de huid, nieren, longen en andere organen de vorming van angiotensine II tot stand brengt. Angiotensine II is een sterke groeifactor van fibroblasten, die een belangrijke rol spelen in de vorming van fibrose. Omdat Japanse sarcoïdose patiënten vaker aandoeningen aan het hart en de huid krijgen dan Nederlandse patiënten, werd er ook een Japanse patiëntengroep met controles vergeleken. Onderzocht werd of chymase genpolymorfismen geassocieerd zijn met het krijgen van hart- of huidaandoeningen. Zoals verwacht verschilden de frequenties van de SNP's tussen Nederlanders en Japanners. Er werden echter geen verschillen gevonden tussen Japanse en Nederlandse patiënten vergeleken met hun respectievelijke controles. Ook waren er geen aanwijzingen dat chymase genpolymorfismen van invloed zijn op de verschillende genotypen van de ziekte. De longfunctie (vitale capaciteit) van Nederlandse patiënten bleek echter beduidend lager te zijn (ongeveer 15%) in aanwezigheid van de T allel van SNP -526 C/T. De aanwezigheid van dit allel bleek overigens geen invloed te hebben op het verloop van de longfunctie over een periode van 4 jaar. Bovendien was er geen aanwijsbare bijdrage van het -526 T allel op de

aanwezigheid van longfibrose op de thoraxfoto. Wellicht dat het -526 C/T polymorfisme invloed heeft op de activiteit van chymase en dus bijdraagt aan een ernstiger soort sarcoïdose in de longen. Het is ook mogelijk dat de vitale capaciteit lager is in gezonde personen in aanwezigheid van het -526 T allel. Hiervoor zullen in de nabije toekomst gezonde personen worden onderzocht.

Hoofdstuk 3: Angiotensin-converting enzyme 2 (ACE2) genpolymorfismen in patiënten met verschillende vormen van sarcoïdose in de longen.

De vorming van angiotensine II door ACE of chymase draagt bij aan fibrose naar aanleiding van weefselschade door bijvoorbeeld ontsteking. Recentelijk is gebleken dat ACE2 deze vorming kan remmen. In dit hoofdstuk worden polymorfismen in het ACE2 gen beschreven die geassocieerd zijn met de ernst van pulmonale sarcoïdose. Omdat het ACE2 gen zich op het X-chromosoom bevindt, werden mannen en vrouwen apart met elkaar vergeleken.

Een bepaalde combinatie van overervende SNP's (haplotype 4) bleek alleen bij mannelijke chronische sarcoïdose patiënten voor te komen en niet bij mannen met acute sarcoïdose. Bovendien droegen mannen met longfibrose vaker het haplotype 5 van het ACE2 gen vergeleken met mannen zonder fibrose. Deze verschillen werden niet gezien bij vrouwelijke patiënten. Het lijkt er dus op dat haplotype 4 een beschermend effect heeft op het ontwikkelen van chronische sarcoïdose in mannen en dat juist het dragen van haplotype 5 bij mannen een verhoogde kans geeft op het ontstaan van longfibrose. Functioneel onderzoek op eiwit niveau naar ACE2 zal moeten uitwijzen of de haplotypen 4 en 5 van het ACE2 gen een tegengesteld effect hebben op het gedrag van het ACE2 enzym. Deze functionele verschillen kunnen hopelijk een beter beeld geven over het effect van ACE2 op de ontwikkeling van chronische sarcoïdose met of zonder fibrose.

Hoofdstuk 4: Transforming growth factor (TGF)-β genpolymorfismen in sarcoïdose patiënten met longfibrose.

Het eiwit Transforming Growth Factor (TGF)-β1 speelt een cruciale rol in ontstekingsreacties en fibrose. Een verhoogde aanwezigheid van dit eiwit wordt zowel in sarcoïdose als in tal van andere fibrotische aandoeningen waargenomen. Een voorbeeld hiervan is idiopathische pulmonale fibrose (IPF) en longschade na blootstelling aan bepaalde cytostatica.

Naast TGF-β1 bestaan er nog twee andere TGF-β varianten: TGF-β2 en TGF-β3. Er wordt vermoed dat de balans tussen TGF-β1 en TGF-β3 van groot belang is voor de progressie of resolutie van littekenvorming.
De genetische variatie van TGF-β1, 2 en 3 werd bij vier groepen onderzocht: gezonde mensen, patiënten met de acute ziekte, patiënten met de chronische ziekte en patiënten waarbij sprake was van fibrose. Er werd geen verschil in frequentie van de verschillende genetische variaties tussen de patiënten en gezonde personen gevonden. Binnen patiëntengroepen werden er echter wel verschillen gevonden. De resultaten wijzen erop dat, in tegenstelling tot TGF-β1, sommige polymorfismen in het TGF-β2 en met name in het TGF-β3 gen bijdragen tot de ontwikkeling van longfibrose als gevolg van sarcoïdose.

Hoofdstuk 5: Z-scores van angiotensin-converting enzyme (ACE) I/D-gecorrigeerde referentiewaarden normale en verhoogde ACE activiteit in sarcoïdose patiënten.
Het I/D (insertie/deletie) polymorfisme in het ACE gen draagt bij aan variatie van ACE waarden. Als gevolg hiervan hebben homozygote DD personen ongeveer 2x zo hoge ACE activiteit als homozygote II personen en liggen de waarden van heterozygoten (ID) hier er tussen in.
Aan de hand van een groep gezonde mannen (n = 100) en vrouwen (n = 100) werd een referentie-interval voor elk ACE I/D polymorfisme vastgesteld (II, ID, DD). Daarnaast werden willekeurige sarcoïdosepatiënten onderzocht. Bij deze groep patiënten werd de ACE activiteit in serum gemeten en het ACE genotype vastgesteld. De ACE waarden van de patiënten werden beoordeeld aan de hand van zowel de conventionele referentie-interval (dus zonder rekening te houden met het ACE genotype) en de ACE I/D genotype-gecorrigeerde referentie-interval. In het laatste principe werden de ACE waarden uitgedrukt als de Z-score. Een Z-score geeft de afwijking van ACE waarden aan als afwijkingen in aantal SD's.
De resultaten toonden aan dat met gebruik van de conventionele ACE referentiewaarden 11 van de 129 onderzochte patiënten misleidende ACE waarden hadden. De ACE waarden van patiënten met het DD genotype werden met name overschat en de ACE waarden van patiënten met het II genotype werden juist onderschat. Om een betrouwbare uitspraak te kunnen doen of een individu een verhoogde dan wel verlaagde ACE activiteit heeft, zal rekening moeten worden gehouden met het ACE I/D polymorfisme.

Hoofdstuk 6: Het mucin-1 568 adenosine/guanine polymorfisme beïnvloedt 'Krebs von den Lungen-6' (KL-6) waarden in serum.

KL-6 (MUC1) is een longepitheel-specifiek eiwit dat verhoogd is in serum bij patiënten met verschillende interstitiële longaandoeningen waaronder sarcoïdose. KL-6 speelt mogelijk ook een rol bij het ontstaan van fibrose. Het functionele 568 A/G polymorfisme in het MUC1 gen werd onderzocht op mogelijke associaties met de hoeveelheid van het eiwit (KL-6) in serum van gezonde personen en sarcoïdose patiënten. Ook werd bekeken of het polymorfisme een mogelijk verband heeft met de radiografische progressie van sarcoïdose in de longen. De gemiddelde KL-6 waarden in serum waren significant hoger in de patiëntengroep dan in controles. Bovendien bleek dat het genotype van het 568 A/G polymorfisme invloed heeft op de hoeveelheid KL-6 in serum in beide groepen. Wanneer de referentiewaarden niet voor het 568 A/G genotype werden gecorrigeerd, bleek ook hier dat met name de KL-6 waarden van patiënten met het 'hoge expressie genotype' werden overschat en die met 'lage expressie genotype' KL-6 waarden werden onderschat (6 van de 74 onderzochte patiënten). Het dragerschap van polymorfisme hield geen verband met de ernst of progressie van de longaandoeningen, al zijn meerdere patiënten nodig om dit te bevestigen. Toekomstige studies zullen uitwijzen of het gebruik van de 568 A/G-specifieke referentiewaarden de interpretatie van KL-6 waarden in serum zullen verbeteren.

Hoofdstuk 7: Het CHI3L1 -329 G/A polymorfisme is geassocieerd met YKL-40 serum waarden.

YKL-40 is een eiwit die wordt geproduceerd door onder anderen kraakbeencellen, ontstekingscellen en kankercellen. YKL-40 is in tal van ziekten verhoogd aanwezig in serum. Hoewel de precieze rol van dit eiwit nog niet helder is wordt ondersteld dat YKL-40 een belangrijke rol speelt in ontsteking en de vorming van fibrose. Recent is gebleken dat YKL-40 sterk verhoogd is in sarcoïdose en dat het mogelijk als merker kan dienen voor de activiteit van de ziekte maar ook als merker voor de aanwezigheid van longfibrose. Omdat het YKL-40 coderende gen (CHI3L1) ook polymorfismen bevat in mogelijk functionele posities, werd gekeken naar de invloed van 6 gekozen SNP's op de hoeveelheid YKL-40 in serum van sarcoïdose patiënten en gezonde personen.

Serum YKL-40 was verhoogd in 79% van de patiënten. YKL-40 bleek bovendien een zeer hoge, negatief voorspellende waarde te hebben (98%).

Dit betekent dat bij een YKL-40 waarde die lager is dan 72.0 ng/ml sarcoïdose met 98% betrouwbaarheid kan worden uitgesloten. Er werd tevens een relatie gevonden tussen YKL-40 waarden en DLco, een longfunctie parameter die vaak verlaagd is bij longfibrose. Op basis van radiografie werd er echter geen verband gevonden tussen YKL-40 waarden en de patiënten met een meer chronisch beeld of fibrose. Hierdoor lijkt de relatie Dlco met YKL-40 niet te verklaren door de aanwezigheid van fibrose.
Het -329 G/A polymorfisme in het CHI3L1 gen bleek van significante invloed te zijn op YKL-40 in serum van gezonde personen. Dit effect was echter niet aantoonbaar in de patiëntengroep. Mogelijk zijn de YKL-40 waarden in de patiënten zo sterk verhoogd door andere (nog onbekende) factoren, dat de invloed van het -329 G/A polymorfisme verwaarloosbaar is. De relevantie van het gevonden verband tussen het polymorfisme en YKL-40 waarden zullen wellicht aan het licht komen in vervolgstudies.

Conclusie
Het effect van een polymorfisme op het in serum gemeten eiwit of op de vatbaarheid en/of verloop van een ziekte zijn in principe gelijke begrippen. De door polymorfismen beïnvloede functionaliteit van het eiwit ligt immers ten grondslag aan de uiting (fenotype) van de ziekte die wordt bepaald door een verzameling van fysieke veranderingen (= parameters). Voorbeelden hiervan zijn de invloed van genetische variaties in het TGF-β3 en ACE2 gen op het ontwikkelen van chronische sarcoïdose en/of longfibrose uitgedrukt in radiografische evolutie. Voor chymase (CMA1) geldt echter dat niet radiografische evolutie maar de vitale capaciteit (VC) verschillend is tussen personen met verschillende allelen van een genpolymorfisme. Het beschrijven van de invloed van polymorfismen op de ontwikkeling van fibrose hangt dus sterk af van de keuze van de soort parameter. Aan de hand van de volgende vragen zal dit worden toegelicht. Als eerste, is de verlaagde VC bij patiënten met de CMA1 variant een indicator van fibrose? Is de relatie tussen hoge serum YKL-40 waarden en verlaagde Dlco bij presentatie een aanwijzing voor transiënte fibrose? Tenslotte, is de radiografische evolutie van sarcoïdose werkelijk een homogene groep of bestaat deze categorie uit verschillende typen longfibrose? Het is dus noodzakelijk om te begrijpen op welke manier deze verschillende parameters een afspiegeling zijn van een ziekteproces. Omdat er aanwijzingen zijn dat fibrose zowel transiënt als progressief kan zijn, zal in sommige gevallen de ene ziekteparameter wel en de andere niet met de

mate of ontwikkeling van longfibrose geassociëerd zijn. Derhalve zal met het gebruik van de juiste parameter voor een specifiek type longfibrose de associaties met genetische variaties mogelijk nauwkeuriger worden vastgesteld.
In tegenstelling tot de conclusies of speculaties die volgen op de gevonden associaties tussen genetische variaties in bepaalde eiwitten en parameters zoals radiografie en longfunctie, is de relatie tussen SNP's in genen die gebruikt worden als sarcoïdosemerkers en de concentraties in bloed een directe aanwijzing voor de functionaliteit van het eiwit. De genotypenafhankelijke waarden van ACE, KL-6 en YKL-40 onderschrijven het idee dat naast variabelen zoals leeftijd, lengte en geslacht, ook de genetische make-up van de merker in ogenschouw zal moeten worden genomen om nauwkeuriger te beoordelen wanneer waarden in het bloed normaal of afwijkend zijn. Omdat dit soort maatregelen een extra belasting kan zijn voor het rapporteren of aflezen van uitslagen, biedt het gebruik van de Z-score (hoofdstuk 3) een uitkomst. Met deze manier van rapporteren kan de betrokken specialist in één oogopslag zien wat normale of afwijkende waarden zijn in een patiënt.
De uitvoering van genetisch onderzoek kan mogelijk een perspectief bieden voor de therapiekeuze voor sarcoïdosepatiënten die met het ontstaan van longfibrose kunnen worden geconfronteerd. Aanvullend onderzoek is nodig om inzicht te verkrijgen in de mechanismen van TGF-β, chymase, KL-6, ACE en ACE2 in longfibrose als gevolg van sarcoïdose.
De meeste genetische studies naar fibrotische ziekten zijn het over één ding eens: fibrose wordt veroorzaakt door een verzameling van genen. Een nadelig gevolg van dit feit is dat de statistische 'power' vermindert wanneer een combinatie van genetische factoren in de associatiestudies moeten worden meegenomen. In dit proefschrift is dit effect ook aanwezig. Het RAAS in het bijzonder is een complex systeem waarin kleine fenotypische variaties veroorzaakt door individuele SNP's tot op zekere hoogte kunnen worden gecompenseerd. De invloed van de combinatie van genetische variaties in verschillende RAAS componenten zal een beter idee geven over de vatbaarheid van een patiënt voor het ontwikkelen van longfibrose. Voor dit soort studies zijn echter veel meer patiënten nodig. Met een grotere groep fibrotische sarcoïdosepatiënten kan bovendien worden onderzocht of de SNP's beschreven in dit proefschrift invloed hebben op de snelheid en/of ernst van de ontwikkeling van longfibrose. HRCT scans zouden mogelijk een uitkomst bieden in het nauwkeuriger indelen van de soorten longfibrose en het bepalen van de genetische variaties op de verschillende typen longfibrose. Zoals

genoemd zijn er in principe drie verschillende categorieën van fibrotische afwijkingen op de HRCT van de long te zien. Echter, een consensus voor HRCT-categorieën zoals die wel bestaat voor de thoraxfoto (Scadding criteria) is nog niet gemaakt waardoor het lastig is om op reproduceerbare manier de verschillende HRCT-patronen te kunnen relateren aan verschillende genetische variaties.

De voortdurende aanvulling van de database met zorgvuldig gefenotypeerde sarcoïdosepatiënten alsmede de ontdekking van nieuwe kandidaat-genen zal ongetwijfeld leiden tot een schat aan nieuwe inzichten in de ontwikkeling van fibrotische sarcoïdose door genetische variaties. Tot slot zullen de resultaten beschreven in dit proefschrift hopelijk aanleiding geven tot het verder in kaart brengen van de invloed van de functionaliteit van de genetische variaties op de pathogenese van longfibrose ten gevolge van sarcoïdose.

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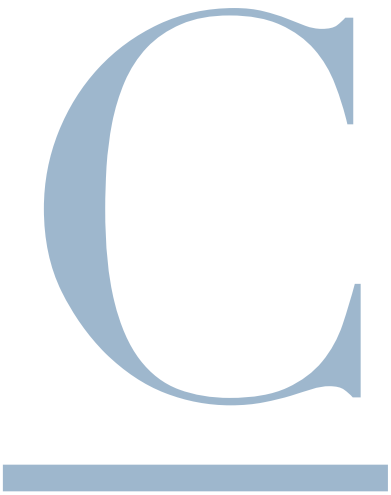
Curriculum VITAE



Adrian Kruit was born August 10, 1972 in Rotorua, New Zealand. Following a roundabout educational path that started with Graphic Art School (Grafische School Haarlem) (1984-1988), he completed his first year (propaedeuse) at the Hogeschool Alkmaar (Hoger Laboratorium School) in 1993.
In 1997, Adrian obtained his Master's degree (MSc) in Medical biology with a specialty in oncology and endocrinology from the Free University in Amsterdam (VU), The Netherlands. From 1998 until 2001, he worked at a privately owned biotech start-up company (Regulon) in Mountain View, California. His work involved research on non-viral gene therapy for cancer treatment, which was a collaborative effort between the department of Urology of the VU Medical Center, Amsterdam (Prof. dr. Donald W.W. Newling and Dr. Albert A. Geldof) and Regulon (Dr. Teni Boulikas).
Before commencing his doctoral studies in May 2003 with Prof. dr. Jules M. M. van den Bosch, Dr. Jan C. Grutters and Dr. Ir. Henk J.T. Ruven at the St. Antonius Hospital in Nieuwegein, he worked as a product specialist in micro-array technology for Amersham Biosciences (GE) and Metrigenix.
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