Pneumoproteins
in interstitial lung diseases

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Pneumoproteins in interstitial lung diseases

Pneumoproteïnen bij interstitiële longziekten

(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

General introduction

R. Janssen

1.1-1 INTERSTITIAL LUNG DISEASES

The interstitial lung diseases (ILD)s are a diverse group of pulmonary disorders that are classified together because of similar clinical, roentgenographic, physiologic, or pathologic manifestations, compromising over 100 different members that have been broadly classified into several categories. The major abnormality in ILDs is disruption of the lung parenchyma. When the lung is injured, epithelial cell basement membranes lose their integrity, heralding the appearance of a variety of inflammatory cells, regenerating type II pneumocytes, and increasing the expression of extracellular matrix components.

Sarcoidosis is the commonest ILD in the western world, with an annual incidence of between 10 and 40 per 100,000 across the whole population in Western Europe and the United States. In our own experience, hypersensitivity pneumonitis (HP) is also quite common in the Utrecht region.

1.1-2 SARCOIDOSIS

Sarcoidosis is a systemic disorder of unknown aetiology associated with an accumulation of CD4+ T-lymphocytes and a T-helper 1 immune response. The disease is pathologically characterized by the presence of non-caseating granulomas in involved organs. It typically affects young adults. Because there is spatial, seasonal, and occupational clustering, it is generally believed that (an) unidentified antigen(s) might be the trigger(s).[1]

The clinical manifestations of sarcoidosis can be widespread. Patients generally present with acute or insidious respiratory problems, variably accompanied by symptoms related to skin, eye, heart, or other organ involvement. In most cases, the clinical presentation of the patient with sarcoidosis is as important as the histopathological information to diagnose the disease. Moreover, positive diagnostic testing, such as biopsy, need to be supported by clinical features. Computed tomography (CT) scan findings that strongly support the diagnosis of sarcoidosis include the presence of mediastinal and hilar adenopathy, lung disease with upper lobe predominance, peribronchial irregularities, and subpleural micronodules.[2;3]

For many patients, systemic treatment is not necessary. The evidence lends support to the use of corticosteroids for patients with symptoms.[4] There are several indications to start treatment: patients with neurological, cardiac, sight-threatening ocular, and serious respiratory involvement, and hypercalcaemia.

The disease appears to vary in incidence among races (much greater frequency in United States (US) blacks than in US whites) and can also aggregate in families.[5;6]

Although part of the familial aggregation in this disease of unknown aetiology may have a non-genetic basis, these characteristics suggests a genetic contribution in the aetiology. Sarcoidosis is genetically a complex disease whose genetic predisposition is determined by the varying effects of several genes. The family pattern does not conform to a simple Mendelian mode of inheritance. The major histocompatibility complex (MHC) class II region of the genome is the most likely target region for identification of disease susceptibility. There is a functional as well as a linkage relevance to investigation of these genes.[7-10] The tumour necrosis factor (TNF)-α complex located adjacent to the MHC class II loci on chromosome 6 might play an important part in determining severity of disease, together with MHC class II alleles. The -308 promoter polymorphism of TNF has proved to be associated with Löfgren's syndrome, which is the form of sarcoidosis with a good prognosis.[11] T-lymphocyte co-stimulatory genes might have a role in sarcoidosis disease susceptibility.[12] In a large family-based and case-control study an association was found between a mutation in a gene possibly involved in T-lymphocyte costimulation (BTNL2) and sarcoidosis.[13] Several residues involved in binding of CD80 to its receptor, cytotoxic T-lymphocyte associated-4, are conserved in BTNL2, which suggests that BTNL2 may have a receptor-binding site similar to that of CD80. The guanine (G) to adenosine (A) transition in the BTNL2 gene leads to the use of a cryptic splice site located four base pairs upstream of the affected wild-type donor site and subsequently to the impairment of the potential T-lymphocyte down-regulatory function of BTNL2. Although genetic and other forms of studies have increased our understanding of the disease, the cause of sarcoidosis remains obscure despite more than fifty years of research.

1.1-3 HYPERSENSITIVITY PNEUMONITIS

HP, or extrinsic allergic alveolitis, is due to a combined type III allergic reaction with the formation of precipitins and a type IV lymphocytic reaction with a granulomatous inflammation in the distal bronchioles and alveoli provoked by the repeated inhalation of certain organic particles or low molecular weight chemicals.[14] An extensive number of etiologic antigens (agricultural dusts, bioaerosols, microorganisms (fungal, bacterial, or protozoal), and certain reactive chemical species) capable of inducing HP have been described and new sources of antigens are still identified.[15-17] Geographical, social and occupational factors determine the particular types of HP found throughout the world. Environmental factors and cofactors may be critical for the pathogenesis of the disease.

HP is a complex syndrome of varying intensity, clinical presentation, and natural history rather than a single, uniform disease.[15;16;18;19] The clinical presentations of HP have been categorized as acute, subacute, or chronic depending upon the frequency, length, and intensity of exposure and upon the duration of subsequent illness. Classical acute HP is the easily recognizable form of HP. This subtype is characterized by the abrupt onset (four to six hours following exposure) of fever, chills, malaise, nausea, cough, chest tightness, and dyspnoea without wheezing. Antigen avoidance in acute HP results in subsiding of symptoms within twelve hours to several days and complete resolution of clinical and radiographic findings within several weeks. The disease may recur with re-exposure. Subacute HP is characterized by the gradual development of productive cough, dyspnoea, fatigue, anorexia, and weight loss. Removal from exposure usually results in complete resolution of findings, although treatment may be required. The improvement takes weeks to months. Patients with chronic HP often lack a history of acute episodes and usually present with cough, dyspnoea, fatigue, and weight loss. Disabling and irreversible respiratory findings due to pulmonary fibrosis are characteristic, and are associated with increase mortality.[20] Differentiating chronic HP from idiopathic pulmonary fibrosis (IPF) may be difficult.[21;22]

The diagnosis HP relies on a constellation of findings: exposure to an offending antigen, characteristic signs and symptoms, abnormal chest findings on physical examination, and abnormalities on pulmonary function tests and radiographic evaluation.[14] The sensitivity of high resolution (HR)CT for the detection of hypersensitivity pneumonitis is greater than that of chest radiography.[23] The finding of poorly defined centrilobular nodules on HRCT scans should prompt consideration of this disease.[23] The pathologic changes are similar regardless of the causative antigen, and depend largely on the disease stage. HP is pathologically characterized by noncaseating granulomas in the interstitium, bronchiolitis with or without organizing pneumonia, and interstitial fibrosis Antigen avoidance is the key element in the treatment. There is often an apparent beneficial response to corticosteroids, although it may be difficult to distinguish between the effects of treatment, the natural course of the disease and the effect of antigen avoidance.[24;25]

Patients who present with HP are a minority of those exposed and do not have apparent increased exposure to the offending agent, compared with exposed, but not ill individuals. Therefore, it appears likely that these patients have a certain genetic predisposition to this disease. The interaction between the host's immune system and external antigen leads, only in genetic susceptible subjects, to a hypersensitivity reaction. Schaaf *et al.* studied polymorphisms of TNF-α promoter in farmers' lung patients and found that the frequency for the TNF-α2 allele was significantly higher in farmers' lung patients than in controls or patients with pigeon breeders' disease.[26]

Camarena *et al.* studied polymorphisms of the MHC class II alleles and TNF-α promoter in patients with pigeon breeders' disease and showed that these patients had a significant increase of the alleles HLA-DRB1*1305, HLA-DQB1*0501 and had an increased frequency of TNF-2^{-308}.[27] A decrease of HLA-DRB1*0802 was also noticed in their pigeon breeders' disease population.

Hill *et al.* genotyped two tissue inhibitor of metalloproteinase (TIMP)-3 promoter variants (-915A/G and -1296thymin (T)/cytosine (C)) in Mexican patients with pigeon breeders' disease.[28] The rare alleles of both variants appeared to be protective against susceptibility to pigeon breeders' disease. Their frequencies differed overall between subjects with pigeon breeders' disease and healthy subjects and this was attributable primarily to the -915G -1296C haplotype. No relationship was seen between the -915G -1296C haplotype and outcome or fibrosis. However, pigeon breeders' disease subjects with the -915G -1296C haplotype did have proportionally fewer lymphocytes in their bronchoalveolar lavage (BAL) fluid than those with the common -915A -1296T haplotype. TIMP-3 variants appear to contribute to susceptibility to pigeon breeders' disease. This may be through the inflammatory reaction rather than the fibrotic reaction. Our group genotyped 41 Dutch bird fanciers' lung patients and found a similar protective effect of the TIMP-3 -915G -1296C haplotype, which adds weight to the association.[29]

1.2-1 SERUM BIOMARKERS

The use of biomarkers in medicine lies in their ability to detect disease and support diagnostic and therapeutic decisions. In general, biomarkers can be used to diagnose a disease, to monitor disease activity and response to treatment, to assess disease severity, and to predict the course of a disease. Novel understanding of the molecular basis of diseases revealed an abundance of exciting biomarkers which present a promise for clinical applications. The serum level of an ideal marker should increase in the presence of the disease (high sensitivity), not increase in the absence of the disease (high specificity), add information about the risk or prognosis, change in accordance with the clinical evolution and treatment, reflecting the current status of disease, or better anticipate clinical changes, *i.e.* indicating the presence of relapse before it becomes obvious at a clinical level, relate to disease burden and extent, be reproducible, and finally, be of easy and cheap determination.[30]

The initial evaluation of a serum biomarker begins with measuring its levels in patients with the disease and in normal controls in order to define sensitivity and specificity. Sensitivity and specificity calculated at various cut-off points give rise to

a receiver operating characteristic (ROC) curve. The clinically most useful biomarker will be one with the largest area under the curve.

A number of novel blood biomarkers of lung disease including cytokines, enzymes, adhesion molecules, collagen relevant products and products of type II pneumocytes, have been studied for their clinical applicability in ILDs.

1.2-2 PNEUMOPROTEINS IN INTERSTITIAL LUNG DISEASES

The proximal conducting airways consist of a pseudostratified epithelium, which is replaced by simple cubical cells in the more distal airways.[31] The airway epithelium is covered with a very thin layer of epithelial lining fluid, which coats more than 95% of the lung-surface area in the alveoli. Multiple different cell types are present in the airways, including ciliated, basal, goblet and Clara cells. The alveolar surface is covered with squamous type I and cubical type II pneumocytes.[31] The lung epithelial cells produce surfactant, mucus and several lung-epithelium-specific proteins. These lung-epithelium-specific proteins can be classified in three separate groups: the mucins, Clara cell protein (CC)10, and the surfactant proteins.[32] Some proteins that are secreted by pulmonary cells are not only present at the surface of the respiratory tract, but are also detectable in the blood. Hermans and Bernard introduced the term *pneumoproteins* for these markers.[33] Because these proteins are produced in the lungs, their presence in serum can only be explained by leakage through the alveolar-capillary membrane into the vascular compartment.[33] The exact routes of passage of macromolecules from the air spaces into the blood are difficult to identify because of the anatomic complexity and cellular heterogeneity of the lung–blood barrier. In order to reach the bloodstream, pneumoproteins must cross the epithelial layer of the airways and/or the alveoli (Figure 1.1).

The alveolocapillary barrier has a surface area estimated at 50 to 100 m^2, which is considerably larger than the surface area of the bronchocapillary barrier, which is estimated at 2.5 m^2. The mean thickness of the air–blood barrier approximates 0.45 mm within the alveoli, but varies from several micrometers to millimeters in the airways, according to the airway level. The distance from the air–liquid interface to the capillaries has therefore been estimated to be on the order of 10-fold thicker in the conducting airways than in the alveoli. Furthermore, the microvascular surface area is very much greater in the alveoli than in the conducting airways. By contrast, for large proteins that are subject to steric hindrance, the route of passage is most likely also determined by the relative size of the pores at the bronchial and alveolar levels. According to heteroporous models, the maximum equivalent pore radius estimated for the alveolar epithelium is on the order of 1 to 5 nm, whereas the large

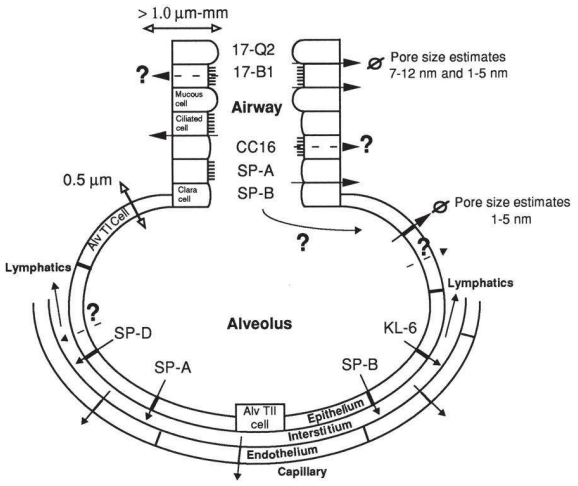

Figure 1.1 Schematic diagram of the pathways of transfer of lung-specific epithelium proteins from the lumen of the respiratory tract into the circulation. (Pore size estimate refers to equivalent pore radius.)[1]

airways are occupied by much larger pores, with radii from 7 to 12 nm, coexisting with small pores of similar size to those of the alveolar epithelium. In addition, the pores of the alveoli are probably not only smaller but also much less numerous, because of fewer intercellular contacts (where the pores are located) owing to the large surface covered by each alveolar type I cell. These anatomic and physiologic characteristics (i.e., surface, thickness, blood flow, estimated pore size) are probably responsible for differences in the amounts of lung secretory proteins leaking into the circulation.

CC10, surfactant protein (SP)-A, SP-D and the mucin associated antigen *krebs von den lungen* (KL)-6 are the most extensively studied pneumoproteins.[32] To date, the exact mechanisms behind increased serum pneumoprotein levels are not clear, but it is likely that they reflect a combination of pulmonary production, epithelial cell injury and lung permeability.[32]

Interestingly, pneumoproteins show variations in the serum of patients with ILDs, suggesting that their assays might represent a new approach in the assessment of these lung disorders. Furthermore, the tests used to detect the respective pneumoproteins in serum are easy to perform.

1.2-3 CLARA CELL PROTEIN 10

CC10 or CC16 is a 16 kDa protein secreted by Clara cells and other nonciliated cells of both the bronchiolar and bronchial epithelium. CC10 is present in high concen-

trations in the respiratory tract secretions, but also in other fluids such as serum. Increased CC10 levels have been found elevated in serum of pulmonary disease patients, probably due to the increased lung permeability caused by disruption of the alveolar-capillary barrier. Although its exact role has yet to be determined, there are arguments to believe that CC10 serves as an immunosuppressive and anti-inflammatory mediator within the airways.[37] CC10 interferes with interferon (IFN)-γ and TNF-α mediated actions and diminishes their biological activity.[38–41] Furthermore, CC10 has anti-fibrotic properties. Lesur *et al.* demonstrated that CC10 is able to inhibit fibroblast chemotaxis *in vitro* by blocking phospholipase A_2 activity, and, therefore, CC10 deficiency may contribute to fibroblast burden activity in fibrosing lung diseases.[42]

Bernard *et al.* demonstrated that serum CC10 is a marker of bronchial dysfunction caused by tobacco smoke. As CC10 appears to be a natural immunosuppressor of the respiratory tract, the decreased CC10 production found in smokers might explain some inflammatory changes associated with smoking.[43]

Serum CC10 can be used to detect exposure to chemicals potentially harmful to the lungs. Smoke inhalation is a well known cause of airways injury in fire fighters. Bernard *et al.* measured serum CC10 in fire fighters from a chemical plant who inhaled smoke from the combustion of polypropylene.[44] The protein was measured immediately after the fire and ten days later. Serum CC10 levels in fire fighters after the fire were significantly elevated compared to controls. Ten days later, serum CC10 from fire fighters had returned to the concentrations found in controls. Acute exposure to smoke results in a transient increase of CC10 in serum most likely due to an increased permeability of the alveolar-capillary membrane.

Silicosis refers to a spectrum of pulmonary diseases caused by the inhalation of silica. Bernard *et al.* measured the concentration of CC10 in the serum of miners exposed to silica.[45] No difference between exposed and control workers could be detected with regard to respiratory symptoms, chest radiographs or lung function tests. However, the concentration of CC10 in serum was decreased in silica-exposed workers compared to controls. The alterations in the serum CC10 levels probably reflect early toxic effects of silica on the respiratory epithelium.

Hermans *et al.* investigated whether sarcoidosis is associated with an increased intravascular leakage of CC10.[46] Serum CC10 concentrations of sarcoidosis patients was significantly increased compared to their matched controls. In nonsmoking patients without significant renal impairment, which also influences serum CC10 levels, CC10 in serum increased with the severity of the chest radiograph and computed tomography changes, and was on average 50-100% higher when parenchymal involvement was present. Sarcoidosis patients had, however, normal levels of CC10 in BAL fluid and an unchanged number of CC10-immunopositive cells in lung biopsy samples, suggesting that an increased secretion of CC10 in the sarcoidosis lung is

very unlikely, and that the elevation of CC10 in sarcoidosis results probably from an increased intravascular leakage of the protein across the air-blood barrier.

Shijubo *et al.* measured CC10 levels in sera and BAL fluids from sarcoidosis patients with progressive and regressive disease.[47] Serum and BAL fluid CC10 levels in the regressive disease group were significantly higher than those in the progressive disease group and healthy subjects. CC10 inhibited, in part, IFN-γ production. The monoclonal antibody TY-5, directed against CC10, restored IFN-γ production by blocking CC10 function. Since sarcoidosis patients with regressive disease showed increased CC10 protein levels in their sera and bronchoalveolar lavage fluids, CC10 may be a regulator of the inflammatory process in sarcoidosis.

1.2-4 *KREBS VON DEN LUNGEN* 6

Mucins are major components of the mucus layer covering the airway epithelium.[48] They consist of high-molecular-weight proteins belonging to an extended family of mucin peptides and characterized by different carbohydrate side chains.[48-51] After translation, these proteins undergo extensive modification by glycosyltransferase, sialotransferase, and sulfotransferase, yielding diverse oligosaccharide structures joined primarily through O-glycosidic linkages to serine (Ser) and threonine (Thr) residues of the protein backbone.[48] Various cells synthesize the same peptide backbone, but, through differential expression of glycosyltransferase, produce oligosaccharidic side chains showing cellular specificity.[52]

KL-6 is a lung-specific sialyted carbohydrate antigen on mucin (MUC)1.[53] In other words, the KL-6 antibody recognizes this specific sugar chain on the MUC1 protein.[53] High concentrations KL-6 are present in BAL fluid, but KL-6 is also detectable in serum.[53] Serum KL-6 is elevated in a majority of ILD patients and normal in patients with bacterial pneumonia or in healthy subjects.[54] Serum KL-6 levels depend mainly on local production in the lungs, pulmonary epithelial cell injury and alveolar-capillary permeability. In lung tissue from patients with idiopathic pneumonitis, the cells stained by the KL-6 monoclonal antibody are type II alveolar cells and alveolar macrophages.[53] However, the KL-6 antibody mainly demonstrated strong reactions on regenerating type II alveolar cells. Inoue *et al.* demonstrated nicely that serum KL-6 is not only dependent on pulmonary production but also influenced by damage to the cells which form the alveolar-capillary barrier.[55] This damage leads to increased pulmonary levels of KL-6 from destructed type II alveolar cells and to increased serum KL-6 levels because of an increased pulmonary permeability.

Hirasawa *et al.* tried to elucidate the pathogenic role of KL-6 in fibrosing lung disease.[56] KL-6 promoted the migration of human lung fibroblasts. Since KL-6 is chemotactic for human fibroblasts, it may also play a functional role in fibrosis.

IPF is the most prevalent form of idiopathic interstitial pneumonia (IIP), which also carries the worst prognosis.[57] Recently, it became apparent that rapid progression of the IPF, as a cause of death, is more common than was once perceived.[58] Yokoyama *et al.* searched for early predictive markers of the therapeutic effects of high-dose corticosteroids (pulse therapy) on patients with rapidly progressing IPF.[59] Serum KL-6 levels decreased significantly in patients who lived, whereas KL-6 levels tended to increase in patients who died. These results suggest that monitoring with KL-6 may contribute to early clinical decisions for alternative therapy in the management of rapidly progressing IPF. Yokoyama *et al.* further studied the prognostic value of serum KL-6 in IPF patients.[60] The patients were categorized by their serum KL-6 levels (as above or below the cut-off level of 1,000 U/ml) and their survival estimated using the Kaplan-Meier method. The difference in median survival between the two groups was significant. The median survival of patients with low KL-6 was more than 36 months, whereas that of patients with high KL-6 was only 18 months. These results suggest that initial evaluation of serum KL-6 level can predict survival in patients with IPF.

Pulmonary alveolar proteinosis (PAP) is a disease characterized by the filling of alveolar spaces with periodic acid-Schiff-positive proteinaceous material and by the hypertrophy of type II pneumocytes in the alveolar interstitium. Takahashi *et al.* measured extremely high serum KL-6 levels in patients with PAP and they were significantly higher than those in patients with ILD in which elevation of serum KL-6 has been recognized.[61] Both serum and BAL-fluid KL-6 levels in patients with PAP correlated well with the disease activity. Nakajima *et al.* demonstrated that the serum KL-6 level decreased after whole lung washing and correlated with symptoms, opacities on the chest radiograph, and arterial blood gas measurements in a patient with PAP.[62] These studies suggest that serum KL-6 level may be a useful marker for PAP.

The acute respiratory distress syndrome (ARDS) is an extreme form of lung injury characterised by disruption to the alveolar epithelium. Ishizaka *et al.* measured KL-6 in epithelial lining fluid and plasma of patients with ARDS.[63] KL-6 concentrations in epithelial lining fluid of patients with ARDS were significantly increased at the onset of the disorder, and KL-6 in epithelial lining fluid at ARDS onset was significantly higher in nonsurvivors than in survivors. Furthermore, plasma KL-6 in nonsurvivors remained significantly higher than in. Sato *et al.* also measured plasma KL-6 levels in ARDS patients and found similar results.[64]

Serum KL-6 can also be used to detect drug-induced pulmonary toxicity. Amiodarone is a drug that is highly effective in suppressing ventricular and supraven-

tricular tachyarrhythmias. The most serious adverse reaction of amiodarone is pulmonary toxicity. Several forms of pulmonary disease occur among patients treated with amiodarone, including chronic interstitial pneumonitis, organizing pneumonia, ARDS, and a solitary pulmonary mass. Measurement of serum concentrations of KL-6 shows promise as a marker of amiodarone pulmonary toxicity. As an example, one report of patients treated with amiodarone described serum concentrations of KL-6 of four to six times the upper limit of normal in patients who developed drug-related pneumonitis but none of the other patients, despite their development of bacterial pneumonia, congestive heart failure, or lung cancer.[65]

Takahashi *et al.* evaluated serum KL-6 levels in farmers with farmers' lung, farmers with positive serum precipitating antibodies to Saccharopolyspora rectivirgula and/or Thermoactinomyces vulgaris without farmers' lung, and farmers without these antibodies.[66] Serum KL-6 concentrations in the farmers' lung group were significantly higher than those in the antibody + and the antibody - groups. In the antibody + group, farmers with high serum KL-6 concentrations had lower permeability coefficients than farmers with normal serum KL-6 concentrations. These results suggest that serum KL-6 concentration can be a useful marker for assessing the activity of farmers' lung and may be able to be used to detect subclinical disease. KL-6 can also be used to detect (sub)clinical HP in another risk group to develop this ILD. Detection of HP in employees involved in the Bunashimeji mushroom industry is difficult. The level of precipitating antibody is not related with the prediction of progression and resolution of HP. Tsushima *et al.* examined the actual prevalence of HP in the Bunashimeji industry and the clinical differences among selected employees.[67] Subjects were divided into office workers, and pickers/packers. The picker/packer group had high serum KL-6 concentrations compared with the office worker group.

Kobayashi *et al.* measured serum KL-6 in 47 patients with histologically confirmed sarcoidosis.[68] Serum KL-6 level was significantly elevated in radiographic stage II and III (with parenchymal involvement) compared with stage 0 and I (without parenchymal involvement). Serum KL-6 level was significantly elevated in patients with CT findings of irregular small opacities, ground-glass opacities, and thickened bronchovascular bundle as compared to those without these findings. Serum KL-6 level was significantly elevated in patients with positive pulmonary accumulation in ^{67}Gallium scintigraphy as compared to those without accumulation. Serum KL-6 level was elevated in patients with a higher CD4+/CD8+ ratio (> or =3) in BAL fluid. These results suggest that serum KL-6 is a useful marker of sarcoidosis activity.

1.2-5 SURFACTANT PROTEIN D

Pulmonary surfactant is a lipid-rich material that prevents lung collapse by lowering surface tension at the air-liquid interface in the alveoli of lung. It is composed of phospholipids, lesser amounts of cholesterol, and several proteins essential for normal respiratory function. The surfactant proteins are mainly composed of four proteins: SP-A, SP-B, SP-C and SP-D.[69] The water soluble surfactant proteins SP-A and SP-D are collectins and appear to contribute to local immune defence by mediating phagocytosis. Potential functions for SP-D include roles in innate immunity on mucosal surfaces and surfactant metabolism.[70] SP-D knock-out mice had a progressive accumulation of surfactant lipids, SP-A, and SP-B in the alveolar space suggesting a role for SP-D in surfactant homeostasis.[70] Progressive development of pulmonary emphysema and subpleural fibrosis have also been described in mice totally deficient in SP-D.[71]

Honda *et al.* prepared monoclonal antibodies against human SP-D and developed an enzyme-linked immunosorbent assay (ELISA).[72] There are several possible mechanisms for producing an elevation of SP-D in serum including increased secretion of SP-D per type II pneumocyte, an increase in the total amount of type II pneumocytes per lung due to diffuse hyperplasia, increased leakage from the airspace to the interstitium, and decreased clearance of SP-D from the vascular compartment. Honda *et al.* demonstrated that the concentrations of SP-D in sera are prominently increased in patients with IPF, IIP associated with collagen disease, and PAP.[72] Serum SP-D levels appeared to reflect the disease activity of IPF and associated with collagen disease and the disease severity of PAP. High levels of SP-D in BAL fluids were shown in patients with PAP, but not with IPF and IIP associated with collagen disease.

Takahashi *et al.* measured serum SP-D levels in patients with progressive systemic sclerosis in order to evaluate their significance in relation to the presence of IIP.[73] Some degree of ILD occurs in more than three-quarters of patients with systemic sclerosis. Fibrosis is commonly preceded by alveolitis. The patients were divided into two groups based on findings by CT. The CT-positive IIP group was further divided into two groups: patients with IIP detectable by chest plain radiography and patients with IIP showing no abnormality. The serum SP-D levels were significantly higher in the CT-positive IIP group than in the CT-negative IIP group. They were also significantly higher in the X-ray-positive IIP group than in the CT-negative IIP group. In the X-ray-negative IIP group, their levels were higher than those of the CT-negative IIP group. Sensitivity of SP-D was high (77%) as well as that of X-ray (80%). Remarkably, five of six patients in the X-ray-negative IIP group had serum SP-D concentrations above the cut-off level, which demonstrates that serum SP-D might contribute to the detection of IIP overlooked by X-ray. Asano *et al.* also measured

serum SP-D levels in patients with systemic sclerosis.[74] Serum SP-D levels in patients with diffuse cutaneous systemic sclerosis were significantly higher than those in patients with limited cutaneous systemic sclerosis. Serum SP-D levels in patients with pulmonary fibrosis were significantly elevated compared with those in patients without pulmonary fibrosis. Moreover, the incidences of decreased diffusing capacity for carbon monoxide and decreased vital capacity were also significantly greater in patients with elevated SP-D levels than in those with normal levels. There was a significant positive correlation between serum levels of SP-D and KL-6. Serum SP-D and KL-6 levels showed almost the same sensitivity and specificity in the diagnosis of IIP. These two markers also predicted pulmonary fibrosis to almost the same degree.

Takahashi *et al.* assessed the values of SP-D in quantifying the extent of disease in IPF and in predicting deterioration in restrictive pulmonary function and survival.[75] SP-D concentrations were significantly correlated with the extent of alveolitis (a reversible change), whereas they did not correlate with the progression of fibrosis (an irreversible change). The SP-D concentration was also related to the extent of parenchymal collapse and the rate of deterioration in pulmonary function. The concentrations of SP-D in patients who died within three years were significantly higher than in patients who were still alive after this time period. Greene *et al.* repeated these Japanese findings in a large North American IPF cohort.[76] Greene *et al.* found that serum SP-D levels were highly predictive of survival in patients with IPF. Serum SP-D may be a useful biomarker in IPF patients, which can be helpful in predicting disease outcome.

Like KL-6, SP-D has been used to detect amiodarone-induced pulmonary toxicity. Umetani *et al.* described two patients with amiodarone-induced pulmonary toxicity who showed abnormally increased serum SP-D concentrations, although their KL-6 level was within the normal range.[77] These cases indicate that SP-D is a useful and early diagnostic marker for amiodarone-induced pulmonary toxicity even when KL-6 is not elevated.

Radiation pneumonitis is the most common complication of radiotherapy for thoracic tumours. Serum SP-D may be of diagnostic value for the detection of radiation pneumonitis. Takahashi *et al.* evaluated the significance of SP-D as serum marker for radiation pneumonitis.[78] Radiation pneumonitis findings detected on chest plain radiography were seen in only three of twelve patients in whom radiation pneumonitis was detected by HRCT. Nevertheless, SP-D concentrations in sera from the patients with radiation pneumonitis were significantly higher than those from the patients without radiation pneumonitis.

Furthermore, SP-D can also be used to detect HP. Tanaka *et al.* reported two Japanese patients with mushroom workers' lung in whom serum SP-D levels were correlated with disease activity.[79]

1.2-6 PNEUMOPROTEINS AND THEIR ENCODING GENES

CC10: The CC10 gene is composed of three short exons separated by a long and a short intron and is mapped to 11q12.3-q13.1.[80-82] Laing *et al.* were the first to describe an A to G substitution in the CC10 promoter.[83] This polymorphism is functional since the CC10 38AA genotype is associated with reduced plasma CC10 levels.[84] Ohchi *et al.* studied the CC10 38G/A single nucleotide polymorphism in Japanese sarcoidosis patients from Hokkaido and found a higher 38A allele frequency in sarcoidosis patients with an odds ratio of 1.6.[85] Furthermore, Ohchi *et al.* divided sarcoidosis patients with follow-up periods of 3 years or more into two subgroups (progressive and regressive disease). The CC10 38A allele frequency in patients with progressive disease was significantly higher than in control subjects. The CC10 38AA genotypes had significantly lower BAL fluid CC10 levels than the 38GG and G/A genotypes. The reporter gene assay showed significantly lower activity in the presence of IFN-γ for the CC10 38A allele.

MUC1: Nine mucin genes, named MUC1 (gene map locus 1q21), MUC2 (gene map locus 11p15.5), MUC3 (gene map locus 7q22), MUC4 (gene map locus 3q29), MUC5AC (gene map locus 11p15.5), MUC5B (gene map locus 11p15), MUC6 (gene map locus 11p15.5-p15.4), MUC7 (gene map locus 4q13-q21), and MUC8 (gene map locus 12q24.3), have been identified.[86] All mucin genes are characterized by repetitive amino acid structures, most commonly long stretches of variable number tandem repeat peptides, which can extend over more than 2,000 amino-acid residues.[48] The tandem repeat units are rich in serine and Thr, and contain at least one proline residue per repeat.

SP-D: The human SP-D gene is localised on the long arm of chromosome 10q22.2-q23.1 and consists of seven coding exons.[87] One of the SP-D polymorphisms resides in the N-terminal region at position 11, where a methionine (Met) is exchanged for a Thr (position 31 from transcription start site).[87] Leth-Larsen *et al.* reported that individuals with the genotype Thr11Thr have significantly lower SP-D serum levels than individuals with the Met11Met genotype.[88] Recently, Heidinger *et al.* sequenced the 5' untranslated region, the coding region and 3' region of the SP-D gene in German blood donors.[89] They found six single nucleotide polymorphisms from which they deduced haplotypes. One SP-D haplotype was associated with reduced SP-D levels.

1.3 AIM OF THE THESIS

The hypothesis underlying this thesis is that pneumoproteins are useful serum biomarkers to diagnose, monitor activity, assess severity and prognosis in ILD. Furthermore, we hypothesized that certain polymorphisms in the pneumoprotein encoding genes have influence on ILD susceptibility and serum protein levels.

In chapter 2 serum KL-6 and SP-D levels are measured in Dutch Caucasian patients with bird fanciers' lung in order to assess their value as diagnostic markers, markers of activity, severity and prognosis. In chapter 3 serum CC10, KL-6 and SP-D levels are measured in Dutch Caucasian patients with sarcoidosis in order to assess their value as diagnostic markers, markers of activity, severity and prognosis. In chapter 4 the MUC1 568 A/G polymorphism is analyzed in a well characterized Dutch Caucasian population (sarcoidosis patients and healthy controls) and to relate genotypes to serum KL-6 levels in order to determine a possible gene-protein relationship. In Chapter 5 the association between the CC10 A38G polymorphism and serum CC10 levels is validated in a clinically well-defined group of Dutch Caucasian sarcoidosis patients in order to make this measurement a more precise instrument to assess pulmonary involvement. Chapter 6 concerns the association between the low CC10 producing 38A allele and sarcoidosis susceptibility in a clinically well-characterised population of Dutch Caucasian and Japanese sarcoidosis patients and controls.

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CHAPTER 2

Analysis of *krebs von den lungen* 6, and surfactant protein D as disease markers in bird fanciers' lung

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ABSTRACT

Background and aim: Krebs von den lungen (KL)-6 and surfactant protein (SP)-D are potential serum markers in interstitial lung diseases. Their discriminative value, and ability to reflect pulmonary disease activity and prognosis in bird fanciers' lung were analyzed.

Methods: We studied 49 patients**,** 38 unexposed and 9 exposed controls. Serum KL-6 and SP-D concentrations were measured at presentation and a second sample, taken after antigen avoidance, was available in 17 patients. Pulmonary function tests were analyzed at presentation and 2-year follow-up.

Results: KL-6 and SP-D were significantly elevated in patients compared to controls ($p < 0.0001$). Receiver operating characteristic curve analysis revealed that both are equally useful in discriminating patients from controls. Analysis of their value as activity markers showed that both correlated with pulmonary function impairment; however, KL-6 correlated best with diffusing capacity. Evaluation of their predictive value showed that higher levels at onset were associated with improvement of diffusing capacity during follow-up. Further, it was noted that KL-6 and SP-D levels decreased after more than one month of allergen avoidance.

Conclusions: KL-6 and SP-D appear useful serum markers in bird fanciers' lung. Since higher levels are associated with more severe lung function impairment at presentation, and better recovery over time, we postulate that in this disease they are especially markers of disease activity.

INTRODUCTION

Bird fanciers' lung (BFL) is a common form of hypersensitivity pneumonitis (HP) induced by the repeated inhalation of antigens from bird feathers and droppings.[1,2] BFL can have a serious impact on patients in terms of morbidity, especially in individuals with chronic BFL where morbidity and mortality are considerable.[3] Early recognition of the disease is of the utmost importance, because antigen avoidance is still the mainstay of therapy. Further, careful disease monitoring and efforts to predict disease course are essential in the management of BFL.

To date, no useful serological disease markers exist in BFL. Lactate dehydrogenase has been suggested, but the use of this molecule is limited because of low lung specificity.[4] Lung epithelium-specific proteins, or pneumoproteins, such as *krebs von den lungen* (KL)-6 and surfactant protein (SP)-D, offer a new perspective as disease markers in interstitial lung diseases (ILD), i.e. in assessing disease activity, severity and prognosis.[5] They can be detected in serum and bronchoalveolar lavage (BAL). To date, the mechanisms behind increased serum pneumoprotein levels are not clear, but it is likely that they reflect a combination of pulmonary production, epithelial cell injury and lung permeability.[5] These pathologic conditions are also thought to be central to the pathogenesis of HP, as demonstrated with electron microscopy and 99mTc-DTPA lung scanning.[6,7]

KL-6 is a mucin-like glycoprotein with a molecular weight of 200 kDa and extensively expressed on the membrane of regenerating type II pneumocytes.[8,9] KL-6 has been found increased in serum of patients with HP and was associated with active pneumonitis.[9-11] SP-D is a member of the C-type lectin superfamily with a molecular weight of 43 kDa and produced by type II pneumocytes.[12] SP-D was found to correlate with disease activity in Japanese patients with mushroom worker's lung.[13] Recently, Ohnishi *et al.* evaluated serum KL-6 and SP-D in patients with various ILDs.[14] However, no patients with HP were included in this study.

The present study was designed to analyze the clinical significance of serum KL-6 and SP-D levels as discriminative, activity and predictive markers in BFL patients. Therefore, we measured KL-6 and SP-D levels in these patients at presentation of disease, and in healthy unexposed and exposed controls. The levels were related to a number of lung function indices. In a subgroup of patients a second serum sample, taken after antigen avoidance, was available, which allowed the evaluation of serial KL-6 and SPD measurements in relation to changes in disease activity. Finally, the predictive value of KL-6 and SPD levels at presentation with regard to lung function recovery was evaluated in a 2-year follow-up study.

MATERIALS AND METHODS

Study population

All patients with BFL, presenting at the Department of Pulmonology of the St Antonius Hospital Nieuwegein, the Netherlands, between 1986 and 1999, were included in this study. All of them experienced respiratory symptoms including dyspnoea or cough, with or without systemic symptoms such as fever and arthralgia after exposure to birds. Additionally, they all had precipitating antibodies to birds and a lymphocytic alveolitis established by BAL, *i.e.* BAL lymphocytes > 15%. None of the patients received corticosteroids, nor had they within the previous three months.

In all patients assessment at time of diagnosis included pulmonary function testing. Serum samples were obtained at first presentation and stored at -80ºC until analysis. In a subset of patients, follow-up serum samples, taken after antigen avoidance, were available. Each sample was analyzed for KL-6 and SP-D, and data was categorized into subgroups based on the time period between blood collection and latest antigen exposure: <24 hours; 2-7 days; 8-30 days; 1-12 months; and > 1 year.[15,16]

The control group comprised healthy subjects with and without exposure to birds, and was used to assess the discriminative value of the serum markers. The study protocol was approved by the Ethical Committee of the St Antonius Hospital.

Krebs von den lungen 6 and surfactant protein D measurements

KL-6 was measured by an enzyme-linked immunosorbent assay (ELISA) technique using a KL-6 antibody kit (ED046; kindly provided by Eisai Co., Tokyo, Japan).[17] SP-D concentrations were measured using specific ELISAs (Yamasa, Chiba, Japan) as described previously.[18] All samples were run in duplicate, and mean values were used for analysis.

Pulmonary function testing

The following pulmonary function tests were performed using ERS recommendations: inspiratory vital capacity (IVC) and forced expiratory volume in one second (FEV_1) by spirometry, total lung capacity (TLC) using bodyplethysmography, and gas transfer measurement using the carbon monoxide diffusing lung capacity single-breath technique ($D_{L_{CO}}$) and $D_{L_{CO}}$ adjusted for alveolar volume ($K_{L_{CO}}$).[19] Pulmonary function data was calculated as percentages of predicted values.

Arterial blood gas levels were measured at rest, and at peak physical performance. The calculated differences in arterial oxygen tension were expressed as ΔpO_2.

Follow-up study

IVC, FEV_1 and $D_{L_{CO}}$ values at 2-year follow-up were compared with those at presentation. Pulmonary function tests at each time point were regarded as normal or impaired: impairment was defined as IVC, FEV_1 and $D_{L_{CO}}$ < 80% of predicted value. Further, for FEV_1 and IVC a change of 15% from baseline value, and for $D_{L_{CO}}$ a change of 10% from baseline value was regarded as significant. Subsequently, patients were categorized as follows: patients with deterioration (*Worse*), stabilization (*Stable*) or improvement (*Better*) of IVC, FEV_1 or $D_{L_{CO}}$, and patients without impairment at presentation or at 2-year follow-up (*No abnormalities*). Serum KL-6 and SP-D levels at presentation were compared between the groups.

Statistical analysis

Data were expressed as median and 25 to 75% quartiles. Correlations between variables were determined using Spearman's rank coefficient. The Mann-Whitney U test was used to compare independent groups and the Wilcoxon test to compare related groups. The KL-6, and SP-D levels were analyzed by receiver operating characteristic (ROC) curves to find cut-off values for optimal diagnostic accuracy. Statistical analyses were performed using the Statistical Package for Social Science for Windows (SPSS; Chicago, IL, USA). Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Clinical characteristics

Forty-nine bird fanciers (29 keeping pigeons, 11 budgerigars, canaries, and/or parrots, and 9 keeping both pigeons and other birds; 25 men and 24 women; mean age 51 years; 44 nonsmokers and 5 smokers), 38 control subjects (19 men and 19 women, mean age 37; all nonsmokers), and 9 exposed but otherwise healthy subjects (7 men and 2 women; mean age 47; all daily exposure to multiple pigeons; all normal chest radiography and lung function) were included in this study.

Serum *krebs von den lungen* 6 and surfactant protein D levels

A strong correlation was observed between serum KL-6 and SP-D levels in BFL patients ($r = 0.7$, $p < 0.0001$; Figure 2.1). Serum KL-6 and SP-D levels in BFL patients (KL-6: 883 U/ml [415-2508] and SP-D: 201 ng/ml [138-352]) were significantly higher than in healthy unexposed controls (KL-6: 177 U/ml [147-209] and SP-D: 68 ng/ml [55-87]; $p < 0.0001$; Figure 2.2a and 2.2b). Healthy exposed subjects showed significantly lower KL-6 levels compared to the patients (371 U/ml [234-594], $p = 0.013$). No

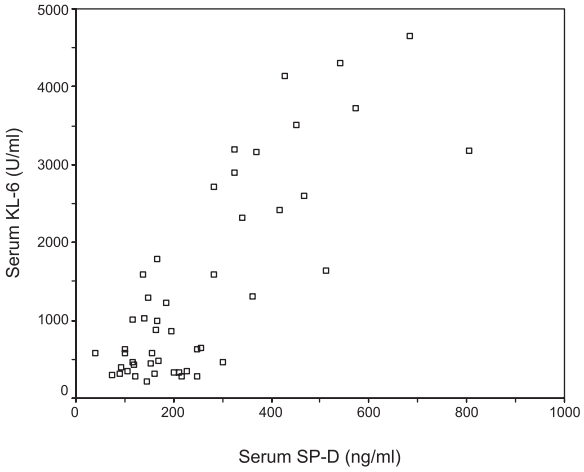

Figure 2.1 Scatter diagram showing the correlation between serum KL-6 (U/ml) and serum SP-D (ng/ml) in 49 BFL patients.

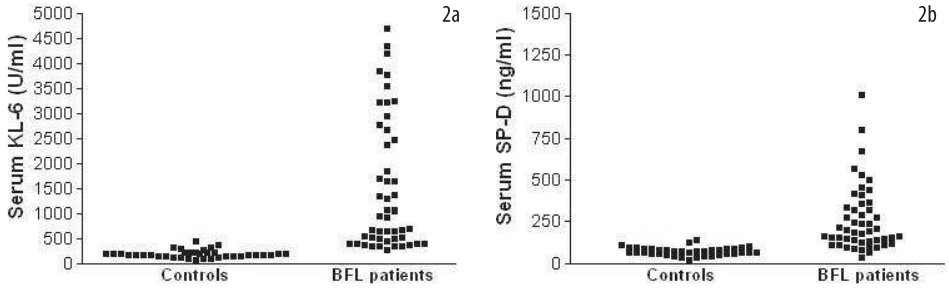

Figure 2.2 Serum KL-6 (a) and SP-D (b) levels in 49 BFL patients and 38 healthy controls.

significant differences in serum KL-6 or SP-D levels were found between subgroups < 24 hours, 2-7 days, and 8-30 days (the first available sample of each patient was used for this analysis).

In 17 patients paired samples, taken before and after at least 1 month of antigen avoidance, were analyzed. The results showed a significant decrease of serum KL-6 and SP-D levels at second measurement, *i.e.* after at least 1 month of antigen avoidance (KL-6: from 1145 U/ml [285-3728] to 332 U/ml [98-693], $p < 0.0001$; SP-D: from 204 ng/ml [39-573] to 105 ng/ml [31-255], $p = 0.001$).

Receiver operating characteristic curve analysis

Results of ROC curve analysis, performed for 44 nonsmokers only, are depicted in Figure 2.3: KL-6 showed largest area under the curve (KL-6 0.98 [95% confidence interval: 0.96-1.0]; SP-D 0.96 [0.91-1.0]; Figure 2.3). Cut-off levels were set as closest

point to 100% sensitivity and 100% specificity: 275 U/ml for serum KL-6 (sensitivity 98%, specificity 87%) and 98 ng/ml for serum SP-D (sensitivity 92% and specificity 90%). No analysis for smokers could be performed because of small numbers.

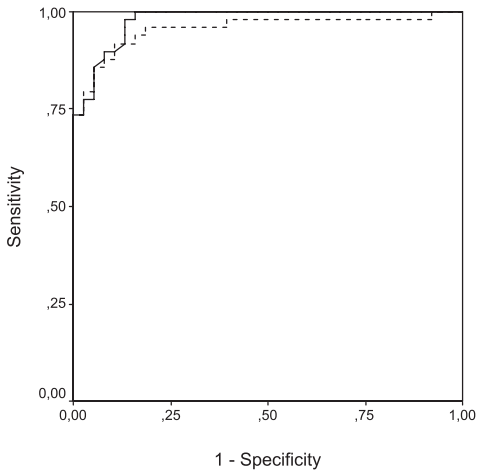

Figure 2.3 ROC curve showing the sensitivity and 1-specificity of serum KL-6 and serum SP-D for the detection of BFL (44 nonsmoking BFL patients included in this analysis). Serum KL-6 (____) and serum SP-D (_ _ _).

Correlation between *krebs von den lungen* 6 and surfactant protein D and pulmonary function tests

The mean D_{LCO} in BFL patients was 68% of predicted [42-90] and the K_{LCO} 68% of predicted [58-83]. The mean ΔpO_2 during maximal exercise was -0.065 kPa [-1.45 - 0.56]. The mean IVC, FEV_1, and TLC were 87% of predicted [65-99], 65% of predicted [49-86] and 89% of predicted [72-100], respectively. The mean FEV_1/IVC ratio was 0.72 [0.59-0.82].

Serum KL-6 and SP-D levels both correlated significantly with Dl_{CO} ($r = -0.8$, $p < 0.0001$ and $r = -0.6$, $p < 0.0001$, respectively), and Kl_{CO} ($r = -0.5$, $p < 0.0001$ and $r = -0.4$, $p = 0.009$, respectively). The association between KL-6 and Dl_{CO} is depicted in Figure 2.4. In addition, serum KL-6 significantly correlated with TLC ($r = -0.7$, $p < 0.0001$) and IVC ($r = -0.5$, $p < 0.0001$), and SP-D with TLC ($r = -0.4$, $p = 0.003$) and IVC ($r = -0.4$, $p = 0.01$). No correlation was found between serum KL-6 and FEV_1, nor between serum SP-D and FEV_1. Further, a correlation was observed between serum KL-6 and serum SP-D and the FEV_1/IVC ratio ($r = 0.6$, $p < 0.0001$ and $r = 0.4$, $p = 0.008$, respectively). Analysis of KL-6 and SP-D in relation to ΔpO_2 during maximal exercise showed that both markers were associated with a decrease in PO_2 (KL-6: $r = -0.7$, $p < 0.0001$; SP-D: $r = -0.5$, $p = 0.02$).

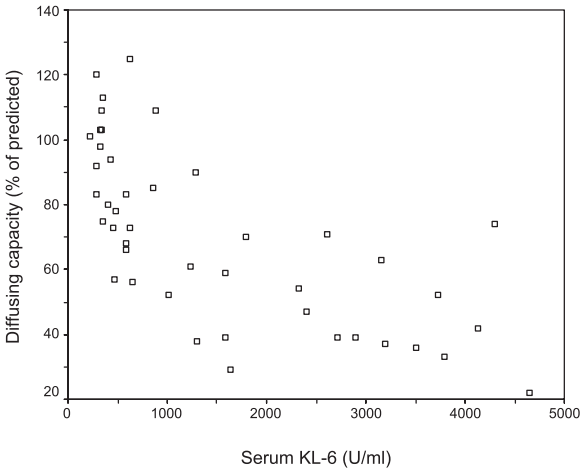

Figure 2.4 Scatter diagram showing the correlation between serum KL-6 (U/ml) and DLCO (percentage of predicted value) in 49 BFL patients ($r = -0.8$, $p < 0.0001$).

Follow-up study

At 2-year follow-up 12 patients showed stable D_{LCO}, and in 5 D_{LCO} deteriorated. Improvement of D_{LCO} was observed in 17 BFL patients, and 12 had no D_{LCO} abnormalities either at presentation or at follow-up. Follow-up D_{LCO} data was missing in three patients.

Significantly higher serum KL-6 and SP-D levels were found in patients having Dl_{CO} abnormalities at presentation but showing improvement during follow-up

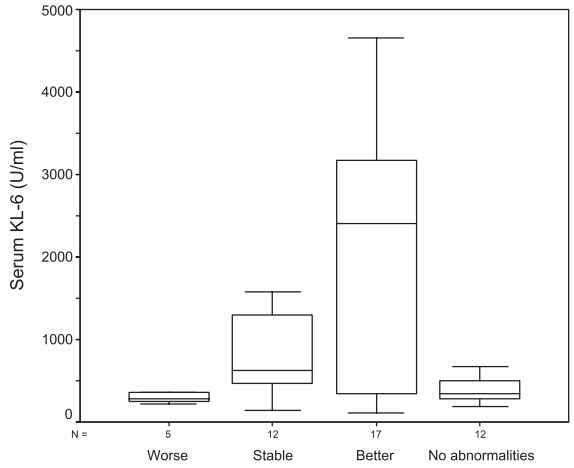

Figure 2.5 Box plot showing relation between KL-6 (a) and SPD (b) levels at presentation and evolution of DLCO. Five patients showed a deterioration (Worse) and 12 stabilization of DLCO (Stable). Improved DLCO was observed in 17 BFL patients (Better), and 12 had no abnormalities either at presentation or at follow-up (No abnormalities).

(KL-6: 2409 [288-3337]; SP-D: 325 [87-439]) in comparison with all patients showing stabilization (KL-6: 619 [472-1442]; SP-D: 208 [125-296]) or deterioration of Dl_{CO} (KL-6: 286 [233-605]; SP-D: 96 [67-221]; KL-6: $p = 0.001$; SP-D: $p = 0.01$; Figure 2.5), and those having no Dl_{CO} abnormalities at presentation or follow-up (KL-6: 344 [267-546]; SPD: 111 [98-187]; KL-6: $p < 0.0001$; SP-D: $p = 0.001$).

No significant results were found when analyzing outcome of FEV_1, IVC and TLC.

DISCUSSION

In this study we have evaluated KL-6 and SP-D as serum markers in BFL. Both were significantly elevated in BFL patients compared to exposed and unexposed controls. ROC curve analysis revealed a larger area under the curve for KL-6 in comparison to SP-D, indicating higher sensitivity and specificity for KL-6, but this difference was not statistically significant. Therefore, KL-6 and SPD appear equally useful as serum markers in distinguishing BFL patients from healthy exposed and unexposed controls. However, except for screening for subclinical HP amongst bird fanciers, farmers and mushroom workers, this is not very helpful for clinical practice.[21] Also it is unlikely that KL-6 and SP-D can be used in differentiating BFL from other forms of ILD, because previous studies have shown increased levels in diseases like sarcoidosis and idiopathic pulmonary fibrosis (IPF).[9,12,20] But measurement of these lung markers in serum might be useful in patients suspected of BFL who have critically impaired lung function and therefore are unable to perform lung function tests.

To date, the diagnosis HP is usually made from a combination of clinical features, radiographic abnormalities, pulmonary function tests, BAL and immunological tests, *i.e.* serum antibodies against specific antigens.[1] The pulmonary function tests typically show an impairment of gas exchange in combination with restrictive lung volumes, although airway obstruction may also occur.[1,22,23] We here demonstrate that serum KL-6 and SP-D negatively correlate with TLC and IVC, and positively with FEV_1/IVC ratio, whereas no correlation was found with FEV_1. In addition, they inversely correlated with Dl_{CO}, and this was in agreement with the finding of increased serum level in those showing desaturation during exercise These results suggest that KL-6 and SP-D reflect severity of restrictive lung function and the degree of impairment of the alveolar-capillary membrane. In general KL-6 showed stronger significances compared to SP-D, indicating the former might be most useful in this respect.

Remarkably, no differences in serum pneumoprotein levels were observed between BFL patients in whom blood samples were taken within 24 hours, between 2 to 7 days, or 8 to 30 days after latest antigen exposure. In striking contrast, a

consistent decrease was observed after 1 month of antigen avoidance. This finding is consistent with the concept that these markers reflect not only disease severity, but also disease activity. Further, it strongly suggests that it takes at least one month without bird contact before active intrapulmonary disease resolves in BFL.

Our results would also suggest that gas transfer reduces while passage of KL-6 across the air-blood barrier into the circulation increases. Diffusing capacity for CO is strongly dependent on the thickness and total area of the alveolar-capillary membrane, whereas KL-6 levels in the serum are thought to depend on the integrity of the air-blood barrier and the extent of type II pneumocyte hyperplasia. Proliferation of this type of cell is a response to alveolar wall damage, often caused by an inflammatory process. The result would be an increase of the amount of KL-6 present on the epithelial cell surface or in the alveolar lining fluid, which indeed could be confirmed in BAL of our patients (data not shown).

Another way of measuring the integrity of the alveolar-capillary membrane is 99mTc-DTPA scanning. Bourke *et al.* used this technique to study alterations in the air-blood barrier in pigeon breeders and showed that rate of 99mTc-DTPA clearance correlated with impairment of lung permeability.[7] Furthermore, this test appeared especially useful for detecting early disease. However, unlike KL-6, 99mTc-DTPA lung scanning is expensive and difficult to apply in large screening surveys. A correlation between Dl_{CO} and 99mTc-DTPA lung scanning has been described in patients with systemic sclerosis.[24] Therefore, it would be interesting to compare KL-6 measurement with Dl_{CO} and 99mTc-DTPA scanning to find out whether these tests can replace each other. If so, KL-6 could be the most cost-effective indicator of impaired lung permeability.

Interestingly, we found that patients with higher levels of pneumoproteins and lower Dl_{CO} had the best recovery over time, suggesting there is an effective type II cell regeneration and healing of the alveolar damage after antigen avoidance in these patients. In other ILDs such as IPF, however, increased serum levels of KL-6 have been associated with an unfavourable prognosis. Further, patients with normal Dl_{CO} and normal pneumoprotein levels at presentation were unlikely to develop serious BFL in the years following. The strong differences between our results and those of studies looking at the prognostic significance of KL-6 and SP-D in IPF are striking.[25-27] However, IPF is a completely different disease and unlike BFL, these patients do not recover and have an unfavourable prognosis by definition. It also suggests the mechanisms responsible for the increase of pneumoprotein in serum differ between diseases or, more likely, their prognostic significance strongly depends on the pathogenetic context. Although additional studies in both HP and IPF patients are needed to confirm these findings, they are in agreement with expert opinion on pulmonary function and prognosis in HP, *i.e.* many patients with highly active disease and

decreased lung volumes and Dl_{CO} often show full recovery over time, provided that antigen exposure can be stopped.[28]

The present study included 49 well-defined BFL patients presenting over a period of 13 years. This is an average of approximately four BFL patients a year, which is representative of the clinical practice in our hospital. Therefore, it is unlikely that this retrospective study was biased by selection. Moreover, we did not observe any differences in serum levels between the first and last quartile of collected samples, making bias due to bio-instability of KL-6 and SP-D epitopes over time unlikely (data not shown). Therefore, in our opinion, the results presented in this study can be extrapolated to the general population of BFL patients.

In conclusion, we have shown that serum KL-6 and serum SP-D are potential markers for BFL, especially reflecting active interstitial inflammation that may be reversible over time (strong inverse association between serum KL-6 levels and diffusing capacity, and best improvement over time after antigen avoidance in cases with highest KL-6/lowest onset Dl_{CO}). To date, pulmonary function tests are major tools in the diagnostic work-up of HP as well as in monitoring the disease course. However, serum KL-6 and SP-D measurements offer a new perspective in (early) detection and monitoring of this disease, especially because these tests are easy to perform.

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CHAPTER 3

Study of Clara cell protein 10, *krebs von den lungen* 6, and surfactant protein D in serum as disease markers in pulmonary sarcoidosis

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ABSTRACT

Study objectives: To determine the discriminative value of serum Clara cell protein 10 (CC10), *krebs von den lungen* (KL)-6, and surfactant protein (SP)-D as markers of interstitial lung diseases, and their ability to reflect pulmonary disease severity and prognosis in sarcoidosis.

Subjects: Seventy-nine patients with sarcoidosis and 38 control subjects.

Measurements: Serum CC10, KL-6, and SP-D concentrations at disease presentation were measured. Pulmonary function tests and chest radiographs were analyzed at presentation and 2-year follow-up.

Results: All markers co-correlated, and a significant difference was found between CC10, KL-6, and SP-D levels in patients with sarcoidosis and control subjects ($p < 0.0001$). Receiver operating characteristic curve analysis revealed largest area under the curve for KL-6. Significantly higher levels of CC10 and KL-6 were found in patients with parenchymal infiltration (stage II, III) compared to patients without parenchymal infiltration (stage I). In concordance, CC10 and KL-6 levels inversely correlated with diffusion capacity and total lung capacity, and KL-6 also with inspiratory vital capacity. Moreover, higher KL-6 levels were weakly but significantly associated with persistence or progression of parenchymal infiltrates at 2-year follow-up.

Conclusion: In this study, KL-6 appears to be the best discriminative marker in differentiating patients with sarcoidosis from healthy control subjects; however, as it is not a specific marker for this condition, this quality is unlikely to be useful as a diagnostic tool. Both CC10 and KL-6 may be of value in reflecting disease severity, and KL-6 tends to associate with pulmonary disease outcome.

INTRODUCTION

Sarcoidosis is a multiorgan granulomatous disorder of unknown aetiology, affecting the lungs in up to 90%.[1] Since the natural history and prognosis of sarcoidosis is highly unpredictable, and the disease tends to wax and wane, it is of the utmost importance to carefully monitor disease development.[1] To date, no single laboratory marker adequately reflects pulmonary disease status.[2] In addition, many of these markers add little to clinical findings, such as erythema nodosum, chest radiography, and pulmonary function tests. Moreover, none of the available markers are able to predict the outcome of sarcoidosis.

Lung epithelium-specific proteins, or pneumoproteins, offer a new perspective in assessing pulmonary involvement in interstitial lung diseases.[3] The tests used to detect pneumoproteins in serum are easy to perform. Among the pneumoproteins, Clara cell protein (CC)10, *krebs von den lungen* (KL)-6, and surfactant protein (SP)-D have been found in serum of patients with sarcoidosis.[4-6] Although each marker has been studied separately, their usefulness as markers in sarcoidosis has never been compared. CC10 is a protein with a molecular weight of approximately 16 kDa produced by bronchiolar Clara cells.[7] Increased CC10 levels have been found in serum of sarcoidosis patients, probably due to the increased lung permeability caused by disruption of the air-blood barrier.[6] KL-6 is a mucin-like glycoprotein with a molecular weight of 200 kDa and extensively expressed on the membrane of regenerating type II pneumocytes.[8,9] Elevated KL-6 levels have also been found in serum of patients with sarcoidosis and were associated with intensity of pneumonitis.[4] Further, SP-D is a member of the C-type lectin superfamily with a molecular weight of 43 kDa produced by type II pneumocytes.[5] This marker has been found mildly elevated in serum of patients with sarcoidosis.[5]

Ohnishi *et al*.[10] evaluated serum KL-6, SP-A, and SP-D in patients with various interstitial lung disease (ILD)s. KL-6 appeared to be best marker in the assessment of interstitial lung diseases; however, no patients with sarcoidosis were included in this study. The potential predictive value of pneumoproteins was recently suggested in a study by Greene *et al*..[11] They found that high SP-A and SP-D levels in serum of patients with idiopathic pulmonary fibrosis (IPF) were associated with decreased survival.

The present study was designed to compare the value of CC10, KL-6, and SP-D as serum markers in pulmonary sarcoidosis; therefore, we assessed the discriminative value of serum CC10, KL-6, and SP-D for patients with sarcoidosis vs. healthy control subjects. Subsequently, serum levels of the pneumoproteins were evaluated in relation to clinical severity parameters in order to establish their value as marker of severity. Finally, a follow-up study was performed to analyze potential predictive value of each pneumoprotein.

MATERIALS AND METHODS

Study subjects

In this study, patients with sarcoidosis diagnosed at the Department of Pulmonology of the St Antonius Hospital Nieuwegein between 1989 and 1999 were studied retrospectively. In all patients, the diagnosis of sarcoidosis was based on compatible clinical findings, histologic evidence of noncaseating epithelioid cell granulomas, and the exclusion of known causes of granulomatous diseases. At time of presentation, none of them were receiving corticosteroids, nor had they within the previous 3 months.

Healthy subjects served as a control group in order to assess the discriminative value of the serum markers. Health was confirmed on the basis of a medical checklist.

In all patients, assessment of sarcoidosis at time of diagnosis included scoring of chest radiography, and pulmonary function testing. Serum samples were obtained at the same time and stored at - 80°C until analysis. Each serum sample was analyzed for CC10, KL-6, and SP-D. The study protocol was approved by the Ethical Committee of the St Antonius Hospital.

Clara cell protein 10, *krebs von den lungen* 6, surfactant protein D and angiotensin-converting enzyme measurements

The concentration of CC10 was determined by an immunoassay relying on the agglutination of latex particles, as described previously.[6] The KL-6 level was measured by a sandwich-type enzyme-linked immunosorbent assay (ELISA) technique using a KL-6 antibody kit (ED046; Eisai; Tokyo, Japan) kindly provided by the manufacturer.[12] The SP-D concentration was measured using enzyme-linked immunosorbent assays (Yamasa; Chiba, Japan).[13] Serum angiotensin-converting enzyme (ACE) was measured using an enzymatic assay (ACE Kinetic; Buhlmann Laboratories; Schönenbuch/Basel, Switzerland) that is used for routine diagnostics in our hospital. All samples were run in duplicate, and mean values were used for subsequent analysis.

Chest radiography

Chest radiographs were scored blind by a chest physician with more than 20 years of experience in ILDs (J.M.M. van den Bosch) according to standard chest radiographic staging.[1] Besides this staging, patients with abnormal chest radiographic findings were also categorized as follows: without parenchymal infiltration (stage I), and with parenchymal infiltration (stage II and III).

Pulmonary function testing

The following pulmonary function tests were performed in agreement with the European Respiratory Society (ERS) recommendations: inspiratory vital capacity (IVC) and forced expiratory volume in one second (FEV_1) by using spirometry, total lung capacity (TLC) by using body plethysmography, and gas transfer measurements were performed using the carbon monoxide diffusing lung capacity single-breath technique ($D_{L_{CO}}$).[14] Pulmonary function data were calculated as percentages of predicted normal values.

Follow-up study

All patients with sarcoidosis included in this study had a follow-up of 2 years in which chest radiographs and pulmonary function tests were repeated at regular time intervals. This provided the opportunity to assess the predictive value of each pneumoprotein concerning functional and radiologic outcome. Radiologic improvement or deterioration was defined as changing of chest radiograph to a lower or higher radiographic stage, respectively. On the basis of these data, we categorized all patients into two groups: patients with deterioration or stabilization of chest radiographic stage (group 1) or patients with improvement of chest radiographic stage (group 2). Furthermore, all patients were categorized on the basis of change in parenchymal infiltrations only, i.e., progression or stabilization (group A), improvement (group B), or no manifestations of parenchymal infiltrations at presentation and follow-up (group C). For this, only obvious changes of pulmonary infiltrates on follow-up chest radiograph in comparison with initial chest radiograph were scored as either "progression" or "improvement," and no or doubtful changes as "stabilization." Change in pulmonary function was expressed as change of percentage of predicted TLC (%TLC), IVC, FEV_1, and $D_{L_{CO}}$ per year: %TLC = (%TLC follow-up – %TLC at presentation)/%TLC at presentation × 100/years.

Finally, as treatment with corticosteroids may influence functional and radiologic outcome, patients with and without treatment were analyzed separately. The decision to treat was based on the following criteria: (1) progressive deterioration of pulmonary function, (2) progressive change on chest radiographs or extensive pulmonary involvement, (3) impairment of organs other than the lung, and (4) persistent symptoms in combination with parameters of disease activity.

Statistical analysis

Data were expressed as median with 25 to 75% quartiles. Correlations between different variables were determined using the Spearman rank coefficient. The Mann-Whitney *U* test was used to compare pneumoprotein levels between two groups. The concentrations of CC10, KL-6, and SP-D were analyzed by using receiver operating

characteristic (ROC) curves in order to find cut-off values for optimal discriminative accuracy. Statistical analyses were performed using the Statistical Package for Social Science for Windows (SPSS; Chicago, IL). Additionally, the Hanley test was used to compare the areas under the ROC curve. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Clinical characteristics

Seventy-nine patients with sarcoidosis (43 men and 36 women; mean age ± SD, 39 ± 12 years; 56 non-smokers and 23 smokers), and 38 control subjects (19 men and 19 women; mean age, 37 ± 10 years; all non-smokers) were studied.

One patient with sarcoidosis presented with radiographic stage 0, 28 patients presented with stage I, 36 patients presented with stage II, 12 patients presented with stage III, and 2 patients presented with stage IV disease. Serum CC10, KL-6, and SP-D values in patients with sarcoidosis, radiographic subgroups, and healthy control subjects are shown in Table 3.1. Since smoking has an effect on pneumoprotein levels, serum values for each marker were calculated for the total group and for subgroups according to smoking habit.[15-17]

Table 3.1 Serum CC10, KL-6 amd SP-D levels in patients with sarcoidosis (smokers and nonsmokers) and healthy control subjects*

	Serum CC10	Serum KL-6	Serum SP-D
Variables	ng/ml	U/ml	ng/ml
Sarcoidosis			
Total (n = 79)	16.7 (12.4 - 20.5)	350 (254 - 548)	100 (71 - 145)
Nonsmokers (n = 56)	16.9 (12.4 - 21.7)	340 (253 - 516)	104 (66 - 143)
Smokers (n = 23)	15.6 (12.7 - 19.2) #	387 (264 - 551) #	92 (71 - 145) #
Stage I			
Total (n = 28)	14.7 (10.0 - 17.1)	269 (233 - 309)	94 (66 - 115)
Nonsmokers (n = 20)	14.7 (9.9 - 18.4)	261 (225 - 303)	94 (61 - 115)
Smokers (n = 8)	14.8 (11.9 - 16.5) #	305 (252 - 545) #	94 (81 - 136) #
Stage II and III			
Total (n = 48)	18.5 (13.4 - 24.0)	451 (313 - 577)	107 (72 - 148)
Nonsmokers (n = 34)	18.3 (13.2 - 25.9)	442 (309 - 586)	110 (83 - 149)
Smokers (n = 14)	18.8 (13.5 - 21.1) #	452 (300 - 621) #	88 (70 - 149) #
Healthy control subjects			
Nonsmokers (n = 38)	10.0 (8.0 - 11.4)	177 (147 - 209)	68 (55 - 87)

*Data are expressed as median (25 - 75% quartiles)
#Nonsignificant difference between nonsmokers and smokers

Receiver operating characteristic curve analysis

A significant difference was found between serum CC10, serum KL-6, and serum SP-D levels in non-smoking patients with sarcoidosis and control subjects ($p < 0.0001$). We did not observe any differences in serum levels between the first and last quartile of collected samples, making bias due to bioinstability of CC10, KL-6, and SP-D epitopes over time unlikely.

ROC curves were used to evaluate the discriminative value of serum CC10, KL-6 and SP-D in sarcoidosis (non-smokers). Serum KL-6 levels resulted in largest area under the curve: CC10, 0.83 (95% confidence interval [CI], 0.74 to 0.91); KL-6, 0.88 (95% CI, 0.81 to 0.96); SP-D, 0.75 (95% CI, 0.65 to 0.85; Figure 3.1). Cut-off levels were set as the closest point to 100% sensitivity and 100% specificity: 12.7 ng/ml for CC10 (sensitivity, 73%; specificity, 84%; and discriminative accuracy, 73%), 223 U/ml for KL-6 (sensitivity, 86%; specificity, 84%;and discriminative accuracy, 86%), and 91.7 ng/ml for SP-D (sensitivity, 66%; specificity, 84%; and discriminative accuracy, 66%). The difference between the area under the curve of CC10 and KL-6, and of CC10 and SP-D was not significant ($p = 0.22$ for both differences), but the difference between the area under the curve of KL-6 and SP-D was significant ($p = 0.018$).

Correlations between serum pneumoprotein levels

All three serum markers were significantly co-correlated in the total and non-smoking sarcoidosis groups (CC10 and KL-6, $r = 0.33$, $p = 0.003$ [total], $r = 0.43$, $p = 0.001$ [non-smokers]; CC10 and SP-D, $r = 0.37$, $p = 0.001$ [total], $r = 0.42$, $p = 0.001$ [non-

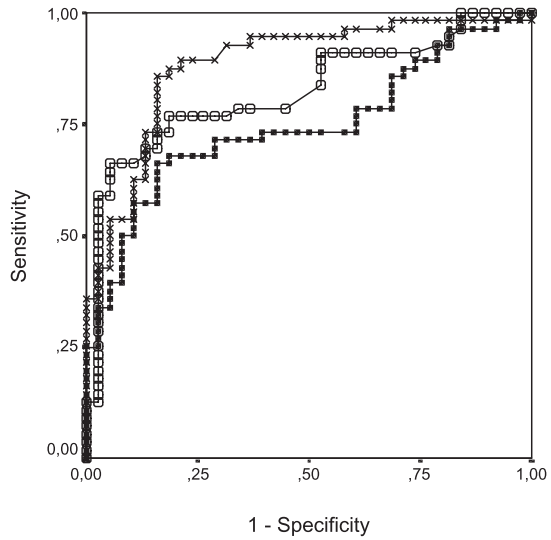

Figure 3.1 ROC curve showing the sensitivity and 1-specificity of CC16 (○), KL-6 (×), and SP-D (■) for the detection of sarcoidosis (nonsmokers).

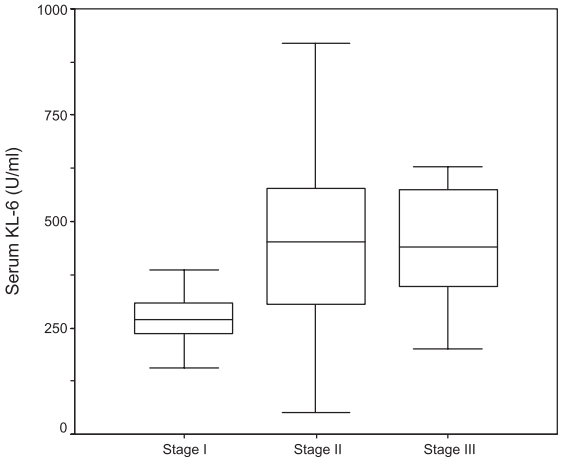

Figure 3.2 Box plot showing the median, interquartile range, and extremes of KL-6 in all patients with stage I (n = 28), stage II (n = 36), and stage III (n = 12).

smokers]; KL-6 and SP-D, $r = 0.40$, $p < 0.0001$ [total], $r = 0.36$, $p = 0.006$ [non-smokers]), but only KL-6 and SP-D were significantly correlated in the smoking sarcoidosis group ($r = 0.44$, $p = 0.038$). In the control group, a significant correlation was found between serum CC10 and serum SP-D ($r = 0.32$, $p = 0.048$). In addition, best correlations were found when combining all non-smoking subjects (patients and control subjects; CC10 and KL-6, $r = 0.58$, $p < 0.0001$; CC10 and SP-D, $r = 0.45$, $p < 0.0001$; KL-6 and SP-D, $r = 0.48$, $p < 0.0001$). No correlation was found between serum ACE levels and any of the tested pneumoproteins.

Analysis of serum CC10, KL-6, and SP-D as markers of severity

Radiography: Analysis of radiographic subgroups in relation to serum levels showed significant higher values of CC10 and KL-6 in patients presenting with stage II or III disease, *i.e.*, parenchymal infiltration, compared to stage I ($p = 0.007$ and $p < 0.0001$, respectively; Figure 3.2). Patients presenting with stage I disease had a median CC10 level of 14.7 ng/ml (range, 10.0 to 17.1 ng/ml), whereas those presenting with stage II/III disease had a median level of 18.5 ng/ml (range, 13.4 to 24.0 ng/ml). For KL-6 these values were 269 U/ml (range, 233 to 309 U/ml) and 451 U/ml (range, 313 to 577 U/ml). No significant difference was found for SP-D. Data for subgroups according to smoking habit are given in Table 3.1.

Pulmonary function: Weak associations were found between CC10 and KL-6 and pulmonary function parameters, but SP-D levels did not correlate with any of the tested parameters. CC10 in non-smoking patients with sarcoidosis correlated with Dl_{CO} ($r = -0.42$, $p = 0.001$; Figure 3.3, *top*) and TLC ($r = -0.34$, $p = 0.011$). Serum KL-6 levels correlated with Dl_{CO} in smoking and non-smoking patients ($r = -0.65$, p =

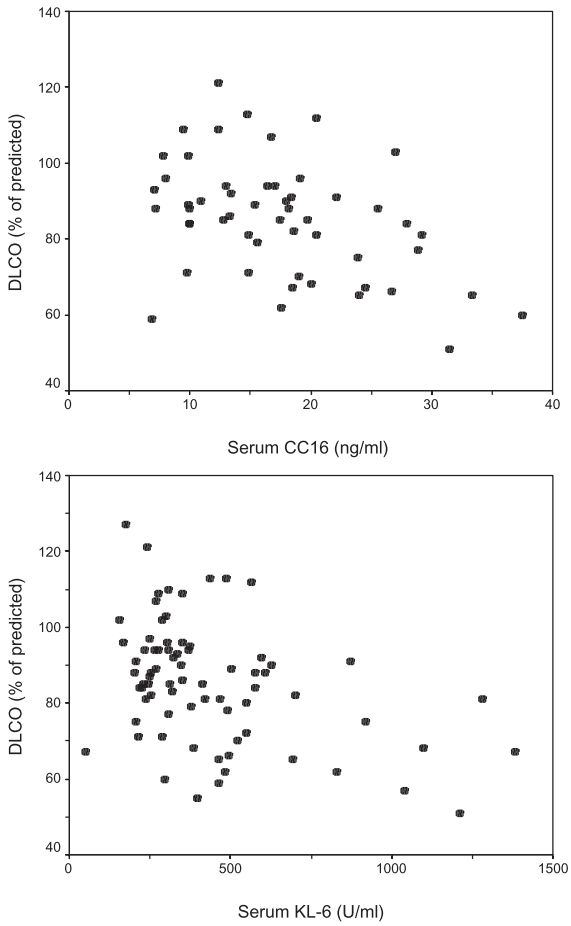

Figure 3.3 Scatter diagrams showing the correlation between CC16 and DLCO in nonsmoking patients (top), and KL-6 and DLCO in the total group of patients with sarcoidosis (Bottom).

0.001 and r = -0.30, p = 0.028, respectively; Figure 3.3, *bottom*), and with IVC only in non-smoking patients with sarcoidosis (r = -0.32, p = 0.017). Further, KL-6 correlated with TLC in non-smokers (r = -0.35, p = 0.008).

Clara cell protein 10, *krebs von den lungen* 6 and surfactant protein D as predictive markers

At follow-up, 8 patients showed a higher chest radiographic stage compared to presentation of disease, and 57 patients showed unchanged radiographic stage (group 1). In 14 patients, a lower follow-up chest radiographic stage was observed (group 2). No significant difference in CC10, KL-6, or SP-D levels were found between groups 1 and 2.

Twenty-three patients with parenchymal infiltrates on their initial chest radiograph showed progression or stabilization of infiltrates, and 1 patient with no pa-

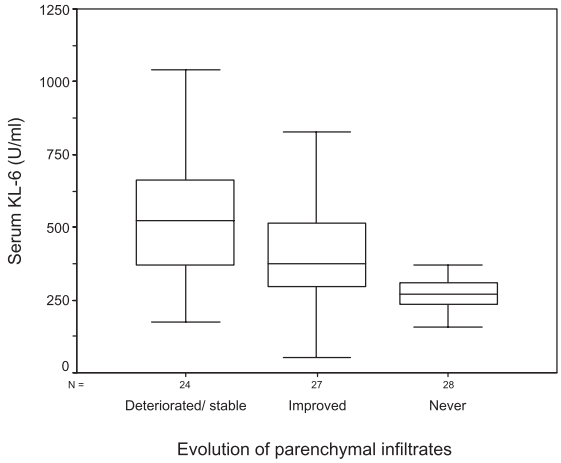

Figure 3.4 Box plot showing the median, interquartile range, and extremes of KL-6 in patients with sarcoidosis and no improvement or progression of parenchymal infiltrates on chest radiograph (group A; n = 24), patients with improvement of parenchymal infiltrates (group B; n = 27), and patients in whom no manifestations of parenchymal disease were detected either at presentation or follow-up (n = 28; group C).

renchymal infiltrates at presentation acquired these during follow-up (group A). Twenty-seven patients with parenchymal infiltrates at presentation showed improvement of the abnormalities during follow-up (group B). Twenty-eight patients with sarcoidosis had no parenchymal disease at presentation or follow-up (group C). Group A showed significantly higher KL-6 levels compared to group B ($p = 0.045$; Figure 3.4), and group B showed significantly higher KL-6 levels than C ($p = 0.008$). For CC10 and SP-D, no significant differences were found between groups A and B, or between groups B and C. Twenty patients were treated with corticosteroids during follow-up, and 59 patients were not treated. Subanalysis of patients with and without corticosteroid treatment showed similar results for KL-6 levels; however, p values between groups A and B dropped below significance. Further, analysis of changes in pulmonary function during follow-up showed no significant correlations between %TLC, percentage of predicted IVC, or percentage of predicted Dl_{CO} and initial levels of CC10, KL-6, and SP-D.

DISCUSSION

In this study, we compared the value of CC10, KL-6, and SP-D as serum markers in pulmonary sarcoidosis. Although all three pneumoproteins were significantly elevated in patients with sarcoidosis compared to control subjects, ROC curve analysis revealed best sensitivity for KL-6. The results on KL-6 are in agreement with a recent study of Ohnishi *et al.*,[10] who demonstrated superiority of serum KL-6 over SP-D

in detecting ILD. However, their study did not include patients with sarcoidosis, and did not evaluate CC10, which is also a potentially novel marker for ILD. Our comparative study on three pneumoproteins now suggests that KL-6 appears to be the most useful serum marker in the assessment of sarcoidosis. However, this is not very helpful for diagnosis. It is unlikely that CC10, KL-6, and SP-D can be used in differentiating sarcoidosis from other forms of ILD, because previous studies[5,9] have shown increased levels in diseases like IPF. Therefore, we believe the value of CC10, KL-6, and SP-D in diagnosing sarcoidosis is limited.

We demonstrated, however, significantly higher levels of CC10 and KL-6 in patients with parenchymal infiltration (stage II and III) compared to patients without parenchymal infiltration (stage I). These results are in agreement with Hermans *et al.*,[6] who observed increased CC10 serum levels in patients with higher radiographic stages, i.e., stage II and III. Kobayashi and Kitamura[4] demonstrated a similar association for KL-6: increased serum levels of KL-6 for sarcoidosis patients with parenchymal disease. Altogether, these results strongly suggest that CC10 as well as KL-6 are useful markers for determining radiologic disease severity in sarcoidosis.

Interestingly, although rather weak, associations were also found between increased serum CC10 and KL-6 levels and lung function severity, i.e., lower IVC and reduced Dl_{CO}. Previous studies[18,19] demonstrated similar findings for KL-6 in beryllosis and systemic sclerosis, but to our knowledge no study has yet described such a correlation for CC10 and KL-6 in sarcoidosis. Apparently, there is some contradiction when gas transfusion reduces while passage of pneumoproteins across the air-blood barrier increases. However, we postulate that Dl_{CO} and serum pneumoproteins are parameters of two distinct processes in sarcoidosis. Gas diffusion is strongly dependent on the thickness and total area of the alveolar-capillary membrane, and leakage of proteins more likely depends on the relation between molecule weight and size of pores in the total pulmonary epithelial-endothelial surface.

Besides their potential value as severity markers, we also looked at the predictive value of each pneumoprotein and found that patients with sarcoidosis and progressive or not improving parenchymal lung disease had slightly but significantly higher KL-6 levels at presentation. As standard chest radiographic staging is limited by reflecting changes in parenchymal infiltrates within stage II or III, this association could only be detected when scoring merely changes in parenchymal infiltrates. This result suggests that higher serum KL-6 levels reflect more severe forms of pulmonary sarcoidosis with higher risk of disease progression, although there was much overlap between the groups. Therefore, we propose that KL-6 levels might add to the predictive value of standard chest radiographic staging; however, further prospective studies (using sophisticated high-resolution computed tomography (HRCT) scanning) are needed.

Although pneumoproteins are potentially useful as markers of inflammation they could, as some reports indicate, also play a role in mediating cellular processes in fibrosis. Hirasawa *et al.*[8] found that purified KL-6 is chemotactic for human fibroblasts *in vitro*, comparable with platelet-derived growth factor, fibroblastic growth factor, and fibronectin. Intriguingly, for CC10 an opposite effect has been described, *i.e.*, CC10 was able to inhibit fibroblast chemotaxis *in vitro*.[20] This suggests that CC10 and KL-6 balance might have influence on the risk of developing fibrotic disease in sarcoidosis. Therefore, studying CC10 and KL-6 levels in serum and BAL fluid in relation to long-term disease outcome could be interesting and help to elucidate the role of these markers in the evolution of sarcoidosis.

In conclusion, KL-6 appears to be best discriminative marker in differentiating patients with sarcoidosis from healthy control subjects; however, as it is not a specific marker, this quality is unlikely to be of use in clinical practice. Both CC10 and KL-6 appear to reflect some aspects of pulmonary disease severity, especially the presence of pulmonary infiltrates on chest radiograph. But further studies are needed to determine the utility of these parameters as true markers of pulmonary disease severity, and to establish if serial measurements correlate with changes in lung function or chest radiographs.

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CHAPTER 4

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 (A) to guanine (G) mucin (MUC)1 gene polymorphism at nucleotide position 568 in a well-characterized white population. Polymorphisms were determined in 327 healthy, white individuals and 74 patients with sarcoidosis, using a polymerase chain reaction-sequence-specific primer assay. The serum KL-6 levels were measured by enzyme-linked immunosorbent assay. 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): AA (195.2 ± 9.9 U/ml; 95% confidence interval [CI], 175.7-214.8), AG (246.0 ± 8.6 U/ml; 95% CI, 229.0-263.1), and GG (302.6 ± 11.8 U/ml; 95%CI, 279.3-326.0). In the patients with sarcoidosis, the results were (mean ± SD): AA (550.1 ± 411.7; 95% CI, 380.2-720.1), AG (716.3 ± 452.4; 95% CI, 547.4-885.2), GG (1,151.0 ± 1122; 95% CI, 610.1-1692.0); $P = 0.02$. Comparison of the KL-6 levels in which the 568 genotype was ignored rendered 6 out of 74 (7.5%) misclassifications of "elevated" versus "normal" KL-6 levels or vice versa. In conclusion, the MUC1 568 A to G polymorphism may be of interest for diagnostic purposes because our study delivered *in vivo* evidence that it contributes to interindividual 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. 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* (KL)-6 offers a new perspective as disease marker in ILDs.[1] Serum KL-6 is elevated in a majority of patients with ILD and is 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] Because 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 (ARDS), and sarcoidosis.[6-9]

The KL-6 antibody recognizes a specific sugar chain on the mucin (MUC)1 protein.[3] There are known variations in the length and structure of the MUC1 protein that result from two known polymorphisms. The variable number of tandem repeats (VNTR) polymorphism present within the coding region codes for a 20-amino-acid motif, resulting in many different alleles that show a bimodal (*i.e.*, small and large) distribution.[10] In addition, the MUC1 pre-mRNA uses one of two neighbouring 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 with smaller proteins.[12]

We hypothesized that the functional MUC1 polymorphisms might contribute to variance in serum KL-6 levels. We analyzed the MUC1 568 A/G polymorphism using an easy-to-perform PCR-sequence-specific primers (PCR-SSPs) assay in a well characterized Dutch white population. The MUC1 568 genotypes were related to serum KL-6 levels to determine a possible gene-protein relationship.

MATERIALS AND METHODS

Healthy control subjects

Venous blood samples were obtained from 327 ostensibly healthy employees of the St Antonius Hospital (210 women [39 ± 12.1 yr of age] and 117 men [42 ± 10.4 yr of age]). By completing a questionnaire, these volunteers provided relevant background information, including medication use, 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 5 pack-years. Ethnicity of both parents was used as the criterion for assuming Dutch white ethnicity of the subject. Exclusion criteria included known pulmonary disease and non-Dutch white ethnicity. The medical ethical committee of this hospital approved the study, 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 yr) 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 American Thoracic Society (ATS)/ European Respiratory Society (ERS)/ World Association of Sarcoidosis and Other Granulomatous Disorders (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.

Serum *krebs von den lungen* 6 measurements

KL-6 concentrations were measured by an enzyme-linked immunosorbent assay (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) 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 the Statistical Package for Social Science for Windows (SPSS; Chicago, IL, USA) and Graphpad Prism (San Di-

ego, CA, USA) software packages. Chi-square 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 sex and smoking as fixed factors and age as a covariate, followed by a post-test for multiple comparisons between groups. Serum KL-6 levels are 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 following 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

Mucin 1 genotypes

The study population was in Hardy-Weinberg equilibrium for the MUC1 568 genotype distribution ($p = 0.44$). In healthy control subjects, 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 patients with sarcoidosis, 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 differences in MUC1 568 genotype or allele frequency distributions were found between patients with sarcoidosis and control subjects.

Relationship between mucin 1 568 adenosine to guanine polymorphism and serum *krebs von den lungen* 6 levels

Healthy control subjects: 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 4.1 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 were observed: AA (195.2 ± 9.9 U/ml; 95% CI, 175.7-214.8), AG (246.0 ± 8.6 U/ml; 95% CI, 229.0-263.1), and GG (302.6 ± 11.8 U/ml; 95% CI, 279.3-326.0). 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). Male subjects tended to have slightly higher KL-6 levels (267.9 ± 8.6 U/ml; 95% CI, 251.0-284.8) than female subjects (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 codependent on age ($p = 0.01$), although grouping the

individuals in age brackets 10-19 did not reveal profound differences in KL-6 levels (age 10-19: female subjects, 186.9 ± 7.2 U/ml and male subjects, 224.9 ± 0 U/ml; age 20-29: female subjects, 213.1 ± 10.0 U/ml and male subjects, 214.2 ± 10.1 U/ml; age 30-39: female subjects, 242.2 ± 9.0 U/ml and male subjects, 256.5 ± 12.0 U/ml; age 40-49: female subjects, 214.2 ± 9.4 U/ml and male subjects, 251.1 ± 10.0 U/ml; age 50-59: female subjects, 255.9 ± 10.3 U/ml and male subjects, 271.9 ± 16.8 U/ml; and age > 60: female subjects, 208.2 ± 7.9 U/ml and male subjects, 217.9 ± 12.9 U/ml). Figure 4.1 illustrates the MUC1 genotype-grouped serum KL-6 levels.

Table 4.1 MUC1 genotype-specific serum KL-6 levels in healthy white subjects (n = 327)

	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 sex.

Patients with sarcoidosis: Figure 4.2 illustrates the influence of the 568 A/G polymorphism on KL-6 levels in patients with sarcoidosis. When the KL-6 levels of the patients were grouped according to the genotype, the results were AA (550.1 ± 411.7; 95%CI, 380.2-720.1), AG (716.3 ± 452.4; 95% CI, 547.4-885.2), and GG (1,151.0 ± 1,122; 95% CI, 610.1-1,692.0; p = 0.02).

Table 4.2 MUC1 genotype-specific serum KL-6 levels in patients with sarcoidosis (n = 74)

	Genotype		
	AA	AG	GG
Number of individuals	25	30	19
Serum KL-6 levels, U/ml	606.5 (136.5)	670.5 (133.9)	1,224.2 (165.3)
95% confidence interval, U/ml	334.0 - 879.0	403.1 - 937.8	894.2 - 1,554.1

SEM are shown in parentheses. Values were corrected forradiographic staging, age, smoking, and sex.

KL-6 levels measured in patients with sarcoidosis 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 (548.9 ± 121.0 U/ml; 95% CI, 307.4-790.3) than those with stages II, III, and IV (1,118.5 ± 133.1 U/ml; 95% CI, 852.9-1,384.2; p = 0.0003).When the 568 A/G genotype influence on KL-6 levels in the patients were corrected for radiographic staging, sex, age, and smoking, significant differences in KL-6 levels between patients grouped according to the MUC1 568 A/G

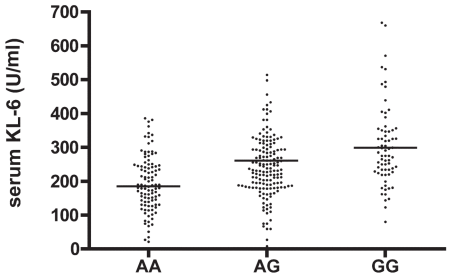

Figure 4.1 Scatterplot illustrating the association between MUC1 568 A/G genotype and serum KL-6 levels (U/ml) in 327 healthy white subjects. Horizontal bars in scatterplots represent estimatedmarginalmean for each group.

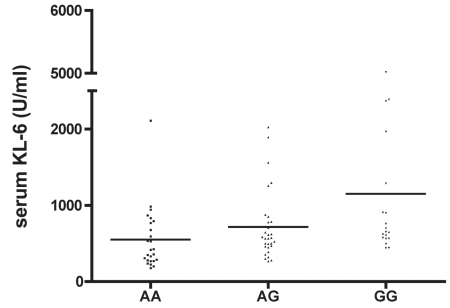

Figure 4.2 Scatterplot illustrating the association between MUC1 568 A/G genotype and serum KL-6 levels (U/ml) in 74 patients with sarcoidosis. Horizontal bars in scatterplots represent mean for each group.

genotype were observed as follows: AA (606.5 ± 136.5 U/ml; 95% CI, 334.0-879.0), AG (670.5 ± 133.9 U/ml; 95% CI, 403.1-937.8), and GG (1,224.2 ± 165.3 U/ml; 95% CI, 894.2-1,554.1; p = 0.003) (Table 4.2). Smoking, sex, or age did not show any significant influence on KL-6 levels (data not shown).

Comparison of the KL-6 levels in which the 568 genotype was ignored rendered 6 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 4.1) and 568 GG genotyped patients (n = 3) changed from having normal (39.8-437.6U/ml) to elevated (> upper reference limit of the GG genotype-specific reference interval in Table 4.1) 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 interindividual differences in serum KL-6 levels. Serum KL-6 levels were higher in 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 patients with sarcoidosis, despite the 3-fold increase of the overall KL-6 levels compared with control subjects. 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 with patients without parenchymal involvement (stages 0/I and patients with Löfgren's syndrome). Discordant classification of KL-6 levels (normal versus elevated) in 7.5% of the patients with evaluated sarcoidosis clearly demonstrates the consequences of the 568 A/G polymorphism on the interpretation of KL-6 levels.

Ligtenberg *et al.* 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 *et al.* reported that determination of the MUC1 568 A/G using PCR-SSPs is an easier way to establish MUC1 haplotypes than determination of the VNTR.[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] The tandem repeat unit is rich in serine and threonine. After 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 *et al.* demonstrated that mucins with a high number of repeat units have more sugar chains compared with those with fewer tandem repeats.[12] Because themonoclonal 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 a diagnostic test is likely to benefit from genotyping for the MUC1 polymorphism at position 568. There may be additional SNPs in the MUC1 gene that were not identified and evaluated in this study and 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 568A/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 patients with IPF.[6] Kohno *et al.* estimated survival in IPF using the Kaplan-Meier method and showed that patients with serum KL-6 levels above 1,000 U/ml have a significantly worse prognosis.[7] In patients with ARDS, KL-6 levels were higher in nonsurvivors 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.

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

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CHAPTER 5

The Clara cell protein 10 adenine38guanine polymorphism influences serum Clara cell protein 10 levels in white controls and sarcoidosis patients

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ABSTRACT

Clara cell protein (CC)10 is one of the most abundantly produced proteins of the lower respiratory tract. Serum CC10 measurements are a useful tool to noninvasively assess the integrity of the air-blood barrier in sarcoidosis. The CC10 adenosine (A) 38guanine (G) promoter polymorphism is known to influence CC10 production. Previously, an association between the CC10 38A allele and low serum CC10 levels has been reported in Japanese sarcoidosis patients. The aim of the present study was to confirm this association in a clinically well characterized population of Dutch white patients with sarcoidosis and control subjects. Seventy-seven white sarcoidosis patients and 330 controls were genotyped using a polymerase chain reaction-sequence-specific primers assay. Serum CC10 levels were measured using an enzyme-linked immunosorbent assay. A significant difference in serum CC10 levels was found between the CC10 A38G genotypes in white controls ($p < 0.0001$) and sarcoidosis patients ($p = 0.005$). In conclusion, serum CC10 levels are significantly associated with CC10 A38G genotype in whites. In order to make a correct interpretation of serum CC10 levels as measure of the alveolar-capillary membrane permeability in sarcoidosis CC10 A38G genotyping should be performed.

INTRODUCTION

Sarcoidosis is a complex granulomatous disease thought to be caused by an unknown antigenic stimulus from the environment in combination with genetic susceptibility.[1] To evaluate the activity and the course of sarcoidosis, several methods, such as chest roentgenogram, pulmonary function testing, gallium-67 lung scan, and bronchoalveolar lavage (BAL), are available. The lung epithelium-specific protein, or pneumoprotein, Clara cell protein (CC)10, produced by nonciliated bronchiolar Clara cells, offers a new perspective as a disease marker in sarcoidosis.[2] Serum CC10 levels in sarcoidosis patients are increased compared to control subjects.[3;4] Since sarcoidosis patients have normal levels of BAL fluid CC10 levels and an unchanged number of CC10-immunopositive cells in lung biopsy samples, an increased secretion of CC10 in the sarcoidosis lung is very unlikely.[3] High serum CC10 levels in sarcoidosis reflect the increased permeability to proteins of the air-blood barrier, which is an important pathologic abnormality associated with sarcoidosis.[3;5]

Laing *et al.* were the first to describe an adenine (A) to guanine (G) substitution in the promoter region of the gene encoding CC10.[6] Some genetic polymorphisms in promoter regions influence regulatory mechanisms and cause interindividual differences in protein production. The CC10 A38G polymorphism is such a functional polymorphism. Laing *et al.* demonstrated that the CC10 38AA genotype is associated with reduced plasma CC10 levels.[7] Ohchi *et al.* demonstrated that the CC10 A38G polymorphism also influences serum CC10 levels in Japanese sarcoidosis patients.[8]

The aim of the present study was to validate the association between the CC10 A38G polymorphism and serum CC10 levels in a clinically well-defined group of Dutch white sarcoidosis patients and healthy controls in order to make this measurement a more precise tool to noninvasively assess the integrity of the alveolar-capillary membrane.

MATERIALS AND METHODS

Subjects

Seventy-seven unrelated Dutch white sarcoidosis patients (44 men and 33 women) were included in the study. In 55 patients, the diagnosis of sarcoidosis was established when clinical findings were supported by histologic evidence, and after exclusion of other known causes of granulomatosis. Twenty-two patients presented with the classic Löfgren's syndrome of fever, erythema nodosum, bilateral hilar lymphadenopathy, and joint symptoms. The diagnosis in these patients was mostly made without biopsy proof in agreement with the statement on sarcoidosis.[1] Eighteen

patients (13 men and 5 women) were active smokers. Venous blood samples were also obtained from 330 ostensibly healthy employees of the St Antonius Hospital (119 men and 211 women). By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases. Ethnicity of the parents of a subject was used as the criterion for assuming Dutch Caucasian ethnicity of the subject. The overrepresentation of women who participated in this study is explained by the predominantly female workforce at this hospital. Sixty-four controls (29 men and 35 women) were current smokers or quite smoking less than a month ago.Verbal and written patient consent was obtained from all subjects and authorization was given by the Ethics Committee of the St Antonius Hospital, Nieuwegein.

Genetic analysis

The CC10 A38G polymorphism was determined using an assay that utilizes sequence-specific primers with 3'-end mismatches and identifies the presence of specific allelic variants through polymerase chain reaction amplification. We used the sequence-specific forward primers 5'-CAG AGA CGG AAC CAG AGA CA and 5'-AGA GAC GGA ACC AGA GAC G in combination with the consensus reverse primer 5'-TCC TGA GAG TTC CTA AGT CC with an expected PCR product size of 150 and 151 base pairs, respectively. In the primer mix, we included the control primers 5'-TGC CAA GTG GAG CAC CCA A and 5'-GCA TCT TGC TCT GTG CAG AT. PCR reactions were run as previously described, in a final volume of 13 µl overlaid with 10 µl of mineral oil.[9] The presence of an allele-specific band of the expected size, in conjunction with a control band, was considered to be positive evidence for each particular allele. The absence of an allele-specific band and the presence of a control band were considered to be evidence for the absence of an allele.

Clara cell protein 10 measurement

CC10 concentrations in serum were measured by an enzyme-linked immunosorbent assay (ELISA) technique using a specific CC10 antibody kit, as described previously.[10] All samples were run in duplicate, and mean values were used for subsequent analysis. We did not observe any differences in serum levels between the first and last quartile of collected samples, making bias due to bio-instability of CC10 epitopes over time unlikely (data not shown).

Chest Radiography

Chest radiographs were scored blind by a chest physician with more than 20 years of experience in sarcoidosis (J.M.M. van den Bosch) according to standard chest radiographic staging.[1] Besides this staging, patients with abnormal chest radiographic

findings were also categorized as follows: without parenchymal infiltration (stage I), and with parenchymal infiltration (stage II and III).

Statistical analysis

The statistical evaluation of our data was performed using the Statistical Package for Social Science for Windows (SPSS; Chicago, IL, USA) and Graphpad Prism (San Diego, CA, USA) software packages.

CC10 levels were expressed as median with 25 to 75 percent quartiles unless otherwise stated. The Mann-Whitney U and Kruskal-Wallis test were used to compare CC10 levels between groups. The reference interval of serum KL-6 in each genotype group was calculated by the following formula: mean ± 1.96 SD.

The genotype frequencies and the frequency of an allele in the chromosomal pool of each stage (allele frequency) were determined by direct counting. Genotype frequencies were tested for Hardy-Weinberg equilibrium. χ^2 contingency table analysis was performed with the appropriate number of degrees of freedom (df) for categorical variables. Fisher exact test was used if expected cell frequencies were lower than 5.

A value of $p < 0.05$ was considered significant for all tests performed.

RESULTS

CC10 genotypes

The study population was in Hardy-Weinberg equilibrium for the CC10 38 genotype ($p = 0.79$). In healthy control subjects, the CC10 allele frequency was 199 (30.2%) for A and 461 (69.8%) for G. The genotype frequency for AA, AG and GG was 31 (9.4%), 137 (41.5%), and 162 (49.1%), respectively. In patients with sarcoidosis, the allele frequency was 51 (33.1%) for A and 103 (66.9%) for G. The genotype frequency for AA, AG and GG was 9 (11.7%), 33 (42.9%), and 35 (45.4%), respectively. No significant differences in CC10 38 genotype or allele frequency distributions were found between patients with sarcoidosis and control subjects, or between male and female subjects.

Relationship between CC10 A38G polymorphism and serum CC10 levels

Since smoking had effect on CC10 levels in previous studies, serum CC10 levels were calculated for the total group and for nonsmokers.[11]

Healthy control subjects: The serum CC10 levels in all healthy individuals was 6.7 [4.9 - 9.13], and the reference interval was 0.4 - 14.3 ng/ml. No significant difference was found between serum CC10 levels in smoking and nonsmoking sarcoidosis pa-

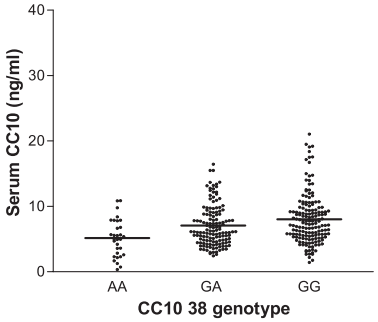

Figure 5.1 Scatter plot showing the association between CC10 A38G genotype and serum CC10 levels in healthy white subjects.

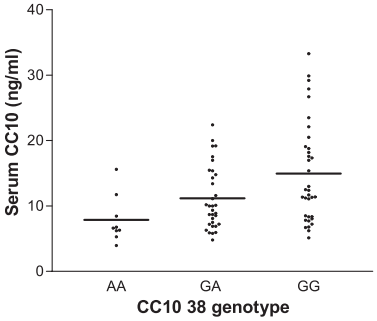

Figure 5.2 Scatter plot showing the association between CC10 A38G genotype and serum CC10 levels in white sarcoidosis patients.

tients (p = 0.56). Table 5.1 summarizes the serum CC10 levels that were categorized according to CC10 A38G genotype in healthy white subjects. A significant difference in serum CC10 levels was found between the CC10 A38G genotypes (χ^2 = 17.8 with 2 df p < 0.0001;Figure 5.1; nonsmoker χ^2 = 14.3 with 2 df p = 0.001). Post-hoc Mann-Whitney U testing revealed differences in serum

CC10 levels between healthy control subjects with 38AA (5.0 ng/ml [2.6 - 7.8]) and 38AG genotype (6.1 ng/ml [4.6 - 8.9]; p = 0.005; nonsmoker p = 0.003), controls with 38AA and 38GG genotype (7.3 ng/ml [5.4 - 9.5]; p < 0.001; nonsmoker p <0.001) and controls with 38AG and 38GG genotype (p = 0.022; nonsmoker p =0.103). Male control subjects had significantly higher serum CC10 levels (7.8 ng/ml [5.6 - 10.9]) than female control subjects (6.1 ng/ml [4.2 - 8.4]; p < 0.0001).

Comparison of the CC10 levels in controls in which the 38 genotype was ignored rendered 8 out of 330 (2.4%) misclassifications of “elevated” versus “normal” CC10 levels or vice versa.

Table 5.1 CC10 38 genotype-specific serum CC10 levels in healthy white subjects

	CC10 38 Genotype		
	AA	AG	GG
Number of individuals	31	137	162
Median [p_{25} - p_{75}], ng/ml	5.0 [2.6 - 7.8]	6.1 [4.6 - 8.9]	7.32 [5.4 - 9.5]
95% CI, ng/ml	4.1 - 6.2	6.5 - 7.6	7.4 - 8.6
Normal range, ng/ml	0 - 10.8	0.9 - 13.2	0.6 - 15.5

Patients with sarcoidosis: Analysis of radiographic subgroups in relation to serum levels showed significant higher values of CC10 in patients presenting with stage II or III disease, *i.e.* parenchymal infiltration, compared to stage I (p = 0.005). Patients presenting with stage I disease had a median serum CC10 level of 8.9 ng/ml [6.8 - 12.5], whereas those presenting with stage II/III disease had a median level of 15.4 ng/ml [7.8 - 20.5]. No significant difference was found between serum CC10 levels in smoking and nonsmoking sarcoidosis patients (p = 0.075). Table 5.2 summarizes the serum CC10 levels that were categorized according to CC10 A38G genotype in white sarcoidosis patients. A significant difference in serum CC10 levels was found between the CC10 A38G genotypes (χ^2 = 10.6 with 2 df p = 0.005; Figure 5.2; non-smoker χ^2 = 8.9 with 2 df p = 0.012). Post-hoc Mann-Whitney U testing revealed differences in serum CC10 levels between sarcoidosis patients with 38AA (6.6 ng/ml [5.8 - 10.1]) and 38AG genotype (10.0 ng/ml [7.21 - 15.1]; p = 0.034; nonsmoker p = 0.068), patients with 38AA and 38GG genotype (12.4 ng/ml [8.4 - 19.1]; p = 0.004; nonsmoker p = 0.009) and patients with 38AG and 38GG genotype (p = 0.05; non-smoker p = 0.056).

Comparison of the CC10 levels in sarcoidosis patients in which the 38 genotype was ignored rendered only 1 misclassification out of 77 (1.3%) of “elevated” versus “normal” CC10 levels.

Table 5.2 CC10 38 genotype-specific serum CC10 levels in white sarcoidosis patients

	CC10 38 Genotype		
	AA	AG	GG
Number of individuals	9	33	35
Median [p_{25} - p_{75}], ng/ml	6.6 [5.6 - 10.1]	10.0 [7.2 - 15.1]	12.4 [8.4 - 19.1]
95% CI, ng/ml	5.1 - 10. 7	9.5 - 12.9	12.3 - 17.6

DISCUSSION

This study found an association between the CC10 A38G polymorphism and serum CC10 levels in white sarcoidosis patients and healthy controls. We demonstrated

that this functional polymorphism contributes to interindividual differences in serum CC10. Serum CC10 levels were higher in individuals carrying the 38 G allele, with 38 A homozygotes having the lowest levels, 38 G allele homozyotes having the highest levels, and heterozygotes having intermediate levels, which is compatible with the gene-dose effect.

A previous study performed in Australian asthmatic - and nonasthmatic children did not find such a clear gene-dose effect. In Laing's study serum CC10 levels in heterozygote asthmatic and nonasthmatic children were almost identical to the CC10 levels in subjects homozygote for the 38G allele as in our study.[7] Ohchi *et al.* also studied the relationship between serum CC10 levels and the CC10 A38G polymorphism in Japanese sarcoidosis patients.[8] They found a significant difference in serum CC10 levels only between sarcoidosis patients with the 38AA and 38GG genotype, however, not between the 38AG and 38GG genotype, or between the 38AA and 38AG genotype. The fact that we found a similar and stronger relationship between the CC10 promoter polymorphism and serum CC10 levels in an ethnical and geographical different adequately large group of subjects adds weight to the gene-protein association.

Serum CC10 levels reflect the integrity of the alveolar-capillary membrane. Ultrastructural alterations in this air-blood barrier are frequently observed in sarcoidosis.[5] Pulmonary permeability can also be assessed by bronchoalveolar lavage, or aerosolized 99mTc-DTPA absorption.[12] Serum CC10 measurements are not as invasive as BAL, and are easier to perform than 99mTc-DTPA lung scanning.

Previous studies demonstrated that serum CC10 levels are associated with radiologic pulmonary disease severity in sarcoidosis.[3;4] Besides its value as severity marker, CC10 might also have a prognostic value in sarcoidosis. Shijubo *et al.* demonstrated that sarcoidosis patients with regressive disease had increased serum CC10 levels.[13] This association might be explained by the fact that CC10 also plays a functional role in mediating cellular processes in fibrosis. CC10 is able to inhibit fibroblast chemotaxis by mechanisms that may be related to a blockage of phospholipase A2 activity.[14] Therefore, CC10 deficiency may contribute to fibroblast burden activity in sarcoidosis. Correcting serum CC10 levels for the CC10 A38G polymorphism may increase its value as a severity and prognostic marker.

Serum CC10 levels are influenced by radiographic stage. In our study, patients with and without parenchymal infiltration were equally distributed between genotype AG and GG, however, the percentage of stage I patients was higher in the genotype AA group. The association between genotype and serum CC10 levels was, therefore, separately analyzed for patients with parenchymal infiltration, which showed similar results as for the total group.

No difference in serum CC10 levels was observed between smoking and nonsmoking control subjects. This finding is in disagreement with Bernard *et al.* who demonstrated reduced numbers of Clara cells in smokers and lower levels of CC10 in serum in comparison with nonsmokers.[11] The difference in CC10 levels between smoking and nonsmoking sarcoidosis patients almost reached significance. However, we could state that in our sarcoidosis patients, correcting for CC10 A38G genotype was more important than correcting for smoking status. Our associations between CC10 A38G genotype and serum CC10 levels were similar in smokers and nonsmokers.

We conclude that serum CC10 levels are significantly associated with CC10 A38G genotype in white sarcoidosis patients and healthy controls. In order to make a more precise interpretation of serum CC10 levels as measure of the permeability of the alveolar-capillary membrane in sarcoidosis CC10 A38G genotyping should be performed.

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CHAPTER 6

The Clara cell protein 10 adenine38guanine polymorphism and sarcoidosis susceptibility in Dutch and Japanese subjects

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ABSTRACT

Clara cell protein (CC)10 (CC16, uteroglobin) is a pulmonary protein postulated to play a counter regulatory role in sarcoidosis pathogenesis. The adenine (A)38guanine (G) polymorphism of the encoding CC10 gene (SCGB1A1) is functional. Recently, an association between the low CC10 producing 38A allele and sarcoidosis susceptibility has been reported in Japanese patients from Hokkaido. The aim of the present study was to confirm this association in a clinically well characterized population of Dutch white and Kyoto Japanese patients with sarcoidosis and control subjects. No difference in genotype or allele frequency was found between patients with sarcoidosis and control subjects in either ethnic population. Remarkably, however, a significant difference was found between the control subjects from Kyoto and Hokkaido, but not between the Japanese groups of patients with sarcoidosis. Furthermore, review of previously published A38G genotyping results showed a consistent difference in CC10 A38G allele frequencies between whites and Japanese subjects. We conclude that the CC10A38G polymorphism does not influence sarcoidosis susceptibility in Dutch whites or in Japanese subjects from Kyoto. This stresses the importance of studying the influence of polymorphisms on disease susceptibility in multiple ethnically and geographically distinct disease and control populations before reaching conclusions.

INTRODUCTION

Clara cell protein 10 (CC10) is a protein with a molecular weight of approximately 10 kDa produced by nonciliated bronchiolar Clara cells.[1] Human CC10 is homologous to Clara cell protein 16 (CC16) and uteroglobin (UGB).[1] Although its exact role has yet to be determined, there are arguments for believing that CC10 serves as an immunosuppressive mediator. CC10 interferes with interferon γ and tumor necrosis factor-α mediated actions and diminishes their biological activity.[2,3] In addition, CC10 inhibits fibroblast chemotaxis and, therefore, CC10 might play a role in the fibrotic response in many inflammatory conditions in the lung.[4]

Sarcoidosis is a complex granulomatous disease thought to be caused by an unknown antigenic stimulus from the environment in combination with genetic susceptibility.[5,6] Higher values of serum CC10 levels were found in patients presenting with pulmonary infiltrates on chest radiograph compared with those without parenchymal involvement.[7,8] Interestingly, high bronchoalveolar lavage (BAL) fluid and serum CC10 levels were found to be associated with a favourable prognosis in this disease.[9]

There is growing evidence for the contribution of genetic polymorphisms to interindividual differences in the regulatory mechanisms of protein production. Therefore, certain cytokine genotypes might increase disease susceptibility. Laing *et al.* were the first to describe an adenine (A) to guanine (G) substitution in the promoter region of the gene encoding CC10 (UGB A38G; current gene symbol: SCGB1A1).[10] Compared with G homozygotes (38GG), those homozygous and heterozygous for the polymorphic variant (38AA and 38AG, respectively) had an increased risk of developing asthma.[10] Furthermore, the 38AA genotype was associated with significantly reduced plasma CC10 levels in both individuals with and without asthma.[11] Also, in our patients with sarcoidosis, 38A-allele carriers showed significantly reduced levels compared with non-A-allele carriers.[12] Therefore, it is believed that the CC10A38G polymorphism is functional and plays a role in regulating pulmonary inflammation. Recently, Ohchi *et al.* investigated the A38G variant in Japanese patients with sarcoidosis.[13] They found an increased carriership of the 38A allele in Hokkaido patients compared with control subjects from the same region in northern Japan, suggesting a contribution in the genetics underlying sarcoidosis susceptibility.[13]

The aim of the present study was to validate this association in clinically well defined groups of patients with sarcoidosis from the Netherlands and from Japan.

Some of the results of this study have previously been reported in the form of an abstract.[12]

MATERIALS AND METHODS

One hundred thirty-eight unrelated and randomly selected Dutch white patients with sarcoidosis (78 men, 60 women; mean age ± SD: 37 ± 10 years) were included in the study. In 97 patients, the diagnosis of sarcoidosis was established when clinical findings were supported by histologic evidence, and after exclusion of other known causes of granulomatosis. Forty-one patients presented with the classic Löfgren's syndrome of fever, erythema nodosum, bilateral hilar lymphadenopathy, and joint symptoms. The diagnosis in these patients was mostly made without biopsy proof.[14] Verbal and written patient consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St Antonius Hospital, Nieuwegein (Utrecht region). The Dutch control subjects comprised 114 white donors from the Blood Transfusion Service in Utrecht, which takes donors mainly from the Utrecht region. All donors were routinely checked for health before donation and gave their written consent.

One hundred unrelated Japanese patients with sarcoidosis from the Kyoto region seen at Central Clinic Kyoto were randomly included in the study. In all patients the diagnosis of sarcoidosis was established in keeping with the diagnostic criteria used for the Dutch patients. One hundred seventeen healthy individuals from the Kyoto region were included as control subjects. All subjects gave their informed consent.

The CC10 A38G polymorphism was determined using sequence specific primers and polymerase chain reaction (PCR) that utilizes sequence-specific primer (SSP)s with 3'-end mismatches and identifies the presence of specific allelic variants through PCR amplification. We used the sequence-specific forward primers 5'-CAG AGA CGG AAC CAG AGA CA and 5'-AGA GAC GGA ACC AGA GAC G in combination with the consensus reverse primer 5'-TCC TGA GAG TTC CTA AGT CC at a final concentration of 30 ng/µl, with an expected PCR product size of 150 and 151 base pairs, respectively. In the primer mix, we included the control primers 5'-TGC CAA GTG GAG CAC CCA A and 5'-GCA TCT TGC TCT GTG CAG AT at a final concentration of 2 ng/µl. PCR reactions were run as previously described at a final volume of 13 µl overlaid with 10 µl of mineral oil.[15] The presence of an allele-specific band of the expected size, in conjunction with a control band, was considered to be positive evidence for each particular allele. The absence of an allele-specific band and the presence of a control band were considered to be evidence for the absence of an allele.

The genotype frequencies and the frequency of an allele in the chromosomal pool of each population (allele frequency) were determined by direct counting for both control and sarcoidosis groups. All genotype frequencies were tested for Hardy-Weinberg equilibrium. A classical χ^2 test was used for a 2 × 2 table, which delivers

(R - 1) (C - 1) = 1 degree of freedom (df) (Statistical Package for Social Science for Windows (SPSS); Chicago, IL, USA). Fisher exact test was used when the underlying criteria for the χ^2 test were not met. A value of $p < 0.05$ was considered significant.

RESULTS

Allele frequencies of the CC10 A38G promoter polymorphism in the Dutch and Kyoto Japanese patients with sarcoidosis and the control subjects are summarized in Table 6.1. Both populations were in Hardy-Weinberg equilibrium for all genotypes. No differences in genotype and allele frequencies were observed between Dutch patients and control subjects or between patients and control subjects from Kyoto. Furthermore, subgroup analysis within the Dutch patients with sarcoidosis did not reveal significant differences in allele frequencies between Löfgren and non-Löfgren cases.

The difference in the Dutch cohort for the CC10 A38G A allele was 0.330-0.338 = -0.008, and the 95% confidence interval of that small difference was 0.074 to -0.091. In the Kyoto cohort this difference was -0.012 with a 95% confidence interval of 0.08 to -0.104; and in the Hokkaido cohort this difference was 0.102 with a 95% confidence interval of 0.16 to -0.04. Equivalence in the width of these intervals, *i.e.*, similar accuracy of the estimates in the three cohorts, indicated sufficient study power to detect differences.

The 38A allele frequency in our Kyoto control subjects was significantly higher compared with the frequency in Hokkaido control subjects reported by Ohchi *et al.* ($\chi^2 = 3.91$ with 1 df, $p = 0.048$; Table 6.1).[13] Notably, studies in Japanese control subjects from the Niigata and Kitakyushu region have shown similar CC10 A38G allele frequencies compared with our Kyoto controls, confirming the deviating 38A allele frequency in control subjects from Hokkaido (Table 6.1).[16,17]

A significant difference was observed in allele frequency distribution between Dutch and Japanese patients with sarcoidosis from Hokkaido ($\chi^2 = 10.54$ with 1 df, $p = 0.0012$; Table 6.1).[13] A similar trend was observed when comparing the allele frequencies in Dutch and Japanese patients with sarcoidosis from Kyoto ($\chi^2 = 3.28$ with 1 df, $p = 0.070$; Table 6.1). However, no significant difference was found when comparing patients with sarcoidosis from Hokkaido and Kyoto.

A review of the literature on the CC10 A38G polymorphism in white control subjects (from Australia, the UK, and Germany) showed results similar to those of the Dutch, further supporting the observation of an essential racial difference in A38G allele frequencies between whites and Japanese subjects (Table 6.1).[10,18,19]

Table 6.1 CC10 A38G allele frequencies in white and Japanese patients with sarcoidosis and control subjects

	Whites				Japanese			
	Dutch	German	British	Australian	Kyoto	Niigata	Kitakyushu	Hokkaido
CC10 A38G								
Controls	(n = 114)	(n = 118)	(n = 194)	(n = 266)	(n = 117)	(n = 196)	(n = 206)	(n = 258)
A	77 (33.8)	73 (30.9)	132 (34.0)	177 (33.3)	100 (42.7)	159 (40.6)	181 (43.9)	180 (34.9)
G	151 (66.2)	163 (69.1)	256 (66.0)	355 (66.7)	134 (57.3)	233 (59.4)	231 (56.1)	336 (65.1)
Sarcoidosis	(n = 138)				(n = 100)			(n = 265)
A	91 (33.0)				83 (41.5)			239 (45.1)
G	185 (67.0)				117 (58.5)			291 (54.9)

DISCUSSION

CC10, the predominant product of Clara cells, lines the bronchiolar epithelium and is thought to protect the respiratory tract from local inflammation through its immunosuppressive actions.[1] The A38G promoter is potentially of great immunogenetic interest, as there is *in vivo* evidence that it accounts for interindividual variations in CC10 production.[11] Recently, a study from Ohchi *et al*. demonstrated that the 38A allele was associated with sarcoidosis susceptibility in Hokkaido Japanese.[13] The present study, however, in clinically well defined sarcoidosis patients from the Netherlands and the Kyoto region in Japan, could not confirm these findings.

Notably, the 38A-allele frequency in Hokkaido control subjects was significantly lower compared with Kyoto control subjects, whereas the 38A-allele frequency in both populations with sarcoidosis was similar. Studies on the same polymorphism in other Japanese populations (from Niigata and Kitakyushu) showed genotyping results similar to those of our Kyoto controls. Niigata, Kitakyushu, and Kyoto are all located in the central and southern part of Japan, and Hokkaido is an island in northern Japan, which may suggest a geographically determined genetic difference within Japan. Alternatively, as already indicated by Iannuzzi, gene environment interactions may be different in northern and southern parts of Japan.[20] But if this is true, then the results found by Ohchi *et al*., and interpreted in the context of the results presented in this paper, indicate that the *absence* of the 38A allele actually *protects* against sarcoidosis in northern Japanese.[13]

Another important finding in this study is the remarkably consistent A38G allele distribution in four different white populations from the Netherlands, Germany, the UK and Australia. This suggests strong genetic homogeneity for CC10 across northern Europe (and Australia, where ancestry is from the same part of Europe). Furthermore, comparing these results with those from the Kyoto control subjects and the Japanese control subjects from Niigata and Kitakyushu showed a clear racial

difference in the A38G distribution between whites and Japanese. This information is crucial for geneticists interested in the unravelling of common complex inflammatory pulmonary conditions, in which counter regulation by CC10 might be important.

Our study highlights one of the pivotal aspects of genetic association studies, *i.e.*, replication of a reported genetic association between a candidate gene and a disease like sarcoidosis. Subsequent testing of the reported findings in one or, even better, more ethnically distinct populations is still an essential strategy in this respect. A thorough review of the various problems of reporting genetic associations with complex diseases, e.g., replication of genetic associations before declaration of evidence as convincing, was recently given by Colhoun *et al*..[21]

We conclude that the CC10 A38G polymorphism does not influence disease susceptibility in Dutch whites or Japanese subjects with sarcoidosis from Kyoto. We show significant differences in the allele frequencies of this polymorphism between Japanese and whites. The fact that no association between the CC10 38A allele and sarcoidosis susceptibility was found, stresses that polymorphisms need to be studied in multiple ethnical and geographic distinct populations before reaching conclusions.

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CHAPTER 7

Summary

Concluding remarks

Samenvatting in het Nederlands

List of publications

Dankwoord

Curriculum vitae

7.1 SUMMARY

The purpose of this thesis was to evaluate the usefulness of pneumoproteins in interstitial lung disease (ILD)s. We emphasized on the pneumoprotein encoding genes and their relationship with serum pneumoprotein levels, and disease susceptibility.

Chapter 1

This chapter offers a brief introduction in ILDs, and emphasizes on sarcoidosis and hypersensitivity pneumonitis (HP). Furthermore, the three pneumoproteins described in the thesis are separately discussed.

Chapter 2

In this chapter, we determined the discriminative value of serum *krebs von den lungen* (KL)-6 and surfactant protein (SP)-D, and their ability to reflect pulmonary disease activity and prognosis in bird fanciers' lung (BFL). KL-6 and SP-D were significantly elevated in patients compared to controls. Receiver operating characteristic (ROC) curve analysis revealed that both are equally useful in discriminating patients from controls. Analysis of their value as activity markers showed that both correlated with pulmonary function impairment; however, KL-6 correlated best with diffusing capacity. Evaluation of their predictive value showed that higher levels at onset were associated with improvement of diffusing capacity during follow-up. Further, it was noted that KL-6 and SP-D levels decreased after more than one month of allergen avoidance. KL-6 and SP-D appeared to be useful serum markers in BFL. Since higher levels are associated with more severe lung function impairment at presentation, and better recovery over time, we postulated that in this disease they are especially markers of disease activity.

Chapter 3

In this chapter, we determined the discriminative value of serum Clara cell protein (CC)10, KL-6, and SP-D as markers of sarcoidosis, and their ability to reflect pulmonary disease severity and prognosis. ROC curve analysis revealed largest area under the curve for KL-6. Significantly higher levels of CC10 and KL-6 were found in patients with parenchymal infiltration (stage II and III) compared to patients without parenchymal infiltration (stage I). In concordance, CC10 and KL-6 levels inversely correlated with diffusion capacity and total lung capacity, and KL-6 also with inspiratory vital capacity. Moreover, higher KL-6 levels were weakly but significantly associated with persistence or progression of parenchymal infiltrates at two year follow-up. In this chapter, KL-6 appeared to be the best discriminative marker in differentiating patients with sarcoidosis from healthy control subjects. Both CC10

and KL-6 may be of value in reflecting disease severity, and KL-6 tended to associate with pulmonary disease outcome.

Chapter 4

The aim of this chapter was to analyze whether serum KL-6 levels are dependent on the functional adenosine (A) to guanine (G) mucin (MUC)1 gene polymorphism at nucleotide position 568 in a well-characterized white population. Polymorphisms were determined in healthy individuals, and patients with sarcoidosis. Significant differences between serum KL-6 levels of healthy subjects who were grouped according to MUC1 568 genotype were observed. A similar genotype-protein correlation was found in sarcoidosis patients. Comparison of the KL-6 levels in which the 568 genotype was ignored rendered 7.5% misclassifications of "elevated" versus "normal" KL-6 levels or vice versa. The MUC1 568 A to G polymorphism may be of interest for diagnostic purposes because our study delivered *in vivo* evidence that it contributes to interindividual variations in KL-6 levels.

Chapter 5

CC10 is one of the most abundantly produced proteins of the lower respiratory tract. Serum CC10 measurements are a useful tool to noninvasively assess the integrity of the air-blood barrier in sarcoidosis. The CC10 A38G promoter polymorphism is known to influence CC10 production. Previously, an association between the CC10 38A allele and low serum CC10 levels had been reported in Japanese sarcoidosis patients. The aim of the present chapter was to confirm this association in a clinically well characterized population of Dutch white patients with sarcoidosis and control subjects. A significant difference in serum CC10 levels was found between the CC10 A38G genotypes in white controls and sarcoidosis patients. Post-hoc testing revealed differences in serum CC10 levels between healthy control subjects with 38AA and 38AG genotype, controls with 38AA and 38GG genotype and controls with 38AG and 38GG genotype. We concluded that serum CC10 levels are significantly associated with CC10 A38G genotype in whites. In order to make a correct interpretation of serum CC10 levels as measure of the alveolar-capillary membrane permeability in sarcoidosis CC10 A38G genotyping should be performed.

Chapter 6

An association between the low CC10 producing 38A allele and sarcoidosis susceptibility has been reported in Japanese patients from Hokkaido. The aim of this chapter was to confirm this association in a clinically well-characterised population of Dutch Caucasian and Kyoto Japanese sarcoidosis patients and controls. No difference in genotype or allele frequency was found between sarcoidosis patients and controls

in either ethnic population. Remarkably, however, a significant difference was found between the controls from Kyoto and Hokkaido, but not between the Japanese sarcoidosis groups. Further review of previously published A38G genotyping results showed a consistent difference in CC10 A38G allele frequencies between Caucasians and Japanese. We concluded that the CC10 A38G polymorphism does not influence sarcoidosis susceptibility in Dutch Caucasians or in Japanese from Kyoto.

7.2 CONCLUDING REMARKS

Biomarkers can be used to diagnose a disease, to monitor disease activity, to assess disease severity, and to predict the course of a disease. We studied these four qualities of pneumoproteins, which are lung specific proteins, in the two most prevalent interstitial lung disease (ILD)s at our department.

Although *krebs von den lungen* (KL)-6 and surfactant protein (SP)-D can help to differentiate bacterial pneumonia from ILD, pneumoproteins have little diagnostic value since these proteins are elevated in various ILDs, and, therefore, lack specificity.[1] Pneumoproteins are for example unable to differentiate sarcoidosis from hypersensitivity pneumonitis (HP; chapter 2 and 3). However, as demonstrated in this thesis, pneumoproteins can be useful to monitor ILD activity and assess disease severity. Although Clara cell protein (CC)10, KL-6 and SP-D had been studied separately in sarcoidosis, we were the first to perform a comparative analysis (chapter 3).[2-4] In sarcoidosis, CC10 and KL-6 reflected disease severity, since they both correlated with radiologic stage and pulmonary function impairment (chapter 3). SP-D did not seem to be useful as serum marker in sarcoidosis (chapter 3). In bird fanciers' lung (BFL), KL-6 and SP-D correlated with disease activity (chapter 2). Serum KL-6 has been used in previous studies to screen for HP in subjects at risk for developing this ILD like farmers and mushroom workers.[5;6] In chapter 2, it was demonstrated that serum KL-6 and SP-D are able to differentiate between subjects exposed to birds without BFL and BFL patients. Therefore, these pneumoproteins could also be used to screen for HP in bird fanciers.

Sarcoidosis is a complex disease thought to be caused by an unknown antigenic stimulus in combination with genetic susceptibility.[7] In the last couple of years many associations have been reported between polymorphisms in various genes and susceptibility to sarcoidosis. Genetic predisposition is, therefore, thought to be determined by the varying effects of several genes. In chapter 6, we did not find a previously described association between sarcoidosis susceptibility and the CC10 adenosine (A)38guanine (G) polymorphism.[8] A review of the literature on the CC10 A38G polymorphism showed a consistently higher percentage of the 38A allele in

healthy Japanese compared to European controls. The healthy Japanese controls from Ohchi's study were the only exception, because their CC10 A38G allele distribution did not differ from European controls. It could be that their control group was not representative for the Japanese population. We believe that, based on the study presented in chapter 6, CC10 is not a sarcoidosis susceptibility gene in Japanese from Hokkaido or Kyoto. In general, it is important to study the influence of a gene polymorphism on disease susceptibility in multiple ethnically and geographically distinct disease and control populations before reaching conclusions. However, it is difficult to publish negative results leading to publication bias. Publication bias is based on the fact that scientists are more likely to submit "positive" results, and journals are less likely to publish "negative" results. Recently, we recognized another bias troubling literature on the genetics of sarcoidosis: citation bias.[9] This bias is caused by the observation that scientists are more likely to cite positive papers, and ignore the conflicting "negative" reports.

Some genetic polymorphisms influence regulatory mechanisms and cause inter-individual differences in protein production. A correlation between serum CC10 and SP-D, and their encoding genes had already been described.[10-12] In chapter 5, we confirmed the CC10 gene-protein association in a Dutch Caucasian population of healthy controls and sarcoidosis patients.[8] Previous studies found a similar association for CC10 in Japanese sarcoidosis patients, and Australian asthmatic children.[8;11] Furthermore, we were the first to demonstrate that serum KL-6 levels are influenced by a mucin (MUC)1 gene polymorphism, and we calculated genotype-specific reference intervals (chapter 4).

Idiopathic pulmonary fibrosis (IPF) is the second most common ILD of unknown origin, which carries a bad prognosis with a five-year survival quite similar to lung cancer. A serum biomarker that predicts clinical outcome in IPF would be useful to stage disease, indicate prognosis and the need for aggressive therapy, and help stratify patients for clinical trials. Previous studies demonstrated that IPF patients with high KL-6 levels have a worse prognosis.[13;14] Future studies are needed to find out whether genotyping the MUC1 568 A/G polymorphism in IPF improves the prognostic value of serum KL-6. In this thesis, we demonstrated some predictive value for serum KL-6 in sarcoidosis (chapter 3), and for both KL-6 and SP-D in BFL (chapter 2). Remarkably, high serum KL-6 levels in sarcoidosis were, similar to the prognostic value in IPF, associated with a less favourable pulmonary disease outcome (chapter 3), while high serum KL-6 levels in BFL were associated with a favourable prognosis (chapter 2).

Interestingly, serum levels of all three studied pneumoproteins in this thesis (CC10, KL-6 and SP-D) are influenced by polymorphisms in their encoding genes. We believe that in the future it will become common practice to use genotype-

specific normal ranges for biomarkers in order to improve their clinical value. The department of clinical chemistry of the St Antonius Hospital already genotypes sarcoidosis patients for an insertion/ deletion polymorphism in the angiotensin-converting enzyme gene in sarcoidosis patients for this purpose. At present, there is too little evidence to recommend pneumoprotein genotyping for clinical purposes. However, we believe that genotype-specific reference intervals should be used in future scientific publications regarding CC10, KL-6 and SP-D.

We conclude that serum pneumoproteins are useful biomarkers for the monitoring of ILD patients. In Table 7.1 we give some recommendations for the use of serum pneumoproteins in ILD. Using genotype-specific reference intervals might improve their clinical value.

Table 7.1 Recommendations for the use of serum pneumoproteins in ILD

	Sarcoidosis	Hypersensitivity pneumonitis	Idiopathic pulmonary fibrosis
Diagnosis	None	None	None
Activity	KL-6	KL-6 and SP-D	KL-6 and SP-D
Severity	CC10 and KL-6	KL-6 and SP-D	KL-6 and SP-D
Prognosis	KL-6	KL-6 and SP-D	KL-6 and SP-D

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7.3 PNEUMOPROTEÏNEN BIJ INTERSTITIËLE LONGZIEKTEN (SAMENVATTING IN HET NEDERLANDS)

De verzamelnaam interstitiële longafwijkingen wordt gebruikt voor aandoeningen van de ruimte tussen de bekleding van de longblaasjes en bloedvaten die verspreid in beide longen tot uiting komen. Het betreft meer dan honderd verschillende ziektebeelden. Sarcoïdose is de meest voorkomende interstitiële longziekte met een geschatte prevalentie van tussen de 10 en 40 per 100.000 inwoners. Extrinsieke allergische alveolitis is een andere, relatief veel voorkomende, interstitiële longziekte verzoorzaakt door de inhalatie van organisch materiaal. In de regio Utrecht zijn vogels een frequente oorzaak van deze longaandoening.

Biomarkers zijn stoffen die kunnen helpen bij het diagnosticeren van een aandoening en/of de activiteit, de ernst en/of de prognose van een ziekte kunnen bepalen. In dit proefschrift hebben we onderzoek gedaan naar zogenaamde pneumoproteïnen. Dit zijn eiwitten die worden geproduceerd in de longen en normaal gesproken slechts in lage concentraties aantoonbaar zijn in het serum. Bij bepaalde ziekteprocessen in het longweefsel kunnen de serum concentraties behoorlijk hoog oplopen.

Het doel van dit proefschrift was het onderzoeken van de bruikbaarheid van deze pneumoproteïnen in het diagnosticeren van interstitiële longziekten en het bepalen van de ziekte activiteit, ernst en prognose. We legden de nadruk op polymorfismen in de genen die coderen voor deze pneumoproteïnen en bestudeerden hun eventuele invloed op serum pneumoproteïnen spiegels.

Hoofdstuk 2

In dit hoofdstuk bepaalden we de waarden van serum kanker van de longen (KL)-6 en surfactant proteïne (SP)-D om patiënten met een vogelhouderslong te onderscheiden van gezonde vrijwilligers met of zonder blootstelling aan vogels. Daarnaast evalueerden we hun vermogen om de activeit van de vogelhouderslong weer te geven en de prognose te bepalen. We analyseerden patiënten met een vogelhouderslong, gezonde vrijwilligers zonder vogelcontact en gezonde vrijwilligers met vogelcontact. KL-6 en SP-D waren significant verhoogd in patiënten vergeleken met gezonde vrijwilligers. Beide markers waren even goed in het onderscheiden van patiënten met een vogelhouderslong en gezonde vrijwilligers. KL-6 en SP-D correleerden met longfunctie parameters. KL-6 correleerde beter met de diffusie capaciteit dan SP-D. Hogere KL-6 waarden waren geassocieerd met verbetering van de diffusie capaciteit na 2 jaar. KL-6 en SP-D daalden na vermijding van vogelcontact. KL-6 en SP-D bleken bruikbare serum markers bij de vogelhouderslong.

Hoofdstuk 3

In dit hoofdstuk bepaalden we de waarde van serum Clara cel eiwit (CC10), KL-6 en SP-D om sarcoïdose te diagnosticeren en hun vermogen om de prognose en ernst van deze ziekte vast te stellen. De drie markers waren onderling gecorreleerd en er werd een significant verschil in serum CC10, KL-6 en SP-D spiegels gevonden tussen patiënten met sarcoïdose en gezonde vrijwilligers. KL-6 differentieerde het beste tussen het al dan niet hebben van sarcoïdose. Significant hogere CC10 en KL-6 spiegels werden gevonden bij sarcoïdose patiënten met parenchym afwijkingen (stadium II en III) vergeleken met patiënten zonder parenchym afwijkingen (stadium I). Daarnaast correleerden CC10 en KL-6 met de diffusie capaciteit en de totale long capaciteit. KL-6 correleerde ook met de inspiratoire vitale capaciteit. Hogere KL-6 spiegels waren zwak maar significant geassocieerd met het persisteren of toenemen van parenchym afwijkingen na twee jaar.

Hoofdstuk 4

Het doel van dit hoofdstuk was het analyseren van een eventuele invloed van het functionele mucine (MUC)1 568 adenosine (A) naar guanine (G) gen polymorfisme op serum KL-6 spiegels in een klinisch goed gekarakteriseerde Nederlands Kaukasische populatie. Een significant verschil werd gevonden in serum KL-6 spiegels van gezonde controles gegroepeerd op basis van het MUC1 568 genotype. Er werden genotype-specifieke referentie waarden voor KL-6 berekend. Een vergelijkbaar verschil werd gevonden in sarcoïdose patiënten. Wanneer geen rekening werd gehouden met het 568 genotype werd 7,5% van de KL-6 spiegels onterecht beoordeeld als verhoogd of normaal. Het MUC1 568 A/G polymorfisme zou van diagnostisch belang kunnen zijn, aangezien onze studie bewijs heeft geleverd dat het bijdraagt aan interindividuele variaties in serum KL-6 spiegels.

Hoofdstuk 5

Serum CC10 kan gebruikt worden om op een noninvasieve manier de integriteit van de lucht-bloed barrière te bepalen in sarcoïdose. Van het CC10 A38G promoter gen polymorphism is bekend dat het de CC10 eiwit productie beïnvloed. In een Japanse studie werd een associatie gevonden tussen het CC10 38A allel en lage serum CC10 spiegels in sarcoïdose patiënten. Het doel van dit hoofdstuk was om deze associatie te bevestigen in een klinisch goed gekarakteriseerde Nederlands Kaukasische populatie. Een significant verschil in serum CC10 spiegels werd gevonden tussen de CC10 A38G genotypes in gezonde vrijwilligers en sarcoïdose patiënten. Er werd een verschil in serum CC10 spiegels gevonden tussen gezonde vrijwilligers met 38AA en 38AG genotype, gezonde vrijwilligers met 38AA en 38GG genotype en gezonde vrijwilligers met 38AG en 38GG genotype. We concludeerden dat serum CC10 spiegels

significant geassocieerd zijn met het CC10 A38G genotype in Nederlanders. Om met behulp van CC10 een goede inschatting te kunnen maken van de doorgankelijkheid van de alveolaire-capillaire membraan in sarcoïdose dient het CC10 A38G polymorfisme gegenotypeerd te worden.

Hoofdstuk 6

In een recente studie werd een associatie gevonden tussen het CC10 A38G gen polymorfisme en de kans op het krijgen van sarcoïdose bij Japanese patiënten uit Hokkaido (een eiland in het noorden van Japan). Het doel van dit hoofdstuk was om deze associatie te bevestigen in een klinisch goed gekarakteriseerde Nederlands Kaukasische en Japanese populatie sarcoïdose patiënten uit Kyoto. Er werd echter geen verschil in genotype - of allel frequencie gevonden tussen deze Japanse en Nederlandse sarcoïdose patiënten enerzijds en gezonde vrijwilligers anderzijds. Het was opmerkelijk dat er wel een significant verschil gevonden werd tussen Japanse controles uit Kyoto en Hokkaido, maar niet tussen de Japanese sarcoïdose patiënten. In de literatuur werd een consistent verschil in CC10 A38G allel frequentie verdeling gevonden tussen tussen Kaukasiërs en Japanners. Onze bevindingen in dit hoofdstuk onderstrepen de noodzaak om de invloed van genetische polymorfismen op vatbaarheid voor een bepaalde ziekte in diverse etnisch en geografisch verschillende populaties te bepalen alvorens conclusies te trekken.

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AstraZeneca®

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Medewerkers St. Antonius ziekenhuis

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7.6 CURRICULUM VITAE

Rob Janssen was born on October 10th in Nijmegen, the Netherlands. From 1988 he received secondary education at the Maas-Waal college in Nijmegen, where he graduated in 1994.

In 1994 he started his medical training at the University Utrecht and was granted the designation of physician in 2000.

From December 2000 until April 2001 he worked as a resident at the department of pulmonology of the St Antonius Hospital, Nieuwegein (head: prof. dr. J.M.M. van den Bosch). Afterwards he worked for two years as a research fellow at the same department, supported by an unrestricted grant from AstraZeneca®. In 2003 he started his official training in respiratory medicine. As part of this he worked as a specialist registrar at the department of internal medicine of Ziekenhuis Hilversum (April 2003-April 2005; head: dr. S. Lobatto), at the department of pulmonology of the St Antonius Hospital (April 2005-May 2006) and at the department of pulmonology of the University Medical Centre Utrecht (May 2006-present; head: prof. dr. J-W. J. Lammers).