Biochemical parameters in monitoring severity of sarcoidosis

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Biochemical parameters in monitoring severity of sarcoidosis

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Biochemical parameters in monitoring severity of sarcoidosis

PROEFSCHRIFT

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Snježana Rothkrantz-Kos
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Promotor

Prof. dr. M.P. van Dieijen-Visser

Co-promotor

Dr. M. Drent

Beoordelingscommissie

Prof. dr. A. Bast (voorzitter)

Prof. dr. P.M. van den Berg-Loonen

Dr. J.M.M. van den Bosch (St. Antonius ziekenhuis, Nieuwegein)

Prof. dr. R.M. Du Bois (Royal Brompton Hospital & National Heart and
Lung Institute, London, UK)

Prof. dr. E.F.M. Wouters

Posvećujem mojim roditeljima i Mauriceu
(Aan mijn ouders en Maurice)

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Abbreviations

All	angiotensin II
A-IIR	angiotensin II receptor
AaPO ₂ max	alveolar-arterial oxygen difference at maximal exercise
ACE	angiotensin converting enzyme
APC	antigen presenting cell
ATS	American Thoracic Society
AUC	area under the curve
BAL	bronchoalveolar lavage
BCGF	B cell growth factor
BCDF	B cell differentiation factor
CD4/CD8 ratio	ratio of T cells bearing predominantly CD4 or CD8 receptor
CSF	colony stimulating factor
DLCO	diffusing capacity for carbon monoxide
ECG	electrocardiogram
EPO	eosinophilic peroxidase
ERS	European Respiratory Society
FVC	forced vital capacity
FEV1	forced expiratory volume in one second
Glc-6-P	glucose-6-phosphate
GR	glutathione reductase
GR-Cr	chromate inhibition test
GPx	glutathione peroxidase
G6PD	glucose-6-phosphate dehydrogenase
GSH/GSSG	glutathione system (GSH, reduced form; GSSG, oxidized form)
H ₂ O ₂	hydrogen peroxide
HLA	human-leucocyte-associated
HRCTtot	high-resolution computer tomography total score
(Hs)-CRP	(high-sensitivity)-C-reactive protein
IFN- γ	interferon γ
IL	interleukin
iNOS	inducible nitric oxide synthase
MAF	macrophage activating factor
MCP-1	monocyte chemotactic peptide-1
MCF	monocyte chemotactic factor

MHC	major histocompatibility complex
MIF	macrophage inhibition factor
MØ	macrophages
MPO	myeloperoxidase
MRI	magnetic resonance imaging
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappaB
NO	nitric oxide
NPV	negative predictive value
O ₂ ^{•-}	superoxide radical
OH [•]	hydroxyl radical
ONOO ⁻	peroxynitrite
ORDAC	over range detection and correction
PaO ₂ rest	arterial oxygen tension at rest
PG-E2	prostaglandin E2
PPV	positive predictive value
RFI	respiratory functional impairment
SAA	serum amyloid A
Sens	sensitivity
SOD	superoxide dismutase
Spec	specificity
sIL-2R	soluble interleukin-2-receptor
TCR Vβ	T-cell receptor Vβ
TNFα	tumor necrosis factor α
VD	activated vitamin D (1α, 25-dihydroxy form)
X ⁻	halide ion (Cl ⁻ , Br ⁻)
WASOG	World Association of Sarcoidosis and Other Granulomatous Disorders

Chapter 1

General introduction

Introduction

It is well established that sarcoidosis is a multisystem disorder of unknown cause(s). To date, no organ is immune to sarcoidosis. Granulomas, usually noncaseating but occasionally necrotizing, are the pathological hallmark of sarcoidosis. Granulomas are structured masses composed of activated macrophages and their derivatives, *i.e.* epithelioid and giant cells^{1,2}. Granulomas are also features of many other (interstitial lung) disorders, *e.g.* extrinsic allergic alveolitis or hypersensitivity pneumonitis, drug-induced pneumonitis, chronic berylliosis, and Crohn's disease. Sarcoidosis subsides in most cases, but it may worsen and become chronic in others. Pulmonary problems may persist, but also devastating extrapulmonary complications may become apparent. Appropriate management of sarcoidosis is mandatory as it predominantly affects fairly young adults (between 20 - 40 years). To date, it requires the attention of pulmonologists as well as specialists from other medical disciplines. Therefore, a multidisciplinary approach is recommended that pays attention to the many aspects of this erratic disorder³.

Sarcoidosis

Etiology and epidemiology

Sarcoidosis is a disseminated, granulomatous disease of unknown etiology that occurs throughout the world. To date, it is the second most common respiratory disease in young adults after asthma. Estimates of prevalence range from 1 to 50 per 100.000 individuals, varying among ethnic and racial groups⁴. The disease is most common among North Americans of African heritage and Northern European Caucasians (The Netherlands and Germany: 40 - 50 cases per 100.000). Various infectious, organic, and inorganic agents are considered to be responsible for a granulomatous reaction in susceptible hosts⁴⁻⁷. It is highly likely that a genetic susceptibility to the development of sarcoidosis exists⁸⁻¹¹.

Clinical presentation

The clinical course of sarcoidosis is highly variable and unpredictable. Virtually every organ can be involved. The lungs are affected in over 90% of patients with sarcoidosis⁴. It also frequently affects the lymph nodes, skin, and eyes.

The acute form is known as Löfgren's syndrome, defined by an acute onset of symptoms of fever, erythema nodosum, hilar lymph adenopathy with or without arthralgia. To date, sarcoidosis patients not only suffer from symptoms related to the lungs (*e.g.* cough, breathlessness), but they may also suffer from a wide spectrum of other symptoms^{4,12}. These symptoms include persistent fatigue, arthralgias, muscle pain, weight loss, skin lesions, eye problems, and neurological as well as cardiological problems^{4,12,13}. These rather non-specific symptoms are disabling for the patient, may become chronic and causing an impaired quality of life¹³⁻¹⁹. Fatigue, as an integral part

of the clinical picture of sarcoidosis, has gained some attention in the literature^{12,20-24}, although the exact mechanism is still unknown.

Generally, spontaneous remission occurs in 75–85% of the cases within two years. However, the disease can also become chronic and progressive, estimated in up to 15–25% of the cases. Failure to regress spontaneously within two years is considered to predict a chronic course of the disease. Overall, the mortality from sarcoidosis is 1–5%. Treatment of sarcoidosis consists largely of immunosuppressive drugs such as steroids, methotrexate, cyclosporin and, more recently, anti-TNF α (Infliximab)^{25,26}.

Diagnostic work-up and follow-up

A pulmonary physician generally coordinates management of sarcoidosis. However, sarcoidosis can involve any organ. The diagnostic work-up and follow-up of the disease is mainly monitored by assessing clinical features and using auxiliary diagnostic procedures. According to the latest international recommendations, the diagnostic work-up includes attention to the medical history, relevant exposures, imaging procedures, laboratory tests, bronchoalveolar lavage²⁷ and, if appropriate, evaluation of biopsy specimens⁴. According to the latest recommendations as published by Baughman *et al.*, diagnosis of sarcoidosis can be established by means of the following criteria:²⁸

- The presence of granulomas in biopsy specimen without evidence of tuberculosis, fungus, malignancy or other causes of granuloma together with clinical features of sarcoidosis support the diagnosis.
- In the absence of biopsy material, clinical features such as bilateral hilar adenopathy on chest radiography, erythema nodosum, uveitis and macular papular skin lesions are suggestive of sarcoidosis.

If there is no evidence of an alternative diagnosis and additional features highly consistent with sarcoidosis are present such as raised angiotensin-converting enzyme (ACE), bronchoalveolar lavage (BAL) fluid lymphocytosis and/or lupus pernio (cutaneous manifestation of sarcoidosis frequently on the nose), the diagnosis can also be confirmed^{4,28}.

The recommended clinical tests in the management of sarcoidosis are presented in Table 1.1. Among others, lung function test results are used to guide the therapeutic approach. Depending on the severity of the inflammation in the lung, lung function tests may reveal restricted lung volumes and/or impaired diffusion capacity and mildly decreased arterial oxygen pressure at exercise. Pulmonary and mediastinal lymph node involvement is found in up to 90% of patients, which is easily recognizable on chest radiography²⁹. Intrathoracic involvement can vary from sole bilateral hilar lymphadenopathy to extensive parenchymal disease and fibrosis. In general, chest radiographic appearances have been divided into four 'stages' or types according to the modified Scadding criteria: stage 0, no lung involvement; stage I, bilateral hilar enlargement alone; stage II, hilar enlargement in association with interstitial lung disease; stage III interstitial lung disease alone; and stage IV (a more recent addition to the original classification), requires radiographic evidence of lung fibrosis³⁰.

Table 1.1. Recommended initial evaluation of patients presenting with sarcoidosis^{4*}.

1.	History	Exposure: - occupational - environmental Symptoms
2.	Physical examination	
3.	Chest radiography (postero-anterior)	
4.	Pulmonary function tests	Spirometry DLCO
5.	Peripheral blood counts	White blood cells Red blood cells Platelets
6.	Serum chemistries	Calcium Liver enzymes: - alanine aminotransferase - aspartate aminotransferase - alkaline phosphatase Creatinine Blood urea nitrogen
7.	Urine analysis	
8.	ECG	
9.	Routine ophthalmologic examination	
10.	Tuberculin skin test	

* In the initial evaluation of sarcoidosis no ACE measurement was recommended.

Disease activity and severity

In general the state of a disease can be classified either by activity and/or severity. Unlike other disorders, activity in sarcoidosis does not necessarily indicate a progressive course, a fatal prognosis, or the need for medical treatment³¹.

Moreover, there is a lot of discussion about the definitions of both, as it is very difficult to make a clear distinction between these two entities. Besides, there is overlap between the two. The activity is defined in the latest World Association of Sarcoidosis and other Granulomatous Disorders (WASOG) statement on sarcoidosis (1999). According to the statement, clinical activity is assessed on the basis of onset, worsening, or persistence of symptoms or signs directly related to sarcoidosis. The statement, however, does not give a clear recommendation on how to define the activity (Table 1.2.)⁴. Unfortunately, the term "activity" has in the past been used freely and not always systematically³².

Inconsistency exists also on how to define severity. Changes in plain chest radiography and lung function tests have traditionally been used as the main indices of change of respiratory function indicating pulmonary severity³². Extrapulmonary manifestations such as cardiac, neurological or ocular lesions often also represent a more severe course of the disease. Many new parameters are evaluated as activity parameters, whereas the concept of severity is left out. Recently, some studies evaluated new parameters with the background of severity³³⁻³⁵. Moreover, among others one genetic study appeared which showed that human leukocyte antigen (HLA)-DQB1 allele *0201 is strongly protective against severe sarcoidosis⁸.

Until now, far less attention has been paid to severity of the disease and the appropriateness of the various markers to detect severity. From a clinical point of view, it is of more importance to depict whether the disease is severe, *i.e.* will have a more or less chronic course than the initial activity. For each appropriate evaluation of the diagnostic accuracy of any marker a clear definition of the condition such as activity or severity is needed. However, a problem arises in case of sarcoidosis.

Therefore, one of the aims of this thesis was to search for new markers, which could possibly reflect disease severity in sarcoidosis. In case of pulmonary sarcoidosis disease severity was defined through the presence of respiratory functional impairment.

The ideal marker(s) of disease severity should be able to predict subsequent organ dysfunction. However, such markers are not identified by now³².

Table 1.2. Markers of disease activity in sarcoidosis⁴.

Clinical	Biochemical or instrumental	Imaging
Fever	Serum ACE	Progressive changes on chest radiographs or lung CT scans
Eye involvement uveitis	Hypercalcemia	Ground-glass attenuation on HRCT scan
Skin involvement - Erythema nodosum - Lupus pernio - Changing scar	Worsening lung function	Positive ⁶⁷ Ga uptake (panda/labda sign)
Joint involvement - Polyarthralgia	BAL fluid: lymphocyte alveolitis and high CD4/CD8 ratio	Fluorescein angiography of the eyes
Splenomegaly	Abnormal ECG, echocardiogram or thallium scan	MRI or CT scan abnormalities
Enlarged lymph nodes (lymphadenopathy)		
Salivary and lacrimal gland enlargement	Abnormal liver function tests	Bone cysts
Myocardial disease		
Unilateral facial paralysis or other neurological symptoms/signs		
Progressive respiratory symptoms (dyspnea, cough)		

Molecular changes in sarcoidosis

Inflammation that does not resolve within a matter of days (by means of “natural immunity”), becomes chronic, as is the case in sarcoidosis. Antigens are than usually presented to the lymphocytes by antigen presenting cells (APC). These APC's (dendritic cells, macrophages and sometimes B cells) trap antigens, concentrate them, prevent their spread and initiate local immune response.

Before an antigen can be presented to a T cell, it must be processed by an APC. This involves the enzymatic degradation of the antigen and anchoring to highly polymorphic class I (variants of HLA -A, -B, -C) or class II (variants of HLA -DP, -DQ and -DR) major histocompatibility complex (MHC) molecules³⁶⁻³⁹. In sarcoidosis associations with the different HLA molecules have been observed, mainly with the subtypes of HLA-B, HLA-DR and HLA-DQ⁸⁻¹⁰. MHC class I interact with CD8⁺ T cells, whereas MHC class II interact with CD4⁺ T cells (see further)³⁶⁻³⁹.

After the presentation of antigen by the APC and reaction with a processed antigen, T cells go through stages of activation, growth and differentiation and secrete a variety of mediators (cytokines). These cytokines are especially important in regulating the function of other lymphocytes. T cells express particular accessory molecules on their cell surface that are important to their function but can also be used to classify them. The T cells expressing CD4 molecules (*i.e.* T-helper cells) are involved in either cell-mediated immunity (Th1 cells, also called delayed hypersensitivity T cells) or promote the activation, growth and differentiation of B cells (Th2 cells). In contrast T cells expressing CD8 molecules, either counterbalance the effects of T-helper cells and suppress immune response (T-suppressor cells) or are involved in the killing of other cells (cytotoxic-T cells). The measurement of T cells expressing either CD4 or CD8 has become clinically important as changes in their balance (CD4/CD8 ratio) may be an indication of a disease³⁶⁻³⁹.

In the sarcoid lung, the earliest pathologic finding is a mononuclear alveolitis composed of increased CD4⁺ T-helper lymphocytes (with an increased CD4/CD8 helper/suppressor ratio), monocyte-macrophages and some B-lymphocytes^{4,40}.

Also in sarcoidosis, the consequence of antigen presentation to specific T-lymphocytes is amplification of the immune response through proliferation of T-lymphocytes and their production of cytokines^{4,28,41}. The APC-lymphocyte conjugation transduces a signal to the lymphocyte to express IL-2 receptors on T-lymphocyte cell surfaces and release IL-2 (Figure 1.1). The binding of IL-2 to IL-2 receptors causes proliferation of IL-2-responsive lymphocytes. The proliferating T-helper cells command the assembly of the granuloma through production of various molecules^{4,28,41,42}. In fact, in the early stage of the disease, the local tissue inflammation in sarcoidosis is dominated by expression of Th1-cytokines (mainly IL-2, interferon- γ (IFN- γ) and tumor necrosis factor- β (TNF- β))⁴³⁻⁴⁹.

Figure 1.1. Pathophysiological processes in patients with pulmonary sarcoidosis (adapted with permission from Nagai *et al.*⁴¹).

MØ = macrophages; IL-1 = interleukin-1; IL-2R = IL-2 receptor; BCGF = B cell growth factor; BCDF = B cell differentiation factor; MCP-1 = monocyte chemotactic peptide-1; MCF = monocyte chemotactic factor; MIF = macrophage inhibition factor; MAF = macrophage activating factor; IFN- γ = interferon γ ; PG-E2 = prostaglandin E2; A-II = angiotensin II; A-II-R = angiotensin II receptor; VD = activated vitamin D (1 α , 25-diOH-form); TCR V β = T cell receptor V β .

Sarcoid lung T-lymphocytes spontaneously release several cytokines such as soluble monocyte chemoattractant factor (MCF) to attract the monocytes. Monocytes are the predominant cellular resource required for granuloma architecture. Recruited monocytes become activated and sequentially differentiate into macrophages, epithelioid cells, and giant cells. These granuloma structures (mainly epithelioid cells) are able to produce angiotensin-converting enzyme and the active form of vitamin D as shown in Figure 1.1. An increased production of oxygen peroxide (indicating presence of oxidative stress) and prostaglandin E2 has also been observed (Figure 1.1)^{41,50}.

The net effect of the Th1 (IFN- γ dominant) response is the organization of the local inflammatory process into the granuloma and an inhibition of fibrogenetic processes⁴⁶. In addition to the T cell activation also some B cell activation occurs which may result in hypergammaglobulinemia⁵¹.

Nevertheless depending on the host susceptibility, a switch to type 2 (IL-4-dominant) may occur in patients with progressive sarcoidosis who evolve towards lung fibrosis^{46,52}. The identity of molecules and receptors that drive the Th1/Th2 balance at sites of disease activity remains controversial⁴⁶.

Outline of the serum markers evaluated in this thesis

Acute phase response

As a consequence of the inflammatory stimuli, IL-1 and IL-6 are produced, which stimulate liver production of C-reactive protein (CRP) and serum amyloid A (SAA), among other proteins.

C-reactive protein

C-reactive protein (CRP) is an acute-phase protein featuring a highly conserved structure⁵³. Expression of CRP is regulated mainly at the transcriptional level with IL-6 being the principal inducer of the gene during the acute phase. It is built up of five identical subunits, aggregated in a symmetric pentameric form by noncovalent binding between the subunits. Each subunit has the ability to bind two calcium ions so that a calcium-dependent specific binding of a ligand is possible. The most avid ligand is phosphocholine (PCh). The PCh is widely distributed in pathogens and in cellular membranes^{53,54}.

CRP is involved in opsonization and phagocytosis through two routes: activation of the classical pathway of the complement and through the direct binding to the Fc fragment of the IgG receptors. Interestingly, expression of Fc receptors was shown to be suppressed in alveolar macrophages from patients with sarcoidosis⁵⁵.

Clearance of CRP is monoexponential and independent of the serum concentration or pathophysiological circumstances, what normally makes the CRP a good marker of disease activity.

The measurement of plasma or serum CRP can help differentiate inflammatory from non-inflammatory conditions⁵³. In rheumatoid arthritis and pancreatitis CRP measurement has prognostic value^{53,56}. Since inflammation is believed to play a role in the pathogenesis of cardiovascular events, measurement of CRP has been proposed for the prediction of the risk of these events^{57,58}. Also in sarcoidosis CRP appeared to be increased especially in acute disease³⁴.

Serum amyloid A

Serum amyloid A (SAA) is a polymorphic protein that consists of four genetically determined isotypes. Isotypes SAA1 and SAA2 are synthesized in response to inflammatory cytokines and present together the SAA response measured as a part of the acute phase reaction^{59,60}. The gene encoding SAA3 is not expressed, whereas SAA4 present a constitutive apolipoprotein in HDL, and it does not behave as acute phase reactant.

SAA (SAA1 and SAA2) synthesis is induced primarily in the liver, by the stimulation of inflammation-related cytokines such as IL-1, IL-6 and TNF- α ^{61,62}. However, it appears that there is also some extrahepatic SAA synthesis. The plasma concentration of SAA changes can be very large due to inflammatory stimuli (1000x increase). The major role of SAA appears to be preserving homeostasis by preventing further progress of injuries or by repairing damaged tissues. This mainly because some of the following

functions have been attributed to SAA: 1. inhibition of antibody production by lymphocytes, 2. inhibition of platelet agglutination; 3. inhibition of the oxidative burst reaction in neutrophils; 4. induction of collagenase and 5. chemotaxis of neutrophils and monocytes^{59,60}.

Immediately after SAA is released from the cells, it binds with high-density lipoproteins (HDL) and circulates throughout the system. The role of HDL is to remove cholesterol from the tissues. Circulating HDL acquires cholesterol by extracting it from cell surface membranes and converts it into cholesterol esters through the action of lecithin cholesterol acyltransferase (LCAT), an enzyme that is activated by the HDL component apoA-I. In the circulation, SAA is rapidly cleared. Inflammatory conditions that are accompanied with high SAA concentrations greatly alter the HDL composition, reducing levels of apoAI, and increasing those of SAA. Reduction of apoAI is due to lowered synthesis in the liver and probably displacement from HDL by SAA^{59,60}, which has been shown in sarcoidosis, too⁶³.

In drawn plasma, almost all SAA is present in high molecular form (most in HDL and little in other lipoproteins). Most SAA assays are directed to SAA in HDL, and not to SAA monomer, which reacts with antibodies differently from the lipid-bound form. Furthermore, for measurement of acute phase SAA, cross-reactivity of antibodies with SAA4 must be avoided.

Clinical value of the SAA has been established in various diseases. During viral infections, SAA appeared to increase more intense as compared to CRP^{59,64,65}. In some diseases, such as Crohn's disease and ulcerative colitis, CRP remained even normal, whereas SAA increased⁶⁶⁻⁷⁰. In sarcoidosis, SAA appeared to be increased in patients with active disease⁶³. To date, one of the strongest advantages of SAA above CRP has been demonstrated in kidney transplantation patients on immunosuppressive steroid therapy. In this later population only SAA appeared to be useful in monitoring acute rejection reactions, because in contrast to CRP, SAA expression is not influenced by corticosteroids^{59,71,72}.

For neither SAA or CRP, no diagnostic accuracy studies or studies aimed to predict fatigue in sarcoidosis have been reported by now.

High intensity alveolitis

Activation of T cells leads to the expression of IL-2 receptors on the cell surface and induces release of soluble IL-2 receptor (sIL-2R) molecules into the circulation. Besides, locally in the lung the redistribution of the cell-types occurs with increase in CD4-cells in the BAL.

Soluble IL-2R

Proliferation of T cells is controlled mainly by IL-2 and the recently discovered IL-15⁷³. For messages to be transmitted from cell to cell, IL-2 must bind to the IL-2R on the receiving cell. The receptor is expressed only on activated T cells and consists of three transmembrane protein chains: α (CD25), β (CD122), and γ (CD132). The α -subunit must associate with the β -subunit to form the IL-2 binding site. Binding of IL-2 with the receptor transduces a signal across the cell membrane and triggers T-lymphocytes to undergo clonal expansion⁷³⁻⁷⁷.

The α -subunit of the IL-2R is expressed on activated T cells and at lower levels on activated B cells, natural killer cells, and tumor cells^{76,77}. The IL-2R immunoassay is designed as an assay to measure this α (CD25) subunit (sometimes also called anti-Tac assay), as a soluble part of the IL-2R complex receptor (sIL-2R).

Increased levels of sIL-2R have been observed in lymphoproliferative disorders, neoplastic diseases and a variety of inflammatory and immunological disorders such as idiopathic pulmonary fibrosis, tuberculosis, leprosy etc.⁵⁰

It has been suggested that sIL-2R might be useful in monitoring pulmonary as well as extrapulmonary involvement in sarcoidosis^{34,35,50,77}. However, no appropriate diagnostic accuracy studies have been performed yet.

Granuloma formation

Angiotensin converting enzyme

Angiotensin I-converting enzyme (ACE), is a dipeptidyl-carboxypeptidase, mostly present on the luminal surface of the vascular endothelium.

The product of the enzymatic action of ACE is angiotension II, which is chemotactic for macrophages and may enhance the phagocytosis and accessory function of alveolar macrophages in sarcoidosis⁵⁰.

Physiologically, ACE is the key-enzyme of the renin-angiotensin system. Angiotensin II is a potent vasoconstrictor, which also stimulates adrenal production of aldosterone (sodium ion retention). Furthermore, ACE is able to cleave vasodilator bradykinin and some other neuronal peptides, inactivating them thereby^{50,78}.

Angiotensin II regulates cellular proliferation, inflammation and endothelial function through the activation of reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases⁷⁹. Namely, angiotensin II activates NAD(P)H-dependent oxidases and these form the most important vascular source of superoxide (O_2^-)^{80,81}, which stimulates nuclear factor- κ B (NF- κ B) to induce proinflammatory cytokines^{79,80}. The NF- κ B appears also to play a role in sarcoidosis⁸²⁻⁸⁴.

In sarcoidosis, ACE is mainly produced by activated granuloma cells (epithelioid cells). Therefore, elevated serum ACE concentrations reflect the total body granuloma burden. However, serum ACE correlates only roughly with the number of organs involved and the number of extrapulmonary sites as assessed by gallium scans⁵⁰. Besides ACE concentrations appeared to have poor predictive value in sarcoidosis^{34,85-87}.

There might be several reasons for that. Firstly, its less good performance might, at least for a part, be explained by the fact that ACE concentrations can be influenced by ACE polymorphism (I/D polymorphism in intron 16 of the ACE gene)^{88,89}. Therefore, adjustment of the reference values for the ACE polymorphism has been suggested^{89,90}. Nevertheless, so far, with respect to ACE polymorphism and susceptibility to disease progression, inconclusive data have been reported^{91,92}.

Secondly, ACE increase can be due to familiar ACE increase⁹³⁻⁹⁵. Thirdly, ACE increase is common in many other diseases, which explains its low specificity for sarcoidosis⁷⁸.

In spite of all of these limitations, ACE is still the only serologic parameter recommended by WASOG in sarcoidosis⁴. ACE is stated to be valuable in several

aspects: confirmation of suspected diagnosis, estimation of granuloma burden of the organism and follow-up of the disease during treatment⁴.

Oxidative stress and sarcoidosis

General facts about oxidative stress

Reactive oxygen and nitrogen species (ROS and RNS) are ubiquitous compounds produced by several types of reactions that can cause extensive damage to lung tissue. Enhanced ROS production was also demonstrated in sarcoidosis^{96-101,102}. The ROS include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$). Among various RNS, one of the most toxic species is peroxynitrite ($ONOO^{\cdot}$), derived from the reaction of $O_2^{\cdot-}$ and the nitric oxide radical ($NO^{\cdot}$)¹⁰³⁻¹⁰⁵. The $NO^{\cdot}$ is produced in high concentrations by the inducible nitric oxide synthase (iNOS), at sites of chronic inflammation.

During inflammation, ROS are produced through the process of so-called respiratory burst. The respiratory burst is an accelerated and increased O_2 uptake by neutrophils and macrophages, which can be ten to twenty times the "resting" O_2 consumption of neutrophils¹⁰⁴. The O_2 uptake is due to the activation of the NADPH oxidase enzyme complex. The activated complex oxidizes NADPH into $NADP^+$ in favor of the reduction of O_2 into $O_2^{\cdot-}$.

In the respiratory burst myeloperoxidase (MPO) and eosinophilic peroxidase (EPO) are important enzymes involved, which produce very potent oxidant hypochlorous acid (HOCl), used for microbial killing.

MPO is an abundant protein of neutrophils, stored in the azurophilic granules of neutrophils and released during phagocytosis^{106,107}. Important lung enzymes like acetylcholinesterase, glutathione-S-transferase and alpha-1-antiproteinase can be modified by MPO in a way that they lose their enzymatic properties¹⁰⁸. An overview of the relationship between the most prominent reactive oxygen or nitrogen species is given in Figure 1.2A.

Under normal conditions, toxic oxygen metabolites are generated at a low level in lung cells by the transfer of a single electron during aerobic metabolism (respiratory chain for ATP production) in mitochondria.

Enzymatic systems such as NADPH oxidase, cytochrome P-450, monoamine oxidase, lipoxygenase and arachidonic acid metabolism represent another type of source of toxic oxygen species.

Lung antioxidants

The lung has to control the redox-balance in order to maintain normal cellular function. To be able to do that, it possesses an integrated antioxidant system.

Superoxide-dismutase (SOD) is an ubiquitous enzyme which catalyses conversion of $O_2^{\cdot-}$ radicals to H_2O_2 . The toxic H_2O_2 can be converted into H_2O in an enzymatic reaction by catalase. However, a central mechanism for reduction of H_2O_2 is the glutathione system (GSH, reduced form; GSSG, oxidized form of glutathione) in a

reaction catalyzed by glutathione peroxidase (GPx). The reaction catalyzed by GPx complements catalase as a reducing system for H_2O_2 but exceeds catalase in its capacity to eliminate additional varieties of toxic peroxides. Therefore, the cells try to maintain a high intracellular GSH:GSSG ratio.

In principle, oxidative stress can result in adaptation or cell injury¹⁰⁴. The adaptation can result in up-regulation of the synthesis of antioxidant defense systems. Such an adaptation has been shown in sarcoidosis patients reflected through an increased expression of catalase and superoxide dismutase^{109,110}.

The enzyme glutathione reductase (GR) maintains high intracellular concentrations of GSH by reducing the oxidized form of glutathione, GSSG, back to the reduced form, GSH. For this reaction, the reducing equivalents are derived from NADPH¹¹¹⁻¹¹⁴. The major pathway to generate NADPH is the hexose monophosphate shunt (HMS). In this pathway, glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme^{115,116}. In erythrocytes this is the only pathway to generate the NADPH. An overview of the enzymatic antioxidants is given in Figure 1.2B.

Additional antioxidants present in the lungs include ceruloplasmin, transferrin, ascorbate (vitamin C), alfa-tocoferol (vitamin E), ferritin, other serum proteins, and small molecules such as bilirubin.

A. Generation of reactive oxygen species

B. Antioxidative enzymatic systems

Figure 1.2. Generation of reactive oxidative species and the relation to antioxidative enzymatic systems.

$\text{O}_2^{\bullet -}$ = superoxide, H_2O_2 = hydrogen peroxide, $\text{OH}^{\bullet}$ = hydroxyl radicals, $\text{ONOO}^{\bullet}$ = peroxynitrite, NADPH = nicotinamide adenine dinucleotide phosphate, MPO = myeloperoxidase, EPO = eosinophilic peroxidase, SOD = superoxide-dismutase, GSH/GSSG = glutathione system (GSH, reduced form; GSSG, oxidized form), GPx = glutathione peroxidase, GR = glutathione reductase, $\text{NO}^{\bullet}$ = nitric oxide radical, X^- = halide ion (Cl^- , Br^-), Glc-6-P = glucose-6-phosphate

Scope and aims of the study

The studies presented in this thesis were all performed in serum samples or in erythrocytes.

When features of sarcoidosis, for example radiological abnormalities and lung function impairment, resolve during treatment, disabling symptoms such as fatigue and pain may persist. Moreover, consensus about laboratory findings to depict disease activity or severity in sarcoidosis is still lacking. Overall this thesis concentrates on severity of sarcoidosis and the concept of activity has been left out from the evaluation.

The first aim of the studies presented in this thesis was to evaluate the clinical value of four different enzymatic markers, C-reactive protein (CRP), serum amyloid A (SAA), soluble interleukin-2-receptor (sIL-2R) and angiotensin converting enzyme (ACE). Firstly, the diagnostic accuracy of these markers to predict sarcoidosis severity was evaluated (CHAPTER 5). Secondly, the relationship of these markers and other clinical data with one of the major problems of sarcoidosis patients, *i.e.* fatigue, was studied (CHAPTER 6).

Prior to this, the methods used to measure these markers were evaluated and presented across CHAPTERS 2-4. In CHAPTER 2 and 3 the performance and agreement between the various CRP and hs-CRP methods were examined. In CHAPTER 4 the analytical performance and reference values for sIL-2R and SAA are presented.

The second aim of the studies presented in this thesis was to study several aspects of oxidative stress and its relationship with sarcoidosis.

Recently, it was hypothesized that oxidative stress might be involved in the pathogenesis of sarcoidosis. Among others, glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GR) are important antioxidant enzymes. These two enzymes are closely related. G6PD is the enzyme responsible for the regeneration of reduced nicotinamide adenine dinucleotide phosphate (NADPH). GR is involved in the regeneration of reduced glutathione (GSH), using the NADPH from the reaction catalyzed by G6PD. Indirectly, NADPH concentration can be measured by chromate-inhibition test. The state of these enzymes and indirectly the NADPH concentrations in the erythrocytes of the patients with sarcoidosis were analyzed in CHAPTER 7.

Previously, it was found that polymorphonuclear neutrophils might explain, at least in part, for severity in sarcoidosis. Myeloperoxidase (MPO) is considered as the most abundant enzyme of the neutrophils. Therefore, two functional polymorphisms, -463 G/A and -129 G/A, able to modify the expression of MPO were examined in sarcoidosis patients. Furthermore, their relationship with the severity of sarcoidosis was examined (CHAPTER 8).

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Chapter 2

High-sensitivity C-reactive protein methods examined

Snježana Rothkrantz-Kos, Maria PJ Schmitz, Otto Bekers,
Paul PCA Menheere, Marja P van Dieijen-Visser

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Abstract

Background

High sensitivity CRP (hs-CRP) is considered as an important risk factor for coronary heart disease. Until now hs-CRP has primarily been used for research purposes, but implementation, as a routine laboratory parameter is obvious. In daily practice one CRP method giving reliable results both in the low and high concentration range is to be preferred.

Methods

A new hs-CRP method developed for the Beckman Coulter IMMAGE[®], called CRPH, and measured by near infrared particle immunoassay (NIPIA) was evaluated and compared to the IMMULITE hs-CRP method from Diagnostic Products Corporation and to the BNA hs-CRP method from Dade Behring. In the higher range the hs-CRP methods were also compared to our routinely used CRP method performed by immunoturbidimetry on the Synchron LX[®]20. Besides, hs-CRP IMMAGE method was also compared to the current IMMAGE[®] CRP method (all three from Beckman Coulter).

Results

In the lower range, 0.2 – 10 mg/L, the CRPH IMMAGE[®] method was linear down to 0.2 mg/L and not influenced by turbidity. Total imprecision (CV) was less than 10% in the range from 0.2 – 40 mg/L. The CRPH IMMAGE[®] method showed good agreement with BNA and IMMULITE hs-CRP methods.

Above 100 mg/L Bland Altman plots showed highly scattered data for all CRP comparisons.

Conclusion

The new IMMAGE[®] hs-CRP is a good method for clinical risk stratification. However, for all CRP and hs-CRP methods poor correlations are seen in the upper part of the manufacturers claimed reference ranges. Therefore, at present the use of two different methods, a hs-CRP for risk stratification and a normal routine CRP method, is required.

Introduction

C-reactive protein (CRP) is a valuable laboratory parameter in the clinical evaluation of infection, inflammation or trauma. Several clinical studies have demonstrated that this acute phase protein also has prognostic value in patients with acute coronary syndromes and is moreover a strong independent predictor of future coronary events in apparently healthy subjects. Rifai and Ridker recently reviewed clinical studies on CRP as a prognostic parameter in coronary heart disease¹.

In most laboratories CRP measurements are performed by immunoturbidimetry on routine clinical chemistry analyzers, having detection limits for CRP of 2–5 mg/L. For studies on CRP as a risk factor for coronary artery disease, a high-sensitivity CRP (hs-CRP) method is required. A variety of methods have now been introduced for measuring hs-CRP and Roberts *et al.* recently compared the different methods^{2,3}. In their first study, comparing four different methods, both normal and increased values in the range of 0–250 mg/L, were compared². In their second study, nine hs-CRP methods were compared in 388 apparently healthy individuals and comparisons were focused on values in the range of 0–10 mg/L³. Hs-CRP assays should indeed distinguish the minor increases from the upper limit of normal. However, until now hs-CRP has primarily been used for research purposes and introduction, as a routine laboratory parameter, is obvious. The laboratory has to be sure to report reliable CRP results regardless of the clinical context; therefore also for the upper part of the manufacturers claimed measuring ranges (varying from 0.2 to 500 or 1000 mg/L), agreement between hs-CRP and CRP methods is required.

We report here the evaluation of a new hs-CRP method for the Beckman Coulter IMMAGE. This new method is compared to two hs-CRP methods (IMMULITE and BNA) and in the higher range also to our routine CRP method, on the routine clinical chemistry analyzer LX20 from Beckman Coulter as well as the current IMMAGE CRP method.

Materials and methods

Samples

Venous blood samples were collected from 291 ostensibly healthy blood donors, 177 male and 114 female, presenting at the Sanguin Blood Bank in Maastricht. Samples for method comparison were collected from 521 patients in whom a CRP was requested for routine analysis. The Medical Ethical Committee of the Hospital approved the procedure followed.

Serum was separated from the red cells by centrifugation and stored at -70°C until analysis⁴. There was no freeze-thawing effect as reported previously⁵.

Methods

Hs-CRP on the IMMULITE Automated Analyzer from Diagnostic Product Corporation is a two-site chemiluminescent enzyme immunometric assay, with one ligand labeled anti-CRP monoclonal antibody and an alkaline phosphatase-labeled anti-CRP polyclonal antibody (Diagnostic Product Corporation, Los Angeles, CA, cat no LKCR1).

Hs-CRP on the BNA nephelometer from Dade Behring is performed by particle enhanced immunonephelometry, using a monoclonal antibody coated to polystyrene particles (Dade Behring, Liederbach Germany, N Hs CRP, cat no OQIY G13; supplement reagent OUMU15).

CRP on the IMAGE[®] Immunochemistry System from Beckman Coulter is measured by peak rate nephelometry with a laser diode at 670 nm. A polyclonal anti-CRP antibody is used (Beckman Coulter, cat no 447280).

The new hs-CRP on the IMAGE[®] (called CRPH) is measured by a near infrared particle immunoassay (NIPIA) with a laser diode at 940 nm, using a polyclonal and monoclonal anti-CRP (goat and mouse) antibody coated to latex particles (Beckman Coulter, Inc. Fullerton, CA, US, cat no 474630).

CRP analysis on the Synchron LX[®]20 System, a routine clinical chemistry analyzer from Beckman Coulter, is based on immunoturbidimetry, using a polyclonal anti-CRP antibody (Beckman Coulter, Inc. Fullerton, CA, US, cat no 465131). In this study, values below 5 mg/L are reported as 4.999 mg/L.

Table 2.1 presents the assay characteristics of the different methods used.

Samples for imprecision studies were prepared from five serum pools in the range from 0.2–50 mg/L, and from two serum pools in the range from 100–350 mg/L as determined on the IMAGE. Within-run precision was obtained by measuring one sample 20 times within a single run. Between-run precision was obtained by measuring each level on 20 consecutive days, based on a single calibration.

Table 2.1. Characteristics of the different CRP methods.

Analyzer	Method	Dilution	Assay range	Manufacturer's claimed	
				Measuring range	Detection limit
IMMULITE BNA	hs-CRP	1:100	0.10 – 500	0.10 – 500	0.10
	hs-CRP	1:20	0.18 – 11	0.18 – 1150	0.18
		1:100	0.88 – 55		
		1:400	3.5 – 220		
IMAGE	hs-CRP	1:2000	17.5 – 1100	0.20 – 1440	0.20
		neat	0.20 – 60		
		1:6	0.20 – 360		
		1:24	0.20 – 1440		
IMAGE	CRP	1:36	– 80	1.0 – 960	1.0
		1:216	> 80		
Synchron LX20	CRP	neat	5 – 200	5.0 – 488	5.0
		ORDAC ^a	150 – 488		

^a ORDAC = Over Range Detection And Correction; when a sample is ORDAC-ed, the LX20 uses a smaller sample size for analysis. Values are given in mg/L.

Linearity studies were performed with two samples containing about 50 mg/L and 250 mg/L CRP. Using the manufacturer's diluent eleven dilutions of each sample were made and measured in five replicates.

The serum index measured on the Synchron LX20 was used to indicate the sample condition in terms of icterus, hemolysis or lipemia. The system monitors the absorbance at 340, 410, 470, 670 and 700 nanometers and solves a set of equations to determine the response for each index. The response is directly proportional to the sample condition. The lipemia index is indicated in different levels 1 – 10. Level 1 is normal, indicating absence of lipemia. Sample turbidity was also assessed by macroscopic examination.

To assess the agreement between serum and plasma for the hs-CRP IMMAGE method, serum, EDTA and heparin plasma samples were collected from 25 patients.

Statistics

hs-CRP distributions were skewed rightward and therefore their values are expressed as medians $\pm$ SD. Agreement between methods was assessed visually by the method of Bland and Altman⁵ and by means of Deming regression analysis⁶. Furthermore, percentile values were estimated and compared. For determination of reference values, the 0.95 nonparametric interpercentile interval was calculated⁷. Gender differences in samples from blood donors were assessed by the Mann-Whitney U test, while median serum versus plasma comparisons were assessed by the Wilcoxon matched pairs signed-ranks test.

Results

Precision and linearity data

The hs-CRP IMMAGE method appeared to be linear over the whole measured CRP concentration range and comparable to results of BNA (Figure 2.1A-B). For five pools with mean concentrations of 0.23, 1.22, 2.18, 16.94 and 44.46 mg/L between run variations of respectively 4.9, 6.7, 2.7, 2.7 and 2.1 % and within run variations of respectively 5.9, 4.3, 3.5, 2.1 and 3.0% were found. So, the total imprecision for the hs-CRP IMMAGE method remained below 7%. For two additional samples with concentrations of 168 mg/L and 342 mg/L within run variations remained below 6.2% for all methods.

Agreement between methods

Figures 2.2A-F show Bland Altman plots for the different method comparisons. Deming regression analysis in the region from 0 – 10 mg/L (Table 2.2) suggests a slightly better agreement of the hs-CRP IMMAGE with BNA ($r = 0.991$) as compared to IMMULITE ($r = 0.986$).

Figure 2.1. Linearity of CRPH IMMAGE® up to 10 mg/L and over the whole measured range.

Linearity results for hs-CRP IMMAGE (A, B) in the low (0 – 10 mg/L) and high (0 – 300 mg/L) CRP concentration range. Dilution samples were run in five replicates. The mean values for each dilution point were plotted vs. expected values and linear regression was performed. Regression analysis results for the respectively low range were: $y = 1.012 \text{ (SE 0.034)} x + 0.201 \text{ (SE 0.118)}$, $r = 0.998$, SEE 0.207; and for the high range: $y = 0.989 \text{ (SE 0.012)} x - 1.335 \text{ (SE 1.133)}$, $r = 0.999$, SEE 3.204.

Table 2.2. Deming regression analysis of the hs-CRP IMMAGE method with other hs-CRP methods.

Analyzer	Slope $\pm$ SD	Intercept $\pm$ SD	SEE ($S_{y x}$)	r
0 – 10 mg/L				
IMMAGE vs. BNA	1.10 ± 0.18	0.01 ± 0.17	0.39	0.991
IMMAGE vs. IMMULITE	1.37 ± 0.25	0.01 ± 0.18	0.54	0.986
IMMULITE vs. BNA	0.80 ± 0.11	-0.01 ± 0.10	0.22	0.989
10 – 300 mg/L				
IMMAGE vs. BNA	1.03 ± 0.14	0.05 ± 0.19	8.09	0.995
IMMAGE vs. IMMULITE	1.16 ± 0.27	0.01 ± 0.22	12.82	0.980
IMMULITE vs. BNA	0.89 ± 0.15	-0.06 ± 0.18	8.92	0.979

For the region from 0 - 10 mg/L, also percentile comparisons of the current CRP and new hs-CRP IMMAGE method to BNA were examined and are shown in the Figures 2.3A-B. The hs-CRP IMMAGE showed excellent agreement with BNA in all quartiles (Figure 2.3A), whereas the current CRP IMMAGE method showed good agreement only with hs-CRP concentrations above 75th percentile (Figure 2.3B). Samples determined on LX20, clear on macroscopic examination, showed no discrepancies with the high-sensitivity methods, whereas this was not the case for turbid samples (data not shown).

Between all hs-CRP methods compared large discrepancies were seen above 100 mg/L (Figure 2.1). Deming regression results are also presented in Table 2.2. Among the hs-CRP methods (Figure 2.2A-F) also for the whole tested range the best agreement was observed between hs-CRP BNA and hs-CRP IMMAGE. Comparison of the hs-CRP

methods with our routinely used CRP method (Synchro LX20) only resulted in a good correlation with the BNA (data not shown).

Figure 2.2 Method comparisons in 521 samples from patients with normal and increased CRP concentrations.

Bland-Altman analysis for 0–10 mg/L (A, C, E) and for 0–300 mg/L (B, D, F). The solid line indicates the mean difference between the methods, and the 95% confidence intervals for the difference are indicated by dashed lines. Mean bias and SD for Bland-Altman plots were –0.1 and 0.4 mg/L (A), 2.0 and 7.9 mg/L (B), 0.6 and 0.6 mg/L (C), 0.2 and 14.3 mg/L (D) –0.7 and 0.4 mg/L (E) and 1.8 and 12.1 mg/L (F).

Closed circles indicate samples showing turbidity assessed by macroscopic examination, whereas open circles indicate completely clear samples.

Serum versus plasma

Mean recoveries ($\pm$ SD) of heparin- and EDTA-plasma related to serum concentrations were: $99.6 \pm 3.6\%$ and $106.4 \pm 11.2\%$ respectively. The results of the regression analysis were as follows: heparin plasma = $0.997 \times \text{serum} - 0.037$, $r = 0.9998$, SEE 0.360; and EDTA-plasma = $0.963 \times \text{serum} - 0.725$, $r = 0.996$, SEE 1.429. No significant differences were found when serum, heparin- and EDTA-plasma were compared for the hs-CRP IMMAGE method (serum *versus* heparin-plasma, $p = 0.52$; serum *versus* EDTA-plasma, $p = 0.09$).

Reference values

Table 2.3 shows patient characteristics and CRP concentrations measured by the hs-CRP IMMAGE in 291 blood donors. The median CRP concentration was 1.57 mg/L and the 0.95 interpercentile interval was 0.20 – 11.24 mg/L for all samples together (males 0.20 – 14.29 mg/L, females 0.20 – 9.84 mg/L). When values above 10 mg/L were omitted, the whole group median value was 1.42 mg/L and the 0.95 interpercentile interval 0.20 – 7.30 mg/L (males 0.20 – 5.40 mg/L, females 0.20 – 8.89 mg/L).

Figure 2.3. Accordance of the current CRP and the new CRPH IMMAGE® method.

Samples collected from 521 patients were analyzed by the current and new hs-CRP method, and the 25th, 50th, 75th and 90th percentiles were determined. A log scale was used for both the *ordinate* and *abscissa* in each panel. Results > 10 mg/L were excluded from each plot. (A), comparison of the results of the hs-CRP IMMAGE and hs-CRP BNA; (B), comparison of the results of CRP IMMAGE and hs-CRP BNA. Closed circles indicate samples showing turbidity assessed by macroscopic examination, whereas open circles indicate completely clear samples.

Table 2.3. CRPH IMAGE and percentiles in blood donors.

Parameter	All	Male	Female
N ^a	291 (282)	177 (170)	114 (112)
Age, years			
Mean	48.3	48.6	47.8
SD	8.10	8.25	7.87
Median	49	50	47
Range	23 - 69	25 - 69	23 - 68
CRP, mg/L			
SD	2.94	3.20	2.48
Percentiles ^a			
2.5 th	0.20 (0.20)	0.20 (0.20)	0.20 (0.20)
25 th	0.67 (0.65)	0.64 (0.62)	0.83 (0.80)
50 th (median)	1.57 (1.42)	1.25 (1.20)	1.86 (1.82)
75 th	3.06 (2.86)	3.03 (2.81)	3.18 (3.00)
90 th	4.98 (4.62)	4.89 (4.30)	5.87 (5.62)
95 th	7.71 (5.50)	6.41 (4.98)	8.11 (7.42)
97.5 th	11.24 (7.30)	14.29 (5.40)	9.84 (8.89)
99 th	15.70 (8.88)	19.94 (6.72)	13.12 (9.74)

^a Values between brackets represent hs-CRP values without samples higher than 10 mg/L.

Discussion

Precision and linearity

The new hs-CRP IMAGE method was found linear in the concentration range of 0.2 – 10 mg/L, which was in agreement with the requirements for hs-CRP assays³. Furthermore, for risk stratification for cardiovascular, cerebrovascular, and peripheral vascular disease, the hs-CRP assay imprecision should be less than 10% at a concentration of 0.2 mg/L^{3,5}, which was also found for the new method. Given the relatively large within-subject variability of about 40%⁵, analytical CVs of 10% or less should be adequate for both clinical and epidemiological studies.

Method comparison and interferences

In the low CRP range (0 – 10 mg/L) the new hs-CRP IMAGE method showed good agreement with hs-CRP methods on BNA and IMMULITE, based on visual inspection of Bland-Altman plots (Figure 2.2A, C, E, G, I and K). In the concentration range from 0.2 – 10 mg/L the new IMAGE hs-CRP method showed excellent agreement with BNA in all quartiles (Figure 2.3A), whereas the current IMAGE CRP method showed good agreement only for concentrations above 75th percentile (Figure 2.3B). Roberts *et al.* performed percentile analysis to allow comparison with a recent study, where the current IMAGE CRP method was included in the comparison². For the percentile comparison, in our study, BNA was taken as a reference method, whereas Roberts *et al.* used the BN II N hs-CRP assay².

In our study, performed on 521 samples, for all hs-CRP methods good agreement was found for concentrations below 100 mg/L.

Above 100 mg/L large discrepancies were seen between the methods, whereas rather good agreement was observed only between LX20 and BNA. Roberts *et al.* found excellent agreement between the compared methods up to 50 mg/L in 50 patients². Furthermore, in our study, above 100 mg/L, the scatter observed was more than 2SD, which could not be explained solely by proportional bias or imprecision data in the higher range, but is more likely due to assay or system related issues (Figures 2.2B, D, F). The scatter that was more than 2SD (above 100 mg/L), was observed for both the CRP IMAGE (data not shown) and the new hs-CRP IMAGE method when compared to BNA (reference method in our study).

Visual inspection of Bland-Altman plots revealed that sample turbidity did not interfere in the hs-CRP methods, as no systematic discrepancies were seen. With Synchron LX20, reliable CRP measurements could only be obtained in sera clear on macroscopic examination (data not shown).

Reference values

The median CRP concentration was 1.57 mg/L and the 0.95 interpercentile interval was 0.20 – 11.24 mg/L for all samples together (males 0.20 – 14.29 mg/L, females 0.20 – 9.84 mg/L). When values above 10 mg/L were omitted, the whole group median value remained higher than median values reported by others (0.58 – 1.13 mg/L)^{2,4}. This is probably due to the already observed weak positive correlation between age and hs-CRP concentration (median age of healthy blood donors in our study was 49 years compared to 32 years reported in previous studies)². It might also be due to eventually unreported episodes of cold within the prior 90 days⁸. The gender difference in hs-CRP was not found significant, neither before ($p = 0.15$) nor after omitting of the values ($p = 0.06$) higher than 10 mg/L, as is in accordance with previous studies^{2,8}.

Conclusion

In conclusion, evaluation of the hs-CRP method on the IMAGE yielded good imprecision results and satisfying linearity as required for hs-CRP methods. The method correlated well with the other two compared hs-CRP methods in the range from 0.2 – 100 mg/L. However, above 100 mg/L poor correlations were found between all different methods, both hs-CRP and CRP. Therefore, the use of two different methods, a hs-CRP for risk stratification and a normal routine CRP method, seems still required. As we are convinced that for the clinical laboratory it is most practical to use one CRP method for the complete measuring range, further investigation to improve agreement between the different methods also in the higher range is required.

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Chapter 3

Evaluation of two new high-sensitivity C-reactive protein methods

Snježana Rothkrantz-Kos, Otto Bekers, Armand Gubbels,
Marjolein Drent, Maria PJ Schmitz, Marja P van Dieijen-Visser

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Abstract

Background

The implementation of a high-sensitivity C-reactive protein (hs-CRP) assay as a routine laboratory parameter may be necessary. It would be most practical to use one CRP method giving reliable results for the whole concentration range. We report here the evaluation of two new hs-CRP methods, which cover both the low and the high concentration ranges.

Methods

The BN ProSpec hs-CRP (Dade Behring) and Synchron LX20 PRO hs-CRP methods were compared with the existing hs-CRP IMMAGE method (taken as a reference) and, for the high concentration range, also with the routine Synchron LX20 CRP method (all from Beckman). Agreement among methods was examined in 521 samples. Reference values were estimated in 291 blood donors. Additionally, the influence of sample turbidity, a major problem of the present Synchron LX20 CRP method, was evaluated.

Results

Measurements of CRP by the BN ProSpec were linear down to 0.2 mg/L, whereas the linearity of Synchron LX20 PRO showed some systematic discrepancies. Over the whole measured range (0.2–250 mg/L), precision (coefficient of variation, CV) was $\leq 3.7\%$ for the BN ProSpec and $\leq 6.1\%$ for LX20 PRO. The Synchron LX20 PRO hs-CRP method was found to be superior to the current routine Synchron LX20 CRP method with regard to precision in the low concentration range and the influence of sample turbidity. Both in the low concentration range and especially in the high concentration range, large discrepancies between methods were observed.

Conclusion

Although acceptable performance was found for the Synchron LX20 PRO hs-CRP method, overall the performance of the BN ProSpec hs-CRP method was superior. However, standardization among assays needs further improvement in both the low and the high concentration ranges.

Introduction

C-reactive protein (CRP), an acute-phase protein, is a valuable laboratory parameter in the clinical evaluation of inflammatory disease. Although CRP is a classical acute-phase reactant, in the absence of infection or trauma its concentration is biologically stable over a long period of time¹. CRP has been introduced as a strong independent predictor of future coronary events in apparently healthy subjects as demonstrated in several clinical studies¹⁻³. Recently, a cardiovascular risk assessment algorithm has been proposed, using high-sensitivity CRP (hs-CRP) in the concentration range 0.7–3.9 mg/L as determined with a nephelometric immunoassay (BN II, from Dade Behring)¹. Although in some studies the limited clinical utility has been discussed⁴, there is still a need for more sensitive assays. Furthermore, implementation of hs-CRP as a routine laboratory parameter is to be expected.

In most clinical laboratories CRP measurements are performed by immunoturbidimetry on a routine clinical chemistry analyzer, with detection limits for CRP of about 2–5 mg/L. These methods are primarily suitable for detection of active inflammation. For studies on CRP as a risk factor for coronary artery disease, a hs-CRP method with a detection limit of about 0.2 mg/L is required⁵. A variety of methods have now been introduced for measuring hs-CRP and their analytical performance has been evaluated and compared⁵⁻⁷. These studies were mainly focused on comparing different methods in the low concentration range of 0–10 mg/L, taking BNII from Dade Behring as the reference method. It was apparent that standardization between the assays still needs improvement in the low range⁵⁻⁸. However, large discrepancies between different hs-CRP methods were also observed in the high concentration range (above 100 mg/L)⁹. The aim of the present study was to evaluate two new hs-CRP methods (Synchron LX20 PRO and BN ProSpec) and compare them to the existing hs-CRP IMMAGE method and also (in the higher range) to our routine Synchron LX20 CRP method. For the laboratory it is most practical to use one CRP method for both the low and the high concentration ranges. Therefore, to find out whether the hs-CRP methods can be used for both risk stratification and detection and monitoring of inflammation, their evaluation was performed over the whole measuring range.

In addition, the influence of sample turbidity, a known major problem of the routine Synchron LX20 method, was examined.

Materials and methods

Samples

Samples for method comparisons were collected from 521 persons either for whom CRP was requested for routine analysis or from a donor population (42 samples). Furthermore, venous blood samples were separately collected from 291 ostensibly healthy blood donors, (177 men and 114 women), who were selected according to the selection criteria of the Sanguin Blood Bank in Maastricht. The medical ethical committee of the hospital approved the procedure that was followed. Serum was

directly separated from the red cells after centrifugation and stored at -80°C until analysis¹⁰.

Methods

The hs-CRP assay on the BN ProSpec from Dade Behring is based on particle-enhanced immunonephelometry (N Hs CRP, cat no OQIY G13; supplement reagent OUMU15; Dade Behring, Liederbach, Germany).

The IMAGE hs-CRP (trade name: IMAGE[®] CRPH) and the LX20 hs-CRP (trade name Synchron LX[®]20 PRO) are both turbidimetric methods based on the peak rate principle³, measured by a near-infrared particle immunoassay, with a laser diode at 940 nm (cat no. 474630 and 378020; Beckman Coulter, Fullerton CA, USA).

CRP was measured on a routine clinical chemistry analyzer, the Synchron LX20 (trade name Synchron LX[®]20, cat no 465131; Beckman Coulter, Fullerton CA, USA): the method is based on immunoturbidimetry.

All the methods were calibrated to CRM 470/RPPHS. According to the manufacturers, the CRP target for CRM 470 is 39.2 mg/L, which was recovered by all instruments within 5% of that target. Table 3.1 presents the assay characteristics of the different methods used.

Table 3.1. Assay characteristics of the methods used.

Analyzer	Method	Dilution (mg/L)	Assay range (mg/L)	Manufacturer's claimed	
				Measuring range (mg/L)	Detection limit (mg/L)
IMAGE	hs-CRP	neat	0.20 – 60	0.20 – 1440	0.20
		1:6	1.2 – 360		
		1:24	4.8 – 1440		
BN ProSpec	hs-CRP	1:20	0.18 – 11		0.18
		1:100	0.88 – 55		
		1:400	3.5 – 220		
		1:2000	17.5 – 1150		
SynchronLX20 PRO	hs-CRP	neat	0.2 – 80	0.20 – 380	0.20
		ORDAC ^a	60 – 380		
Synchron LX20	CRP	neat	5 – 200	5.0 – 488	5.0
		ORDAC ^a	150 – 488		

^a ORDAC, over range detection and correction; the LX20 uses a smaller sample size for analysis of such samples. hs-CRP, high-sensitivity C-reactive protein.

The serum index measured on the Synchron LX20 was used to indicate the sample condition in terms of lipemia. A lipemic index above 1 was used to indicate turbidity. Sample turbidity was also assessed by macroscopic examination and by measuring triglycerides. Samples with triglyceride concentration above 3 mmol/L were classified as turbid samples.

Samples for precision studies were prepared from 11 serum pools in the concentration range from 0.2 – 250 mg/L, as determined on the BN ProSpec, taking into account cardiovascular risk assessment algorithm cut-off points³. Within-run precision was obtained by measuring one sample 20 times within a single run. Between-run precision

was obtained by measuring each concentration on 20 consecutive days, based on a single calibration (one lot number). Since the method agreement experiment was performed on the basis of one lot number (four calibrations), no lot-to-lot stability investigations during the between-run precision experiment were performed.

Statistics

Agreement between methods was assessed visually by the method of Bland and Altman^{11,12} and by Deming regression analysis¹³. For evaluation of blood donor samples, the 0.95 non-parametric inter-percentile interval was calculated¹⁴. Gender differences in samples from blood donors were assessed by the Mann-Whitney U test. Median serum versus plasma comparisons was assessed by Wilcoxon matched pairs signed-ranks test.

Results

Precision and linearity studies

Precision data for BN ProSpec and LX20 PRO are presented in Table 3.2. The mean value of the lowest pool (0.25 mg/L) as measured on the BN ProSpec was below the detection limit of the LX20 PRO and therefore these data cannot be reported. In the hs-CRP concentration range 0.7 to 3.9 mg/L, which has been proposed for cardiovascular risk assessment, between-run coefficients of variation (CVs) ranged from 1.1 to 3.3% for the BN ProSpec and from 2.5% to 5.4% for LX20 PRO. Precision of BN ProSpec was better ($CV \leq 3.7\%$) than that of the LX20 PRO ($CV \leq 6.1\%$) over the measured range (0.2 – 250 mg/L, as measured on the BN ProSpec).

Linearity results are presented as percentage deviation from expected values (Figure 3.1). Expected values were calculated from two initial pools containing 60 and 310 mg/L CRP. No systematic deviation from expected values for the BN ProSpec could be detected over the whole tested range. However, for LX20 PRO, the linearity of the method was less good (below 10 mg/L). Deviation from expected values changed from positive (0 – 10 mg/L) to negative (above 10 mg/L) values. No specific discrepancies in linearity were observed at concentrations for which automatic dilution is performed (Table 3.1).

Influence of sample turbidity on the CRP and hs-CRP methods on the LX20

To evaluate whether the hs-CRP LX20 PRO method is less sensitive to sample turbidity than the present LX20 CRP method, lipemic index, triglycerides and sample turbidity assessed by macroscopic examination were determined in all samples. Both methods were compared to the hs-CRP IMMAGE method (Figure 3.2). Below 20 mg/L ($n = 270$), 12 (4.4%), 29 (10.7%) and 100 (37%) samples were classified as turbid by means of lipemic index, triglycerides and macroscopic examination, respectively. Therefore, assessment of sample turbidity by means of macroscopic examination

appeared to be more sensitive than the use of either lipemic index or measurement of triglycerides.

Because a good relation was found between LX20 PRO and IMMAGE, LX20 PRO appears insensitive to sample turbidity, whereas on the LX20 a reliable CRP analysis seems possible only for clear serum samples.

Table 3.2 Summary of precision data.

Pool	Synchron LX20 PRO			BN ProSpec		
	Mean hs-CRP concentration (mg/L)	CV _B ^a (%)	CV _w ^b (%)	Mean hs-CRP concentration (mg/L)	CV _B (%)	CV _w (%)
0	- ^c	-	-	0.25	3.1	3.7
1	0.36	4.5	6.1	0.40	3.3	3.5
2	0.77	5.4	4.8	0.64	2.5	2.7
3	1.65	3.8	3.4	1.30	1.5	1.5
4	2.67	3.1	1.8	2.18	1.1	0.6
5	4.35	2.5	1.6	3.86	1.4	0.9
6	7.06	5.5	1.7	6.74	1.6	1.0
7	14.7	2.1	2.1	15.0	1.6	1.0
8	51.4	2.1	2.0	45.1	1.4	1.0
9	162	2.5	2.9	133	2.3	0.8
10	318	3.1	2.0	246	1.4	1.0

^{a, b} The concentration of high-sensitivity C-reactive protein (hs-CRP) are based on between-run results.

^a CV_B, between-run coefficient of variation (n = 20).

^b CV_w, within-run coefficient of variation (n = 20).

^c -, below detection limit.

Agreement between methods

Bland-Altman plots are presented in Figure 3.3, and the statistics are summarized in Table 3.3. The percentage plots¹⁵ were used, as the method differences appeared correlated with their mean values. Large discrepancies were observed both in the low and high concentration ranges.

For the BN ProSpec method, the observed bias was negative across the whole concentration range. However, in the low concentration range average bias was approximately 2.5 times higher than in the high concentration range (-19.3% versus -7.1%). As can be seen from percentage plots for the range 0 – 10 mg/L, a change in the slope occurs at 1.6 mg/L. Thus bias and limits of agreement were calculated for the range 1.6 to 10 mg/L (Table 3.3). Very broad limits of agreement were found, indicating poor agreement (-40 to 1.8%). Because of the proposed cardiovascular assessment algorithm, a separate Deming regression analysis was performed for concentrations below 1.6 mg/L. Deming comparison (0 – 1.6 mg/L range) of the BN ProSpec and IMMAGE methods gave a mean (standard deviation, SD) slope of 1.37 (0.22), an intercept of -0.09 (0.11) and an $S_{y|x}$ of 0.06 ($r = 0.979$).

For LX20 PRO method, in the range from 0 – 10 mg/L, the observed bias was negative (-12.6%), whereas above 10 mg/L the observed bias was positive (8.9%). Again, very broad limits of agreement (-30 to 5%) were observed in the low concentration range.

Above 10 mg/L, the bias for LX20 PRO *versus* IMMAGE was higher and positive (8.9%) compared with BN ProSpec *versus* IMMAGE (-7.1%). Overall, the plots in Figure 3.3 suggest better agreement with the BN ProSpec. Moreover, above 100 mg/L there were 23% BN ProSpec samples, compared with 38% LX20 PRO samples having absolute differences with the reference method of more than 30 mg/L.

Comparison of the hs-CRP methods with our routinely used CRP method (Synchron LX[®]20) above 15 mg/L, resulted in a good agreement only with BN ProSpec: less good agreement was found with both IMMAGE and LX20 PRO (data not shown).

Figure 3.1. Linearity of the evaluated high sensitivity C-reactive protein (hs-CRP) methods for the CRP range 0 - 10 mg/L (A) and 10 - 300 mg/L (B).

Linearity is presented as the average percentage deviation together with standard deviation: $\text{deviation (\%)} = \frac{[\text{measured} - \text{expected}]/\text{expected}] \times 100$. Open circles indicate LX20 PRO results, closed circles the BN ProSpec hs-CRP results and square open symbols the hs-CRP IMMAGE results. The linearity data for the hs-CRP IMMAGE method were published previously⁹.

Evaluation of blood donor samples

The hs-CRP concentrations of 291 serum samples collected from apparently healthy adult blood donors were measured on the LX20 PRO and the BN ProSpec. A frequency histogram with percentile values is shown in Figure 3.4. On the LX20 PRO, the median CRP concentration was 1.81 mg/L with a 0.95 inter-percentile interval of 0.26 - 12.72 mg/L, whereas for the BN ProSpec a median CRP concentration of 1.35 mg/L and a 0.95 inter-percentile interval of 0.26 - 12.51 mg/L were found. No significant gender differences were found ($p < 0.053$ for LX20 PRO, $p < 0.132$ for BN ProSpec). In Figure 3.4 percentile values are reported for samples with CRP concentration < 10 mg/L (3.4% of cases omitted). The inter-percentile intervals found were 0.26 - 7.42 mg/L for LX20 PRO and 0.26 - 7.24 mg/L for BN ProSpec.

Figure 3.2. Influence of sample turbidity on C-reactive protein (CRP) and high sensitivity CRP (hs-CRP) measurements using the LX20 and LX20 PRO, respectively.

Samples with a lipemic index < 1 (A, B), triglycerides concentrations < 3 mmol/L (C, D) or turbidity assessed by macroscopic examination (E, F) are indicated by closed circles. The dashed line indicates unity.

Serum versus plasma comparison

To compare sample types, serum, heparin- and EDTA-plasma hs-CRP concentrations for 25 patients were compared for the two new methods. Only slight differences were seen between serum and plasma samples. For BN ProSpec, mean (SD) recoveries in heparin- and EDTA-plasma were 98.3 (3.4)% ($p < 0.02$) and 99.9 (10.5)% ($p < 0.18$), respectively, 100.4 (5.0)% ($p < 0.90$) and 108.5 (10.4)% ($p < 0.04$), respectively, for the hs-CRP LX20 PRO.

Figure 3.3. Method comparison in 521 patient samples by means of Bland-Altman analysis for the high-sensitivity C-reactive protein (hs-CRP) concentration ranges 0 – 10 mg/L (A, C) and 10 – 300 mg/L (B,D).

The dashed lines indicate zero difference between the methods. Closed symbols indicate samples showing turbidity assessed by macroscopic examination, open symbols indicate completely clear samples.

Table 3.3. Comparison of the LX20 PRO and BN ProSpec methods with the IMMAGE method.

	0 – 10 mg/L		10 – 350 mg/L	
	LX20 PRO (y) versus IMMAGE (x)	BN ProSpec (y) versus IMMAGE (x) ^a	LX20 PRO (y) versus IMMAGE (x)	BN ProSpec (y) versus IMMAGE (x)
<i>Deming regression analysis</i>				
Slope±SD	1.15 ± 0.17	0.96 ± 0.11	1.16 ± 0.12	1.11 ± 0.08
Intercept±SD	-0.10 ± 0.17	0.73 ± 0.03	-2.75 ± 3.42	-1.52 ± 2.49
r	0.995	0.996	0.995	0.997
S _{y x}	0.23	0.19	7.59	5.10
<i>Bland-Altman analysis</i>				
Bias (in %)	-12.6	-19.3	8.9	-7.1
Limits of agreements (in %)	-30 to 5	-40 to 1.8	-7.4 to 2.4	-18 to 4
Wilcoxon's test (z, p)	-12.6 p < 0.0001	-13.0 p < 0.0001	-13.7 p < 0.0001	-11.0 p < 0.0001

^a Deming regression and Bland-Altman analysis were performed on data for 1.6 – 10 mg/L (results for 0 – 1.6 mg/L are presented in the text). SD, standard deviation.

Figure 3.4. Evaluation of blood donor samples.

C-reactive protein (CRP) values were quantified in 291 blood donors (177 men and 114 women) of median age 49 years. Percentile values are based on samples with CRP concentrations of less than 10 mg/L. 2.5th, 25th, 50th, 75th and 97.5th percentile values for the BN ProSpec were 0.26, 0.67, 1.35, 2.64 and 7.24 respectively, and for the LX20 PRO they were 0.26, 0.77, 1.81, 3.12 and 7.42 respectively.

Discussion

Agreement among the methods

Two distinct populations of data could be identified from the Bland-Altman plots (*i.e.* those below and above 10 mg/L). Regression analysis and bias estimates were therefore performed separately for these two measuring ranges. In the percentage plots, mean bias (mean of relative differences) appeared to be larger in the low concentration range (-12.6% LX20 PRO, -19.3% BN ProSpec) than in the higher concentration range (8.9% LX20 PRO, -7.1% BN ProSpec) when comparing the methods to the hs-CRP IMMAGE method. Moreover, in the low concentration range BN ProSpec (nephelometric assay) showed a non-linear relationship with the IMMAGE (turbidimetric assay), with a slope change at around 1.6 mg/L, probably caused by lack of linearity of the LX20 PRO (see Figure 2.1). Hamwi et al. noticed similar disagreement when comparing four turbidimetric assays to the BN II method⁷.

However, in the higher range, regardless of the lower percentage bias, the differences found are clinically significant. Above 100 mg/L as measured by the hs-CRP IMMAGE method, there were 23% of BN ProSpec samples and 38% of LX20 PRO samples for which the absolute differences with the reference method was >30 mg/L. These results suggest that, above 100 mg/L there is better agreement between the BN ProSpec and the IMMAGE methods compared with that between the LX20 PRO and the IMMAGE methods. The observed results are in accordance with previous studies, in which at concentrations above 100 mg/L, the same large discrepancies were observed between the compared methods^{5,9}.

Since all method agreement studies were performed using reagents of only one lot number, influence of lot-to-lot instability could *a priori* be excluded. The differences between the methods might also have been caused by improper automatic dilution, but

this does not appear to be the case (Table 3.1). Furthermore, poor linearity might also cause poor agreement. Inspection of linearity results reveals that the LX20 PRO method is not linear, whereas the BN ProSpec appears linear cross the whole concentration range. To be able to follow the influence of linearity on the agreement among the methods, previously published linearity results of the IMAGE method were presented as percentage deviation from expected values in the same graph (Figure 3.1). The same trend in deviation from linearity seen for the LX20 PRO was seen for the IMAGE. This deviation does not appear to be dependent on precision. Although slightly higher CVs were obtained below 1 mg/L, good precision data were observed for LX20 PRO and BN ProSpec across the whole range examined. Although the performance of BN ProSpec is better with respect to precision, the hs-CRP assay precision for risk stratification of vascular disease should be less than 10% at a concentration of 0.2 mg/L⁵, which was achieved for both methods.

All methods were calibrated to CRM 470/RPPHS. However, even assays calibrated to the same reference material may yield different results. The difference may be related to suboptimal value transfer. Alternatively, inappropriate curve-fitting or matrix differences between the system specific calibrators may be responsible for the observed bias⁸. It is obvious that standardization among assays should be improved not only in the low range, but also for the whole measuring range.

From our results it is clear that agreement between methods needs further improvement. Moreover, for clinical risk stratification, concordance between assays is needed, especially at cut-off values separating risk groups⁸.

Samples from blood donors

Samples from blood donors appeared to have higher median values (1.81 mg/L on the LX20 PRO, 1.35 mg/L on BN ProSpec) than those reported by others (range 0.58 – 1.13 mg/L^{5,16}, but were similar to the findings reported previously¹⁰. Methodology-related differences cannot be excluded. Besides, a weak positive correlation between age and hs-CRP concentration has been observed^{10,16}. Since in our population the median age was 49 years, compared to 32 years reported in the earlier study, this might also contribute to the higher values found⁵. The hs-CRP values higher than 10 mg/L (3.4% of blood donors) might be due to existence of some subclinical inflammatory disease^{5,10,17}.

Influence of sample turbidity

In this study, parallel comparison of the hs-CRP LX20 PRO method and the routine CRP turbidimetric method with the IMAGE method revealed that turbidity of samples results in considerable overestimation of CRP concentrations measured with our routine CRP LX20 method. The samples that were used were randomly taken from hospital population (mostly fasting) and from donor samples (non fasting), the latter group to obtain more samples in the low range (<10 mg/L). As a consequence, there are relatively more donor samples (non-fasting), and thus turbid samples, in the lower concentration range (16.3% lower than 10 mg/L compared with 0.8% above 10 mg/L). An effect of turbidity is of course present for the whole concentration range, but in our study this was more pronounced in the lower range caused by the sample population.

Although the reagent insert from Beckman indicates that turbidity can interfere in the test and ultracentrifugation of turbid samples is required, we showed that the influence of non-specific interferences with the Synchron LX20 CRP is greatly underestimated. This is very important, assuming that in order to allow stat analysis, CRP is still measured in many laboratories using the immunoturbidimetric Synchron LX20 method, which according to the manufacturer is reliable for clear samples with values above 5 mg/L. Besides, lipemic index, the automatic screening procedure for selection of turbid samples on the LX20, appeared inadequate.

Conclusion

Use of hs-CRP as a routine laboratory parameter appears inevitable. Therefore, for the laboratory, it would be most practical to use one CRP method giving reliable results for the whole measuring range. The BN ProSpec method appears superior to the Synchron LX20 PRO method and suitable for the whole measuring range. The Synchron LX20 PRO is to be preferred to the current Synchron LX20 CRP method, regarding precision in the low concentration range and influence of sample turbidity. However, the Synchron LX20 PRO needs some improvement with respect to linearity. Furthermore, better standardization among hs-CRP assays is still needed.

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Chapter 4

Analytical evaluation and determination of reference values of soluble interleukin-2-receptor and serum amyloid-A

Snježana Rothkrantz-Kos, Marjolein Drent, Maria P Schmitz,
Paul PPC Menheere, Marja P van Dieijen-Visser

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Abstract

Background

There is a growing interest in both, serum amyloid A (SAA) as an acute phase reactant and soluble IL-2 receptor (sIL-2R) as a T cell activation parameter. In this study, we evaluated the analytical performance of SAA and sIL-2R assays and we report the reference values in an adequate reference population.

Materials and methods

SAA was determined by particle-enhanced immunonephelometry on the BN Prospec from Dade Behring, whereas sIL-2R was determined on the IMMULITE Automated Analyzer, by means of a two-site chemiluminescent enzyme immunometric assay. Reference intervals were established in 282 adult blood donors.

Results

For sIL-2R the coefficients of variation (CV) for within- ($n = 20$) and between-run ($n = 20$) assay precision were $< 3.8\%$ and $< 7.3\%$, respectively, and for the SAA $< 9.2\%$ and $< 10.8\%$. Both methods were linear, sIL-2R below 1000 kU/L and SAA below 854 mg/L. For sIL-2R reference intervals were found to be 241 – 846 kU/L (2.5th – 97.5th percentile). For SAA sex differences were observed and the reference values in males were 0.99 – 9.87 mg/L and in females 0.84 – 11.4 mg/L. The sIL-2R concentration appeared not related to age. However, SAA concentrations tended to a moderate increase with age.

Conclusions

Both assays, SAA on the BNProSpec and sIL-2R of the IMMULITE appeared to be useful in clinical practice.

Introduction

Serum amyloid A (SAA) is an acute phase protein. The liver is mainly responsible for the production upon stimulation by interleukins IL-1 and IL-6, respectively. Like C-reactive protein (CRP), SAA belongs to the quick responding acute phase proteins.

The diagnostic value of these two different acute phase proteins is influenced by the underlying disorder. For example, during viral infections, SAA appeared to increase more intense as compared to CRP¹⁻³. In some diseases, such as rheumatoid arthritis, Crohn's disease and ulcerative colitis, CRP remained even normal, whereas SAA increased⁴⁻⁸. Increased CRP was shown to be associated with inflammation in sarcoidosis⁹, whereas SAA appeared to be increased in patients with active sarcoidosis¹⁰. To date, one of the strongest advantages of SAA above CRP has been demonstrated in kidney transplantation patients on immunosuppressive steroid therapy. In this later population only SAA, but not CRP appeared to be useful in monitoring acute rejection reactions^{2,11,12}.

Another inflammatory parameter of clinical importance is soluble interleukin-2-receptor (sIL-2R). Interleukin-2 stimulates activation of T cells through a complex interleukin-2 receptor molecule¹³. It concerns a heterodimeric receptor, which is partly released as a soluble form of the 55 kD chain, the so called soluble IL-2 receptor^{14,15}. sIL-2R was found to be a good prognostic marker, related to pulmonary as well as extrapulmonary manifestations of sarcoidosis¹⁶⁻¹⁹. Recently, it has been demonstrated by various groups that sIL-2R appeared to be more useful as compared to the traditionally used angiotensin-converting enzyme and might even replace it¹⁸⁻²⁰.

Before an assay can be used for clinical evaluation, a critical examination of assay performance is required. In this paper we report an extensive evaluation of the new SAA assay on the BNProSpec, from Dade Behring. Although evaluation of sIL-2R on the IMMULITE is available, no reference values were reported until now²¹. Therefore, the reference values estimated in a population of healthy blood donors for both, SAA, determined on the BN ProSpec from Dade Behring, and for sIL-2R, determined on the IMMULITE are presented.

Materials and methods

Study population

To allow determination of reference values, between 1999 – 2002 venous blood samples were collected from 282 ostensibly healthy blood donors presenting at the Sanguine Blood Bank in Maastricht. The Medical Ethical Committee of the Hospital approved the procedure followed.

Serological measurements

Samples were obtained by venipuncture in serum tubes and clotted blood was centrifuged (2500 g at 20°C) for 15 minutes to obtain serum. Serum was separated and stored at -70°C for further analysis. The whole procedure took less than an hour.

Soluble IL-2 receptor (sIL-2R) was determined on the IMMULITE Automated Analyzer, by means of a two-site chemiluminescent enzyme immunometric assay, with a detection limit of 50 kU/L, and a measuring range 50 – 7500 kU/L (Diagnostic Product Corporation, Los Angeles, CA, cat no LKIP1). IL-2R reagent (LIP2) consists of alkaline phosphatase conjugated to rabbit polyclonal anti-IL-2R antibody.

Serum amyloid A (SAA) was determined on the BN Prospec from Dade Behring, by particle-enhanced immunonephelometry. The detection limit for SAA was 3 mg/L, with a measuring range of 3 – 1000 mg/L, depending on dilution (Dade Behring, Liederbach Germany, N SAA reagent, cat no OQMP 11). N SAA reagent (OQMP 11) consists of lyophilised polystyrene particles coated with sheep antibodies to human SAA.

For both assays, within-run precision was obtained by measuring one sample 20 times within a single run. Between-run precision was obtained by measuring each level on 20 consecutive days, based on a single calibration.

For sIL-2R, samples for precision studies were prepared from two serum pools in the range from 200 – 1000 mg/L. The linearity studies were performed with one sample containing about 1000 kU/L of sIL-2R. Using the manufacturer's diluent (IL-2R Sample Diluent (LIPZ)) eleven dilutions of each sample were made in two replicates and measured.

With respect to SAA, samples for precision studies were prepared from nine serum pools in the range from 0.98 – 821 mg/L, as determined on the BN ProSpec. The linearity studies were performed with two samples containing about 854 mg/L and 150 mg/L SAA. Using the manufacturer's diluent (N Diluent (OUM T65)) nine dilutions of each sample were made in five replicates and measured.

Statistical analysis

Statistical analysis was performed using the SPSS10.0 for Windows (SPSS, Chicago, IL, USA). The distributions of the SAA and sIL-2R were positively skewed. Therefore, the reference values are presented as an interpercentile interval (2.5th – 97.5th range). Linear regression was used to test the relationship with age. Sex-differences were tested by means of Mann-Whitney U test. All p-values were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Imprecision and linearity of sIL2R assay

In two pools with mean concentrations of sIL-2R of 193 and 978 kU/L between run variations of respectively 3.6 and 7.2% and within run variations of respectively 2.7 and 3.7% were found. The IMMULITE sIL-2R assay appeared to be linear below 1000 kU/L. The results of the regression analysis are presented in Figure 1A. The

percentage deviation of the observed values as compared to expected values varied from -2.3% – 13.6% (mean: 4.8%).

Imprecision and linearity of SAA assay

Since to the best of our knowledge this report concerns, the first evaluation of the SAA assay on the BN ProSpec, slightly more extensive evaluation was performed as compared to the sIL-2R evaluation. For nine pools with mean concentrations of 0.98, 4.31, 7.40, 14.7, 21.64, 85.44, 134, 452 and 821 mg/L between run variations of respectively 10.7, 9.3, 4.4, 4.3, 4.1, 4.8, 4.7, 6.9 and 6.6% and within run variations of respectively 8.9, 9.2, 5.1, 6.3, 2.6, 3.3, 7.4, 5.0 and 5.2% were found. So, the total imprecision for the SAA BN ProSpec method remained below 10.7%. The method appeared to be linear below 854 mg/L. Linearity results are presented in Figure 4.1B. The percentage deviation of the observed values as compared to expected values varied from -21.8% – 17.6% (mean: 2.4%).

Figure 4.1. Linearity of sIL-2R (IMMULITE) and SAA (BN ProSpec) assays.

The values for each dilution point are plotted vs. expected values and linear regression was performed. Linearity results of sIL-2R IMMULITE (A) and SAA BN ProSpec (B) method are presented for the whole tested range. For sIL-2R, dilution samples were run in duplicate in 2 independent experiments and the results of the one of these experiments are plotted, whereas the SAA linearity was determined in a single experiment in four replicates (SAA). The linearity equation is presented with the standard errors for the intercept and the slope within parentheses.

Determination of blood donor samples

The sIL-2R and SAA concentrations of 282 serum samples collected from apparently healthy adults are presented in Table 4.1. Overall, the median sIL-2R concentration was 448 kU/L with a 0.95 interpercentile interval of 241 – 846 kU/L. The median SAA concentration (0.95 interpercentile interval) was 2.49 (0.90 – 10.22 kU/L). For sIL-2R no gender differences were found (Mann-Whitney $U = 8665$, $p = 0.23$), whereas for SAA significant gender differences were found (Mann-Whitney $U = 7285$, $p = 0.013$) as shown in Table 4.1. Furthermore, no relationship with age was found for sIL-2R ($p = 0.91$). However, the SAA showed a moderate relationship with age ($r = 0.14$, $p = 0.02$), as visible from Figure 4.2.

Analytical performance of the two evaluated assays

With respect to sIL-2R, the results were comparable to a previous study on imprecision and linearity²¹. As the assay performance was previously evaluated, we performed only a limited evaluation. Ledue *et al.* reported for sIL-2R concentrations of ca. 850 kU/L and 2000 kU/L, between run variations of respectively 3.7% and 7.0% and within run variations of respectively 3.3% and 5.1%²¹. In the present study, we additionally reported the imprecision for a pool with mean concentration of 193 kU/L. In line with the previous study²², our analyses also revealed a low imprecision (< 4%).

To the best of our knowledge, this is the first study reporting imprecision and linearity results on the BN Prospec for SAA. As shown in the section *Results* we extensively evaluated the measuring range. Below 4.5 mg/L, the imprecision (both within- and between run) was ranging from 8.9 – 10.7%. However, the imprecision between 4.5 – 821 mg/L SAA, remained below 7.4%, suggesting a good performance of the assay. In an earlier study SAA was evaluated on the Behring nephelometer II (BNII), a previous generation of Dade Behring assays. The coefficients of variation (CV) found, for intra- and inter-assay precision were below 5.2% and 8.5%, respectively, for the three samples at concentrations representing low, normal and high²². Linearity for each method was within 5% of the expected values throughout the calibration range. These results are in agreement with our findings, suggesting that the performance of the SAA assay on the BN ProSpec is comparable to the same assay on the BNII. However, the agreement studies are needed to confirm the accordance between the methods, as shown in case of CRP^{23,24}.

Table 4.1. Evaluation of soluble interleukin-2-receptor (sIL2R) and serum amyloid A (SAA) in blood donor samples.

		sIL-2R (kU/L) ^a			SAA (mg/L)		
		All	Males	Females	All	Males	Females
Mean		468	475	456	3.22	2.96	3.60
SD		150	148	153	2.31	2.13	2.51
Minimum		189	189	208	0.77	0.77	0.78
Maximum		1058	944	1058	13.80	12.20	13.80
Percentiles	2.5	241	246	226	0.90	0.99	0.84
	25	354	368	341	1.65	1.58	2.00
	50	448	451	440	2.49	2.27	2.76
	75	557	551	563	4.03	3.51	4.84
	95	744	745	749	7.94	7.66	8.67
	97.5	846	860	812	10.22	9.87	11.4

^a sIL-2R values were quantified in 282 (172 males and 110 females) blood donors with median age of 49 years.

Figure 4.2. sIL-2R and SAA reference values in relation with age.

Open symbols present female values, whereas closed symbols present male values. The horizontal lines present reference values in the whole group.

Discussion

Reference values of sIL-2R and SAA

With respect to sIL-2R we found no sex differences and no relationship with age. The latter is in accordance with a previous study in Japanese patients²⁵. As far as we know, this is the first study reporting sIL-2R values in a large population. Previous studies, often clinical trials, all used small populations (sample size < 40) to define the control group values²⁶⁻²⁹. Moreover, mostly no clear definition on the criteria used to select the control patients were given.

The reference values observed for SAA were comparable with the results of the previous studies^{22,30}. The upper level of the reference interval (97.5th percentile) found in this study was 10.22 mg/L, whereas the median value of 2.49 mg/L is slightly higher as compared to the study of Ledue *et al.*²². For SAA, significant sex differences were found ($p = 0.013$), which were not reported previously. Ledue *et al.* established reference intervals on BNII in 261 adult blood donors (aged 36.2 ± 9.0 years) with 2.5th, 50th, and 97.5th percentiles of < 0.84, 2.10 and 9.70 mg/L for SAA²². Yamada *et al.* reported the normal range of 0.17 – 10.0 mg/L with a self developed method based on the measurement of SAA by kinetic nephelometry³⁰.

In accordance with a previous study of Ledue *et al.*, we observed a weak relationship of SAA with age²². However, in the present study this relationship appeared to be only present in males. One could argue that the sex differences observed and the sex-dependent relationship with age are simply sample related. Nevertheless, we used a large sample to establish the reference values in healthy blood-donors, in accordance to the requirements for the establishment of reference values^{31,32}. The donor population was selected according to the criteria used by the Sanguine Blood Bank in Maastricht. The participants were asked to fill in a questionnaire consisting of questions considering the presence of acute or chronic illness or trauma, medication, pregnancy etc. Accordingly, donors meeting any of these criteria were excluded. The same reference population was used to establish the CRP reference values. The results were highly

consistent with a larger study using the tenfold higher sample size³³. Therefore, we considered our sample population representative for the establishment of the reference values.

In conclusion, evaluation of the sIL-2R method on the IMMULITE and SAA method on the BN ProSpec yielded good imprecision results and satisfying linearity. The reference values established in 282 ostensibly healthy blood donors for sIL-2R were 241 – 846 kU/L. Moreover, these latter values appeared to be sex and age independent. The reference values for SAA (0.99 – 9.87 mg/L in males and 0.84 – 11.4 mg/L in females) on BN ProSpec were sex dependent and showed relationship with age.

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Chapter 5

Usefulness of inflammatory markers to depict respiratory functional impairment in sarcoidosis

Snježana Rothkrantz-Kos, Marja P van Dieijen-Visser,
Paul GH Mulder, Marjolein Drent

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Abstract

Background

Sarcoidosis is a multiorgan inflammatory granulomatous disorder of unknown origin for which adequate markers to monitor disease severity are lacking. The aim of this study was to evaluate the clinical usefulness of serological markers of inflammation [(high-sensitivity C-reactive protein (hs-CRP) and serum amyloid A (SAA)], T cell activation [soluble interleukin-2 receptor (sIL-2R)], and granuloma formation [angiotensin-converting enzyme (ACE) for monitoring pulmonary severity.

Methods

Of the 185 sarcoidosis patients who visited the Sarcoidosis Management Center between 1999-2002, we selected 144 non-smoking patients: 73 untreated (group I) and 71 treated (group II). Subgroups of the untreated patients [group Ia (non-chronic group with time since diagnosis ≤ 2 years) and group Ib (chronic group with time since diagnosis > 2 years)] were evaluated separately. ROC-curves and logistic regression analyses were used to compare the diagnostic accuracy of different markers to assess disease severity. Pulmonary disease severity was defined by lung function test results.

Results

In untreated subgroup Ia and the total untreated group (group I), sIL-2R had the largest areas under the curves (AUCs: 0.891 and 0.799, respectively), and the highest sensitivity (82% and 64%), specificity (94% and 88%), and positive (82% and 70%) and negative (94% and 88%) predictive values among the evaluated markers in both untreated groups. Nevertheless, the confidence intervals for sIL-2R AUC, sensitivity, and specificity were broad and partly overlapped those of ACE, hs-CRP, and SAA. In the treated group (group II), all four markers appeared to have comparable AUCs ranging from 0.645 for SAA to 0.711 for sIL-2R.

Conclusion

sIL-2R appears to be useful for monitoring respiratory disease severity in sarcoidosis. We recommend sIL-2R measurement in the follow-up of patients with sarcoidosis.

Introduction

In young adults, pulmonary sarcoidosis is the second most common respiratory disease after asthma. Sarcoidosis is a systemic granulomatous inflammatory disease that primarily affects the lungs and lymphatic system of the body^{1,2}.

Sarcoidosis is characterized by a hyperimmune response to an unknown agent at the lesion sites^{1,3}. In sarcoidosis, inflammatory stimuli generally lead to activation of monocyte-macrophages, which in turn produce cytokines, e.g., tumor necrosis factor- α , and interleukins, e.g., interleukin-1 (IL-1) and IL-6^{4,5}. As a consequence, IL-1 and IL-6 concentrations increase and stimulate hepatic production of acute-phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA)⁶. CRP has been shown to be a rather stable marker of systemic inflammation⁷. Recently, Drent *et al.*⁸, using a traditional, less sensitive CRP method, demonstrated that a moderate increase in serum CRP is implicated in sarcoidosis. High-sensitivity CRP (hs-CRP) methods have recently been introduced to accurately monitor minor increases in serum CRP⁹, but no studies evaluating hs-CRP in sarcoidosis have been reported. Increased SAA has also shown been shown to be independently associated with sarcoidosis activity¹⁰. SAA appears to be less sensitive to immunosuppressive drugs, such as corticosteroids, and therefore has been recommended in the follow-up of patients to whom such drugs have been administered⁶.

Cytokines produced by activated monocytes-macrophages, mainly IL-1 and IL-6, also stimulate the production of IL-2. Production of IL-2 leads to T cell activation¹¹. Activated T cells express an IL-2 receptor (55-kDa/75-kDa heterodimer) on their cell surface and release a soluble form of the 55 kDa chain, the so called soluble IL-2 receptor (sIL-2R)¹². sIL-2R was found to be increased in patients with active sarcoidosis^{13,14}.

In sarcoidosis, inflammation does not resolve, but leads to granuloma formation. Angiotensin-converting enzyme (ACE) is a product of granuloma (of epithelioid cells that are derivatives of the activated macrophages). Despite its shortcomings, ACE is mostly used in the assessment and the follow-up of sarcoidosis¹⁵.

All of the above markers have been shown to be related to sarcoidosis activity, but their relationships with the severity of this disease have not yet been fully established. A relationship between sIL-2R and severity of sarcoidosis has recently been suggested^{16,17}, but ROC curve analysis was lacking in both studies.

From a clinical point of view it is even more important to know whether sarcoidosis is severe, rather than active. Lung function tests provide information about the presence of respiratory functional impairment (RFI), which is one of the indicators of disease severity^{1,18}. Furthermore, RFI is one of the reasons to initiate treatment, which is aimed at preventing irreversible fibrotic changes¹.

The aim of the present study was to determine the diagnostic accuracy of sIL-2R, ACE, hs-CRP, and SAA to predict the severity of pulmonary sarcoidosis, as indicated by RFI.

Materials and Methods

Study population

Between 1999 – 2002, 185 sarcoidosis patients visited the Sarcoidosis Management Centre of the University Hospital Maastricht, a Dutch referral center for sarcoidosis. Out of these patients, 144 non-smoking patients were prospectively included in this study. The diagnosis of sarcoidosis was based on consistent clinical features and bronchoalveolar lavage fluid analysis, according to the American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders (ATS/ERS/WASOG) guidelines^{1,19}. The diagnosis was confirmed histologically in 85% of the cases. No comorbidity was present in any of these patients. Informed consent was obtained from all participants.

In sarcoidosis, spontaneous remissions without treatment can occur. For that reason, a period of observation of 2 years is justified if patient is relatively asymptomatic. Hence, sarcoidosis is generally considered chronic if present for more than 2 years²⁰. Accordingly, subgroup analyses were performed with the untreated group divided in two groups: a subgroup with a time since diagnosis ≤ 2 years (group Ia, non-chronic group) and a subgroup with a time since diagnosis > 2 years (group Ib, chronic group). To determine reference values, we collected venous blood samples (serum) from 282 ostensibly healthy blood donors presenting at the Sanguin Blood Bank in Maastricht. The Medical Ethical Committee of the Hospital approved the procedure followed.

Serological measurements

Simultaneously with lung function tests, blood samples were taken, and serum was stored at -20°C until actual measurement, which occurred for all samples within 2 months after storage. In addition, frozen aliquots of control sera were always checked. No influence on the stability of the evaluated markers was found for the samples that were treated in this way.

Soluble IL-2R was determined by a two-site chemiluminescent enzyme immunometric assay (cat. no. LKIP1; Diagnostic Product Corporation) on the IMMULITE Automated Analyzer. The detection limit of the assay is 50 kU/L, and the measuring range is 50 – 7500 kU/L. The within- and between-run imprecision of the assay was $< 7.2\%$, and the reference interval for sIL-2R was 241 – 846 kU/L.

hs-CRP and SAA were measured by particle enhanced immunonephelometry on the BN ProSpec (Dade Behring). The detection limit for hs-CRP is 0.175 mg/L, and the measuring range is 0.175 – 1100 mg/L, depending on dilution (N Hs CRP; cat. no. OQIY 13; supplement reagent OUMU; Dade Behring). The detection limit of SAA is 3 mg/L, with a measuring range of 3 – 1000 mg/L, depending on dilution (N SAA reagent, cat no OQMP 11; Dade Behring). The imprecision of the SAA BN ProSpec method was $< 11\%$ and the reference interval was 0.90 – 10.22 mg/L.

Evaluation of the hs-CRP assay on the BN ProSpec has been reported previously²¹. The reference interval was 0.26 – 7.24 mg/L.

Serum ACE (ACE) was measured by colorimetric method (cat. nr. FU 116; Fujirebio Inc.). ACE acts on a substrate p-hydroxybenzoyl-glycyl-L-histidyl-L-leucine and separates p-hydroxybenzoylglycine, which is converted in two subsequent reactions into quinoneimine dye. The absorbance of the quinoneimine dye is measured at 505 nm to evaluate ACE activity. The imprecision of the ACE assay was < 5.6%, and the reference interval for ACE was 9 – 25 U/L.

The tests and the measurements in sarcoidosis patients were evaluated by one professional analyst or a PhD student trained by this analyst; both were blinded to the patients' histories.

Evaluation of severity of Sarcoidosis Pulmonary Disease

Chest radiographs were graded according to the radiographic staging of DeRemee (0 to III), with stage IV, the end stage of lung fibrosis, added^{1,22}.

Lung function indices, including the forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC), were measured with a pneumotachograph. The diffusing capacity for carbon monoxide (DLCO) was measured by the single-breath method. Both measurements were performed on a Masterlab (Jaeger, Würzburg, Germany). The intrasession CV for DLCO was 4 – 6%, and the intersession CV was 9%. Values were expressed as a percentage of those predicted²³.

Both the radiographic staging and pneumotachography tests were performed and interpreted by two professionals who were blinded to the patients' histories.

Statistical analysis

Statistical analysis was performed with SPSS10.0 for Windows (SPSS). For all selected patients, non-missing and interpretable results were obtained for both the laboratory tests and the lung function tests. The distributions of the explanatory variables CRP, SAA, ACE, and sIL-2R were positively skewed, therefore, the data are presented as medians and interquartile ranges. A log transformation was applied to normalize the data before further analysis, if appropriate. Logistic regression was used to test the discriminatory effect of the (log-transformed) explanatory variables simultaneously by use of likelihood ratio tests. A weighted sum of explanatory variables with the estimated log odds ratios as weights served as linear predictor score in a ROC analysis. Areas under the ROC curves were compared using a paired nonparametric test described by DeLong *et al.*²⁴. The optimal cut-off point (for the predictor) coincides with the point on the ROC curve where the sum of sensitivity and specificity was maximal. At this point the slope to the ROC curve equals unity, which is under certain conditions the result of minimization of the total costs attributable to false-positive and false-negative outcomes²⁵. All p-values were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Definition of severity criteria and group description

Clinical characteristics of the study group are presented in Table 5.1. Only non-smoking patients were included in the study because smoking can cause, or at least worsen, lung inflammation. Sarcoidosis patients were divided in two groups, untreated (group I) and treated (group II).

Table 5.1. Clinical characteristics of the studied sarcoidosis population (n=144) ^a

	Group I	Group II
n	73	71
Gender ^b , M/F	33 / 40	46 / 25
Age ^d , years	40.8 ± 11.0	43.8 ± 10.4
Serologic markers ^a		
sIL-2R ^b , kU/L	733 (464 – 1244)	584 (400 – 832)
ACE, U/L	20.0 (16.0 – 27.0)	20.0 (16.0 – 27.0)
hs-CRP, mg/L	3.60 (1.68 – 8.36)	3.03 (1.28 – 6.79)
SAA, mg/L	5.33 (2.28 – 9.13)	5.90 (3.14 – 11.70)
Lung function tests		
RFI ^c , n (%)	22 (30)	39 (55)
DLCO ^d , %	87.5 ± 18.6	82.3 ± 18.0
FVC ^d , %	99.2 ± 21.5	91.0 ± 20.4
FEV1 ^d , %	92.2 ± 23.2	81.7 ± 23.1
Chest radiographic staging, n (%)		
Stage < II	33 (45)	22 (31)
Stage ≥ II	40 (55)	49 (69)

^a Patients were selected according to the criteria described in Materials and methods: Group I, all untreated patients; Group II, all treated patients.

^{b,c} Group I vs. group II: ^b $p < 0.05$; ^c $p < 0.01$.

^d Variables presented as mean (SD) follow a gaussian distribution.

^a Serologic markers are presented as median with range (25th – 75th percentiles) within parentheses.

RFI was defined as DLCO < 80%, FVC < 80%, or FEV1 < 80% (percentage of predicted). Patients without RFI were those for whom all three indices were ≥ 80%, according to standard recommendation¹. A cross-tabulation of the test results with respect to the reference standard (RFI) is presented in Figure 5.1. Treated patients appeared to have significantly lower sIL-2R concentrations compared with the untreated patients ($p < 0.05$). The differences in sIL-2R between the treated and untreated group also remained significant after correction for pulmonary function tests, *i.e.* presence of RFI ($p < 0.05$). Moreover, 55% of the treated patients presented with RFI compared with only 30% of untreated patients ($p < 0.01$).

The untreated group was further divided into two subgroups: a subgroup with time since diagnosis ≤ 2 years (group Ia, non-chronic group) and a subgroup with time since diagnosis > 2 years (group Ib, chronic group). Clinical characteristics of the two subgroups are presented in Table 5.2. There was no relationship of inflammatory

markers with time since diagnosis, but we observed significant difference in DLCO and FVC between the two groups ($p < 0.05$).

Diagnostic accuracy of evaluated inflammatory markers

The ROC analysis results of the untreated and treated groups are presented in Figure 5.2. Overall, the areas under the curves (AUCs) were significantly different from the null-hypothesis, true area = 0.5 (meaning no discrimination). In the group of untreated patients (group I, $n = 73$), the AUC for sIL-2R was significantly larger than the AUC for ACE ($p = 0.033$), but we found no significant differences for sIL-2R compared with hs-CRP ($p = 0.236$) or SAA ($p = 0.180$). The AUC's for hs-CRP and SAA were comparable ($p = \text{NS}$), and although smaller, both were not significantly different from the AUC for ACE (Figure 5.2A).

In treated patients (group II, $n = 71$), the evaluated inflammatory markers had comparable AUCs, which were all < 0.720 (Figure 5.2B). However, in all three tested groups, the AUC for sIL-2R was the largest. All ROC curves were significantly different from the AUC of 0.5.

Figure 5.1. Distribution of ln-transformed test results with respect to the reference standard.

RFI was used as a reference standard (RFI⁺, RFI present; RFI⁻, RFI absent). RFI was defined as present if DLCO was $< 80\%$, or FEV1 was $< 80\%$, or FVC was $< 80\%$ of the predicted value and as absent if DLCO was $\geq 80\%$, FEV1 was $\geq 80\%$, or FVC was $\geq 80\%$ of the predicted value. Open circles indicate untreated patients; closed circles indicate treated patients. In each group, the horizontal line represents the upper limit of the reference interval (97.5th percentile).

In addition, the logistic regression was used to test the discriminatory effect of explanatory variables simultaneously. The various combinations of markers yielded different logistic regression models giving different linear prediction scores for the construction of AUCs.

The linear prediction score based on the combination of all four markers yielded AUCs (SE) of 0.812 (0.055) for group I and 0.744 (0.058) for group II. The combinations of sIL-2R and hs-CRP, sIL-2R and SAA, and sIL-2R and ACE yielded AUCs (SE) of 0.812 (0.054), 0.803 (0.056), and 0.803 (0.057), respectively, for group I and 0.733 (0.059), 0.732 (0.059), and 0.708 (0.061) for group II. However, none of these models appeared to be significantly different from the AUCs for sIL-2R alone [(0.799 (0.058) for group I and 0.711 (0.061) for group II)].

Table 5.2. Clinical characteristics of the subgroups of untreated sarcoidosis patients (n = 73)^a.

	Group Ia	Group Ib
n	42	31
Gender, M/F	21 / 21	12 / 19
Age ^b , years	41.1 ± 11.8	40.3 ± 10.1
Serologic markers ^c		
sIL-2R, kU/L	825 (520 – 1437)	618 (454 – 1203)
ACE, U/L	19.5 (16.0 – 27.0)	20 (14 – 27)
hs-CRP, m/L	3.55 (1.62 – 7.29)	3.90 (1.68 – 8.68)
SAA, mg/L	5.50 (2.33 – 9.29)	4.67 (2.16 – 9.16)
Lung function tests ^d		
RFI, n (%)	11 (26%)	11 (36%)
DLCO ^d %	89.6 ± 14.7	84.8 ± 22.7
FVC ^d %	101.4 ± 16.5	96.2 ± 26.9
FEV1 ^d %	94.7 ± 19.1	88.8 ± 27.8
Chest radiographic stage, n (%)		
Stage <II	22 (53%)	11 (35%)
Stage ≥II	20 (48%)	20 (65%)

^a Patients were selected according to the criteria described in Materials and methods. Group Ia, untreated patients with time since diagnosis ≤ 2 years, Group Ib, untreated patients with time since diagnosis > 2 years.

^b Variables are presented as mean ± SD, as they follow a gaussian distribution.

^c The values of serological parameters are presented as median with range (25th – 75th percentiles) within parentheses.

^d p < 0.05 for group Ia vs. Ib.

Subgroup analysis

The ROC results for the subgroups of untreated patients are presented in Figure 5.3. In the group Ia (non-chronic group, n = 42), only sIL-2R (p < 0.0001) and ACE (p < 0.04) had an AUC significantly different from the null-hypothesis; the p values for the AUCs for hs-CRP and SAA were 0.141 and 0.074, respectively. In group Ia, the AUC for sIL-2R did not differ significantly from the AUC for ACE (p = 0.111).

Figure 5.2. ROC curves for the inflammatory markers to determine RFI in untreated (A) and treated (B) patients.

Closed circles indicate ACE, open circles sIL-2R, closed squares SAA, and open squares indicate hs-CRP. The diagonal line indicates an AUC of 0.5 (no discrimination between the two states). RFI was defined as present if DLCO was $< 80\%$, FEV1 was $< 80\%$, or FVC was $< 80\%$ of the predicted value and as absent if DLCO was $\geq 80\%$, FEV1 was $\geq 80\%$, or FVC was $\geq 80\%$ of the predicted value. (A). For Group I (all untreated patients), the AUCs (95% confidence intervals) were 0.799 (0.686 – 0.913) for sIL-2R, 0.650 (0.504 – 0.795) for ACE, 0.708 (0.583 – 0.832) for hs-CRP, and 0.701 (0.580 – 0.821) for SAA. (B). For Group II (all treated patients), the AUCs (95% confidence intervals) were 0.711 (0.592 – 0.829) for sIL-2R, 0.671 (0.541 – 0.801) for ACE, 0.681 (0.556 – 0.806) for hs-CRP, and 0.645 (0.518 – 0.773) for SAA.

In group Ib (chronic group, $n=31$), the AUCs for sIL-2R ($p=0.043$), hs-CRP ($p=0.019$), and SAA ($p=0.035$) all were significantly different from the AUC of 0.5 ($p < 0.05$), in contrast to ACE ($p=0.536$). The data for group Ib are presented in Figure 5.3B.

The linear prediction score based on a combination of all four markers yielded AUCs (SE) of 0.886 (0.056) for group Ia and 0.773 (0.084) for group Ib. The combinations of sIL-2R and hs-CRP, sIL-2R and SAA, and sIL-2R and ACE yielded AUCs (SE) of 0.812 (0.067), 0.889 (0.055), and 0.880 (0.058), respectively, for group Ia and 0.777 (0.083), 0.723 (0.093), and 0.723 (0.043) for group Ib. However, they also were not significantly different from the AUCs for sIL-2R alone [0.891 (0.054) for group Ia and 0.723 (0.043) for group Ib].

For untreated group of patients, the optimal cut-off points were defined, as were their sensitivity/specificity pairs and predictive values. The combined results are presented in Table 5.3. Somewhat overlapping sensitivity and specificity confidence intervals were observed, but sIL-2R had the highest combination of positive and negative predictive values among the markers: 70% and 85% respectively, for group I, 82% and 94% for group Ia, and 67% and 84% for group Ib. In group Ib, however, the negative predictive values for both SAA and hs-CRP was 85% and the positive predictive value was only 50% for the chosen cut-offs.

Figure 5.3. ROC curves of the inflammatory markers in untreated subgroups of patients.

Closed circles indicate ACE, open circles sIL-2R, closed squares SAA, and open squares indicate hs-CRP. The diagonal line indicates an AUC of 0.5 (no discrimination between the two states). RFI was defined as present if DLCO was $< 80\%$, FEV1 was $< 80\%$, or FVC was $< 80\%$ of the predicted value and as absent if DLCO was $\geq 80\%$, FEV1 was $\geq 80\%$, or FVC was $\geq 80\%$ of the predicted value. (A). Group Ia (nonchronic group). AUCs (95% confidence intervals) were 0.891 (0.786 – 0.997) for sIL-2R, 0.720 (0.523 – 0.917) for ACE, 0.651 (0.466 – 0.836) for hs-CRP, and 0.683 (0.518 – 0.848) for SAA. (B). Group Ib (chronic group). AUCs (95% confidence intervals) were 0.723 (0.521 – 0.924) for sIL-2R, 0.568 (0.351 – 0.786) for ACE, 0.759 (0.591 – 0.927) for hs-CRP, and 0.732 (0.552 – 0.912) for SAA.

Prognostic value of sIL-2R for untreated group of patients

Although this study was not designed to be a prognostic study, we looked how many patients of the non-chronic untreated group, which is the most interesting for the prediction of the future outcome, were finally treated with respect to the sIL-2R values. Only 7 out of 31 patients with low sIL-2R (≤ 1300 kU/L) values, as compared to 8 out of 11 patients with high sIL-2R values (> 1300 kU/L) needed treatment. This indicates that 73% of the cases with high values had a less favorable outcome compared to 23% with low sIL-2R levels.

Table 5.3. ROC curve analysis results for the inflammatory markers in relation to RFI in the untreated (sub)groups^a.

	Selected cutoff	Sensitivity (95% CI), ^b %	Specificity (95% CI), %	PPV, %	NPV, %
Group I, n = 73					
ACE	21 U/L	68 (45 – 86)	75 (60 – 86)	54	84
sIL-2R	1200 kU/L	64 (41 – 83)	88 (76 – 96)	70	85
hs-CRP	3.0 mg/L	91 (71 – 99)	53 (39 – 67)	46	93
SAA	2.5 mg/L	96 (77 – 99)	37 (24 – 52)	40	95
Group Ia, n = 42					
ACE	21 U/L	73 (39 – 94)	77 (59 – 90)	53	89
sIL2R	1300 kU/L	82 (48 – 98)	94 (79 – 99)	82	94
hs-CRP	3.5 mg/L ^c	82 (48 – 98)	58 (39 – 76)	41	90
SAA	8.0 mg/L ^c	55 (23 – 83)	77 (59 – 90)	46	83
Group Ib, n = 31					
ACE	21 mg/L ^c	64 (30 – 89)	70 (46 – 88)	54	78
sIL-2R	750 kU/L	73 (39 – 94)	80 (57 – 94)	67	84
hs-CRP	3.5 mg/L	82 (48 – 98)	55 (32 – 77)	50	85
SAA	4.0 mg/L	82 (48 – 98)	55 (32 – 77)	50	85

^a Group I, all untreated patients; Group Ia, nonchronic untreated patients (time since diagnosis ≤ 2 years); Group Ib, chronic untreated patients (time since diagnosis > 2 years).

^b CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

^c The AUCs of the ROC curves had wide confidence intervals (see legends of Figures 5.2 and 5.3.) and were not significantly different from 0.5.

Discussion

Diagnostic performance of the evaluated markers

This study evaluated the diagnostic accuracy of inflammatory markers to predict respiratory severity (RFI) in sarcoidosis. The present study provided a clear definition of the reference standard and used ROC-curves in the assessment of the test performance as proposed by Zweig *et al.*²⁵. The respiratory severity was assessed by lung function test results. In the whole untreated sarcoidosis patient group as well as in the subgroups of untreated group divided according to time since diagnosis (group Ia, the non-chronic group, and group Ib, the chronic group), ROC curves and logistic regression analysis indicated that sIL-2R had the highest ability to determine pulmonary severity. Comparable ROC-curves for ACE, SAA, and hs-CRP were found in both untreated groups, independent of time since diagnosis. In the treated group (group II), all markers showed the same, weak ability to predict severity in sarcoidosis. Furthermore, their lines were far from the ideal ROC shape, giving several possible (sub)optimal cut-off points. Logistic regression analysis yielded linear predictor scores based on different combinations of markers, which were used to construct the ROC curves in the various groups, but the ROC curves of the obtained models were comparable to the sIL-2R ROC curve.

Although the sensitivity confidence intervals for ACE and sIL-2R were largely overlapping, positive predictive values were higher for sIL-2R than for ACE. In addition, the specificity confidence intervals for sIL-2R and ACE only partly

overlapped (Table 5.2). Because these markers were correlated with each other, the DeLong method (non-parametric method) was used to avoid overemphasizing the differences between the AUCs²⁴. It could definitely be confirmed that sIL-2R was the strongest predictor of RFI in both untreated groups by means of logistic regression analysis (parametric method). These results are in agreement with results reported for previous clinical studies with comparable numbers of patients^{16,17}. The study of Grutters *et al.* also suggested that extrapulmonary manifestations are accompanied by increased sIL-2R values¹⁷. Although extrapulmonary manifestations of sarcoidosis were beyond the scope of this study, this underlines the importance of sIL-2R in sarcoidosis.

In line with results reported by others, this study demonstrated that ACE concentrations have poor predictive value in sarcoidosis^{16,26-28}. The reason for its poorer performance compared with sIL-2R might, at least for a part, be explained by the fact that ACE concentrations can be influenced by an ACE polymorphism (I/D polymorphism in intron 16 of the ACE gene)^{29,30}. Therefore, adjustment of the reference values for the ACE polymorphism has been suggested^{30,31}. Nevertheless, with respect to the ACE polymorphism and susceptibility to disease progression, inconclusive data have been reported^{32,33}.

In the present study, the usefulness of hs-CRP and SAA to predict RFI in sarcoidosis was evaluated. The confidence intervals for hs-CRP and SAA sensitivity and specificity were broad and only partly overlapped with the confidence interval for sIL-2R. In addition, their positive predictive values were much lower than those of sIL-2R. These results are in agreement with a previous study, which found that the mean CRP concentrations of patients with stable or progressing disease (indicating severe disease) did not differ significantly from those in controls, in contrast to sIL-2R¹⁶. It appears that acute-phase response, reflected through increased CRP and SAA concentrations, can be expected only in patients with active disease, including Löfgren syndrome^{8,10,16}.

SAA has been shown to be less sensitive to immunosuppressive drugs (*i.e.*, corticosteroids) and has been recommended for monitoring of patients to whom such drugs have been administered⁶. However, this could not be confirmed by our study. Indeed, in treated patients, all four markers had comparable, rather low, AUCs. Because corticosteroids might affect the concentrations of the markers differently, we selected only those patients who had been on treatment for at least several months. Presumably this is the only way to gather information about the usefulness of the evaluated markers to reflect RFI in patients with sarcoidosis under treatment in general. To date, ACE has not appeared to be useful in the follow-up of sarcoidosis patients during corticosteroid treatment³⁴. Similar results were demonstrated for sIL-2R, CRP, and SAA in the present study.

Definition of severity

The recommendations of the STARD group for the evaluation of diagnostic accuracy studies were followed as far as possible in the present study³⁵. Pulmonary disease severity is usually evaluated by lung function tests and chest radiography^{1,18}, but there is no gold standard. In addition, there is only a weak correlation between lung function tests and chest radiographic stage^{1,36}. The most common indicators of RFI are DLCO and FVC¹, which give information on actual state of the lungs. Both indicate mutually

restrictive and/or obstructive pulmonary function abnormalities in sarcoidosis¹. Abnormal FVC, DLCO, and FEV1 values are traditionally used as indicators for treatment in case of pulmonary involvement³⁶.

Conclusion

In conclusion, to initiate treatment it is crucial to know whether sarcoidosis is severe, rather than active. Hence, in this study, we examined whether the evaluated markers were able to predict sarcoidosis severity. Sarcoidosis severity was defined through RFI. In the untreated group of patients, sIL-2R appeared to be the best marker for predicting disease severity, whereas the traditionally used ACE appeared comparable to hs-CRP and SAA. We therefore recommend the measurement of sIL-2R, in addition to the standard measurement of ACE, to monitor disease severity and follow-up in sarcoidosis.

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Chapter 6

The relationship between fatigue and clinical parameters in acute pulmonary sarcoidosis

Jolanda De Vries, Snježana Rothkrantz-Kos,
Marja P. van Dieijen-Visser, Marjolein Drent

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Abstract

Background

Studies on the relationship between fatigue and clinical parameters are sparse. In the present study this relationship was examined in a systematic way.

Methods

Patients with time since diagnosis ≤ 2 years, visiting the outpatient clinic of the University Hospital Maastricht ($n = 60$; 34 untreated, 26 treated) were clinically evaluated and completed the Fatigue Assessment Scale (FAS). A representative sample of the Dutch population ($n = 1893$) also completed the FAS. Pulmonary disease severity was estimated from lung function test results and measures of metabolic derangement. Acute phase response markers high-sensitivity C-reactive protein (hs-CRP), serum amyloid A (SAA) and sarcoidosis activity parameters, soluble interleukin-2-receptor (sIL-2R), and angiotensin-converting enzyme (ACE) were also measured.

Results

Only 27% of the sarcoidosis patients were diagnosed as non-fatigued (FAS score < 22), compared to 80% in the control population ($n = 1893$). In the sarcoidosis patients no sex differences and no differences in fatigue scores between the treated and the untreated groups were found. Patients with fatigue (FAS-score ≥ 22) had lower DLCO values ($p < 0.05$). However, none of the tested clinical or serological parameters appeared to be a significant predictor of fatigue.

Conclusion

In the present study, it was confirmed that fatigue is a major problem in sarcoidosis. The extent of fatigue could not be explained by clinical parameters. Thus, up to now, no clinical or physiological variable seems useful in predicting which patients are fatigued. In this light, the Fatigue Assessment Scale might be considered as a supplementary tool in sarcoidosis.

Introduction

Sarcoidosis is a disorder of unknown origin most frequently occurring in the lung. Although sarcoidosis occurs worldwide, the prevalence and the course of the disease vary across countries and ethnic group¹. In the Netherlands the prevalence is estimated to be 40–50/100000².

Clinical manifestations of sarcoidosis depend on the intensity of the inflammation and organ systems affected. Pulmonary sarcoidosis may present itself in a variety of ways with symptoms related directly to the chest such as coughing, dyspnea on exertion, retro-sternal chest pain, chest discomfort, and wheezing^{3,4}. Furthermore, fatigue, arthralgia, and erythema nodosum are common features of sarcoidosis, which vary however, across countries^{4–7}. Fatigue appeared to be a major problem in 30 to 90 per cent of the patients^{3,3,8–15}. This fatigue can be substantial and persistent^{3–5,9,10,12,14,15}. Fatigue is disabling for the patient, causes an impaired quality of life (QOL), and may become chronic¹⁶. In a study among 64 sarcoidosis patients, even patients who did not suffer from fatigue had an impaired QOL compared to healthy persons. However, patients who reported fatigue had the most impaired quality of life¹⁵. In addition, fatigue is a predictor of depressive symptoms^{17,18} as well as of pain⁷. Because of the capacity of fatigue to cause impaired QOL, it is important to measure and study fatigue.

An acute phase response evidenced by moderate increase of the C-reactive protein (CRP) level was found in sarcoidosis patients who suffered from fatigue and other related symptoms¹². Besides, in the same study, a presence of metabolic derangement was shown to be related to fatigue and to the CRP concentrations. In line with this, a relation between elevated CRP concentrations and fatigue was demonstrated in rheumatoid arthritis, Crohn's disease, and ulcerative colitis patients¹⁹.

Nowadays more sensitive parameters have been introduced to monitor inflammation such as high-sensitivity C-reactive protein (hs-CRP) and serum amyloid A (SAA). Besides, a new questionnaire has been developed, allowing to measure fatigue in sarcoidosis patients more in-depth^{20,21}, as compared to the single item fatigue question and the facet Energy and Fatigue of the WHOQOL-100 questionnaire, used previously¹⁵. Therefore, the aim of this study is to examine whether the more sensitive measures used to monitor inflammation can be used to explain fatigue in patients with acute manifestations of pulmonary sarcoidosis.

Materials and Methods

Participants

From January 2000 to October 2002, 60 (34 untreated and 26 treated) patients who visited the outpatient clinic of the University Hospital Maastricht, a referral center for sarcoidosis, with a time since diagnosis of $\leq$ two years ("acute" sarcoidosis patients) were included in this study. Patients were diagnosed with sarcoidosis based on consistent clinical features and bronchoalveolar lavage (BAL) fluid analysis results, according to the WASOG guidelines. Comorbidity was defined as any medical problem

not related to sarcoidosis. Disorders or conditions considered as comorbidity included cardiovascular disease, thyroid disease, diabetes, anemia, cancer, muscle weakness and immobility due to musculo-skeletal disorders. Extrapulmonary localisations of sarcoidosis were not considered as comorbidity but as sarcoidosis related. None of the patients had any significant medical history or co-morbidity.

Informed consent was obtained from all participating patients. A control group of persons also completed the fatigue questionnaire. This group, comprising of 1893 persons, was a representative sample of the Dutch population²⁰. They completed a computer-administered questionnaire. The respondents of the latter sample were all involved in an internet-based panel. Every week this panel received a questionnaire directly on their personal computer. With regard to the demographics of the reference sample, there were 1128 males and 765 females and the mean age of the reference sample was 44.7 (SD = 15.3; range 16 – 87). This sample was not analyzed as part of this study but was used as a reference group.

Lung function testing

Lung function measurements, including forced expiratory volume in one second (FEV1) and forced vital capacity (FVC), were measured with a pneumotachograph. The diffusing capacity for carbon monoxide (DLCO) was measured by the single-breath method (both Masterlab, Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted²².

Chest radiographs

Chest radiographs were graded according to the radiographic staging of DeRemee (0 to III), adding stage IV, the end stage of lung fibrosis^{3,23}. A radiologist, blinded to the patient's history, performed all interpretations.

Serum markers

Simultaneously with lung function tests and fatigue assessment, blood samples were taken and serum was stored at -20°C until actual measurement of inflammatory markers. Serum IL2 receptor (sIL-2R) was determined on the IMMULITE Automated Analyser, by means of a two-site chemiluminescent enzyme immunometric assay, with a detection limit of 50 kU/L, and a measuring range 50 – 7500 kU/L (Diagnostic Product Corporation, Los Angeles, CA, cat no LKIP1). The imprecision of the assay (both within- and between run) appeared to be below 7.20%. The reference range for sIL-2R was 241 – 846 kU/L.

Hs-CRP and SAA were determined by particle-enhanced immunonephelometry, on the BN Prospec from Dade Behring. The detection limit for hs-CRP was 0.175 mg/L and the measuring range was 0.175 – 1100 mg/L, depending on the dilution (Dade Behring, Liederbach Germany, N Hs CRP, cat no OQIY 13; supplement reagent OUMU).

The detection limit of SAA was 3 mg/L, with a measuring range of 3 – 1000 mg/L, depending on the dilution (Dade Behring, Liederbach Germany, N SAA reagent, cat no OQMP 11).

The imprecision of the SAA BN ProSpec method appeared to be below 10.7%. The reference values were 0.90 – 10.22 mg/L.

Evaluation of the hs-CRP assay on the BN ProSpec was reported previously. Reference values were 0.26 – 7.24 mg/L²⁴.

Serum ACE (ACE) was measured by a colorimetric method (Fujirebio Inc., Tokyo, Japan, cat. nr. FU 116). ACE acts on a substrate p-hydroxybenzoyl-glycyl-L-histidyl-L-leucine and separates p-hydroxybenzoyl-glycine, which is converted in two subsequent reactions in quinoneimine dye. To evaluate the ACE activity, the absorbance of quinoneimine dye is measured at 505 nm. The imprecision of the ACE assay appeared to be below 5.6%. Reference values of ACE were 9 – 25 U/L.

Metabolic measures

Body composition was measured by single frequency bioelectrical impedance analysis (RJL systems, Detroit, USA) in the supine position on the right side. Fat-free mass (FFM) was calculated from $[(\text{height})^2/\text{resistance}]$ and body weight using the Lukaski formula. Resting energy expenditure (REE) was measured after an overnight fast under standardized conditions²⁵ by indirect calorimetry using a ventilated hood (Oxycon beta, Mijnhardt, Bunnik, The Netherlands). REE was adjusted for FFM and gender, by analysis of covariance²⁶.

Questionnaire

The Fatigue Assessment Scale (FAS)^{20,21} was used to measure fatigue (Appendix). This questionnaire consists of ten questions. The response scale is a 5-point scale (1 never to 5 always). Scores on the FAS can range from 10 to 50. The psychometric properties are good also in sarcoidosis. Moreover, the FAS appeared to be unidimensional in a large sarcoidosis population^{20,21}.

Table 6.1. Demographics data of the evaluated sarcoidosis patients.

Variables	Total group	Untreated patients	Treated patients
Number of cases	60	34	26
Sex: male/female	31 / 29	15 / 19	16 / 10
Age ^a , years	40.8 ± 10.4 (19 – 66)	40.3 ± 10.5 (23 – 66)	41.5 ± 10.5 (19 – 58)
Time since diagnosis ^a , months	10.2 ± 9.6 (0 – 24)	8.9 ± 9.0 (0 – 24)	12.0 ± 10.2 (0 – 24)
Smoking: no/yes ^b	53 / 7	27 / 7	26 / 0

^a The data are presented as mean ± standard deviation with range in parentheses.

^b Significant difference ($p < 0.01$) between the treated and untreated patients.

The cut-off score of the FAS (22) was derived from two large representative samples of 1) the Dutch working population and 2) the general population. In both samples, 80% of the participants had a FAS score below 22²⁷. In addition, this result was compared with other fatigue measures such as the emotional exhaustion subscale of the Dutch Maslach Burnout Inventory (UBOS)²⁸ to verify the appropriateness of the found FAS cut-off score.

In a large study among 1046 sarcoidosis patients, the FAS appeared to be a unidimensional scale. The content validity (factor analysis and mokken scale analysis), construct validity (factor analysis comparing with the Beck Depression Inventory), and internal consistency (Cronbach's alpha) of the FAS were good. The test-retest reliability was 0.89^{20,21}. A change in score of 5 or more is considered clinically significant (minimal clinical difference for score to be significant).

Patients also completed the Bath Breathlessness Scale (BBS)²⁹. This is a 35-item adjective subjective breathlessness (dyspnea) scale measuring four aspects of breathlessness. In addition, patients can be asked to indicate on an 8-point response scale (range 0 to 7) how breathless they felt during the last two weeks. In the present study, only the question about perceived severity of breathlessness was used.

Statistical Analysis

For all of the selected patients non-missing and interpretable results were obtained for the laboratory tests, lung function tests, metabolic derangement measures and the FAS. The comparison between the sarcoidosis group and the reference sample, was performed by means of one sample t-tests. The distributions of the explanatory variables CRP, SAA, ACE and sIL-2R were positively skewed. Therefore, the data are presented as median with interquartile range. Patients were divided into two groups: non-fatigued (FAS score < 22) and fatigued (FAS score $\geq$ 22). Differences between the fatigued and non-fatigued groups were tested by means of Student t-tests or Mann-Whitney U test, depending on the distribution (normal or skewed) of the tested (explanatory) variables, *i.e.* serum markers, metabolic derangement measures and lung function test results.

A log transformation (ln) was applied to normalize the data prior to further analyses. First, the relationship between fatigue (as continuous variable) and the laboratory parameters was examined by means of Pearson correlations. Second, univariate logistic regression was used to test the discriminatory effect of each explanatory variable with regard to being fatigued or non-fatigued. Finally, multivariate logistic regression was used to assess simultaneously the effect of the three clinical parameters with the lowest p-values from the univariate analyses. The lung function test results were firstly treated as continuous variables and thereafter the values were dichotomized (the abnormal values were considered those with the %predicted of < 80%). The metabolic derangement measures were treated as continuous variables. The hs-CRP, CRP, SAA, sIL-2R and ACE levels were not treated as continuous variables in the logistic regression analyses, but were dichotomized. The reason was that the distribution of the data was highly skewed and all levels below the lower detection limit of these assays were recorded as the lower detection limit value. As the upper reference limit can be influenced by the presence of some subclinical inflammation and, therefore gives outliers, the values of the 90th percentile of the reference values were used to dichotomize the laboratory parameters. The likelihood ratio tests (-2 LL) were used. All p-values were two-tailed and $p < 0.05$ was considered statistically significant. Statistical analyses were performed using the SPSS10.0 for Windows (SPSS, Chicago, IL, USA).

Results

General and clinical characteristics of sarcoidosis patients

General characteristics of sarcoidosis patients ($n = 60$) are presented in Table 6.1. In the group of untreated patients ($n = 34$) there were 7 smokers (20.6%), while all patients of the treated group ($n = 26$) were non-smokers.

Clinical characteristics of the patients are presented in Table 6.2. With respect to lung function data, the FEV1 ($p < 0.01$) and FVC ($p < 0.05$) values appeared lower in the treated group as compared to the untreated group. No differences in radiographic staging or the DLCO between the two groups were found. Also, with regard to serological data and the metabolic derangement measures, no differences were observed between the treated and untreated group.

The perceived severity of dyspnea was also assessed. During the previous two weeks prior to the study, the sarcoidosis patients had a mean score of 3.28 (SD = 1.71) on a possible range of 0 to 7. This score represents a moderate level of dyspnea. Compared with a group of idiopathic pulmonary fibrosis patients from a previous study (mean = 3.80, SD = 2.0)³⁰ the sarcoidosis group scored significantly but not extremely lower.

Table 6.2. Clinical characteristics of the sarcoidosis patients.

Variables	Total group ($n = 60$)	Untreated patients ($n = 34$)	Treated patients ($n = 26$)
Lung function tests ^c			
DLCO	87.6 $\pm$ 17.3	90.1 $\pm$ 13.4	84.2 $\pm$ 21.4
FEV1 ^b	90.1 $\pm$ 22.6	94.2 $\pm$ 18.4	84.8 $\pm$ 26.5
FVC ^a	99.0 $\pm$ 20.2	102.3 $\pm$ 16.14	94.6 $\pm$ 24.3
Radiographic stages			
0/I/II/III/IV	14 / 15 / 11 / 18 / 2	6 / 11 / 8 / 9 / 0	8 / 4 / 3 / 9 / 2
Serological parameters ^d			
ACE (U/L)	21.0 (17.0 – 30.0)	21.0 (17.0 – 31.0)	22.0 (18.0 – 31.0)
CRP (mg/L)	9.0 (5.0 – 15.0)	3.7 (1.4 – 12.7)	3.8 (1.3 – 8.5)
hs-CRP (mg/L)	3.5 (1.3 – 10.5)	7.0 (2.0 – 15.3)	10 (6.0 – 15.5)
SAA (mg/L)	6.3 (3.3 – 11.7)	5.9 (2.6 – 11.1)	6.9 (4.0 – 11.8)
sIL-2R (kU/L)	711 (405 – 1066)	737 (427 – 1269)	697 (454 – 865)
Metabolic derangement measures			
REE (kcal/24h)	95.9 $\pm$ 10.1	94.7 $\pm$ 11.7	97.5 $\pm$ 7.5
REE/FFM (kcal/kg FFM)	31.0 $\pm$ 4.5	30.8 $\pm$ 4.9	31.3 $\pm$ 4.1
Fatigue measure			
FAS score ^c	27.5 $\pm$ 8.6 (10 – 48)	25.7 $\pm$ 9.0 (10 – 45)	29.7 $\pm$ 7.7 (13 – 48)

^a Significant difference ($p < 0.05$) between the treated and untreated patients.

^b Significant difference ($p < 0.01$) between the treated and untreated patients.

^c The data are presented as mean $\pm$ standard deviation.

^d Because of the non-gaussian distribution of the data, the values are presented as median with range (25th – 75th percentiles).

Evaluation of the FAS (fatigue) score

The whole patient group ($n = 60$) had a mean FAS score of 27.5 ($SD = 8.6$), whereas the reference group ($n = 1893$) had a mean score of 18.0 ($SD = 5.7$) ($t = -8.51$, $p < 0.001$). Only 27% of sarcoidosis patients had a FAS score < 22 (mean $\pm$ SD: 17.1 ± 3.6), compared to 80% in the control population.

Table 6.3. shows that the difference between both groups on the total FAS score reflects substantial differences for each individual item. In each instance, the sarcoidosis patients are more fatigued than the reference group. The only exception is question 10 ('When I am doing something I can concentrate quite well'). As this question is not recoded, a high score on this question indicates a low score for fatigue. In other words the reference group has more problems with staying concentrated.

In sarcoidosis patients, no sex differences were found with respect to the FAS score. In contrast, in the control group females had higher fatigue scores as compared to males ($t = -6.1$, $p < 0.001$). No differences in fatigue scores between the treated and the untreated groups were found (Table 6.2).

Table 6.3. A comparison between the sarcoidosis group and the reference group at the FAS item level.

Question/Item	Sarcoidosis patients ^a	Reference group ^a	t-value ^b
1	3.53 ± 1.19	2.13 ± 0.88	9.17
2	3.47 ± 1.21	1.90 ± 0.94	10.0
3	2.75 ± 1.20	1.78 ± 0.87	6.25
4 ^c	2.62 ± 1.19	3.74 ± 1.14	-7.28
5	2.92 ± 1.12	1.72 ± 0.79	8.24
6	2.43 ± 1.13	1.79 ± 0.81	4.43
7	2.10 ± 1.07	1.47 ± 0.67	4.57
8	2.40 ± 1.11	1.77 ± 0.72	4.41
9	2.15 ± 0.99	1.48 ± 0.71	5.25
10 ^c	3.67 ± 1.13	1.72 ± 0.79	13.34

^a The data are presented as mean $\pm$ standard deviation.

^b $p < 0.001$ for each of the ten items, between the sarcoidosis group and reference group.

^c These questions are not recoded, meaning that a high score reflects low score for fatigue.

FAS score in relation to clinical data

Since with regard to the laboratory parameters, metabolic derangement measures, radiographic staging and lung function data (except for FVC and FEV1) no differences were observed between the treated and untreated groups (Table 6.2), grouping of the data was allowed. Figure 6.1 shows the distribution of the clinical data in fatigued and non-fatigued groups in respectively untreated and treated groups of patients.

Figure 6.1. Scatter of serological parameters and metabolic derangement measures.

Patients were divided into two groups with respect to their FAS score. The group with fatigue had a FAS score ≥ 22 , whereas group without fatigue had a FAS score < 22 . The horizontal line represents the mean values of the (logarithmic transformed, except for the DLCO, FEV1 and FVC) data, divided according to treatment.

Open circles indicate untreated patients, whereas closed circles indicate with prednisone treated patients. For the DLCO and FVC there was one missing case in treated non-fatigued group.

These results further suggest that prednisone treatment at the moment of 'sampling' probably had no influence on the results of clinical or serological tests (Figure 6.1). Therefore, for the further evaluation of the FAS score with respect to clinical data, patients were divided into two groups: the group without fatigue (total FAS-score < 22) and the group with fatigue (total FAS score ≥ 22).

Patients with fatigue (FAS-score ≥ 22) had lower DLCO values ($p < 0.05$). However, with regard to FEV1, FVC and the radiographic staging no differences between the fatigued and non-fatigued groups were observed. Also regarding laboratory parameters and metabolic derangement measures no significant differences between fatigued and non-fatigued groups were present.

Among the laboratory markers, only sIL-2R and SAA correlated with the metabolic derangement measures ($p < 0.05$). The sIL-2R correlated with REE% ($r = 0.30$, $p = 0.023$), whereas SAA correlated with REE/VVM ($r = 0.31$, $p < 0.02$). sIL-2R and SAA correlated also with each other ($r = 0.39$, $p < 0.01$). sIL-2R appeared to correlate with ACE to a large extent ($r = 0.60$, $p < 0.0001$).

Prediction of fatigue (FAS-score ≥ 22) using clinical data

First, a serie of univariate logistic regression analyses was completed. Table 6.4. shows the odds ratios with their corresponding confidence intervals. As can be seen from the Table 6.4, no statistically significant predictors were found. For each single variable the analyses were repeated correcting for prednisone use (*data not shown*).

Table 6.4. Prediction of fatigue assessed by Fatigue Assessment Scale (FAS) by clinical data in patients with acute pulmonary sarcoidosis.

Variables	Univariate OR (95% CI)	p-value	Multivariate OR (95% CI)	p-value ^c
Prednisone use				
Yes	3.0 (0.8 – 10.8)	0.078	2.7 (0.7 – 10.0)	
Severity of disease ^a				
RFI Present	2.1 (0.6 – 7.5)	0.249	1.3 (0.3 – 5.4)	
Presence of hypermetabolism				
REE	1.0 (1.0 – 1.1)	0.852		
REE/FFM	1.0 (0.9 – 1.2)	0.827		
Serum markers ^b				
ACE > 24.4 U/L	0.7 (0.2 – 2.1)	0.495		
sIL-2R > 685 kU/L	1.0 (0.3 – 3.2)	0.969		
SAA > 6.9 mg/L	3.0 (0.8 – 10.8)	0.078	2.6 (0.6 – 10.0)	
hs-CRP > 5 mg/L	0.9 (0.2 – 3.3)	0.860		
CRP > 5 mg/L	1.3 (0.4 – 4.3)	0.712		
				0.1633

^a Severity of disease. RFI Present, if DLCO < 80% or FEV1 < 80% or FVC < 80%. RFI absent if all of these parameters ≥ 80 .

^b The serum markers were dichotomised according to the 90th percentile of the reference values.

^c The p-value in the multivariate analysis is for the prediction of fatigue by the SAA, corrected for prednisone use and the presence of respiratory functional impairment.

The p-values correspond to the -2LL test in the respectively univariate or multivariate analysis.

However, no significant results were obtained. The three variables with the lowest *p*-values from the univariate analysis: prednisone use ($p = 0.078$), presence of respiratory functional impairment ($p = 0.249$) and the SAA values ($p = 0.078$) were used in the multivariate logistic regression analysis. However, no significant model could be obtained ($p = 0.163$).

Discussion

The present study clearly showed that fatigue is a major problem in sarcoidosis. Seventy-three percent of the sarcoidosis patients were fatigued, compared with 20% in the control population. No differences were found in fatigue scores between the treated and the untreated group and between male and female patients. The prednisone treatment at the moment of 'sampling' appeared not to have influence on the results of clinical or serological tests, which allowed for the grouping of the patients. Subsequently, patients were divided with respect to the FAS score in the fatigued and non-fatigued groups. The fatigued patients tended to have decreased lung function test results, although only the DLCO ($p < 0.05$) appeared to be significantly decreased. With regard to radiographic staging no differences between the groups concerning fatigue were observed. With respect to serological data and metabolic derangement measures patients with fatigue tended to have increased values, albeit not significantly. The serological parameters SAA and sIL-2R correlated well with metabolic derangement measures, whereas ACE, CRP, and hs-CRP showed no relationship with metabolic derangement measures. Among the clinical parameters, none of the explanatory variables were able to predict whether patients were fatigued or not and no combined model could be found.

Fatigue

Compared with a representative sample, the sarcoidosis patients had much higher fatigue scores. The finding that fatigue is a major problem in sarcoidosis is in accordance with previous studies^{3,5,8-15}. However, besides the fact that the fatigue was assessed by means of a more appropriate questionnaire, this was the first time that fatigue scores in sarcoidosis patients were compared with a healthy population. Another study that used the FAS, examined a large group of Dutch persons working at least 20 hours per week ($n = 765$)²¹. In the latter study about 80% of the persons from the working population sample had a FAS score of < 22 , comparable to the control sample used in the present study. This emphasizes the large difference with the sarcoidosis population and provides a further underpinning of the severity of fatigue in sarcoidosis. The present study differs from the previous studies in the way the fatigue was assessed. In this study, a fatigue questionnaire (FAS) was used. In the other studies, fatigue was measured with a single dichotomous (yes/no response) fatigue question. The FAS provides information on the severity of fatigue instead of merely presence or absence of fatigue. Furthermore, it is known that single items as used in previous studies are less reliable³¹.

Fatigue in relation to clinical data

In disorders like COPD it was appreciated that, at least in part, in hypermetabolic patients the resting energy expenditure (REE) was attributed to the level of the systemic inflammation^{25,32}. The study of Drent *et al.* indicated that an acute phase response (reflected through increased CRP values), in combination with metabolic derangement (reflected through increased REE) might provide some insight into fatigue in sarcoidosis¹². Even those cases without signs of pulmonary involvement appeared to have increased REE values¹². Therefore, assessment and inclusion of REE (corrected or not for the fat-free mass, FFM) was considered to have an additional value in the management and follow-up of sarcoidosis patients. For that reason, clinical data indicating lung function and metabolic derangement together with serological data were used to see whether a combined model could be found that was able to predict fatigue.

Among all the tested markers, SAA was the strongest ($p = 0.078$ (Table 6.4.), if corrected for prednisone use, $p = 0.108$) but non-significant positive univariate predictor of fatigue. The lack of significance at 0.05 level might be due to the fact that the present sample size was too small. Therefore, a careful interpretation of this result is needed. In fatigue, the concentration of brain 5-hydroxytyptamine (5-HT or serotonin) was shown to be increased^{33,34}. Interestingly, in a murine model it was shown that an increase of 5-HT upon stimulation of interleukin-6 was accompanied by the increase of SAA³⁴. Nevertheless, larger studies are needed to validate the results of this study and confirm whether the SAA can indeed be used to monitor fatigue.

With respect to corticosteroid treatment, some trend between corticosteroid use and fatigue appeared to exist, although the result of logistic regression analysis seemed not significant. As the sample size is rather small, careful interpretation of this result is needed. In a logistic regression analysis we corrected for the effect of prednisone, as we indeed considered the OR for prednisone of 3, with a p -value of 0.078 rather high. In a larger sample, this p value might become significant (the same holds for the SAA). However, no differences in fatigue scores and most of the clinical parameters (Table 6.2) between the treated and the untreated groups were found. Therefore, it might be assumed that in our sample the influence of corticosteroid therapy is limited.

Neither the CRP nor hs-CRP were able to predict fatigue. Overall, the CRP values were higher as compared to hs-CRP values. This only emphasizes the discrepancies between the two methods as shown earlier²⁴. Moreover, activity markers such as ACE and sIL2R also showed no relation to fatigue. Besides, in contrast with previous findings¹², in the present study no relationship with the measures of metabolic derangement and the CRP or hs-CRP could be found. Despite the discrepancies between the methods (CRP and hs-CRP), the lack of relationship of the CRP or hs-CRP with the measures of metabolic derangement is difficult to explain. It is probably population related. Moreover, the SAA and sIL-2R appeared to correlate with metabolic derangement measures, nevertheless confirming the relationship of the acute phase response with metabolic derangement in sarcoidosis, at least partly, in agreement with a previous study¹².

As mentioned before, one of the limitations of this study is the small sample size. Since we expected low correlations, a large sample size would be needed to make firm statements. However, large populations of sarcoidosis patients are difficult to recruit for any study since the disease is not extremely common. In comparison to our previous

study¹², a different type of statistical analysis was now used with the aim to predict fatigue (logistic regression analysis). Although both, the previous as the present study show some overlap in clinical parameters (REE, CRP), in the present study we added additional clinical (presence of respiratory functional impairment) and laboratory parameters (sIL-2R, SAA and ACE). In the multivariate analysis we combine all these parameters. Furthermore, the CRP concentrations reported in the previous study were measured with a less sensitive CRP method, which appeared to be very sensitive to sample turbidity and might give unreliable results²⁴. It was to be expected that the CRP concentrations would only slightly be increased in sarcoidosis patients^{12,35}. To measure these minor increases from the upper reference level, the more sensitive CRP method is needed. Therefore, in this study we used the so-called high-sensitivity CRP method to accurately measure the CRP concentrations.

Although it was beyond the scope of this study, it might have been important to compare the exercise test and its relationship with the FAS. Changes in gas-exchange with exercise were previously demonstrated to be the most sensitive physiologic measurement to assess the extent of pulmonary disease in early radiographic stages of sarcoidosis^{32,33}. Accordingly, a significant maximal exercise limitation in sarcoidosis patients without significant pulmonary impairment was shown by Delobbe *et al.*³⁴. In line with this, a recent study, further confirmed the value of exercise testing. Namely, HRCT findings appeared to be much more sensitive in depicting respiratory disability, especially abnormal gas exchange, as compared to radiographic staging³⁵. Currently, a prospective study is conducted to evaluate the possible relationship of impaired gas exchange with fatigue and dyspnoea more carefully. Preliminary results showed only a moderate relationship with the FAS score between the subscore mental fatigue and desaturation during exercise ($r=-0.23$, $p<0.05$)³³.

The results of this study emphasize the difficulties that clinicians face when trying to objectify fatigue. Fatigue, as an integral part of the clinical picture of sarcoidosis, is disabling for the patient and causes an impaired quality of life^{3,4,12}. The course of the disease is mainly monitored by assessing clinical features and using auxiliary diagnostic procedures. However, an objective system for the assessment of disease activity and fatigue is still lacking. Moreover, the non-specific symptoms including fatigue are often hard to objectify, as confirmed by this study. Up to now, no clinical or physiological variable seems useful in predicting which patients are fatigued. The feeling of fatigue is a subjective experience that is associated with many diseases and states. However, the large ostensibly healthy population was screened by means of the FAS for the presence of fatigue and the cut-off of 22 was carefully chosen²¹. Besides, the FAS was constructed in a way to minimize the intersubjective agreement, the well-known source of error and bias in human judgement³⁶.

Conclusion

The present study confirmed that fatigue is a major problem in sarcoidosis. None of the tested variables such as lung function data, metabolic derangement measures and laboratory parameters of inflammation, T cell activation or granuloma formation seemed useful in predicting which patients are fatigued. In this light, the Fatigue Assessment Scale can be considered as a supplementary tool in sarcoidosis to assess fatigue.

Appendix

The Fatigue Assessment Scale (FAS)^{a, b}

	Never	Sometimes	Regularly	Often	Always
1. I am bothered by fatigue	1	2	3	4	5
2. I get tired very quickly	1	2	3	4	5
3. I don't do much during the day	1	2	3	4	5
4. I have enough energy for everyday life ^c	1	2	3	4	5
5. Physically, I feel exhausted	1	2	3	4	5
6. I have problems to start things	1	2	3	4	5
7. I have problems to think clearly	1	2	3	4	5
8. I feel no desire to do anything	1	2	3	4	5
9. Mentally, I feel exhausted	1	2	3	4	5
10. When I am doing something, I can concentrate quite well ^c	1	2	3	4	5

^a The following ten statements refer to how well the person usually feels. Per statement one out of five answer categories can be chosen, varying from Never to Always. 1 = Never, 2 = Sometimes; 3 = Regularly; 4 = Often; 5 = Always.

^b Based on large representative samples of the Dutch population, the cut-off score of the FAS is 21, i.e., scores of ≥ 22 are considered to represent substantial fatigue. A change in the FAS score of 5 points is considered to be clinically relevant.

^c Scores on questions 4 and 10 should be recoded (1=5, 2=4, 3=3, 4=2, 5=1). Subsequently, the total FAS score can be calculated by summing the scores on all questions (the recoded scores for questions 4 and 10)

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Chapter 7

Decreased redox state in red blood cells from patients with sarcoidosis

Snježana Rothkrantz-Kos, Marjolein Drent, Herma Vuil,
Martin De Boer, Aalt Bast, Emiel F.M. Wouters, Dirk Roos,
Marja P. van Dieijen-Visser

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Abstract

Background

The glutathione system has a key role in the defense against oxidative stress. To function properly, this system needs NADPH to maintain glutathione (GSH) in its reduced form. We hypothesized that the clinical problems associated with sarcoidosis might be related to a decreased anti-oxidant defense and we therefore measured the activity of the NADPH-generating enzyme glucose-6-phosphate dehydrogenase (G6PD), the GSH-regenerating enzyme glutathione reductase (GR) and indirectly the level of NADPH in red blood cells from patients with sarcoidosis.

Methods

In a population of sarcoidosis ($n = 88$) patients, G6PD, GR and GR activity after incubation with chromate (GR-Cr) were measured in erythrocytes. A decreased concentration of NADPH was revealed by an increased GR-Cr (> 0.6 IU/g Hb). To exclude a mutation in the G6PD gene, sequencing was performed in cases with an abnormal GR-Cr. Sarcoidosis pulmonary disease severity was evaluated by means of laboratory data, radiographic staging, HRCT scoring, pulmonary function- and exercise capacity testing.

Results

Fourteen (29.2%) females and one male (2.5%) demonstrated an increased GR-Cr test, indicative of a decreased NADPH level. Patients with an abnormal test result demonstrated also significantly increased ACE and GR values ($p < 0.05$). Only one female case (of 6 tested) appeared to have a mutation in the G6PD gene.

Conclusion

In a considerable percentage of female patients with sarcoidosis, a decreased level of NADPH in the erythrocytes was found.

Introduction

Sarcoidosis, a disease predominantly affecting young adults, is the commonest diffuse lung disease, with a population prevalence ranging from 1.4 to 64 per 100,000 population¹. The disease etiology remains undetermined, although genetic predisposition is likely to play an important role^{1,2}. Environmental factors, which are mostly unknown, may determine the disease course. A consequence of environmental factors is often oxidative stress, inducing the production of free radicals³.

During oxidative stress, either intracellular in origin or initiated by drugs or environmental chemicals, reduced glutathione (GSH) is critical for protection of the cells^{4,5}. GSH is involved in the detoxification of reactive oxygen species through its donation of reducing equivalents. Furthermore, GSH reduces oxidized protein thiol groups back to reduced thiol groups (-SH)^{5,6}. The enzyme glutathione reductase (GR) maintains high intracellular concentrations of GSH by reducing the oxidized form of glutathione (GSSG) back to GSH. For this reaction, the reducing equivalents are derived from reduced nicotinamide adenine dinucleotide phosphate (NADPH) ($\text{GSSG} + \text{NADPH} + \text{H}^+ \leftrightarrow 2\text{GSH} + \text{NADP}^+$)⁶⁻⁹. The only pathway to generate NADPH in erythrocytes is the hexose monophosphate shunt (HMS). In this pathway, glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme^{5,10}.

There is clearly lack of international data about the relation of cellular redox state and sarcoidosis. We hypothesized that the malfunctioning of this protective system might be involved in sarcoidosis, and recognition of this phenomenon might be of clinical importance. Avoiding certain drugs and other causes of oxidative stress might then limit the extent of the inflammatory response in sarcoidosis.

Therefore, the erythrocyte capacity to maintain NADPH concentrations was evaluated in a population of sarcoidosis patients by means of the chromate inhibition test^{11,12}.

Materials and Methods

Study population

Eighty-eight mainly Caucasian sarcoidosis patients were included in this study. These patients visited the Sarcoidosis Management Center of the University Hospital Maastricht, a Dutch referral center for sarcoidosis, between January 2000 and January 2001. The patients' diagnosis was based on consistent clinical features and bronchoalveolar lavage fluid analysis¹³, according to the ATS/ERS/WASOG guidelines¹. The diagnosis was histologically confirmed in 91% of the cases. Informed consent was obtained from all participating subjects. Patient characteristics are summarized in Table 7.1.

Erythrocyte redox-state evaluation

The activities of G6PD and GR, as well as the chromate inhibition test (GR-Cr) were measured spectrophotometrically in hemolysates of washed erythrocytes according to

the method of Zürcher *et al.*¹¹, with minor modifications as described by Jonges *et al.*¹². G6PD activity was measured to screen for possible G6PD deficiency and GR for its capacity to regenerate GSH, both by means of a direct spectrophotometric method^{11,12}. To screen for levels of NADPH in the erythrocytes, the chromate inhibition test was used. This test is based on the inhibition by chromate (Cr^{6+}) of GR activity in erythrocytes with a normal level of NADPH, but not in erythrocytes with decreased levels of NADPH. Chromate inactivates GR with reduced, catalytically active (-SH) thiol groups (this reduction is dependent on NADPH concentration) in the active center of the enzyme, but not GR with oxidized (-S-S-) thiol groups. The remaining glutathione reductase activity, will be thus higher in NADPH-deficient cells than in normal cells, and is thus a reflection of the intracellular redox state.

Table 7.1. General characteristics of the studied sarcoidosis patient population.

	Female	Male	Total
Number of cases	48	40	88
Age at diagnosis (yr) ^a	36.1 ± 13.0 (12 – 68)	36.8 ± 8.3 (25 – 55)	36.4 ± 11.1 (12 – 68)
Smoking (no / yes)	42 / 6	38 / 2	80 / 8
Use of prednison (no / yes)	41 / 7	22 / 18	63 / 25
Race (caucasian / negroid)	41 / 7	37 / 3	78 / 10

^a Data are mean ± SD with range in parenthesis

Molecular genetic studies

Nucleotide sequence analysis of the G6PD gene was performed in 6 cases with an increased GR-Cr (> 0.6 IU/g Hb). Genomic DNA was isolated from 5 ml of K_3 -EDTA anti-coagulated whole blood (buffy coat) with the High Pure PCR Template Preparation Kit, cat no. 1796828 (Roche, Mijdrecht, The Netherlands). The whole G6PD coding sequence was amplified from genomic DNA by polymerase chain reaction (PCR) with primers complementary to the intron regions, to ensure analysis of all exon and intron splicing regions of the G6PD gene (Table 7.2). Aliquots of 50 – 200 ng genomic DNA were amplified in the Air Thermo-cycler 1605 (Idaho Technology, Inc., Idaho Falls, ID, USA). The protocol used was as follows: denaturation at 95°C for 5 minutes, followed by 40 – 50 cycles of annealing at 60°C for 30 seconds and extension at 72°C for 15 seconds. For nucleotide sequencing, the dideoxynucleotide chain termination method was used, with an ABI PRISM Big Dye Terminator Cycle sequencing kit on the ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Norwalk, CT, USA).

Evaluating Sarcoidosis Pulmonary Disease Severity

Serum ACE was measured with a colorimetric method (Fujirebio Inc., Tokyo, Japan, cat. nr. FU 116). ACE hydrolyzes p-hydroxybenzoyl-glycyl-L-histidyl-L-leucine and generates p-hydroxybenzoyl-glycine, which is converted in two subsequent reactions in quinoneimine dye. The absorbance of the quinoneimine dye is measured at 505 nm to evaluate the ACE activity.

Serum calcium concentration was measured by indirect potentiometry on a Synchron LX®20 with a detection limit of 0.5 mmol/L and a measuring range of 0.5 to 5.0 mmol/L (Beckman Coulter, Inc. Fullerton, CA, US; electrolyte buffer reagent (P/N 467915) and electrolyte reference reagent (P/N 467935)).

Table 7.2. Primers used for the sequencing of the G6PD gene.

Primer sequences ^a (5' to 3')	Amplified fragment bp from ... to...(size)	G6PD protein part
PRS CTCTGCAGGCCCGCGGAAGCTCGGT	2015 – 2443 (428)	Promotor region
PRAS CCGCTGCCGCTGCTCTGCATCCCC A		
1S CGGCGATGGGGATGCGGGAGCACTA	2364 – 2773 (409)	Exon I: no coding sequence
1AS GCGCAGCGCGGGACAGTACGCTCCT		
2S AGGAACTCTCAAGAAAGGGGCTAAC	3264 – 3533 (269)	Exon II: Met1 – Ser 39
2AS AAAAGCTGAGGCATGGAGCAGGCAC		
3S AAGGGTGGAGGATGATGTATGTAGG	13253 – 13623 (370)	Exon III: Gly41 – Trp53 Exon IV: Leu54 – Lys88
4AS TGGGGGCTGGTAGAGAGGGCAGAAC		
5S CTGGGGCAGAACACACACGGACTCA	14042 – 14403 (361)	Exon V: Ala89 – Ile162
5AS ATAGAGTGGTGGGAGCACTGCCTGG		
6S TGGGAGGGCGTCTGAATGATGCAGC	14920 – 15227 (307)	Exon VI: Gly163 – Arg215
6AS GGCCAGGTGAGGCTCCTGAGTACCA		
7S GGGTGACCCCTCACATGTGGCCCT	15268 – 15517 (249)	Exon VII: Phe216 – Arg257
7AS GGCTCTGCCACCCTGTGCCAGCCT		
8S GTTTGGGGTCCCCATGCCCTTGAAC	15756 – 15979 (223)	Exon VIII: Asp258 – Lys288
8AS CAGATGGGCCTGCGACAGGGCATGC		
9S TGCACATCTGTGGCCACAGTCATCC	16294 – 16619 (325)	Exon IX: Val289 – Asp350
9AS TGCCCGCACACAGGGCATGCCCAGT		
10S GCTCCCACTGAGACACTCACGCACT	16624 – 16982 (358)	Exon X: Gly351 – Lys429
10AS GGCCAGGGCCGCCACCCTCCACA		
11S GTGGGGCCCGGGGACTCCCATGGT	16975 – 17162 (187)	Exon XI: Asn430 – Ser455
11AS ACCCCATAGCCACAGGTATGCAG		
12S GGGGTGGCCTTTGCCCTCCCTCC	17157 – 17388 (231)	Exon XII: Asp456 – Ser486
12AS GGCATGAGGTAGCTCCACCCTCAC		
13S AGGAAAGGGTGGGGGCTGGGGACAGA	17318 – 17590 (272)	Exon XIII: Arg487 – Leu515
13AS GTCAATGGTCCCGAGTCTCCCGA		

^a Primers were designed based on the Genbank sequence with accession number X55448. (Homo sapiens gene for G6PD, glucose-6-phosphate dehydrogenase) and the numbering of amino acids was deduced from the cDNA accession number X03674.

Soluble IL2 receptor (sIL-2R) was measured on the IMMULITE automated analyzer, which is a two-site chemiluminescent enzyme immunometric assay with a detection limit of 50 kU/L and a measuring range of 50 – 7500 kU/L (Diagnostic Product Corporation, Los Angeles, CA, cat no LKIP1).

Chest radiographs were graded according to the radiographic staging of DeRemee (0 to III), adding stage IV, *i.e.* the end stage of lung fibrosis^{1,14}.

By means of high-resolution computer tomography (HRCT), thin-section scans with 1-mm collimation were obtained at 10-mm intervals through the chest. The scanning parameters included 137 kVp, 255 mA, and 1-second scanning time. Both mediastinal

(width, 400 HU; level, 40 HU) and lung (width, 1,600 HU; level, -800 HU) window images were obtained. The semiquantitative HRCT scoring system was used as described previously¹⁵. This HRCT score measures parenchymal, as well as lymphatic, and pleural involvement and grades each of these pathological features (six in total) into four categories. The total HRCT score was obtained by counting the six individual scores together, obtaining a maximal score of 18.

Lung function parameters, including the forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC), were measured with a pneumotachograph. The diffusing capacity for carbon monoxide (DLCO) was measured by the single-breath method (both Masterlab, Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted¹⁶.

Patients performed a symptom-limited incremental exercise test as described previously³. Breath-by-breath gas exchange was measured by a ventilated hood system (Oxyconbeta; Jaeger, Bunnik, The Netherlands). In rest and during maximal exercise, arterial blood samples were taken for blood gas analyses. The alveolar-arterial oxygen differences at rest ($PO_{2\text{rest}}$) and at maximal exercise ($AaPO_{2\text{max}}$) were calculated.

Statistics

All analyses were performed with the Statistical Package for Social Science (SPSS) for Windows (SPSS version 10.0, Chicago, IL, USA). Group comparisons were performed by means of parametric tests (Independent Samples t-test) for continuous data and by means of non-parametric tests (Mann-Whitney U test) for categorical data. A two-sided p-value of less than 0.05 was considered to be statistically significant.

Results

Table 7.3. shows clinical and laboratory data of the studied sarcoidosis population. Male patients appeared to have a worse clinical picture than female patients with respect to radiographic staging, total HRCT score, FEV1 and FVC. They also appeared to have significantly lower GR activities in their erythrocytes than female patients.

To assess whether normal levels of NADPH were present in the erythrocytes, the chromate inhibition test was performed. Abnormal GR-Cr values were found in 17.1% of sarcoidosis patients, 14 of 48 females (29%) and 1 of 40 male patients (2.5%), indicating decreased levels of NADPH.

In Table 7.4 the clinical data of the female patients have been divided into two groups, one with GR-Cr test results lower than or equal to 0.6 IU/g Hb (normal) and another with a GR-Cr test result higher than 0.6 IU/g Hb (abnormal). Among clinical parameters, only GR and ACE appeared to be significantly higher ($p < 0.05$) in the group with the abnormal GR-Cr test results, even after correction for the use of prednisolone.

Table 7.3. Clinical and laboratory findings of the studied sarcoidosis population^a

Parameter (normal range)	Female (n = 48)	Male (n = 40)	Total (n = 88)
ACE (9-25 U/L)	22.8 ± 9.6 (88 – 60)	20.1 ± 8.4 (38 – 41)	21.6 ± 9.1 (38 – 60)
sIL-2R (241 – 846 kU/L)	861 ± 561 (2168 – 2271)	735 ± 432 (2368 – 2243)	804 ± 507 (2168 – 2271)
TotalCa ²⁺ (2.1 – 2.6 mmol/L)	2.4 ± 0.1 (2.38 – 2.6)	2.5 ± 0.1 (2.38 – 2.7)	2.4 ± 0.1 (2.3 – 2.7)
GR-Cr (≤ 0.6) / > 0.6)	34 / 14	39 / 1	73 / 15
G6PD (4.8 – 7.2 IU/g Hb)	5.1 ± 1.0 (1.58 – 8.6)	4.8 ± 0.4 (3.98 – 5.7)	5.0 ± 0.8 (1.58 – 8.6)
GR ^b (2.4 – 4.8 IU/g Hb)	4.5 ± 1.1 (2.78 – 7.1)	3.9 ± 0.7 (2.58 – 5.4)	4.2 ± 1.0 (2.58 – 7.1)
FEV1, % pred ^c	91.2 ± 19.9 (298 – 135)	77.4 ± 20.9 (268 – 108)	85.2 ± 21.3 (268 – 135)
FVC, % pred ^c	99.3 ± 19.2 (498 – 152)	86.7 ± 15.3 (498 – 110)	93.9 ± 18.6 (498 – 152)
DLCO, % pred	81.9 ± 15.7 (378 – 112)	84.7 ± 20.7 (398 – 124)	83.2 ± 18.0 (378 – 124)
DVA, % pred	76.5 ± 13.3 (468 – 105)	81.8 ± 13.8 (538 – 107)	78.9 ± 13.7 (468 – 107)
PO ₂ rest (8.7 – 13.1 kPa)	11.6 ± 1.7 (7.8 – 15.0)	11.3 ± 1.6 (6.48 – 15.6)	11.4 ± 1.6 (6.48 – 15.6)
AaPO ₂ max, (0 – 4.7 kPa)	4.1 ± 1.8 (0.8 – 8.7)	4.4 ± 1.6 (0.98 – 7.5)	4.2 ± 1.7 (0.88 – 8.7)
Radiographic stages ^b , 0/II/III/IV	10 / 7 / 18 / 6 / 3	3 / 4 / 14 / 9 / 5	13 / 11 / 32 / 15 / 8
HRCTtot ^b (0 – 18)	4.6 ± 3.4 (0 – 12)	6.7 ± 3.6 (8 – 13)	5.5 ± 3.6 (8 – 13)

^a Data are mean ± SD with range in parenthesis, except for GR-Cr and radiographic stages.^b p < 0.05 (males vs. females).^c p < 0.01 (males vs. females).**Table 7.4. Clinical parameters in female sarcoidosis patients with abnormal chromate-inhibition test as compared to those with normal test result^a**

Parameter (normal range)	GR-Cr < 0.6 IU/g Hb (n = 34)	GR-Cr > 0.6 IU/g Hb (n = 14)
ACE ^b (9 – 25 U/L)	20.4 ± 8.2 (8 – 47)	28.4 ± 10.6 (17 – 60)
sIL-2R (241 – 846 kU/L)	843 ± 571 (216 – 2271)	902 ± 553 (373 – 1891)
Total Ca ²⁺ (2.1 – 2.6 mmol/L)	2.4 ± 0.1 (2.3 – 2.6)	2.4 ± 0.1 (2.3 – 2.5)
G6PD (4.8 – 7.2 IU/g Hb)	5.2 ± 0.9 (4.0 – 8.6)	5.2 ± 1.3 (1.5 – 6.7)
GR ^b (2.4 – 4.8 IU/g Hb)	4.1 ± 1.0 (2.7 – 6.7)	5.3 ± 1.0 (4.1 – 7.1)
FEV1, % pred	93.9 ± 14.6 (58 – 128)	84.8 ± 28.5 (29 – 135)
FVC, % pred	101.1 ± 13.8 (63 – 128)	95.3 ± 28.2 (49 – 152)
DLCO, % pred	83.4 ± 13.3 (58 – 112)	78.6 ± 19.8 (37 – 104)
DVA, % pred	76.6 ± 12.9 (57 – 105)	76.4 ± 14.6 (46 – 100)
PO ₂ rest, kPa (8.7 – 13.1 kPa)	11.8 ± 1.7 (7.8 – 15.0)	11.0 ± 1.4 (9.4 – 13.6)
AaPO ₂ max, kPa (0 – 4.7 kPa)	4.2 ± 1.8 (2.3 – 8.7)	3.8 ± 1.9 (0.8 – 6.1)
Radiographic stages ^c 0/II/III/IV	6 / 7 / 14 / 3 / 1	4 / 0 / 4 / 3 / 2
HRCTtot (0 – 18)	4.4 ± 3.1 (0 – 12)	5.0 ± 4.1 (0 – 12)

^a Data are mean ± SD with range in parenthesis.^b p < 0.05 (normal vs. abnormal GR-Cr test group).^c p < 0.01 (normal vs. abnormal GR-Cr test group).

Since an abnormal GR-Cr test result is usually caused by G6PD deficiency (leading to decreased levels of NADPH), we also measured the G6PD activity in the patients' erythrocytes. In only one patient a strongly reduced G6PD activity and highly abnormal GR-Cr test result was found, with 1.5 IU/ g Hb for G6PD and 5.1 IU/ g Hb for GR-Cr. To investigate due to which genetic defects in the G6PD gene the abnormal chromate inhibition test results were obtained, DNA was sequenced in 6 of 14 randomly chosen female patients with an abnormal GR-Cr test result (42.8% of cases), as shown in Figure 7.1.

Of the 14 females, two were negroid, whereas all other females with an abnormal GR-Cr test result were Caucasian and from Dutch origin. Only in one negroid female (patient with G6PD activity of 1.5 IU/ g Hb for G6PD and GR-Cr result of 5.1 IU/g Hb) a mutation in the G6PD gene was found. This was a combination of 202G→A (Met68 to Val) and 376A→G (Asp126 to Asn) called G6PD A-, the most common G6PD mutation in the negroid race. The male patient with an abnormal GR-Cr result was Caucasian.

Figure 7.1. G6PD in relation to GR-Cr test and patients selected for sequencing.

Open triangles present patients with normal GR-Cr test values (≤ 0.6 IU/g Hb), whereas closed triangles present patients with abnormal GR-Cr test values (> 0.6 IU/g Hb). Remaining patients were those with abnormal GR-Cr test values, which were sequenced (star symbol). Only one patient of 6 sequenced with GR-Cr = 5.1 IU/g Hb appeared to be a carrier of a G6PD A-.

Discussion

The lung, through its large contact surface with the environment, is one of the most oxygen-exposed organs. Therefore, a causal role for oxidative stress in the inflammatory process involved in various pulmonary disorders, including sarcoidosis, has been considered^{17,18}. Pulmonary antioxidant defenses are widely distributed and include both enzymatic and non-enzymatic systems. The major enzymatic antioxidants are superoxide-dismutase, catalase and GSH peroxidase. However, the GSH peroxidase activity is dependent on GR for the generation of GSH, a reaction for which NADPH is required. The only pathway to generate NADPH in erythrocytes is the hexose monophosphate shunt (HMS). In this pathway, glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme^{5,10}.

Prompted by earlier observations, it was hypothesized that decreased levels of NADPH might be involved in the pathogenesis of sarcoidosis^{19,20}. This was indirectly established

by means of the chromate inhibition test (GR-Cr), which gave abnormal results in 29% of female sarcoidosis patients, whereas only one male patient (2.5%) appeared to have an abnormal test result. Although most studies suggest a slightly higher disease rate for women¹, it is hard to find the explanation for this large sex difference.

The chromate inhibition test is considered to be a sensitive biochemical assay that indirectly indicates cellular NADPH concentrations, and is used for the detection of G6PD deficiency in female carriers of this disease^{11,12}. In the West-European, Caucasian population, G6PD deficiency is found with a prevalence of less than 0.1%²¹, and the GR-Cr test yields an abnormal value in a similar percentage of this population (unpublished data). Thus, our finding of 15 abnormal GR-Cr tests in 73 sarcoidosis patients (17%), *i.e.* in 13 of 68 Caucasians (19%), is very deviant from the values, which are to be expected. One possible reason for the abnormal GR-Cr test could be G6PD deficiency. However, these results could not be explained by genetic mutations in the G6PD genes of the sarcoidosis patients. Only one patient of negroid origin appeared to be a carrier of G6PD A-. G6PD A- represents a mild form of G6PD deficiency, found largely in the African population, but also in other parts of the world^{22,23}.

Among clinical parameters, only GR and ACE appeared to be significantly higher ($p < 0.05$) in the group with the abnormal GR-Cr test results. Metabolic stress can lead to increased riboflavin uptake by erythrocytes²⁴. In modified form (as FAD), riboflavin is incorporated into GR²⁴. Slightly decreased NADPH might possibly also cause this phenomenon and lead as compensation to increased GR concentrations, as observed in G6PD deficiency^{24,27}. Also ACE appeared to be significantly higher ($p < 0.05$) in the group with the abnormal GR-Cr test results, which might indicate an increased granuloma burden in this patient subgroup²⁸.

Increased NADPH consumption in erythrocytes of sarcoidosis patients could be a reflection of oxidative stress, which develops during pulmonary damage. NADPH might indirectly be used for instance in protection of hemoglobin from oxidation. Methemoglobin reductase (NADH diaphorase) catalyzes methemoglobin reduction²⁹. Through the reduction of methemoglobin the cellular level of NADH decreases. There is an exchange of reducing equivalents between NADH and NADPH (catalyzed by NAD:NADP transhydrogenase), which could subsequently lead to a decrease in NADPH concentration. Decreased NADPH will lead to abnormal chromate-inhibition test results.

Conclusion

A substantial, unexpectedly high number of patients suffering from sarcoidosis appeared to have an abnormal chromate inhibition test, indicating decreased NADPH concentrations in their erythrocytes. Possibly, an increased consumption of reduced NADPH is involved in the inflammatory process triggered by oxidative stress in sarcoidosis.

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Chapter 8

Relationship of myeloperoxidase promotor polymorphism and disease severity in sarcoidosis?

Snježana Rothkrantz-Kos, Marjolein Drent, Abraham Rutgers,
Peter Heeringa, Jolanda De Vries, Marja P. van Dieijen-Visser,
Jan-Willem Cohen Tervaert

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Abstract

Background

Previously, we demonstrated that the number of polymorphonuclear neutrophils (PMNs) in the bronchoalveolar lavage fluid (BALF) is useful in distinguishing sarcoidosis patients with a more favourable outcome from those having a more severe course of disease. Neutrophils contain the oxidant generating enzyme, myeloperoxidase (MPO). Cellular levels of MPO can be influenced by functional promotor polymorphisms, -463G/A and -129G/A, which might modulate disease severity.

Methods

In the present study, we investigated two MPO promotor polymorphisms in 110 sarcoidosis patients and in 191 ethnically matched controls. Pulmonary disease severity was evaluated by means of radiographic staging, HRCT scoring, lung function- and exercise capacity testing.

Results

No significant differences were found between sarcoidosis patients and healthy controls, with regard to either polymorphism. Furthermore, no association was observed between -463 G/A and -129 G/A polymorphism, and the severity of sarcoidosis.

Conclusion

Functional MPO promotor polymorphisms, -463G/A and -129G/A, did not explain for disease severity in the studied sarcoidosis population. Future studies are needed to identify predictive features useful in guiding therapeutic strategies and to determine difficult to treat cases.

Introduction

Sarcoidosis, a disease predominantly affecting young adults, is the most common diffuse lung disease with a population prevalence of around 40:100.000^{1,2}. Sarcoidosis is an immune mediated inflammatory disease with unknown etiology, although genetic predisposition is likely to be important^{2,3}. The main pathologic feature of the disease is a chronic inflammation resulting in non-caseating granuloma formation. The prognosis of sarcoidosis is rather variable. A great deal of uncertainty exists on how to predict the natural course of the (untreated) disease, and identify those individual cases who should be treated immediately. More important, as it is still not possible to identify the cause of sarcoidosis, therapy is directed at controlling the inflammatory reaction⁴.

Bronchoalveolar lavage fluid (BALF) analysis appears to reflect the severity of sarcoidosis⁵⁻⁹. Notably, the number of polymorphonuclear neutrophils (PMNs) is increased in advanced, chronic sarcoidosis^{5-7,9}. In line with these findings, interleukin 8 (IL-8), a potent chemoattractant and activator of neutrophils, in BALF appears to be highly elevated in sarcoidosis patients who showed progressive disease^{8,10}. Myeloperoxidase (MPO) is an abundant protein in PMNs and monocytes, stored in the azurophilic granules of neutrophils and released during phagocytosis^{11,12}. MPO in BALF originates from lung PMNs and can be used to estimate the presence and/or activation of PMNs¹³. Indeed, a strong correlation between the MPO level and the PMNs count in BALF has been demonstrated in patients with Wegener's granulomatosis¹⁴. The MPO concentrations appear to be associated with two described functional promotor polymorphisms, namely the -463 G/A MPO promotor polymorphism^{15,16} and the recently described -129 G/A promotor polymorphism¹⁷. Several diseases were reported to be associated with -463 G/A promotor polymorphism^{16,18-22}, whereas no association studies of -129 G/A have yet been reported.

MPO appears to be involved in tissue damage through production of potent oxidant HOCl, which is able to oxidize many cellular compounds such as thiols and NADPH and lead to chlorination of DNA bases and tyrosine residues in proteins^{11,12,23-25}. Therefore, it can be assumed that decreased availability of MPO related to functional MPO promotor polymorphisms, has a protective effect by decreasing the chance of tissue damage.

It was hypothesized that MPO polymorphism might contribute, at least in part, to disease severity in sarcoidosis and make it possible to determine those cases who might benefit from agents controlling the inflammatory reaction at an early stage. Hence, the incidence of two MPO promotor polymorphisms (-463 G/A and -129 G/A) was analyzed in a Dutch Caucasian sarcoidosis population as well as a possible relationship with disease severity.

Materials and Methods

Study population

Caucasian sarcoidosis patients ($n = 110$), who visited the Sarcoidosis Management Center of the University Hospital Maastricht, a Dutch referral center for sarcoidosis between January 2000 and April 2001, were included in this study. Patients were diagnosed based on consistent clinical features and BALF fluid analysis, according to the ATS/ERS/WASOG guidelines^{2,26}. The diagnosis was histologically confirmed in 85% of the cases. The control population consisted of 191 healthy individuals. Informed consent was obtained from all participating subjects. Patient characteristics are summarized in Table 8.1.

Table 8.1. Characteristics of the Dutch Caucasian sarcoidosis patients and controls genotyped for -463 G/A and -129 G/A myeloperoxidase (MPO) promotor polymorphism.

	Sarcoidosis patients ($n = 110$)	Control subjects ($n = 191$)
Male / female, n	55 / 55	100 / 91
Age ^a , years	40.7 ± 11.0 (17 – 70)	36.4 ± 14.4 (18 – 82)
Time since diagnosis ^a , years	4.25 ± 5.67 (0 – 37)	-
Smoking (no / yes), n	101 / 9	-
Prednison (no / yes), n	78 / 32	-

^a Data are expressed as mean $\pm$ SD with range in parentheses.

Evaluating Sarcoidosis Pulmonary Disease Severity

Chest radiographs were graded according to the radiographic staging of DeRemee (0 to III), adding stage IV, the end stage of lung fibrosis^{2,27}. With high-resolution computer tomography (HRCT), thin-section scans with 1-mm collimation were obtained at 10-mm intervals through the chest. The scanning parameters included 137 kVp, 255 mA, and 1-second scanning time.

Both mediastinal (width, 400 HU; level, 40 HU) and lung (width, 1600 HU; level, -800 HU) window images were obtained. The semiquantitative HRCT scoring system was used as described previously²⁸.

Lung function parameters, including the forced expiratory volume in 1 s (FEV1) and forced vital capacity (FVC), were measured with a pneumotachograph. The diffusing capacity for carbon monoxide (DLCO) was measured by the single-breath method (both Masterlab, Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted²⁹.

Serum ACE (sACE) was measured by colorimetric method (Fujirebio Inc., Tokyo, Japan, cat. nr. FU 116). ACE acts on a substrate p-hydroxybenzoyl-glycyl-L-histidyl-L-leucine and separates p-hydroxybenzoyl-glycine, which is converted in two consequent reactions in quinoneimine dye. The absorbance of quinoneimine dye is measured at 505 nm to evaluate the ACE activity.

Soluble Interleukin-2 receptor (sIL-2R) was measured on the IMMULITE Automated Analyzer, which is a two-site chemiluminescent enzyme immunometric assay with a detection limit of 50 kU/L and a measuring range of 50 – 7500 kU/L (Diagnostic Product Corporation, Los Angeles, CA, cat no LKIP1).

Molecular studies

The -463 G/A polymorphism was determined as previously described^{16,18,19}. The -129 G/A polymorphism was determined as described by Hoy *et al.*¹⁷. The most important features are presented in Table 8.2. When screening for -129 G/A polymorphism, we identified a 129G to A specific ApaI restriction site within the 278 bp PCR product. PCR products were digested with 20 units of ApaI (Roche, Mijdrecht, The Netherlands) for two hours or overnight at 30°C, separated on a 2%-agarose gel and stained with ethidium-bromide.

Table 8.2. Sequences of primers and digestion fragments length used for myeloperoxidase (MPO) genotyping.

MPO Promotor	Primers	Digestion of PCR product	Genotype (characterized by fragments in bp)
-463 G/A	5'-CGGTATAGGCACACAATGGTGAG 5'R-GCAATGGTTCAAGCGATTCTTC	Aci I	GG (168, 121, 61) GA (289, 168, 121, 61) AA (289, 61)
-129 G/A	5'- CCTCCACAGCTCACCTGATAT 5'R- CGCTTGAACCATTCACATCA	Apa I	GG (124) GA (154)

Statistics

All analysis were performed using the SPSS10.0 for Windows (SPSS, Chicago, IL, USA). Agreement with Hardy-Weinberg-equilibrium and differences in allele and genotype frequencies between groups were examined using chi-square tests. Because multiple comparisons were performed, a probability value divided by number of comparisons was considered statistically significant (Bonferroni's correction). Univariate logistic regression analysis was used to calculate odds ratios with 95% confidence intervals. Group comparisons were performed by means of Independent Samples t-test or Mann-Whitney U test.

Results

The two functional MPO polymorphisms fulfilled Hardy-Weinberg expectations in both sarcoidosis and control subjects. Genotype distributions associated with the -463 G/A or -129 G/A polymorphism did not differ significantly between patients with sarcoidosis and healthy controls (Table 8.3). Furthermore, for both polymorphisms sex-specific analysis showed no differences of allele or genotype frequencies (data not shown).

Base-line clinical data of the patients with sarcoidosis are presented in Table 8.4.

Additionally, it was tested whether any association could be found with MPO polymorphisms. For this purpose, for -463 G/A polymorphism, the GA and AA genotypes were grouped together and compared to the GG genotype. No relation with age at diagnosis was found. Furthermore, severity of the disease was evaluated in relation to genotype. The results of this analysis are presented in Table 8.5. As can be seen no significant differences in the severity as presented by clinical data could be found, the finding which remained also after correction for prednisone use. The same analysis as shown in Table 8.5 was repeated with respect to -129 G/A polymorphism and no relation to clinical data was found (data not shown).

Table 8.3. Genotype frequencies of the myeloperoxidase (MPO) promotor polymorphisms in the Dutch Caucasian sarcoidosis and control population^a.

Polymorphism		Sarcoidosis Patients (n = 110)	Control Subjects (n = 191)	OR (95% CI)	p-value ^b
-463 G/A					
Genotype	GG	67 (60.9%)	113 (59.2%)	1.0	0.942
	GA ^c	37 (33.6%)	68 (35.6%)	1.01 (0.35 – 2.91)	
	AA ^c	6 (5.5%)	10 (5.2%)	0.91 (0.56 – 1.52)	
Allele	G	171 (77.7%)	294 (77.0%)	1.0	0.829
	A ^c	49 (22.3%)	88 (23%)	0.95 (0.64 – 1.42)	
-129 G/A^d					
Genotype	GG	97 (88.2%)	167 (87.4%)	1.0	0.849
	GA ^c	13 (11.8%)	24 (12.6%)	0.93 (0.45 – 1.91)	
Allele	G	207 (94.1%)	358 (93.7%)	1.0	0.854
	A ^c	13 (5.9%)	24 (6.3%)	0.93 (0.46 – 1.88)	

^a Data are expressed as absolute numbers with percentages in parentheses.

^b p-values are for overall comparison between cases and control subjects (Chi-square analysis). Odds ratios (OR) for genotypes/alleles were calculated by logistic-regression analysis.

^c GG genotype or G allele is the reference group.

^d AA genotype was absent from both sarcoidosis and control group.

Table 8.4. Evaluation of severity in Dutch Caucasian sarcoidosis patient population^a

	Female (n = 55)	Male (n = 55)	Total (n = 110)
ACE (9 – 25 U/L)	20 (17 – 26)	20 (15 – 28)	20 (16 – 27)
sIL-2R (241 – 846 kU/L)	550 (404 – 937)	767 (409 – 1180)	667 (408 – 1085)
FEV1, % pred ^b	97.0 (84 – 106)	87.0 (63 – 99)	92.0 (69.8 – 103)
FVC, % pred ^b	105 (94.2 – 115)	91.0 (77.5 – 102)	98.5 (84.5 – 110)
DLCO, % pred	89 (78 – 96)	85.5 (72.5 – 97.5)	87.0 (76.5 – 96.5)
Radiographic stages 0/I/II/III/IV ^b	17 / 8 / 14 / 14 / 2	4 / 8 / 14 / 20 / 9	21 / 16 / 28 / 34 / 11
HRCTtot (0 – 18) ^b	4.0 (1 – 6)	6.0 (3 – 9)	5.0 (3 – 8)

^a Data are median with interquartile range (25th – 75th percentile) in parenthesis, except for radiographic staging.

^b p < 0.01 comparing males and females.

Table 8.5. Relationship of the -463 G/A myeloperoxidase (MPO) promotor polymorphism and disease severity in a Dutch Caucasian sarcoidosis population (n = 110)^a

Diagnostic parameter	Female		Male	
	GG (n = 32)	GA/AA (n = 23)	GG (n = 35)	GA/AA (n = 20)
sACE (9-25 U/L)	20 (18 – 26)	20 (16 – 27)	23 (15 – 27)	18.5 (15.3 – 32.8)
sIL-2R (241-846 kU/L)	511 (397 – 806)	732 (414 – 1581)	801 (409 – 1180)	664 (409 – 1181)
FEV1, % pred	99 (88 – 107)	92 (71 – 106)	79 (61 – 100)	90 (73 – 96)
FVC, % pred	105 (96 – 115)	105 (88 – 114)	89 (75 – 105)	95 (86 – 100)
DLCO, % pred	89 (81 – 95)	87 (72 – 99)	83 (69 – 96)	92 (80 – 102)
Radiographic stages 0/I/II/III/IV	11/8/6/7/0	6/0/8/7/2	3/5/7/13/7	1/3/7/7/2
HRCTtot (0-18)	3 (1 – 5)	6 (2 – 7)	7 (4 – 9)	6 (3 – 9)

^a Data are median with interquartile range (25th – 75th percentile) in parenthesis, except for radiographic staging

Discussion

This study showed that distribution of two functional myeloperoxidase (MPO) promotor polymorphisms (-463 G/A and -129 G/A) was similar in sarcoidosis patients as compared to controls. Furthermore, no relation was found between these polymorphisms and disease severity. To exclude a possible bias due to corticosteroid use, treated and untreated sarcoidosis patients were also considered separately. The results in both subgroups appeared to be similar to those in the total population, *i.e.* no relation with MPO genotype (also after stratification for sex and age) was found.

Among the cells present in bronchoalveolar lavage fluid (BALF), polymorphonuclear neutrophils (PMNs) are considered to be a key mediator indicative of severity of the disease. In cases with more extensive long term radiographic features, impaired lung function parameters, having a worse prognosis and sometimes corticosteroid resistant chronic disease, PMNs were found to be increased in BALF^{5-7,9}. Moreover, a strong correlation exists between the myeloperoxidase level and the PMN count in BALF¹⁴.

Some could argue that the neutrophils might just be an epiphenomenon, coming in when disease is extensive and fibrotic. However, it was recently demonstrated that the number of neutrophils in BALF even at first presentation is indicative of prognosis and disease outcome^{5,9}.

GG genotype of -463 G/A polymorphism was previously found to correlate with higher levels of MPO mRNA and higher protein levels, as shown by Western blotting, than the GA or AA genotype¹⁵. In contrast, when circulating levels of MPO were measured in serum by means of enzyme immunoassay, no such correlation was found with -463 G/A polymorphism, but only with -129 G/A polymorphism¹⁷.

It was recently demonstrated that -463 G/A MPO promotor polymorphism influences diseases severity and expression in chronic granulomatous disease²² and Wegener's granulomatosis²⁰, both granulomatous diseases different from sarcoidosis. In the present study, however, no association of MPO promotor polymorphisms with sarcoidosis were found. One possible explanation could be, that disease expression in chronic granulomatous disease and Wegener's granulomatosis is modulated by infections with

extensive PMN involvement. Indeed, co-trimoxazol (antibacterial drug) positively influences the course of disease in both chronic granulomatous disease³⁰ and Wegener's granulomatosis^{31,32}, whereas this has never been shown in sarcoidosis.

Granuloma formation in sarcoidosis appears to be a strictly T cell dependent process³³. Since MPO was shown to inhibit T cell proliferation^{34,35}, one can postulate that MPO promotor polymorphisms might also influence the T cell dependent disease process in sarcoidosis. Therefore, the role of MPO and its polymorphisms seem very complex and not completely defined in the different diseases that are characterized by granulomatous inflammation.

In conclusion, the PMN count in BALF appears to be useful in determining chronic and difficult to treat sarcoidosis patients. MPO plays a key role in the immunopathogenesis of several granulomatous disorders and MPO release appears to be related to MPO polymorphism(s). However, in the Dutch Caucasian sarcoidosis population studied, similar frequencies of functional MPO promotor polymorphisms (-463 G/A and -129 G/A) were found as compared to control subjects. Furthermore, no association between -463 G/A and -129 G/A MPO promotor polymorphisms, and the severity of sarcoidosis, including radiographic features, impaired lung function, time since diagnosis and corticosteroid use was found in our study population. Given the inability to identify the cause of sarcoidosis, therapy is directed to control the inflammatory reaction. Future studies are needed to determine those cases with a more advanced chronic course of the disease who might benefit from early treatment of the inflammatory reaction.

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Chapter 9

General discussion

General discussion

Sarcoidosis is characterized by an abundant immunological response, which in most cases spontaneously resolves. Nevertheless, in some cases the disease has a more or less severe course. Finally, it may lead to end-stage lung fibrosis with signs of respiratory functional impairment. One of the most important issues to deal with for clinicians is to identify those cases that will deteriorate and eventually develop fibrosis. Those cases should be monitored more closely and receive appropriate and timely treatment in order to prevent fibrosis development.

In general, the state of a disease can be classified either by activity or severity. Unlike other disorders, activity in sarcoidosis does not necessarily indicate a progressive course, a fatal prognosis, or the need for medical treatment¹. Many markers have been searched to reflect the disease activity. Unfortunately, the term “activity” has in the past been used unsystematically, resulting in the fact that no good marker of activity could be found². Previously, the term activity in pulmonary sarcoidosis has been related to the presence of T cell activation (alveolitis in case of pulmonary sarcoidosis), whereas until now no good consensus on activity exists (CHAPTER 1). Angiotensin converting enzyme (ACE) is still recommended by World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) as a marker of activity in sarcoidosis, despite of its shortcomings³⁻⁵.

So far little attention has been paid to the appropriateness of markers to detect severity. From a clinical point of view, it is more important to depict whether the disease is severe, *i.e.* will have a more or less chronic course and will end-up with functional impairment rather than the initial activity. No proper marker of severity in sarcoidosis exists, which enables to predict deterioration. Moreover, this is complicated by the fact that an appropriate and sufficient definition of activity and severity is still lacking.

There is a lot of discussion going on about the definitions of both terms, as it is difficult to make a clear distinction between these two entities. Besides, there is overlap between the two. Activity is a sign of inflammation. Severity, reflected through respiratory functional impairment, might be accompanied by inflammation (so activity may be present) as well. However, finally an end-stage situation without signs of an active inflammation can be found.

For each appropriate evaluation in the form of a diagnostic accuracy study for any marker a clear definition of the condition such as activity or severity is needed. In this thesis we introduce the definition of respiratory functional impairment to define pulmonary severity. We suggest the usage of this term, as it is as such of more interest to pulmonary physicians. Furthermore, we feel that severity, indicating functional impairment is of more clinical relevance.

Accordingly, one of the aims of this thesis was to search for new markers, which would enable to reflect disease severity in sarcoidosis. Respiratory functional impairment was used to depict the severity of the disease.

Four markers which have been claimed or suggested to be good markers of disease activity were evaluated for their ability to reflect disease severity: C-reactive protein

(CRP), serum amyloid A (SAA), soluble interleukin-2-receptor (sIL-2R) and angiotensin-converting enzyme (ACE).

These markers were also related to one of the most common and bothering symptoms, *i.e.* fatigue.

Prior to clinical evaluation of different biomarkers, the methods used to measure these markers were evaluated.

A previous study showed that CRP was associated with metabolic derangement and fatigue in sarcoidosis, using our routine CRP method⁶. CRP is also used as a marker of prediction of risk of future arteriosclerosis. From that aspect, several papers indicated that the routine CRP measurement as used among many laboratories could not be used in the low range because of the poor performance and too high detection limit (mostly between 2 and 5 mg/L). Moreover, in case of sarcoidosis, CRP changes were for a considerable part confined to the lower range⁶.

Our routine CRP method (detection limit 5 mg/L) indeed appeared to suffer from serious shortcomings in the low range. Moreover, the method appeared to be extremely sensitive to sample turbidity, making the results obtained in such samples unreliable. This underlined further the need for a more appropriate method for the CRP measurement. The methods able to measure consistently very low CRP concentrations are called high-sensitivity CRP (hs-CRP) methods. Several hs-CRP methods were evaluated for their performance together with their sensitivity to turbidity of a sample. Based on the extensive evaluation of hs-CRP and CRP methods in CHAPTER 2 and 3, we decided to use BN ProSpec hs-CRP method for further evaluation of sarcoidosis patients.

Based on several previous studies it was assumed that SAA and sIL-2R might potentially be useful in sarcoidosis⁷⁻⁹. We evaluated these two new assays for analytical performance and determined the reference values as shown in CHAPTER 4. Evaluation of the sIL-2R method on the IMMULITE and the SAA method on the BN ProSpec yielded good imprecision results and satisfying linearity. The reference values for sIL-2R appeared to be sex and age independent. The reference values for SAA on BN ProSpec were sex dependent and showed a relationship with age.

The aim of the study presented in CHAPTER 5 was to evaluate the clinical usefulness of serological markers, of which the analytical performance was evaluated across the previous chapters. Thus, the diagnostic accuracies of hs-CRP, SAA and sIL-2R were established in sarcoidosis patients and they were compared to the traditionally used ACE in monitoring disease severity. Hundred forty-four non-smoking, 73 untreated (Group I) and 71 treated (Group II) patients were selected. The subgroups of the untreated patients, Group Ia (non-chronic group with time since diagnosis ≤ 2 years) and Group Ib (chronic group with time since diagnosis > 2 years) were evaluated separately. ROC-curves and logistic regression analyses were used to compare the diagnostic accuracy of different markers to assess disease severity. Respiratory functional impairment *i.e.* pulmonary disease severity was defined by means of lung function test results. The major finding of this chapter was that among the evaluated markers, in untreated patients, sIL-2R appeared to be the best marker to monitor

respiratory functional impairment and thus pulmonary severity. Furthermore, it appeared to be the most suitable marker for the follow-up. In line with others, sIL-2R was clearly shown to be superior to usually used ACE to monitor not only the activity but also pulmonary severity⁸⁻¹⁰. However, the difference between the approach used in this chapter and other studies is that we performed an appropriate diagnostic accuracy study. For relationship between the markers, a fact that is often overlooked, was statistically corrected. Moreover, the value of the four markers was examined in an extensive way in non-treated as well as treated sarcoidosis patients. Finally, an attempt was made to give a reproducible and clear definition of criteria used to define pulmonary severity based on lung function test results as follows: DLCO < 80 or FVC < 80 or FEV1 < 80. We suggest that the criteria as defined in this chapter could be used more broadly; at least as long as there is a lack of official definition of severity criteria.

Patients with sarcoidosis most often suffer from symptoms related to the lungs, but may also suffer from a wide spectrum of other symptoms. One of the most often reported general symptoms is fatigue. As studies on the relationship between fatigue and laboratory and clinical parameters are sparse, in CHAPTER 6 this relationship was examined.

Previously it was shown that CRP appeared to be associated with metabolic derangement and fatigue in sarcoidosis, by means of less sensitive routine CRP measurement⁶. Our evaluation in CHAPTERS 2 and 3 clearly showed that this routine method has serious shortcomings. Therefore, new hs-CRP method on the BN Prospec, the SAA and sIL-2R were measured among the serum markers in addition to traditionally used ACE. As factors that might predict fatigue also lung function test results reflecting respiratory functional impairment and the measures of metabolic derangement were included.

Patients with a time since diagnosis ≤ 2 years, ($n = 60$; 34 untreated, 26 treated) were clinically evaluated and completed the Fatigue Assessment Scale (FAS)¹¹. It was confirmed that fatigue is a major problem in sarcoidosis. A representative sample of the Dutch control population ($n = 1893$) also completed the FAS. Only 27% of the sarcoidosis patients were classified as not being bothered by fatigue (FAS score < 22), compared to 80% in the control population. Moreover, in the sarcoidosis patients no sex differences and no differences in fatigue scores between the treated and the untreated groups were found.

The extent of fatigue was neither related to the new tested markers nor to ACE, metabolic derangement measures or lung function data. As long as there is a lack of 'measurable' clinical parameters to objectively assess fatigue, the FAS is useful and recommended as a supplementary tool to characterize the extent of fatigue in sarcoidosis.

The first part of this thesis was used to evaluate inflammatory markers and their ability to define respiratory functional impairment and fatigue in sarcoidosis. In the second part attention was paid to the systems, directly and/or indirectly involved in oxidative stress and microbial killing.

Prompted by earlier observations, it was hypothesized that oxidative stress probably plays an important role also in sarcoidosis and can eventually modify severity of the disease. Therefore, enzymatic systems, which take care of normal cellular redox-state glucose-6-phosphate dehydrogenase, G6PD and of continuous production of reduced glutathione (GSH) such as glutathione reductase (GR), were assessed in CHAPTER 7. The possibility was raised that some subclinical G6PD-deficiency might be present in patients with sarcoidosis that may modify disease severity. As the G6PD-gen lies on the X-chromosome, G6PD-deficiency will be clearly visible in males through the measurement of G6PD activity, but it will be masked in heterozygous females. Consequently, in females the degree of G6PD deficiency might vary enormously. Therefore, the chromate inhibition test (GR-Cr) as a more reliable test was used to assess the G6PD-deficiency. In the Netherlands, the GR-Cr test is the standard test used to establish the G6PD deficiency. This test is based on the inhibition of GR activity by chromate in erythrocytes with a normal level of reduced nicotinamide adenine dinucleotide phosphate (NADPH), but not in erythrocytes with decreased levels of NADPH, which might be a consequence of G6PD inactivity.

Decreased levels of NADPH, indicating G6PD deficiency, were found in 29% of the female sarcoidosis patients and in one male patient (2.5%). No explanation for this sex difference could be given. Six patients with abnormal chromate-inhibition test were chosen and their G6PD gene was completely sequenced, including non-coding and promotor region of the gene. The selection of patients for sequencing was random, although representing as well extremely abnormal as nearly normal GR-Cr values. Only one female of Negroid origin appeared to have a mutation in the G6PD gene. This same patient also had enormously decreased G6PD activity, so that even based on solely G6PD activity the diagnosis of G6PD deficiency could be considered. In the remaining patients no mutation in the G6PD gene was found.

Taken together, the GR-Cr test appeared inappropriate to reflect G6PD-deficiency in patients with sarcoidosis, due to its high-degree of false-positivity. One explanation for these findings is that an increased consumption of reduced NADPH is involved in the inflammatory process triggered by oxidative stress in sarcoidosis.

However, the false-positivity of the test might also be caused by the increased GR activity on its own, so that the inhibition is less efficient. The GR appears to be present in two forms, the active form, which is associated with flavine adenine dinucleotide (FAD) and the inactive form, not associated with FAD. GR is almost completely saturated with FAD in red cells in patients with severe metabolic disorders or G6PD deficiency. In line with that, increased GR activity with a high degree of saturation with FAD can be found in red cells of severe uremia, cirrhosis of liver and G6PD deficiency^{12,13}. In the young red cells (reticulocytes) GR activity can also be increased and is sometimes used to correct for the presence of young cells, which in general have the activities of the most enzymes increased.

The major problem associated with G6PD deficiency is hemolytic anemia. Hemolytic anemia is the decreased ability of red blood cells to transport oxygen throughout the body, which can have rather serious consequences. Many (oxidative) drugs, infections, or fava beans can cause this. When any one of these agents enters the red blood cell,

hemoglobin becomes denatured, thus destroying its function as the principle oxygen-carrying molecule.

Usually, G6PD deficiency is accompanied with rather large restrictions in drugs and nutrients, which may be taken to avoid hemolytic crisis due to G6PD deficiency. Therefore, it is very important to have a reliable test to establish the G6PD deficiency. As shown in CHAPTER 7, the GR-Cr test cannot be used to establish G6PD deficiency in sarcoidosis patients. The question, which arises, is whether the same test can be used in other “comparable” inflammatory conditions. Anyhow, a careful revision of the test is needed.

In CHAPTER 8, the role of another enzyme involved in non-specific immunity, related to oxidative stress, has been examined. It was previously demonstrated that the number of polymorphonuclear neutrophils (PMNs) in the bronchoalveolar lavage (BAL) fluid is useful to distinguish patients with a more favorable outcome from those having a more severe course of disease in sarcoidosis¹⁴. The major content of neutrophils is the oxidant-generating enzyme, myeloperoxidase (MPO). Cellular levels of MPO can be influenced by functional promotor polymorphisms, -463 G/A and -129 G/A that might modulate disease severity. These two MPO promotor polymorphisms were investigated in 110 sarcoidosis patients and in 191 ethnically matched controls. For both polymorphisms no significant differences were found between sarcoidosis patients and healthy controls. Furthermore, no association was observed between both polymorphism, and the severity of sarcoidosis.

Directions for future research

The study presented in CHAPTER 8 showed that two functional polymorphisms, which were previously shown to be related to diseases with comparable clinical presentation, were not associated with sarcoidosis. Also other studies looked at many different polymorphisms. However, although some of them found an association¹⁵⁻¹⁹, many showed a lack of any association with sarcoidosis^{3,20}. Therefore, the search for a combination of polymorphisms, which might predict the course and progression of the disease (severity) is warranted.

Recently, a new unrecognized neurological complication of sarcoidosis, small-fiber neuropathy has been demonstrated²¹. For some disorders the link between decreased redox-state and cellular changes has already been established, such as neurons or cardiomyocytes²²⁻²⁵. Sarcoidosis has been suggested to trigger an oxidative stress response as also indicated by an increased activation of nuclear regulatory factor-kappaB (NF- κ B)^{26,27}.

The possibility exists that the decreased redox-state in patients with inflammatory disorders including sarcoidosis might contribute to nerve-cell changes, which in turn can lead to small-fiber neuropathy. This is an interesting area with great clinical implications, which should be further explored.

As shown in CHAPTER 6, although helpful, a questionnaire is always at least partly a subjective measure to evaluate fatigue. Therefore, other markers able to objectify fatigue should be explored. Sarcoidosis manifests mostly in active young adults of age 20 - 40. The possibility exists that a stressful life accompanied with prolonged cortisol production can somehow evoke the sarcoidosis appearance. Possibly some hormonal dysbalance can also be a potentially useful measure of fatigue in sarcoidosis and should further be explored.

In sarcoidosis, there appears to be extra-renal production (by granuloma) of active form of vitamin D. As the active form of vitamin D has influence on calcium metabolism, the calcium metabolism can largely be deregulated in patients with sarcoidosis²⁸. The mechanisms behind this deregulation as well as the clinical impact are largely unknown and need further to be explored.

Finally, one of most interesting areas to be explored and of interest in sarcoidosis is proteomics, which offers the possibility to look at the whole spectrum of serum or BAL fluid proteins at the same time. The first step has been set by group of Grünewald *et al.*²⁹. The proteomics might offer the possibility to find markers that will be able to distinguish acute from chronic patients and possibly to predict patients which will deteriorate due to fibrotic changes from those who will not end up with fibrosis. This new approach might give answers to many questions, which the clinicians are facing for a very long time. This underlines once more the importance of cooperation between clinicians and laboratories to explore the insight in this whimsical disorder sarcoidosis.

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Chapter 10

Samenvatting

Samenvatting

Sarcoidose

In HOOFDSTUK 1 is een inleiding in sarcoïdose (ook wel bekend als de ziekte van Besnier-Boeck-Schaumann), een aandoening waarvan de oorzaak nog niet bekend is gegeven. Sarcoïdose kan zich op allerlei plaatsen in het lichaam manifesteren maar treedt het meest op in de longen (90%). De aandoening treft vooral volwassenen tussen de 20 en 40 jaar. Naar schatting hebben in Nederland ongeveer 40 van de 100.000 inwoners sarcoïdose.

Het kenmerk van sarcoïdose is een granuloom ofwel een chronische ontstekingshaard. Dit is een opeenhoping van immuuncellen, zoals (alveolaire) macrofagen en geactiveerde T-lymfocyten, in de aangedane weefsels. Hoewel tot op heden geen eenduidige oorzaak is gevonden, suggereert het ziektemechanisme dat er sprake is van één of meerdere oorzaken van buiten het lichaam in samenhang met een erfelijke aanleg.

Afhankelijk van onder anderen welke orgaansystemen zijn aangetast, kunnen patiënten met sarcoïdose zich met allerlei klachten aandienen, zoals kortademigheid, oogproblemen, huidafwijkingen, gewrichtsklachten, duizeligheid, hoofd- en/of spierpijn. Ook zijn er patiënten met niet-specifieke klachten, zoals algehele malaise, gewichtsverlies en koorts. De meest gerapporteerde klacht is echter moeheid. Deze kan lang blijven bestaan, zelfs nadat tekenen van ziekteactiviteit genormaliseerd zijn.

Het verloop van sarcoïdose is onvoorspelbaar. Een variant, het syndroom van Löfgren gaat bijvoorbeeld gepaard met koorts, huidafwijkingen en/of gewrichtsklachten. Deze vorm heeft een acute presentatie en in het algemeen een gunstig verloop. Wanneer sarcoïdose zich manifesteert in het hart, het centrale zenuwstelsel en/of met uitgebreide longafwijkingen zijn de vooruitzichten ongunstiger.

Voor de begeleiding van sarcoïdosepatiënten en het inschatten van de prognose is het belangrijk dat er een duidelijk beeld wordt verkregen van de omvang van de ziekte. Deze omvang wordt onder meer vastgesteld met behulp van laboratoriumonderzoek, beeldvormende technieken, longfunctietesten, visueel onderzoek van de luchtpijp (bronchoscopie) en een bronchoalveolaire lavage (het spoelen van een gedeelte van de long).

Aangezien de oorzaak van sarcoïdose nog steeds niet bekend is, bestaat de behandeling in het algemeen uit symptoombestrijding die bij milde klachten bestaat uit het gebruik van eenvoudige ontstekingsremmers en bij een ernstiger verloop uit immuunsuppressiva (zoals o.a. prednison). Verder zijn leefregels met betrekking tot het leren omgaan met de moeheid van groot belang.

Evaluatie van markers om de ernst van sarcoïdose te voorspellen

In het algemeen worden gedurende een ontstekingsreactie door de lever in zeer korte tijd grote hoeveelheden C-reactive protein (CRP) en serum amyloid-A (SAA) aangemaakt. Beide eiwitten worden dan ook acute fase eiwitten genoemd. Bij bepaalde

aandoeningen is de productie niet explosief maar er vindt wel een constant licht verhoogde productie van deze eiwitten. CRP is belangrijk voor de initiatie van het opruimen van bacteriën (fagocytose). SAA zorgt voor het beperken en repareren van toegebrachte schade aan de weefsels (door bijvoorbeeld bacteriën). Als gevolg van bepaalde ontstekingsreacties, zoals sarcoïdose, worden T-lymfocyten heel sterk gestimuleerd tot delen. In dat geval komen veel van de zogenaamde IL-2 receptoren (IL2R) aan hun oppervlak voor, die ertoe bijdragen dat deze deling van de T-lymfocyten verder wordt gestimuleerd. Deze IL2R komen deels vrij en zijn in de circulatie meetbaar als oplosbare (soluble) interleukin-2-receptoren (sIL2R). Speciale cellen (epithelioid cellen) die deel uit maken van granulomen bij sarcoïdose zijn ook in staat angiotensin-converterend enzyme (ACE) te produceren en deze is ook in de bloedbaan te meten.

Tot op heden is er geen goede marker om de ernst van sarcoïdose te bepalen. Daarom werden in het eerste deel van dit proefschrift laboratoriumparameters sIL2R, ACE, CRP en SAA, geëvalueerd als potentiële markers bij sarcoïdose.

Voordat een parameter in de dagelijkse praktijk toegepast kan worden als marker, dient deze zorgvuldig onderzocht te worden (HOOFDSTUK 2 t/m 4). Hierbij spelen twee factoren een essentiële rol; ten eerste moet de meetmethode betrouwbaar zijn en ten tweede dienen patiënten in verschillende, goed te onderscheiden, groepen te worden ingedeeld (bijvoorbeeld, actief/niet-actief, ernstig/niet-ernstig verloop).

Betreffende het eerste punt, werd in HOOFDSTUK 2 en 3 met name veel aandacht geschonken aan de evaluatie van de CRP methodieken. De huidige toegepaste CRP methodiek bleek ongeschikt te zijn voor het meten van lage concentraties en verder extreem gevoelig voor troebeling in het bloed, veroorzaakt door (bijvoorbeeld) verhoging van trygliceriden (vetten) in niet-nuchter afgenomen bloedmonsters. Aangezien uit eerdere studies is gebleken dat CRP een rol kan spelen bij de beoordeling van de ernst van sarcoïdose, werd een betrouwbare methodiek gezocht voor het meten van lage concentraties.

In HOOFDSTUK 4 zijn de laboratoriumtechnische aspecten van de SAA en sIL2R geëvalueerd, omdat het nieuwe bepalingen betreft. De referentiewaarden voor sIL2R bleken niet afhankelijk te zijn van leeftijd en geslacht. De referentiewaarden voor SAA bleken iets hoger te zijn bij vrouwen. Bovendien werd een relatie met leeftijd gevonden, namelijk iets oudere mannen bleken iets hogere waarden te hebben dan de jongere mannen.

Het onderscheiden van patiënten groepen (actief/niet-actief, ernstig/niet-ernstig verloop) is in relatie tot sarcoïdose niet eenvoudig. Noch voor de activiteit, noch voor de ernst is er een goede definitie beschikbaar.

In HOOFDSTUK 5 werd een studie opgezet waarbij aan de hand van internationale richtlijnen criteria zijn gedefinieerd om patiënten in de groepen “ernstig” en “niet-ernstig” in te delen. Deze indeling vond plaats op basis van de aan- of afwezigheid van longfunctiestoornissen. Deze criteria waren zo gedefinieerd dat ze eenduidig te interpreteren en toe te passen zijn. Uit de studie is gebleken dat van de eerder genoemde laboratoriumparameters, sIL2R de meest geschikte marker voor het voorspellen van de ernst van sarcoïdose is. Opvallend genoeg bleek dat ACE, de klassieke sarcoïdose marker, niet bruikbaar is om ernst te voorspellen.

Het voorspellen van moeheid

Uit eerdere studies is gebleken dat moeheid een belangrijk symptoom is van patiënten met sarcoïdose. In HOOFDSTUK 6 werd gekeken of de bovengenoemde laboratoriumparameters in staat zijn om moeheid bij sarcoïdose te voorspellen. Geen van de geëvalueerde laboratoriumparameters, noch de gebruikte longfunctieparameters, bleken goede markers van moeheid te zijn. Alleen minder objectieve hulpmiddelen, zoals vragenlijsten, bleken in staat moeheid te voorspellen.

Oxidatieve stress en sarcoïdose

Naar aanleiding van eerdere studies werd in het tweede deel van het onderzoek gekeken of oxidatieve stress, direct of indirect, mogelijk een rol speelt bij sarcoïdose. Oxidatieve stress is een toestand waarbij er geen goede balans bestaat tussen de vorming (pro-oxidatieve werking) en de eliminatie (anti-oxidatieve werking) van toxische zuurstofverbindingen.

Twee aspecten die met de gevoeligheid voor oxidatieve stress te maken hebben werden onderzocht.

In HOOFDSTUK 7 werd gekeken of een bepaald enzym te weten G6PD, in staat is om voor voldoende anti-oxidatief (reducerend) vermogen te zorgen. Een enzym is een eiwit dat chemische reacties doet versnellen. G6PD zorgt dat er voldoende van een bepaald stof (anti-oxidatief vermogen) dat afgekort NADPH heet, aangemaakt wordt. Een genetische of biochemische verandering van G6PD heeft tot gevolg een verlaging van NADPH, wat G6PD-deficiëntie wordt genoemd. In Nederland wordt G6PD-deficiëntie vastgesteld met behulp van de zogenaamde “chroom-inhibitie test”. De test berust op het principe dat een enzym, het glutathione reductase (bijna) volledig geremd wordt door chroom in normale rode bloedcellen maar niet in G6PD-deficiënte cellen.

Tijdens het onderzoek bleek dat bijna één derde van de vrouwelijke sarcoïdose patiënten en slechts één mannelijke patiënt verlaagde NADPH waarden hadden (Hoofdstuk 7). Er konden echter geen mutaties in het G6PD-gen worden gevonden. De chroom-inhibitie test bleek minder geschikt voor screening op G6PD deficiëntie omdat die veel fout positieve uitslagen opleverde. De door deze test aangetoonde verlaagde NADPH concentraties is een aanwijzing dat de oxidatieve stress een rol speelt bij sarcoïdose. Toekomstige studies dienen de klinische relevantie verder te evalueren en mogelijke therapeutische consequenties te onderzoeken.

In HOOFDSTUK 8 werd gekeken of twee puntmutaties (polymorfismen), in het gen coderend voor het enzym myeloperoxidase (MPO), geassocieerd zijn met sarcoïdose en of ze gerelateerd zijn aan een ernstiger verloop.

MPO is een enzym dat schadelijke zuurstof verbindingen kan genereren en is een belangrijk enzym in een bepaalde soort witte bloedcellen, de neutrofielen. Eerder is aangetoond dat een toename van het aantal neutrofielen in de longen een indicatie is voor een slechtere prognose van sarcoïdose en een verhoogde kans op het ontwikkelen van longfibrose. Dit zou kunnen samenhangen met de hoeveelheid MPO die door deze cellen geproduceerd worden (het niveau van MPO expressie) en haar pro-oxidatieve werking. Twee verschillende functionele polymorfismen werden bekeken in de

promotor van het MPO gen, namelijk de -463 G/A and -129 G/A. Er werd verondersteld dat een verminderde aanmaak van dit enzym een "beschermend" effect kan hebben op de ontsteking bij sarcoïdose, en zodoende een minder ernstig verloop kan geven. Echter, er werd geen verband gevonden tussen dit polymorfisme en sarcoïdose. Bovendien werd er geen verschil in de ernst van sarcoïdose ernst tussen dragers van dit polymorfisme en de personen die dit polymorfisme niet hebben.

Sarcoïdose is een intrigerende aandoening aangezien er ondanks grote onderzoeksinspanningen nog veel vragen onbeantwoord zijn, met name wat betreft de mogelijke oorzaken, het voorspellen van het ziekteverloop en de waarde van laboratoriumonderzoek in de diagnostiek en follow-up. Tevens is er nog geen adequate therapie voorhanden. Gezien het feit dat de oorzaak nog niet bekend is, bestaat de behandeling vooralsnog voornamelijk uit symptoombestrijding.

Toekomstig onderzoek, met name in de richting van genoom onderzoek en proteomics (eiwit profilering), zal misschien antwoord kunnen geven op de vele openstaande vragen.

Chapter 11

Sažetak

Sažetak

Sarkoidoza

U POGLAVLJU 1 dat je uvod u sarkoidozu (bolest poznata i pod imenom Besnier-Boeck-Schaumann-ova bolest). Etiologija ove bolesti nije poznata. Zahvaća različite organe i tkiva, ali pretežno pluća, u 90% slučajeva. Najčešće obolijevaju mlađe osobe u dobi od 20 do 40 godina. Sarkoidoza se javlja u otprilike 40 na 100.000 ljudi, premda taj broj varira ovisno o zemljopisnom području.

Glavno obilježje sarkoidoze su granulomi odnosno kronična upalna žarišta. Stvaraju se kao posljedica nakupljanja upalnih stanica u oboljelim tkivima, a posebno bijelih krvnih stanica, kao što su makrofagi i aktivirani T-limfociti. Premda kod sarkoidoze uzročnik bolesti nije poznat, mehanizam bolesti sugerira da bi taj uzročnik ipak morao biti vanjski faktor u kombinaciji s nekim genetskim faktorom.

Simptomi s kojima se bolesnici javljaju ovise o organu koji je zahvaćen (otežano disanje, kašalj, očni problemi, promjene na koži, zglobovima, vrtoglavica te glavobolja i/ili bol u mišićima). Bolesnici se također mogu tužiti na nespecifične tegobe kao što su opća slabost, gubitak na težini i povišena tjelesna temperatura. Najčešće se tuže na umor, simptom koji je vrlo teško objektivizirati, a zaostaje čak i nakon što su se ostali parametri aktivnosti normalizirali.

Tijek sarkoidoze varira. Tako akutni oblik sarkoidoze, poznat još i pod imenom Löfgrenov sindrom, koji se manifestira kao kombinacija povišene tjelesne temperature, bolova u zglobovima i specifičnih kožnih promjena (nodozni eritem), općenito ima povoljniji tijek. Sarkoidoza srca, središnjeg živčanog sustava ili vrlo proširena plućna sarkoidoza imaju nepovoljan tijek i prognozu.

Za praćenje bolesnika i procijenu prognoze vrlo je važno dobiti jasnu kliničku sliku. Proširenost bolesti obično se procjenjuje pomoću laboratorijskih pretraga, rentgenoloških i CT pretraga, testova plućne funkcije, vizualnog pregleda dušnika (bronhoskopija) te bronhoalvolarnog ispiranja (aspirirana fiziološka otopina nakon pranja plućnog reznja, koja daje informacije o vrsti i količini upalnih stanica).

S obzirom da uzrok sarkoidoze još uvijek nije poznat, terapija se uglavnom sastoji u olakšavanju simptoma u slučaju blažih tegoba (ne-steroidnim inhibitorima upale) ili se u slučaju težih tegoba koriste sredstva koja inhibiraju cjelokupni tjelesni obrambeni sustav (kao npr. prednizon). Osim toga, promjena načina života i pridržavanje određenih pravila je jako važno za olakšavanje tegoba kao što je umor.

Evaluacija markera sarkoidoze u procijeni težine bolesti

U upalnoj reakciji jetra obično proizvodi velike količine C-reaktivnog proteina (CRP) i serum amiloida A (SAA). Oba proteina se jos nazivaju i proteini akutne faze. Kod nekih oboljenja ta proizvodnja ipak nije eksplozivna, nego je konstantno blago povišena. CRP je važan u inicijaciji procesa uništavanja bakterija ili nekog drugog uzročnika upale (fagocitoza). SAA se brine za to da upala bude ograničena i da dođe do reparacije (popravka) tkiva. U nekim upalnim reakcijama, kao što je sarkoidoza, T-limfociti se

ubrzano dijele i umnožavaju. Pri tome se na površini T-limfocita javljaju receptori, tzv. IL2-receptori (IL2R), koji omogućuju to umnožavanje. Jedan dio tog IL2R receptora se oslobađa i može se mjeriti u cirkulaciji, i naziva se topljivi IL-2R (sIL2R). Specijalne stanice (epitelioidne stanice), koji također čine granulom, su u stanju proizvoditi angiotenzin-konvertirajući enzim (ACE), koji je također mjerljiv u cirkulaciji.

Do danas ne postoji niti jedan laboratorijski parametar koji bi se mogao koristiti za procijenu težine bolesti. Radi toga su u prvom djelu ove dizertacije, laboratorijski parametri kao što su sIL2R, ACE, CRP i SAA, bili evaluirani kao potencijalni markeri sarkoidoze.

Prije nego što se neki parametar može primjeniti u dijagnostici, mora se temeljito ispitati. Pri tome su jako bitna dva faktora : 1. metoda koja se koristi za mjerenje dotičnog parametra mora biti pouzdana (POGLAVLJA 2 do 4) i 2. bolesnici se moraju moći svrstati u dvije različite grupe (npr. s aktivnom nasuprot inaktivnom bolešću, ili bolesnici s težim oblikom bolesti nasuprot onih s blažim oblikom bolesti).

Što se tiče prvog zahtjeva, u POGLAVLJIMA 2 i 3, osobita je pažnja bila posvećena evaluaciji različitih CRP metoda. Dosadašnja, općenito prihvaćena CRP metoda pokazala se sasvim nepouzdanom za mjerenje niskih koncentracija i ekstremno osjetljiva na zamućenost uzorka. S obzirom da su prijašnje studije pokazale da su niske CRP koncentracije tipične za sarkoidozu, bilo je potrebno naći metodu kojom bi se niske CRP koncentracije mogle pouzdano mjeriti.

U 4 POGLAVLJU SAA i sIL2R su bili evaluirani budući da predstavljaju nove mjerne metode. sIL2R referentne vrijednosti pokazale su se neovisnima o životnoj dobi i spolu. Nasuprot tome, SAA referentne vrijednosti su se pokazale višima u žena. Osim toga, u starijih muških osoba nađene su više SAA vrijednosti nego u mlađih muških osoba.

Što se tiče drugog zahtjeva, situacija je složenija. Bolesnike sa sarkoidozom nije lako svrstati u grupe (bolesnike s aktivnom nasuprot onih s inaktivnom bolešću, ili bolesnike s težim oblikom bolesti nasuprot onih s blažim oblikom bolesti). Razlog tome je da niti za aktivnost niti za “težinu” bolesti ne postoje jasno definirani kriteriji.

U 5 POGLAVLJU, da bi izveli studiju diagnostičke točnosti za četiri gore navedena laboratorijska parametra, sami smo definirali kriterije kako bi bolesnike svrstali u grupe “teži oblik bolesti” nasuprot “blaži oblik bolesti”, na temelju internacionalnih preporuka. Ta podjela je učinjena na temelju prisutnosti tj. odsutnosti plućnih poremećaja. Kriteriji su tako definirani da se daju jednoznačno interpretirati i primjeniti. Pokazalo se da je od evaluiranih parametara, sIL2R najpouzdaniji prediktor težine bolesti, dok se tradicionalno prihvaćen i korišten ACE pokazao manje pouzdanim.

Predikcija umora

Iz prijašnjih studija pokazalo se da je umor jedan od najčešćih nespecifičnih simptoma u bolesnika sa sarkoidozom, koji je teško objektivno mjeriti. U 6 POGLAVLJU, cilj je bio vidjeti da li neki od parametara upale, evaluiranih u prethodnom poglavlju, je u stanju statistički predvidjeti umor. Niti jedan od evaluiranih laboratorijskih parametara ili parametara plućne funkcije, bilo pojedinačno ili u kombinaciji, nije se pokazao dobrim u predviđanju umora. Radi toga se još uvijek savjetuje korištenje psiholoških upitnika, premda manje objektivnih sredstava, u svrhu evaluacije takvih nespecifičnih simptoma.

Psihološki upitnici su se u prijašnjim studijama kao jedini pokazali u stanju predvidjeti umor.

Oksidativni stres i sarkoidoza

Cilj drugog dijela ove dizertacije, bio je istražiti kakvu ulogu ima, bilo direktno ili indirektno, oksidativni stres u sarkoidozi. Oksidativni stres je stanje neravnoteže između stvaranja (pro-oksidativnog djelovanja) i eliminacije (anti-oksidativnog djelovanja) toksičnih kisikovih spojeva. Dva aspekta, koja su u vezi s osjetljivošću na oksidativni stres, su bila istražena.

U 7 POGLAVLJU predmet istraživanja bila je glukoza-6-fosfat dehidrogenaza (G6PD). G6PD je enzim odgovoran za opskrbu stanica dovoljnim količinama anti-oksidativnog spoja, nikotina-adenina-dinukleotid fosfata (NADPH). Genetske ili biokemijske promjene u G6PD imaju za posljedicu sniženje koncentracije NADPH, što se još ponekad naziva i G6PD-deficijencija. U Nizozemskoj se G6PD-deficijencija određuje biokemijski, pomoću indirektnog testa inhibicije GR pomoću kromata (tzv. kromat-inhibicijski test). Abnormalni rezultat sugerira sniženu celularnu koncentraciju NADPH. Gotovo trećina ženskih pacijenata i jedan muški pacijent sa sarkoidozom su imali abnormalne rezultate kromat-inhibicijskog testa. Usprkos tome, samo je kod jednog pacijenta rezultat biokemijskog testa mogao biti potvrđen genetskom mutacijom u G6PD genu. Pokazalo se da kromat-inhibicijski test ne može biti primjenjen kod pacijenata sa sarkoidozom za dijagnosticiranje G6PD deficijencije zbog visokog postotka lažno-pozitivnih rezultata. Ipak, snižena celularna koncentracija NADPH upućuje na prisutnost intracelularnog oksidativnog stresa. Klinička relevancija te činjenice se mora dalje evaluirati kao i njezin utjecaj na terapiju.

U 8 POGLAVLJU, bilo je istraženo da li dvije točkaste mutacije (polimorfizmi) utječu na ekspresiju gena koji kodira za enzim mijeloperoksidazu (MPO) pri sarkoidozi, te da li prisutnost polimorfizma ima utjecaja na prezentaciju ("težinu") bolesti. MPO je enzim koji je u stanju generirati toksične kisikove spojeve i najobuhvatniji je enzim bijelih krvnih stanica (neutrofila). Već prije je pokazano da porast broja neutrofila u plućima najavljuje lošiju prognozu i povećanu vjerojatnost za razvitak plućne fibroze. Ta činjenica je vrlo vjerojatno u vezi sa količinom MPO koju stanica proizvede (sa nivoom MPO ekspresije) i njezinog pro-oksidativnog djelovanja. Radi toga su predmet istraživanja bila dva funkcionalna polimorfizma: -463 G/A i -129 G/A u promotoru gena koji kodira enzim mijeloperoksidazu (MPO). Veza između polimorfizama i sarkoidoze nije bila utvrđena. Osim toga nije bilo nikakve razlike u kliničkoj slici između nositelja i onih koji nisu nositelji tih dvaju polimorfizama.

Zaključak

Sarkoidoza je interesantna bolest budući da usprkos svim istraživačkim naporima još preostaje puno otvorenih pitanja, prije svega onih koja se tiču uzroka bolesti, predviđanja tijeka bolesti i vrijednosti laboratorijskih istraživanja u dijagnostici i slijeđenju tijeka bolesti. Budući da uzrok još uvijek nije poznat, nema adekvatne terapije, već se ona uglavnom sastoji u suzbijanju simptoma. Buduća istraživanja,

osobito istraživanja genoma te profiliranja bjelančevina pomoću masene spektrometrije (proteomics), možda će dati odgovore na neka od tih pitanja.

Dankwoord

Dankwoord

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Snježana

Curriculum vitae

Curriculum vitae

Snježana Kos was born on April 10th, 1973 in Zagreb (Croatia). After she finished secondary school in 1991, she started with her academic education on the faculty of Pharmacy and Medical Biochemistry at the University of Zagreb. There she specialized in Medical Biochemistry and graduated in February 1996. In September 1996 she started with the study of Health Sciences at the University of Maastricht. She graduated in September 1998 specializing in Biological Health Sciences. From October 1998 she worked on the research project of protein translation initiation under supervision of Prof. dr. R. Boelens at the Department of Biomolecular NMR of the Bijvoet Center at Utrecht University. In January 2001 she started as a researcher at the Department of Clinical Chemistry at the University Hospital Maastricht under supervision of Prof. dr. M. P. van Dieijen-Visser and Dr. M. Drent (Department of Respiratory Medicine). The result of that work is presented in the present thesis. She is currently engaged with the training in Clinical Chemistry in the same hospital.

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List of publications

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