

Chapter 5

Potential biomarkers for diagnosis of sarcoidosis using proteomics in serum

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Abstract

Background

Sarcoidosis is a multi-systemic inflammatory disorder, which affects the lungs in 90% of the cases. The main pathologic feature is chronic inflammation resulting in non-caseating granuloma formation. Until now there is no satisfying biomarker for diagnosis or prognosis of sarcoidosis. This study is focused on the detection of potential biomarkers in serum for the diagnosis of sarcoidosis using Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.

Methods

For detection of potential biomarkers, protein profiles of anion exchange fractionated serum of 35 sarcoidosis patients and 35 healthy controls were compared using Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Sensitivities and specificities of the potential biomarkers obtained with Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry generated with decision tree algorithm were compared to the conventional markers Angiotensin Converting Enzyme and soluble Interleukin-2 Receptor.

Results

Optimal classification was achieved with metal affinity binding arrays. A single marker with a mass-to-charge value of 11,955 resulted in a sensitivity and specificity of 86% and 63%, respectively. A multimarker approach of two peaks with mass-to-charge values of 11,734 and 17,377 resulted in a sensitivity and specificity of 74% and 71%, respectively. These sensitivities and specificities were higher compared to measurements of Angiotensin Converting Enzyme and soluble Interleukin-2 Receptor. Identification of the peak with a mass-to-charge value of 17,377 resulted in the alpha-2 chain of haptoglobin.

Conclusions

This study acts as a proof-of-principle for the use of Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry in the detection of new biomarkers for sarcoidosis. The peak of the multimarker with a mass-to-charge value of 17,377 was identified as the alpha-2 chain of haptoglobin.

Introduction

Sarcoidosis is a systemic granulomatous disorder of unknown cause characterized by its pathological hallmark, the noncaseating granuloma^{1,2}. The clinical presentation of sarcoidosis is highly variable³. Involvement of the lungs or intrathoracic lymph nodes becomes clinically evident in 90% of the symptomatic patients during their disease and up to 30% show spontaneous remission⁴. A chronic course occurs in 10-30% of the patients, at times resulting in significant impairment of lung function^{5,6}. Unfortunately, till now there is no good marker for both diagnosis and prognosis of sarcoidosis.

It is obvious that there is a great need to discover novel biomarkers for sarcoidosis and translate them to routine clinical use⁷, where they can play an important role in both diagnosis and follow-up. Proteomics analysis has been successfully employed in the discovery of new biomarkers in different human diseases⁸. Surface-Enhanced Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) is a promising new technology in the search for new biomarkers and was first introduced by Hutchens and Yip⁹. The ProteinChip system manufactured by Ciphergen Biosystem Inc. indicated as SELDI-TOF-MS has the potential to discover useful biomarkers faster than any existing technology. For the detection of potential biomarkers, protein profiles of disease and control groups are compared to find distinctive patterns, which can clearly discriminate the disease and control group. Protein peaks can be either up- or down regulated in the disease group versus the control group.

Biomarker Patterns is a decision tree algorithm that easily can deal with the large amount of proteins that can be detected in the serum samples. Biomarker patterns, software program for pattern analysis, is very useful for the multimarker approach, which is used for the classification. Because of the multifactorial nature of sarcoidosis, it is very likely that a combination of several markers will be necessary to effectively detect and diagnose sarcoidosis. Since the study of Petricoin et al.¹⁰ on protein profiling to detect ovarian cancer, the use of SELDI-TOF-MS protein profiling as a diagnostic tool, has become an important subject of investigation¹¹. Until now this approach has been suggested for different diseases, for example ovarian^{10,12-16} prostate¹⁷⁻²¹ and lung²² cancer research, but also inflammatory diseases^{23,24}. Recently, Kriegova et al.²⁵ reported protein profiles of bronchoalveolar lavage fluid (BALF) from patients with pulmonary sarcoidosis using SELDI-TOF-MS. Furthermore, these protein patterns in sarcoid BALF were linked to particular disease course, i.e. stages of sarcoidosis. Sarcoidosis markers were also detected with a two-dimensional gel electrophoresis (2-DE) approach in BALF^{26,27} and serum²⁸. The group of Song et al.²⁹ collected sarcoidosis tissue to detect sarcoidosis markers using a protein immunoblot assay. This is the first study that detected markers in serum using SELDI-TOF-MS.

The aim of this study is focused on the detection of potential serum biomarkers for the diagnosis of sarcoidosis using SELDI-TOF-MS. In this study, the sensitivity and specificity obtained with the SELDI-TOF-MS technique will be compared with the sensitivities and specificities of the angiotensin converting enzyme (ACE) and soluble Interleukin-2 Receptor (sIL-2R) concentration measurements, because these two blood markers are often used in the assessment and the follow-up of sarcoidosis³⁰⁻³²

Materials and methods

Patients and samples

Sarcoidosis patients who visited an education meeting held at the University Hospital Maastricht February the 24th 2005 were asked to participate. Of the 120 attendees 100 agreed to participate. The diagnosis sarcoidosis was based on consistent clinical features and BAL fluid analysis results, according to the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) guidelines³³ with a biopsy confirming sarcoidosis in 80% of cases. A healthy control group consisted of partners of the patients and employees of the hospital without any relevant medical history. Finally, out these 100 sarcoidosis patients 35 cases were enrolled who met the following inclusion criteria: a time since diagnosis of less than ten years (12 patients with a time since diagnosis longer than ten years were excluded from the study), non smoker (18 patients which were smokers at the moment of blood collection were excluded from the study), using no medication and not suffering from other diseases (31 patients which used corticosteroids at the moment of blood collection and 25 patients who suffered from other diseases, like diabetes, heart failure, which were not related to sarcoidosis, were excluded from the study). Out of the healthy control also 35 persons were selected matched for sex and age. The Medical Ethical Committee of the Hospital approved the procedure followed. Informed consent was obtained from all participants.

Characteristics of the studied population are illustrated in Table 5.1. Collection of serum samples was standardized by using a clotting time of 30 min at room temperature, spinning for 15 min at a speed of 4000 RPM (3000 g) and storage of the samples in aliquots within one hour at -80°C after blood collection³⁴. All fractionated serum samples had only two thaw steps from collection to analyzing. Serum ACE activity was measured by colorimetric method (Cat. nr. FU 116; Fujirebio Inc.) and sIL-2R was determined by a two-site chemiluminescent enzyme immunometric assay (Cat. nr. LKIP1; Diagnostic Product Corporation) on the IMMULITE Automated Analyzer. Serum was also used for analyses of the possible biomarker haptoglobin detected during this study. Total haptoglobin was measured by particle-enhanced immunonephelometry on the BN Prospec (Dade Behring).

Lung function indices, including the 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 measurements were performed on a Masterlab (Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted³⁵. Chest radiographs were graded according to the radiographic staging of DeRemee (0 to III), with stage IV, the end stage of lung fibrosis, added^{36,37}. Both the radiographic staging and pneumotachography tests were performed and interpreted by two professionals who were blinded to the patient's histories.

Table 5.1 Clinical characteristics of the studied population.

	Sarcoidosis patients	Healthy controls
n	35	35
Gender, M/F	18 / 17	16 / 19
Age ^a , years	45.6 ± 8.8	47.3 ± 9.8
Biopsy, n, (%)	27 (77.1%)	
Time since diagnosis, years, (mean)	1 - 9 (5)	
Serological markers ^b		
sIL-2R, U/mL	632.0 (437.5 - 859.5)	506.0 (394.0 - 597.5)
ACE, U/L	22.0 (16.0 - 24.0)	13.5 (12.0 - 17.8)
Lung function tests ^a		
DLCO, %	85.9 ± 16.3	
FVC, %	106.9 ± 16.5	
FEV ₁ , %	100.1 ± 16.8	
Chest radiographic stage, n		
Stage 0/I/II/III/IV	3/9/15/8/0	

ACE: angiotensin converting enzyme, DLCO: diffusing capacity for carbon monoxide, FEV₁: forced expiratory volume in 1 s, FVC: forced vital capacity, sIL-2R: soluble Interleukin-2 Receptor. ^a Variables presented as mean ± SD, as they follow a Gaussian distribution. ^b Serological markers are presented as median with range (25th - 75th percentiles) within parentheses.

Statistical analysis

Statistical analysis was performed with SPSS 11.0 for Windows (SPSS). ROC curve analyses³⁸ were performed for the ACE activity and sIL-2R concentrations. The optimal cut-off point coincides with the point on the ROC curve where the sum of sensitivity and specificity is maximal. When there were more cut-off point with the same sum, the one with the highest sensitivity was chosen. 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 outcomes. Groups comparisons were performed by means of parametric tests (Independent Samples t-test). A two-sided p-value of less than 0.05 was considered to be statistically significant.

SELDI-TOF-MS analysis

An anion exchange procedure was used, which allowed high-throughput fractionation of serum based on the biophysical properties of proteins³⁹. Serum was separated into six different fractions (pH9 + flow through, pH7, pH5, pH4, pH3, and organic wash). The anion exchange fractionation was standardized according to the Ciphergen protocols. For the anion exchange fractionation, we used 20 μ l serum and 30 μ l (9M urea, 2% Chaps, 50 mM Tris-HCL pH9) as starting material according to the Ciphergen protocols. Linke et al.⁴⁰ illustrated that fractionation greatly increases the number of peptide and protein ion signals, when compared to both unfractionated as well as albumin-depleted samples and this will increase the chance to find potential biomarkers.

In the screening experiment, each fraction of eight sarcoidosis and eight healthy controls was applied to three different ProteinChip array surfaces (Ciphergen Biosystems Inc.) to find out the optimal array and fraction. The weak cation exchange (CM10), the immobilized metal affinity capture coupled with copper (IMAC-Cu²⁺) and normal phase (NP20) arrays were used.

For ProteinChip array binding, the spots on the IMAC arrays were preloaded with 50 μ l CuSO₄ (0.1 M). The CM10 and IMAC arrays were equilibrated twice with 100 μ l of binding buffer (Na-phosphate buffer 50 mM, 0.5 M NaCl (pH7) for the IMAC arrays and Na-acetate (pH4) for the CM10 arrays). Ten μ l of each fraction was diluted in 90 μ l of the specific binding buffer as suggested by Ciphergen and added to each well of the bioprocessor and mixed for 45 min. After removing the remaining sample, the CM10 and IMAC arrays were washed and mixed three times with 150 μ l binding buffer for 5 min followed by one brief wash with 200 μ l Hepes buffer (pH7). For the NP20 ProteinChip arrays, the fractions were diluted with Milli-Q (1:1 v/v) and added directly on each spot. The arrays were incubated in a humid chamber for 30 min, followed by a brief water rinse. All steps were performed at room temperature.

The sinapinic acid solution (SPA) as energy absorbing matrix was prepared according to the recommendations of the manufacturer, with the exception that trifluoroacetic anhydride (TFAH) as a solvent component was used instead of trifluoroacetic acid (TFA). After all the arrays were dried, 1 μ l SPA solution was applied. The spotting of SPA was repeated once and the ProteinChip arrays were stored at room temperature in the dark until further use.

The CM10, IMAC and NP-20 arrays were read on a Protein Biosystem Ilc (PBS Ilc) instrument. External calibration of the instrument was performed using the All-in-1 peptide and the All-in-1 protein molecular mass standards (Ciphergen Biosystems) on the NP-20 array. A well-defined protocol for calibration of the PBS Ilc, implementation of quality control (QC) samples and acceptance criteria for QC was used in this study, which was described in a previous study of Bons et al.³⁴.

Data analysis

For the detection of sarcoidosis biomarkers, serum protein profiles of sarcoidosis patients and healthy controls were compared. Peaks were auto detected using Ciphergen Express Data manager 3.0.6 (Ciphergen Biosystems, Inc.). The biomarker wizard clusters were exported to Biomarker Patterns Software (BPS) 5.0.2 (Ciphergen Biosystems Inc.). BPS uses the peak information generated by the training set of known samples to build a binary decision tree algorithm. The algorithm functions by assigning each sample in the data set into one of the two groups or nodes with a rule based on the intensity of a particular peak or splitter. Each sub node has a different rule that further divides the data set and this process continues until all cases are assigned into terminal nodes. This results in correct classification percentages of the so-called "learn set". The software generates and tests the models, using a process of cross-validation by randomly picking 10% of the samples. The peaks that formed the main splitters of the tree with the highest prediction rates in the cross-validation analysis were then selected to make a final decision tree with the greatest possible predictive power and this results in correct classification percentages of the so called "test set". So the classification ability of the decision tree algorithms in the learning set to distinguish between sarcoidosis and controls was validated after cross-validation, which results in a test set.

A Bayesian approach was used to calculate the expected probabilities of each class in each terminal node. Sensitivity was calculated as the ratio of the number of correctly classified disease samples to the total number of disease samples. Specificity was calculated as the ratio of the number of control samples correctly classified to the total number of control samples⁴¹.

Identification

SDS-PAGE and 2-DE

Twenty μ l of samples in which the peaks of the three biomarkers were present, but also samples in which the peaks of the three biomarkers were absent, were loaded on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. For the two-dimensional gel electrophoresis (2-DE), 40 μ l of the same samples used for the SDS-PAGE were loaded for the first dimension. The SDS-PAGE, 2-DE and the digestion procedure were performed as described in the studies of Bouwman et al.^{42,43} with minor modifications.

MALDI-TOF-MS

For Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS), 1.5 μ l of each peptide mixture and 0.5 μ l matrix solution (2.5 mg/ml alpha-cyano-4-hydroxy cinnamic acid (CHCA) in 50% acetonitrile/0.1%

TFA) were spotted automatically onto a 96-well-format target plate. The spots were allowed to air dry for homogeneous crystallization. Spectra were obtained using a M@LDI-LR mass spectrometer (Waters). The instrument was operated in positive reflector mode.

The peptide mass list was searched with ProteinLynx Global Server v2.0 (Waters) or Mascot search engine against the Swiss-Prot database. One miss-cleavage was tolerated, carbamidomethylation was set as a fixed modification and oxidation of methionine as an optional modification. The peptide mass tolerance was set to 100 ppm. No restrictions were made on the protein M_r and the pI. A protein was regarded as identified when it had a significant ProteinLynx or Mascot probability score ($p < 0.05$), a minimum of four peptides were required to match.

Results

Serological markers

The ROC curve analysis of the patient and control groups for the ACE and sIL-2R measurements are presented in Table 5.2. For both measurements, the areas under the curves (AUCs) were significantly different from the null-hypothesis, true area=0.5. For ACE the sensitivity and specificity percentages were 70.6 and 71.4, respectively. For sIL-2R the sensitivity and specificity percentages were 62.9 and 57.1, respectively.

Table 5.2 ROC curve analysis results for the inflammatory markers, ACE and sIL-2R

	AUC (95% confidence intervals)	Selected cut-off	Sensitivity (%)	Specificity (%)
ACE	0.779 (0.668 – 0.891)	16.5	70.6	71.4
sIL-2R	0.667 (0.539 – 0.795)	515.5	62.9	57.1

ACE: Angiotensin Converting Enzyme, AUC: Area Under Curve, sIL-2R: soluble Interleukin-2 Receptor.

SELDI-TOF-MS analysis

For choosing the optimal fraction and array, the number of significant mass-to-charge ratio (m/z) values and the cluster plots were evaluated for each condition with the Ciphergen Express software. For single charged molecules, the m/z value is equal to the molecular weight (MW) in dalton (Da). The cluster plots illustrate the distribution of the intensities for each m/z value. With an increased number of significant m/z values ($p < 0.05$) and superior cluster plots, the chance to detect a potential biomarker in a larger sample set is theoretically higher. A cluster plot is superior when there is minor or no overlap between the data points of the sarcoidosis patients compared to the control samples, which means that there is a good discrimination between both groups. By comparing the different chips and

fractions in the screening experiments with eight sarcoidosis patients and eight healthy control samples, we detected the most significant m/z values ($p < 0.05$) with superior cluster plots in fraction 3 ($n=5$) and 4 ($n=7$) on the IMAC array, these two conditions were used for performing the IMAC experiment with 35 sarcoidosis and 35 control samples.

By repeating the IMAC experiment with 35 sarcoidosis and 35 control samples, fraction 4 on the IMAC array resulted in two significant m/z values ($p < 0.05$) and fraction 3 on the IMAC arrays resulted in seven significant ($p < 0.05$) m/z values. By using Biomarkers Patterns, decision trees were made and the decision trees generated from the fraction 3 samples resulted in a superior classification of the sarcoidosis and control samples, compared to the results from fraction 4 samples on the IMAC array.

For analysis of fraction 3 samples on the IMAC array, the following settings were chosen: laser intensity 220 and detector sensitivity 7. Biomarker Patterns generated a decision tree using 1 splitter with a m/z value of 11,955 and a cut-off value of 1.113 that classified cases in two terminal nodes. The peak was upregulated in the samples of sarcoidosis patients. The control terminal node contained 28 samples (23 control and five sarcoidosis samples) and the sarcoidosis terminal node contained 42 samples (30 sarcoidosis and 12 control samples). This single marker could correctly classify 30 of 35 (86%) sarcoidosis patients and 23 of 35 (66%) controls in the training set. The ability of the decision tree algorithms to distinguish between sarcoidosis and controls was validated after cross-validation, which is called the test set. In the test set, 30 of 35 (86%) samples were correctly classified as sarcoidosis and 22 of 35 (63%) samples were correctly classified as controls (Table 5.3). With a lower laser intensity setting; laser intensity 190 and detector sensitivity 7, Biomarkers Patterns generated a decision tree using two splitters with m/z values of 11,734 and 17,377 and cut-off values of 3.060 and 0.45, respectively, which classified patients and controls in three terminal nodes. The peak with a m/z value of 11,734 was upregulated and the peak with a m/z value of 17,377 was downregulated in the sarcoidosis patient samples. The first control terminal node, which was based on the first splitter, contained 21 samples (17 control and four sarcoidosis samples). The other 49 samples were divided in a sarcoidosis and control terminal node using the second splitter. The sarcoidosis terminal node contained 32 samples (26 sarcoidosis and six control samples) and the control terminal node contained 17 samples (12 control and five sarcoidosis samples). In the training set, 26 of 35 (74%) sarcoidosis and 29 of 35 (83%) controls were correctly classified and in the test set, 26 of 35 (74%) samples were correctly classified as sarcoidosis and 25 of 35 (71%) samples were correctly classified as controls (Table 5.3). The SELDI-TOF-MS protein profiles and gel views of the three potential biomarkers are indicated in Figure 5.1. There is a variation in protein expression between patients and

controls. This is seen in the cluster plots of the three m/z peak splitters used in the Biomarker Patterns analysis (Figure 5.2).

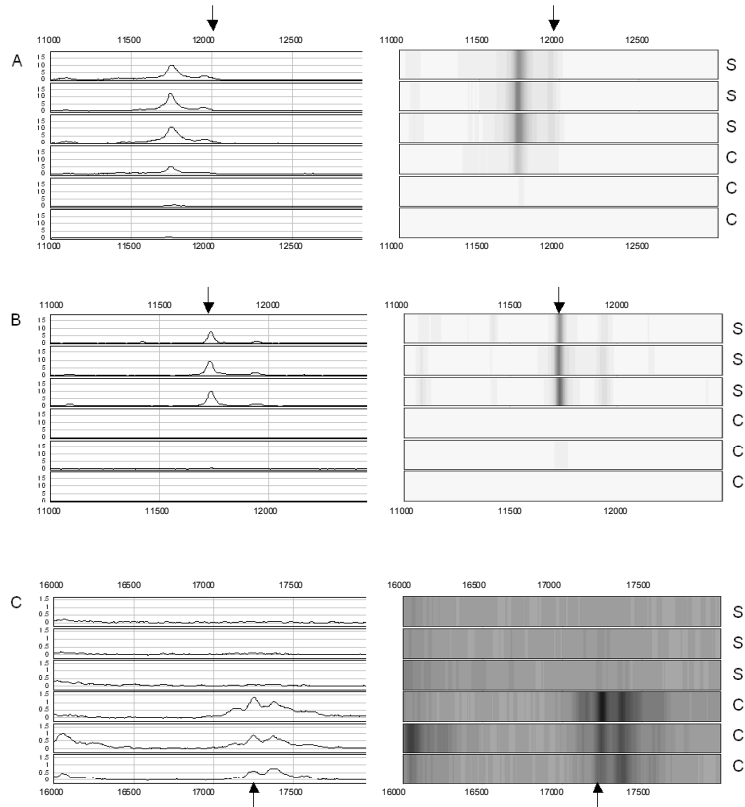


Figure 5.1 SELDI-TOF mass spectra and gel views of the three potential biomarkers with m/z values of 11,955 (A), 11,734 (B), and 17,377 (C). Representative protein spectra of three sarcoidosis (S) and three control (C) samples are illustrated per marker. The marker is indicated with a vector. The mass is given as m/z values on the x-axis and the intensity is displayed along the y-axis.

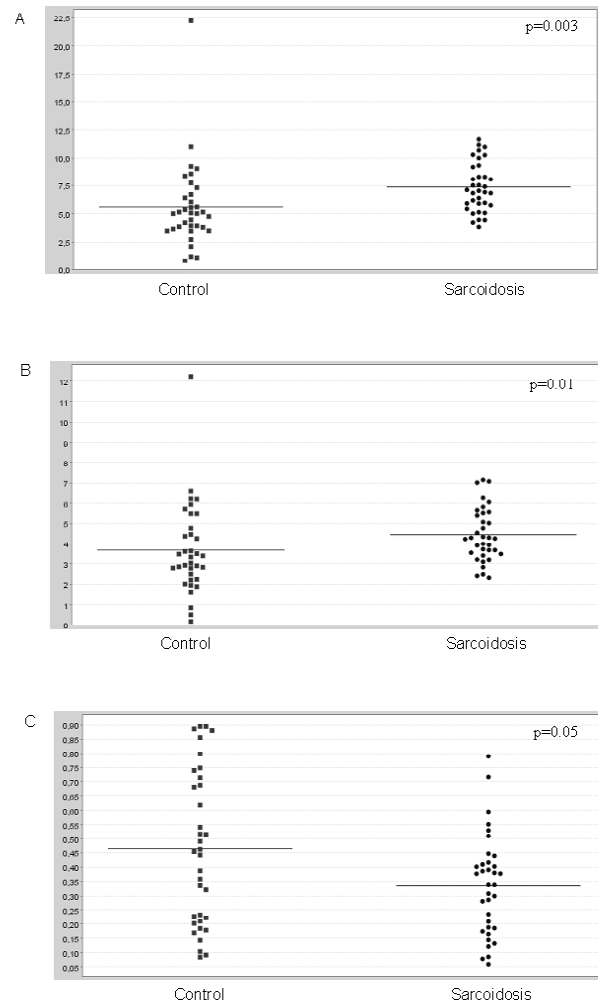


Figure 5.2 The cluster plots of the resulted peak splitters after Biomarker Patterns analysis; m/z 11,955 (A), m/z 11,734 (B), m/z 17,377 (C). The groups are indicated on the x-axis and the normalized intensities are indicated on the y-axis. The horizontal bars present the mean intensities for the control and the sarcoidosis group.

Table 5.3 The m/z values of the peak splitters, the cut-off values of the peak intensities, the p-values and the results of the learn and test set for the sarcoidosis and control samples per laser setting. The spectra were calibrated with the protein low calibration on the NP-20 array.

Laser setting	m/z value protein-low calibration (NP-20)	Cut-off peak intensity	p-value	Correct classification of the learn set	Correct classification of the test set
LI 220 SE 7	11,955	1.113	0.003	S : 30/35 = 86% C : 23/35 = 66%	S : 30/35 = 86% C : 22/35 = 63%
LI 190 SE 7	11,734 17,377	3.060 0.450	0.01 0.05	S : 26/35 = 74% C : 29/35 = 83%	S : 26/35 = 74% C : 25/35 = 71%

LI: laser intensity, m/z : mass-to-charge ratio, SE: detector sensitivity, S: sarcoidosis, C: control

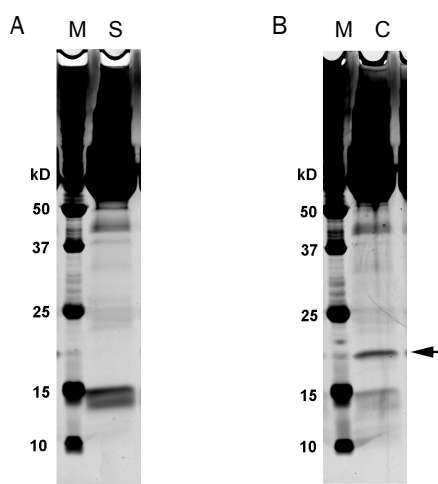


Figure 5.3 Silver stained band pattern of one fraction 3 sarcoidosis sample (A) and one fraction 3 healthy control sample (B) separated by SDS-PAGE. The left lane shows the marker and the right lane shows the sarcoidosis sample (S) in Figure 5.3A and the healthy control sample (C) in Figure 5.3B. In Figure 5.3A there is no band visible at MW 17 kDa. Figure 5.3B clearly shows a band at 17 kDa and this band is indicated with a vector.

Identification

SDS-PAGE and 2-DE were used for isolation and purification of the potential biomarkers and MALDI-TOF-MS was used for identification. We only tried to identify the three most discriminating markers described in our study. For the biomarker with a m/z value of 17,377 a sarcoidosis and a healthy control sample were applied on a SDS-PAGE gel. In Figure 5.3 is illustrated that the band at a molecular weight (MW) of 17 kDa, which represents the peak at m/z 17,377, is not visible in the sarcoidosis patient sample (A), but clearly visible in the healthy control

sample (B). This also indicates that this peak is downregulated in the sarcoidosis patient versus the healthy control. This agrees with the SELDI-TOF-MS results. Identification of the 17 kDa peak, after excising this peak out of the SDS-PAGE gel revealed the following protein as the best candidate of database searches: alpha chain of haptoglobin (P00738) with a probability Mascot score (probability based mowse score) of 86. Protein scores greater than 67 are significant ($p < 0.05$). In the 2-DE gels was seen that there were more spots around the MW of 17 kDa for the healthy control sample (Figure 5.4), but unfortunately in this study we were unable to identify the excised spots from the 2-DE gels. For the biomarkers with a m/z value of 11,734 and 11,955, also a sarcoidosis and control sample were applied on the SDS-PAGE gel, but it was also not possible to identify the bands or spots with MW around 11 kDa which represent the peaks at m/z 11,734 and 11,955 on either the SDS-PAGE gels or the 2-DE gels.

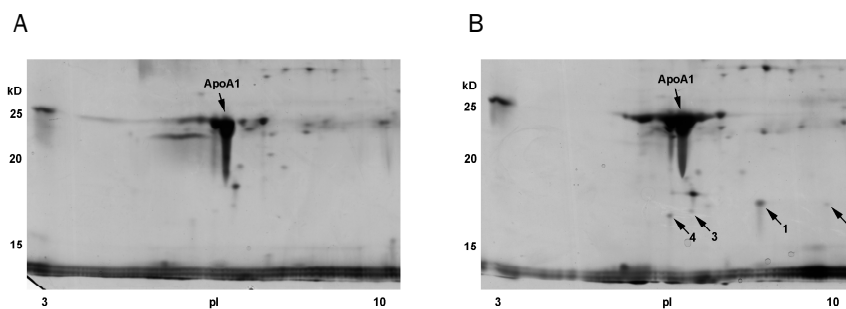


Figure 5.4 Silver stained spot pattern of one fraction 3 sarcoidosis sample (A) and one fraction 3 healthy control sample (B) separated by 2-DE. In both 2-DE gels ApoA1 was identified with a MW of 25 kDa. Figure 5.4 B clearly shows that below the Apo A1 spot, there are 4 protein spots around a MW of 17 kDa with different pI values in the 2-DE gel. These protein spots are not visible in Figure 5.4 A.

In Figure 5.3A and Figure 5.3B there are also two bands visible around 15 kDa. These peaks are not the potential biomarkers with m/z values 11,734 and 11,955. The peaks corresponding to the bands at 15 kDa gave no significant difference between sarcoidosis and control and were also not used for the Biomarker Patterns tree algorithm. Excising these peaks out of the SDS-PAGE revealed the following protein as the best candidate of database searches: Haemoglobin beta subunit (P68871) with a Mascot score (probability based mowse score) of 100.

Total haptoglobin measurement

Because the peak of the multimarker at m/z 17,377 was identified as the alpha chain of haptoglobin, the total haptoglobin was measured. The means and standard deviations (SD) for the haptoglobin concentration (g/l) were 1.32 ± 0.48

for the sarcoidosis group and 1.31 ± 0.43 for the healthy control group. There was no significant difference in total haptoglobin concentration between the sarcoidosis and healthy control group ($p=0.9$).

Discussion

This is the first study that shows that protein profiling in serum using SELDI-TOF-MS can be used as a diagnostic tool for sarcoidosis. It was demonstrated that the diagnostic value of the biomarkers obtained with SELDI-TOF-MS appeared to be favourable compared to the diagnostic value of ACE and sIL-2R in the same study population. At present, there still is no appropriate parameter to assess the activity of sarcoidosis. Clinical activity is assessed based on onset, worsening or persistence of symptoms, or signs directly related to sarcoidosis and a combination of clinical parameters³³.

Despite its shortcoming, ACE is mostly used in the assessment and the follow-up of sarcoidosis³¹. Recently some studies reported that sIL-2R appeared to be a better marker to predict disease severity compared to the traditionally used ACE, but there is a great overlap in the sensitivity and specificity results of both serum markers^{30,32,44}. Although both ACE and sIL-2R are used in the assessment and the follow-up of sarcoidosis, they still appeared to be inferior to the SELDI-TOF-MS data in our study.

In the present study was also demonstrated that there appeared to be a great variety in the protein patterns between patients, but also within controls. This variation is caused by the biological variation and analytical variation of SELDI-TOF-MS³⁴. Potential sources of variability that arise during SELDI-TOF-MS profiling include spot-to-spot variation of chip surfaces, laser detector variability over time, pipetting variability⁴⁵ and the crystallization process of the energy absorbing matrix^{46,47}. But in spite of this variation in intensities and biological variability, we were able to find potential biomarkers with the Biomarker Patterns software. A sensitivity of 86% and a specificity of 63% was reached with a single marker with a m/z value of 11,955 (upregulated in sarcoidosis versus control) and a sensitivity of 74% and a specificity of 71% was reached with a multimarker using two peaks with m/z values 11,734 (upregulated in sarcoidosis versus control) and 17,377 (downregulated in sarcoidosis versus control). We only tried to identify the three most discriminating markers described in our study. The other m/z peaks were not identified. Using Mascot search engine against the Swiss-Prot database we found that the peak at 17 kDa excised from the SDS-PAGE gel was the alpha chain of haptoglobin. Haptoglobin consists of two different polypeptide chains; the α -chain and the β -chain and these are linked by disulfide bonds. Three Haptoglobin (Hpt) phenotypes, Hpt 1-1 (structural formula: $(\alpha^1\beta)_2$), Hpt 2-1 (structural formula: $[(\alpha^1\beta)_2 + (\alpha^2\beta)_n]$) and Hpt 2-2 (structural formula: $(\alpha^2\beta)_n$), are genetically determined

by a polymorphism with two alleles: Hpt-1 and Hpt-2. This polymorphism results in variants in alpha-chains: α^1 (8.9 kDa) and α^2 (16 kDa). The α^1 is monomeric and the α^2 is partially dimeric (probably the result of incomplete genetic crossover). The β -chain (40 kDa) is heavier than the α -chain and is identical in all haptoglobin phenotypes⁴⁸. The marker at 17 kDa, which was detected in our study, is compatible with the alpha-2 chain of haptoglobin.

It is known that in the presence of oxidative stress, glucose-6-phosphate dehydrogenase (G6PD) deficiency leads to hemolysis⁴⁹, which causes a decrease in haptoglobin, which as such might clarify the lower haptoglobin fragment peaks in sarcoidosis patients. Therefore, total haptoglobin of the sarcoidosis and healthy control serum samples were compared. There was no significant difference between both groups. This can be caused due to the fact that the 17 kDa peak is only the alpha-2 chain of haptoglobin and the total haptoglobin assay measures the total haptoglobin complex. Therefore, specific antibodies against the alpha chain of haptoglobin should be used to compare both groups.

Another explanation might be the fact that the 17 kDa peak is not a single marker, but it is part of a multimarker. The 2-DE gel of the healthy control also clearly shows that there are four protein spots around a MW of 17 kDa with different pI values in the 2-DE gel. These spots are not visible in the 2-DE gel of the sarcoidosis samples. This indicates that there are more proteins with the same MW, but these proteins have another pI value.

Recently, Kriegova et al.²⁵ compared protein profiles from sarcoidosis patients with healthy controls in bronchoalveolar lavage fluid (BALF). In line with our findings, they found a peak with a MW of 11.7 kDa upregulated in sarcoidosis versus controls. However, no further analyzing or identifying of this sarcoidosis associated peak was performed. Because the calibration procedure is not described in the study of Kriegova et al., we are not absolutely sure if we detected the same peak. However, in most SELDI-TOF-MS studies, the All-in-1 peptide and the All-in-1 protein molecular mass standards (Ciphergen Biosystems) are used for the external calibration, like in our study.

In the present study serum was used for the protein profiling experiments, because collection of blood is much less invasive compared to collection of BALF. Although the results seem promising, one of the major limitations was the rather small sample size. Future studies, in a larger cohort of well-defined sarcoidosis patients, are necessary to evaluate the clinical value of this observation. The collection of well-defined sarcoidosis samples is time consuming. Moreover, to establish the diagnostic value a population of patients suffering from other interstitial lung diseases should be included as well. Further attempts will be undertaken, because the identification of these two markers will lead to a better understanding of sarcoidosis and would be of great interest. In future studies, we will enlarge the sample group and we will also validate our markers with a blind sample set. After this validation, we can search for disease activity markers. Eventually,

implementation of a quantitative immunoassay, is needed, to give a good prediction of the disease state and disease severity.

In conclusion, the present study showed that protein profiling in serum could be used in the management of sarcoidosis. The peak of the multimarker at m/z 17,377 was identified as the alpha-2 chain of haptoglobin.

References

1. Baughman RP, Lower EE, Du Bois RM. Sarcoidosis. *Lancet* 2003;361:1111-1118.
2. Kataria YP, Holter JF. Sarcoidosis: A Model of Granulomatous Inflammation of Unknown Etiology Associated with a Hyperactive Immune System. *Methods* 1996;9:268-294.
3. Judson MA, Baughman RP, Thompson BW, Teirstein AS, Terrin ML, Rossman MD, Yeager H Jr, McLennan G, Bresnitz EA, DePalo L, Hunninghake G, Iannuzzi MC, Johns CJ, Moller DR, Newman LS, Rabin DL, Rose C, Rybicki BA, Weinberger SE, Knatterud GL, Cherniak R. Two year prognosis of sarcoidosis: the ACCESS experience. *Sarcoidosis Vasc Diffuse Lung Dis* 2003;20:204-211.
4. Rybicki BA, Maliarik MJ, Major M, Popovich J Jr, Iannuzzi MC. Epidemiology, demographics, and genetics of sarcoidosis. *Semin Respir Infect* 1998;13:166-173.
5. Baughman RP, Winget DB, Bowen EH, Lower EE. Predicting respiratory failure in sarcoidosis patients. *Sarcoidosis Vasc Diffuse Lung Dis* 1997;14:154-158.
6. Arcasoy SM, Christie JD, Pochettino A, Rosengard BR, Blumenthal NP, Bavaria JE, Kotloff RM. Characteristics and outcomes of patients with sarcoidosis listed for lung transplantation. *Chest* 2001;120:873-880.
7. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics* 2002;1:845-867.
8. Srinivas PR, Verma M, Zhao Y, Srivastava S. Proteomics for cancer biomarker discovery. *Clin Chem* 2002;48:1160-1169.
9. Hutchens TW, Yip T. New Desorption strategies for the mass spectrometric analysis of macromolecules. *Rapid Commun Mass Spectrom* 1993;7:576-580.
10. Petricoin III EF, Mills GB, Kohn EC, Liotta LA. Proteomic patterns in serum and identification of ovarian cancer. *The Lancet* 2002;360:170-171.
11. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA. Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 2002;359:572-577.
12. Kozak KR, Amneus MW, Pusey SM, Su F, Luong MN, Luong SA, Reddy ST, Farias-Eisner R. Identification of biomarkers for ovarian cancer using strong anion-exchange ProteinChips: potential use in diagnosis and prognosis. *Proc Natl Acad Sci U S A* 2003;100:12343-12348.
13. Zhang Z, Bast RC Jr, Yu Y, Li J, Sokoll LJ, Rai AJ, Rosenzweig JM, Cameron B, Wang YY, Meng XY, Berchuck A, Van Haaften-Day C, Hacker NF, de Bruijn HW, van der Zee AG, Jacobs IJ, Fung ET, Chan DW. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004;64:5882-5890.
14. Vlahou A, Schorge JO, Gregory BW, Coleman RL. Diagnosis of Ovarian Cancer Using Decision Tree Classification of Mass Spectral Data. *J Biomed Biotechnol* 2003;2003:308-314.
15. Ye B, Cramer DW, Skates SJ, Gygi SP, Pratomo V, Fu L, Horick NK, Licklider LJ, Schorge JO, Berkowitz RS, Mok SC. Haptoglobin-alpha subunit as potential serum biomarker in ovarian cancer: identification and characterization using proteomic profiling and mass spectrometry. *Clin Cancer Res* 2003;9:2904-2911.
16. Rai AJ, Zhang Z, Rosenzweig J, Shih Ie M, Pham T, Fung ET, Sokoll LJ, Chan DW. Proteomic approaches to tumor marker discovery. *Arch Pathol Lab Med* 2002;126:1518-1526.
17. Banez LL, Prasanna P, Sun L, Ali A, Zou Z, Adam BL, McLeod DG, Moul JW, Srivastava S. Diagnostic potential of serum proteomic patterns in prostate cancer. *J Urol* 2003;170:442-446.
18. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, Semmes OJ, Schellhammer PF, Yasui Y, Feng Z, Wright GL Jr. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. *Cancer Res* 2002;62:3609-3614.
19. Petricoin EF 3rd, Ornstein DK, Paweletz CP, Ardekani A, Hackett PS, Hitt BA, Velasco A, Trucco C, Wiegand L, Wood K, Simone CB, Levine PJ, Linehan WM, Emmert-Buck MR, Steinberg SM, Kohn EC, Liotta LA. Serum proteomic patterns for detection of prostate cancer. *J Natl Cancer Inst* 2002;94:1576-1578.

20. Qu Y, Adam BL, Yasui Y, Ward MD, Cazares LH, Schellhammer PF, Feng Z, Semmes OJ, Wright GL Jr. Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. *Clin Chem* 2002;48:1835-1843.
21. Li J, White N, Zhang Z, Rosenzweig J, Mangold LA, Partin AW, Chan DW. Detection of prostate cancer using serum proteomics pattern in a histologically confirmed population. *J Urol* 2004;171:1782-1787.
22. Zhukov TA, Johanson RA, Cantor AB, Clark RA, Tockman MS. Discovery of distinct protein profiles specific for lung tumors and pre-malignant lung lesions by SELDI mass spectrometry. *Lung Cancer* 2003;40:267-279.
23. Poon TC, Hui AY, Chan HL, Ang IL, Chow SM, Wong N, Sung JJ. Prediction of liver fibrosis and cirrhosis in chronic hepatitis B infection by serum proteomic fingerprinting: a pilot study. *Clin Chem* 2005;51:328-335.
24. Zhu XD, Zhang WH, Li CL, Xu Y, Liang WJ, Tien P. New serum biomarkers for detection of HBV-induced liver cirrhosis using SELDI protein chip technology. *World J Gastroenterol* 2004;10:2327-2329.
25. Kriegova E, Melle C, Kolek V, Hutyrova B, Mrazek F, Bleul A, du Bois RM, von Eggeling F, Petrek M. Protein Profiles of Bronchoalveolar Lavage Fluid from Patients with Pulmonary Sarcoidosis. *Am J Respir Crit Care Med* 2006;173:1145-1154.
26. Magi B, Bini L, Perari MG, Fossi A, Sanchez JC, Hochstrasser D, Paesano S, Raggiaschi R, Santucci A, Pallini V, Rottoli P. Bronchoalveolar lavage fluid protein composition in patients with sarcoidosis and idiopathic pulmonary fibrosis: a two-dimensional electrophoretic study. *Electrophoresis* 2002;23:3434-3444.
27. Sabounchi-Schutt F, Astrom J, Hellman U, Eklund A, Grunewald J. Changes in bronchoalveolar lavage fluid proteins in sarcoidosis: a proteomics approach. *Eur Respir J* 2003;21:414-420.
28. Sabounchi-Schutt F, Mikko M, Eklund A, Grunewald J, AStröm J. Serum protein pattern in sarcoidosis analysed by a proteomics approach. *Sarcoidosis Vasc Diffuse Lung Dis* 2004;21:182-190.
29. Song Z, Marzilli L, Greenlee BM, Chen ES, Silver RF, Askin FB, Teirstein AS, Zhang Y, Cotter RJ, Moller DR. Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J Exp Med* 2005;201:755-767.
30. Ziegenhagen MW, Rothe ME, Schlaak M, Muller-Quernheim J. Bronchoalveolar and serological parameters reflecting the severity of sarcoidosis. *Eur Respir J* 2003;21:407-413.
31. Muller-Quernheim J. Serum markers for the staging of disease activity of sarcoidosis and other interstitial lung diseases of unknown etiology. *Sarcoidosis Vasc Diffuse Lung Dis* 1998;15:22-37.
32. Rothkrantz-Kos S, van Dieijen-Visser MP, Mulder PG, Drent M. Potential usefulness of inflammatory markers to monitor respiratory functional impairment in sarcoidosis. *Clin Chem* 2003;49:1510-1517.
33. ATS/ERS/WASOG Committee. Statement on sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 1999;16:149-173.
34. Bons JA, de Boer D, van Dieijen-Visser MP, Wodzig WK. Standardization of calibration and quality control using surface enhanced laser desorption ionization-time of flight-mass spectrometry. *Clin Chim Acta* 2006;366:249-256.
35. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;16:5-40.
36. Hunninghake GW, Costabel U, Ando M, Baughman R, Cordier JF, Du Bois R, Eklund A, Kitaichi M, Lynch J, Rizzato G, Rose C, Selroos O, Semenzato G, Sharma OP. ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. *Sarcoidosis Vasc Diffuse Lung Dis* 1999;16:149-173.
37. DeRemee RA. The roentgenographic staging of sarcoidosis. Historic and contemporary perspectives. *Chest* 1983;83:128-133.

38. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988;44: 837-845.
39. Solassol J, Marin P, Demetree E, Rouanet P, Bockaert J, Maudelonde T, Mange A., Proteomic detection of prostate-specific antigen using a serum fractionation procedure: potential implication for new low-abundance cancer biomarkers detection. *Anal Biochem* 2005;338:26-31.
40. Linke T, Ross AC, Harrison EH. Profiling of rat plasma by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, a novel tool for biomarker discovery in nutrition research. *J Chromatogr A* 2004;1043:65-71.
41. Liu W, Guan M, Wu D, Zhang Y, Wu Z, Xu M, Lu Y. Using tree analysis pattern and SELDI-TOF-MS to discriminate transitional cell carcinoma of the bladder cancer from noncancer patients. *Eur Urol* 2005;47:456-462.
42. Bouwman F, Renes J, Mariman E. A combination of protein profiling and isotopomer analysis using matrix-assisted laser desorption/ionization-time of flight mass spectrometry reveals an active metabolism of the extracellular matrix of 3T3-L1 adipocytes. *Proteomics* 2004;4:3855-3863.
43. Bouwman F, Suylen D, Renes J, Mariman E. Evaluation and improving the success rate of protein identification by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2005;19:2465-2468.
44. Grutters JC, Fellrath JM, Mulder L, Janssen R, Van Den Bosch JMM, van Velzen-Blad H. Serum sIL2R measurement in sarcoidosis patients: a clinical evaluation. *Chest* 2003;124:186-195.
45. Koopmann J, Zhang Z, White N, Rosenzweig J, Fedarko N, Jagannath S, Canto MI, Yeo CJ, Chan DW, Goggins M. Serum diagnosis of pancreatic adenocarcinoma using surface-enhanced laser desorption and ionization mass spectrometry. *Clin Cancer Res* 2004; 10:860-868.
46. Jock CA, Paulauskis JD, Baker D, Olle E, Bleavins MR, Johnson KJ, Heard PL. Influence of matrix application timing on spectral reproducibility and quality in SELDI-TOF-MS. *Biotechniques* 2004; 37:30-32, 34.
47. Cordingley HC, Roberts SL, Tooke P, Armitage JR, Lane PW, Wu W, Wildsmith SE. Multifactorial screening design and analysis of SELDI-TOF ProteinChip array optimization experiments. *Biotechniques* 2003;34:364-365, 368-373.
48. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem* 1996;42:1589-1600.
49. Dhaliwal G, Cornett PA, Tierney LM Jr. Hemolytic anemia. *Am Fam Physician* 2004;69:2599-2606.