VILNIUS UNIVERSITY

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Peculiarities of the Course of Sarcoidosis and Factors Determining Its Development

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Regina Aleksonienė

Sarkoidozės eigos ypatumai ir jos raidą lemiantys veiksniai

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CONTENT

1. INTRODUCTION

Sarcoidosis is a systemic inflammatory disease caused by the combined effects of genetic susceptibility and environmental exposures. Multiple potential infectious, non-infectious, organic, and inorganic environmental etiologic agents, unfortunately without any definitive demonstration of causality, have been proposed for sarcoidosis. It is likely that in predisposed individuals, sarcoidosis is the result of the interplay between different etiologic agents and the immune system.

Although the phenotypic expression of the disease appears to vary in different populations, in general, patients with sarcoidosis tend to improve over time. Many patients enter clinical remission within a few years of diagnosis; however, approximately one third of them experience chronic disease. Outcomes are generally less favourable for parenchymal pulmonary sarcoidosis compared with lymph node limited disease.

Established experimental sarcoidosis models are still lacking, while studies on pathophysiologic mechanisms as well as the search for diagnostic indicators are hampered by high variability in the course of the disease. One of the recent studies using mass spectrometry based proteomics identified 4,306 proteins in BAL cells, of which 272 proteins were differentially expressed in sarcoidosis compared with controls, and 121 were differentially expressed comparing progressive vs. non-progressive sarcoidosis subjects.

The prognosis of sarcoidosis is highly disparate in ethnic and genetic factors, the initial presentation, and other contributors. There is still no consensus on which tools are necessary to predict the progression of the disease or how to monitor its activity. If present during an early phase, ongoing inflammation and interstitial fibrosis, specific to the chronic phase of the disease, may alert the presence of a more serious form and, consequently, a tendicity to chronicity. Compared with patients with persistently active non-fibrotic disease, the potential complications and long-term outcomes are worse for

those with fibrotic sarcoidosis. The extent of fibrosis on computed tomography is an independent predictor of mortality. Since the mechanisms driving the transition from inflammation to fibrosis in sarcoidosis are poorly understood and the evolution and severity of the disease vary substantially, it is difficult to predict the disease course. It is not known if a fibrosis cascade is activated at disease onset or if it is a response to poorly controlled inflammation.

Some cytokines, chemokines as well as immune cells derived mediators were proposed to be useful for the diagnosis and follow-up of sarcoidosis patients; however, not a single biomarker with proven unequivocal prognostic value has yet been identified. Up to now, only one serum factor, angiotensin-converting enzyme (ACE), has been mentioned in international guidelines. However, its sensitivity and specificity are low. In addition, a recent study has demonstrated that its clinical value as a biomarker can be further reduced for patients treated with ACE inhibitors. Some other serum factors also have been proposed for sarcoidosis biomarkers, including activated macrophages and neutrophils secreted chitotriosidase, lysozyme, produced by monocyte-macrophage system and epithelioid cells and involved in granuloma formation, mucin protein Krebs von den Lungen-6.

Bronchoalveolar lavage fluid (BALF) examination has been used as a diagnostic tool for forty years; the best known indicators for sarcoidosis are lymphocytosis and an increased CD4+ to CD8+ lymphocyte ratio. BAL cell patterns and lymphocyte phenotyping often provide information useful for differentiation of interstitial lung diseases, and most of the studies have shown that a CD4+/CD8+ ratio greater than 3.5 has high diagnostic specificity for lung sarcoidosis. However, its sensitivity is low; moreover that specificity appears to be lower in advanced radiographic stages of the disease.

Consequently, there is still great interest in cellular biomarkers, which seem to be related to inflammation, immune cell migration, and fibrosis, including cell surface molecules or their ligands. We performed a literature search looking for cellular markers that could help to predict the course of sarcoidosis and selected four molecules,

i.e. CD31, CD38, CD44, and CD103. Many inflammatory cells and tissue cells with various, sometimes even opposite, effects may be positive for CD31, CD38, CD44, and CD103, but as sarcoidosis is driven by T-cell mechanisms, particularly the accumulation of activated CD4 T cells in lungs, allowing for T-cell attachment and transmigration through the endothelium, the research was performed on T-cell subpopulations CD4+ and CD8+.

CD31, also known as platelet endothelial cellular adhesion molecule-1 (PECAM-1), is an integral membrane protein expressed by endothelial cells, platelets, dendritic cells, and blood cells, including T lymphocytes. CD31 is known to be involved in leukocyte-leukocyte interactions, as well as in interactions between lymphocytes and vascular endothelium and migration to the inflamed tissues through intercellular junctions. The ligand to the CD31 molecule is transmembrane glycoprotein CD38, a multifunctional receptor and co-receptor involved in transmembrane signalling and cell adhesion, also contributing to the modulation of antigen-mediated T-cell responses. The human CD38 molecule is expressed by immature hematopoietic cells, downregulated on mature cells, and re-expressed at high levels on activated lymphocytes, such as T cells, B cells, dendritic cells, and natural killer (NK) cells. CD38 ligation is followed by an increase in intracellular Ca^{2+}, cell activation, proliferation, differentiation, and migration, as well as by the production and secretion of a panel of cytokines, such as interleukin (IL)-6, IL-10, interferon-γ, granulocyte macrophage colony stimulating factor (GM-CSF), and by proliferative effects. CD44 is a family of cell surface glycoproteins, also termed hyaladherin (HA), belonging to the group of cell adhesion molecules. HA is involved in cell-to-cell and cell-to-matrix interactions and participates in the regulation of hyaluronic metabolism, activation and migration of lymphocytes, as well as the release of cytokines in the areas of inflammation. CD44 participates in a wide variety of cellular functions, including lymphocyte activation, recirculation and homing. CD44 expression is known to be greater in the areas of granuloma formation and fibrosis. One more biomarker investigated in

sarcoidosis patients is integrin αEβ7 (CD103), an adhesion molecule expressed on 95% of intraepithelial CD4+ lymphocytes in the mucosa, but on less than 2% of circulating peripheral blood lymphocytes. Differential expression of this marker on BAL CD4+ lymphocytes has been demonstrated, and CD103 has been proposed as a diagnostic marker of granulomatous lung disorders, such as sarcoidosis, albeit with controversial results.

The aim of our study was to evaluate the course of sarcoidosis based on various radiological features, lung function test parameters and cellular markers of the immune response in bronchoalveolar lavage fluid, blood and lung biopsy tissue with the expectation to detect prognostic factors.

1.1 Aim

The aim of our study was to evaluate the course of sarcoidosis based on various radiological features, lung function test parameters and cellular markers of the immune response in bronchoalveolar lavage fluid, blood and lung biopsy tissue with the expectation to detect prognostic factors.

1.2 Research novelty

To our knowledge, a comprehensive radiological examination, advanced lung function test parameters, and cellular markers of the immune response in blood, bronchoalveolar lavage fluid and lung biopsy tissue of patients with pulmonary sarcoidosis and a study of the course of the disease in a study were performed for the first time. The study provided unique data on the cellular immune response in several tissues of the human body in newly diagnosed pulmonary sarcoidosis.

2. MATERIALS AND METHODS

2.1 Subjects

This study was approved by the Vilnius Regional Biomedical Research Ethics Committee (No. 158200-12-5591160).

We conducted the study in two phases.

In 2012 (the first phase), the study sample consisted of 80 consecutive patients newly diagnosed with pulmonary sarcoidosis at the Centre of Pulmonology and Allergology, Vilnius University Hospital Santaros Klinikos. Patients (41 females and 39 males with a mean age of 39.0 years) underwent diagnostic testing as part of routine clinical investigation.

A total of 81 consecutive patients (37 females and 44 males with a mean age of 37.2 years) with newly diagnosed pulmonary sarcoidosis and 25 healthy control patients (8 females and 17 males with a mean age of 40.0 years) were enrolled in the study carried out from 2013 to 2015 (the second phase), and blood and BALF analyses were performed. Bronchoscopic lung biopsies were performed in 35 patients with sarcoidosis. In addition, five samples were taken from biopsies that exhibited no histological changes, and these were used as a control for tissue analyses.

All study patients were Caucasian. None of the patients (including controls) had any relevant medical history or comorbidity (e.g., tuberculosis) or a history of exposure to organic or mineral dusts known to cause granulomatous lung disease, or any immunosuppressive therapy – steroids, cytostatics, biotherapy.

Patients were grouped into stages according to chest radiography and high-resolution computed tomography (HRCT). Comparisons were made between the groups according to smoking history and the presence of Löfgren syndrome. All patients underwent diagnostic testing as part of the routine clinical investigation, including chest radiography, HRCT examination, pulmonary function test, fibreoptic bronchoscopy with bronchoalveolar lavage (BAL), bronchoscopic

lung biopsy, and BALF cell and blood examination. All study tests were performed within an average of two weeks (in all cases, less than in one month) from the first visit to our centre. The diagnosis was confirmed according to the Joint Statement of the American Thoracic Society, the European Respiratory Society and the World Association for Sarcoidosis and Other Granulomatous Disorders. A signed informed consent form was obtained from each participant.

2.2 Chest radiography and computed tomography

Chest radiographic staging was performed according to the Scadding criteria as follows: stage 0, normal chest radiograph; stage I, mediastinal lymphadenopathy only; stage II, mediastinal lymphadenopathy with parenchymal lesion; stage III, parenchymal disease only; and stage IV, pulmonary fibrosis. HRCT scans were performed using 64-slice CT (GE LightSpeed VCT, GE Healthcare, Milwaukee, Wisconsin). Scan parameters were as follows: collimation of 64 × 1.25 mm; tube voltage, 120 kV; tube current, 660 mAs.

The following patterns were evaluated: micronodule, macronodule, consolidation, ground glass opacity and linear opacities. A nodule profusion score was based on the number of nodules per zone: 0 = no nodules, 1 = 1–5 nodules, 2 = 6–10 nodules, 3 = 11–15 nodules, and 4 = >15. The sum of the scores and the mean values of each parameter were defined for each patient. For each parameter, a patient might have had a maximum of 24 points.

The presence of typical (i.e. hilar, mediastinal (right paratracheal), bilateral, symmetric, ≥15 mm in diameter) and atypical (i.e. unilateral, isolated, anterior and posterior mediastinal) localisation of lymph nodes and the occurrence of lymph node calcification were determined.

2.3 Pulmonary function tests

Pulmonary function testing (PFT) included the measurement of forced vital capacity (FVC) and forced expiratory volume in one second (FEV1) and the determination of total lung capacity (TLC), vital capacity (VC), residual volume (RV) with body plethysmography, and diffusing capacity of carbon monoxide (DLCO) with standard single-breath technique Vmax Encore (Viasys® Healthcare, USA). Results were expressed as percentages of predicted normal values.

The first phase of the study showed statistically significant differences in several pulmonary function parameters between the patient groups with different radiographic stages of sarcoidosis. Radiographic stage I patients had better pulmonary function tests as compared with those in stages II and III. A decrease of DLCO was the most commonly observed impairment of the lung function in our patients. Although we observed a decrease in all pulmonary function parameters (FVC, FEV1, FEV1/FVC, TLC, VC and DLCO) among smokers as compared with non-smokers, the differences were not statistically significant.

In the second phase of the study, spirometry was normal in patients with pulmonary sarcoidosis, only DLCO corresponding to mild gas diffusion impairment was observed in stage II and III of sarcoidosis. RV was higher in smokers than in non-smokers ($p < 0.01$).

2.4 Fibreoptic bronchoscopy, bronchoalveolar lavage and bronchoscopic lung biopsy

Fibreoptic bronchoscopy, BAL, and bronchoscopic lung biopsy were performed as described elsewhere. The subjects were premedicated with atropine, and lidocaine was delivered topically via an atomiser. The bronchoscope was inserted transnasally (in most cases) or orally and passed to segmental or subsegmental bronchus. BAL was performed in the right middle lobe, lingual, or in the area of

the greatest radiologic abnormality. Sterile isotonic saline at room temperature was instilled in two 50-ml aliquots (as standard procedure in our centre). Each aliquot was retrieved with gentle manual aspiration (usually 30–40 ml in total). The second aliquot was used to analyse BALF cells and lymphocyte subpopulations.

Bronchoscopic lung biopsy was performed under fluoroscopic guidance as follows: 6 to 10 pieces (3–4 mm^2 each) of lung tissue were taken from suspicious areas for histopathological examination at the National Centre of Pathology subordinate to Vilnius University Hospital Santaros Klinikos.

2.5 Immunostaining and flow cytometry of peripheral blood and bronchoalveolar lavage fluid samples

Peripheral blood and BALF samples were taken on the same day, and both were analysed at the Laboratory of Clinical Immunology and Blood Transfusion at the Centre of Laboratory Medicine (Vilnius University Hospital Santaros Klinikos). BALF for cell analysis was filtered through a 70 μm pore filter to remove mucus. The total cell numbers (Neubauer chamber) were determined, and the cellular material was sedimented by centrifugation (300 g for 7 min. at 4°C). Trypan blue dye (0.4%) was used for the assessment of BALF cell viability (the viability of the cells was 95±5%). Cell differentials were obtained by counting at least 600 cells by light microscopy after staining with May-Grünwald-Giemsa stain (Merck, Darmstadt, Germany). The monoclonal antibodies (BD Biosciences, San Jose, CA, USA) used for lymphocyte characterisation were as follows: peridinin-chlorophyll-protein-complex (PerCP) conjugated anti-CD3; phycoerythrin (PE)-conjugated anti-CD4 and anti-CD8; and fluorescein-isothiocyanate (FITC)-conjugated anti-CD31, anti-CD38, anti-CD44, and anti-CD103. For both blood and BALF lymphocyte subpopulation analyses, 100 μl of the cell suspensions (1×106 cells) were incubated with monoclonal antibodies for 15 min.: blood at room temperature, BALF at 4°C in the dark. Afterwards, peripheral blood

erythrocytes were lysed using 2 ml of lysing solution (incubation for 10 min. at room temperature in the dark), and then the suspension was centrifuged at 300 g for 7 min. at room temperature. The supernatant was discarded, and the remaining pellet washed by adding 2 ml of phosphate buffered saline (PBS) and centrifuging at 300 g for 7 min. at room temperature (blood) or 4°C (BALF). Then the supernatant was discarded, and the cells were re-suspended in 500 μl of 1% paraformaldehyde. Following cell fixation, flow cytometry was run on an FACS Calibur (BD Biosciences, San Jose, CA, USA). After proper instrument settings, calibration and compensation, the results were analysed using CellQuestPro software (BD Biosciences, San Jose, CA, USA). Fixed cells were analysed within 24 hours. Cells were sequentially gated on lymphocytes (based on forward scatter (FSC) versus side scatter (SSC)) and on T lymphocytes (based on SSC versus CD3 PerCP histogram); after that, markers of interest on CD4+ and CD8+ subpopulations were determined. Isotype control γ1/γ2a/CD3 was used for negative marker settings; these settings were applied for subsequent lymphocyte analysis with a minimum of 10000 events. Peripheral blood and BALF flow cytometry dot plots of representative patient are shown in Figure 1.

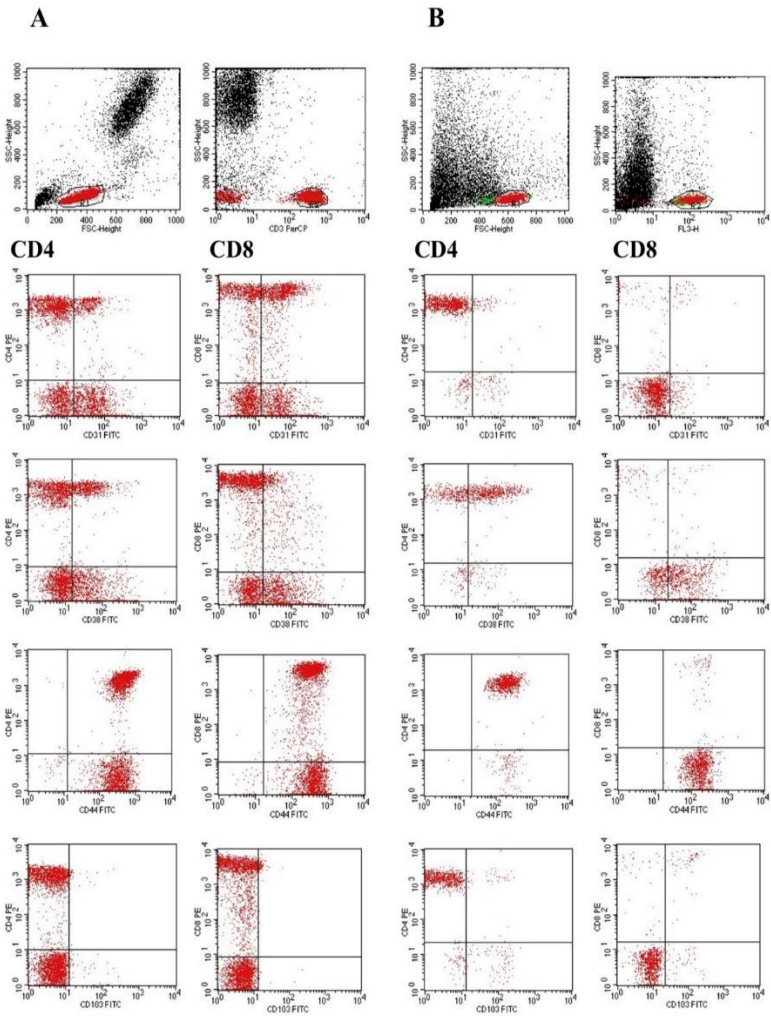


Figure 1. Flow cytometry dot plots demonstrating gating strategy in peripheral blood (dot plots A) and in BALF (dot plots B). Side scatter vs. forward scatter gate for lymphocytes (R1), followed by a second gate on CD3+ T-cell component (R2), CD3+ lymphocyte CD4+ and CD8+ subpopulations and finally CD31+, CD38+, CD44+ and CD103+ expression on CD4+ as well as CD8+ lymphocytes.

2.6 Tissue preparation and digital image analysis of histology specimens

Biopsy specimens were fixed in formalin and stained with Hematoxylin-Eosin (HE) for histological investigation, and Picro-Sirius Red was used for fibrosis assessment. Immunohistochemistry was performed using formalin-fixed paraffin-embedded sections, which were cut 3 μm thick and mounted on positively charged slides (Figure 2). Immunohistochemistry for CD8+, CD38+, CD44+, and CD103+ on the whole tissue sections was performed using the Ultra View Universal DAB detection kit on the Ventana Benchmark Ultra staining system (Ventana Medical Systems, Tucson, Arizona, USA). Epitope retrieval was performed on the slides using the Ventana Ultra CC1 buffer at 95°C for 64 min. The sections were then incubated with DAKO anti-Human CD8+ mouse monoclonal antibody (C8/144B, 1:100 dilution), Cell Margue anti-CD38 rabbit monoclonal antibody (SP149, 1:100 dilution), Abcamanti-CD44 rabbit monoclonal: antibody (EPR1013Y, 1:100 dilution), and Abcamanti-Integrin alpha E rabbit monoclonal antibody (EPR4166, 1:500 dilution) at 37°C for 32 min. using the Ventana Ultraview DAB detection kit. Finally, the sections were developed in 3,3′-Diaminobenzidine as the chromogen at 37°C for 8 min., counterstained with Mayer's hematoxylin, and mounted.

Immunohistochemistry for CD4+ was performed using the EnVision FLEX+ visualisation system on the DAKO Autostainer Link 48+ system (Agilent DAKO, USA). Epitope retrieval was performed on the slides using the DAKO TRS High pH buffer at 97°C for 20 min. with the PT-link module (Agilent DAKO, USA). The sections were then incubated with DAKO anti-Human CD4+ mouse monoclonal antibody (4B12, 1:150 dilution) at 22°C for 30 min. using the EnVision FLEX+ detection kit, developed in DAB at 22°C for 10 min., counterstained with Mayer's hematoxylin, and mounted. Digital images were captured using the Aperio Scan-Scope XT Slide Scanner

(Aperio Technologies, Vista, CA, USA) under 20× objective magnification (0.5 μm resolution).

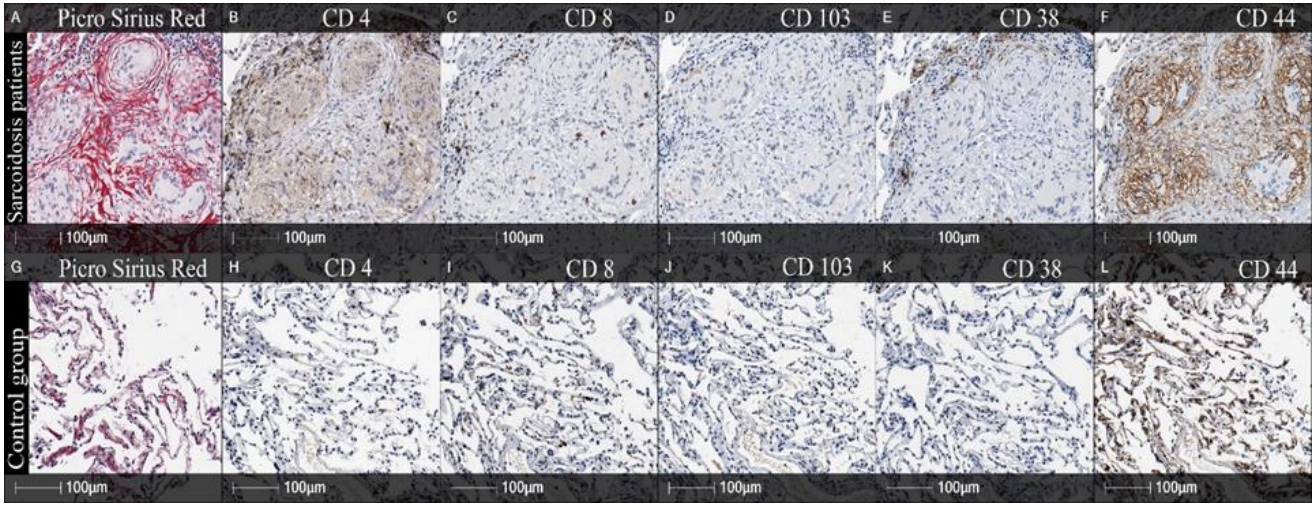


Figure 2. Examples of lung tissue controls and sarcoid patients stained for Picro Sirius Red and CD4+, CD8+, CD103+, CD38+, CD44+ immunohistochemistry. A–F - lung biopsy tissue containing non-necrotising granulomatous inflammation from sarcoid patients. Picro Sirius Red staining reveals greater fibrosis in granulomatous inflammation areas compared with control tissues. CD4+, CD8+, CD103+, CD38+ immunohistochemistry shows slightly higher total numbers of lymphocyte populations in sarcoidosis patients, however only the percentage of CD4+ and CD103+ cells was found to be significantly higher.

2.7 Digital image analysis

Digital image analysis (DIA) was performed using the HALOTM Classifier Module and Cyto Nuclear v1.5 algorithms (IndicaLabs, NM, USA) with a manually selected region of interest (ROI) enclosing the tissue section. The software enabled automated recognition of selected tissue areas and cell segmentation in scanned images. The nuclear/cytoplasmic analysis was calibrated to enumerate positive and negative inflammatory cell profiles in the lung tissue. The positivity thresholds of DIA were monitored and individually calibrated by visual inspection in each immunohistochemical stain. The result of various inflammatory cell densities was calculated by cell counts in the lung tissue per square millimetre. Fibrosis areas were calculated using the HALOTM Area Quantification v1.0 algorithm (Figure 3).

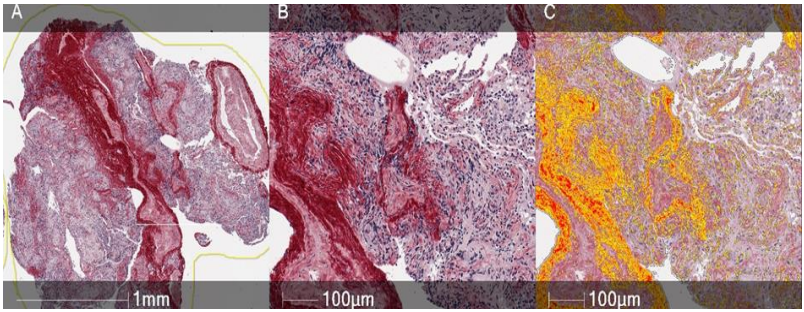


Figure 3. Examples of lung tissue histochemistry, with staining Picro Sirius Red analysis by digital image analysis (DIA).
Pictures A and B (sarcoidosis patient biopsy) show the scanned Picro Sirius Red histochemistry without analysis at different magnification levels. Picture C illustrates the automated detection of collagen, labelled in yellow, orange, and red colours, and outlined by the previously calibrated HALO Area Quantification v1.0 algorithm.

2.8 Statistical analysis

Statistical analysis was performed using SPSS software, Version 20.0 (Statistical Package for Social Sciences, IBM, USA) to present the mean, standard deviation (SD), and the available number of observations of quantitative variables. The nonparametric Kruskal-Wallis criterion was used to evaluate all the parameters. For normality of the quantitative variables, the Shapir-Wilks test was used. In order to test hypothesis for between-group comparison of the quantitative variables, the Student's t-test or the nonparametric Mann-Whitney U test was used as appropriate. In order to test hypothesis for between-group comparison of categorical variables, the Pearson's or Spearman's rank correlation coefficient was used as appropriate to determine the correlation. The cut-off values of the quantitative factors were estimated based on the ROC curves. In addition, sensitivity and specificity for these values were presented, including AUC with 95% CI. These cut-off values were used to categorise the variables and calculate their Odds Ratios (OR) with 95% CI.

3. RESULTS

3.1 Blood vs. BALF lymphocyte subsets in the control and different radiographic stages of sarcoidosis

We compared control and sarcoidosis groups, and no significant difference was found in the blood CD4+ T lymphocyte count (41.9%±7.3% vs. 41.1%±8.5%). However, the CD8+ T lymphocyte count in the sarcoidosis group was found to be significantly lower (32.0%±8.5% vs. 27.1%±9.0%, $p<0.05$). In BALF, on the contrary, significant differences were found (Figure 1).

Blood vs. BALF T-lymphocyte subsets in different radiographic stages of the sarcoidosis and control groups are presented in Figure 4 (CD4+ T cells) and Figure 5 (CD8+ T cells). The percentage of CD31+ and CD38+ peripheral blood cells of the control group was found to be significantly higher compared with BALF ($p<0.0004$) on both CD4+ and CD8+ T lymphocytes, whereas CD44+ was expressed equally on blood and BALF CD4+ T lymphocytes, but was significantly lower ($p<0.02$) on BALF CD8+ T cells. The CD103+ cell percentage in BALF was significantly higher compared with peripheral blood ($p<0.00001$ for the control group CD4+ and CD8+ T lymphocytes).

The percentage of the studied marker in blood was found to be sarcoidosis-stage-dependent. Specifically, CD4+CD38+ ($p=0.004$) and CD4+CD103+ ($p=0.003$) T cell numbers were found to be significantly higher in sarcoid patients in radiographic stage II compared with controls (Figure 4). The opposite was true for the activation marker percentage on CD8+ T lymphocytes (Figure 5): CD44+ in the stage III group ($p=0.035$) was significantly lower than controls.

The percentages of several BALF lymphocyte subsets in sarcoid patients were significantly different from the controls. The percentages of BALF CD4+CD31+, CD4+CD38+, and CD4+CD44+ T cells were found to be significantly higher, while CD8+CD44+ T

cells were significantly lower in the sarcoidosis group compared with the control group. Some markers were found to be sarcoidosis-stage-dependent. Specifically, the expression of CD31+ on CD4+ T cells was found to increase with a sarcoidosis stage ($p=0.002$ for stage II vs. the controls). CD4+CD38+ T cell numbers in stage I ($p=0.001$) and stage II ($p<0.0001$) were found to be significantly higher compared with controls and tended to decrease in more advanced stage III sarcoid patients. The CD44+ marker had significantly higher expression on CD3+CD4+ cells of sarcoid patients in all three stages ($p<0.0001$).In contrast , the number of CD4+CD103+ T cells was found to be significantly higher in the control group compared with stage I sarcoid patients (12.6%±8.8% vs. 5.1%±4.8%, $p=0.006$) and tended to increase with a radiographic stage (8.1%±7.7% for stage II ($p=0.028$) 19.7%±9.7% for stage III, $p=0.054$). For CD8+ T cells, no clear tendency in its CD38+ percentage changes could be found, while the percentage of CD31+, CD44+ and CD103+ was significantly lower in sarcoid patients ($p=0.035$ for CD31+, $p=0.001$ for CD44+ and $p=0.011$ for CD103+ comparing stage I sarcoid patients with the controls).

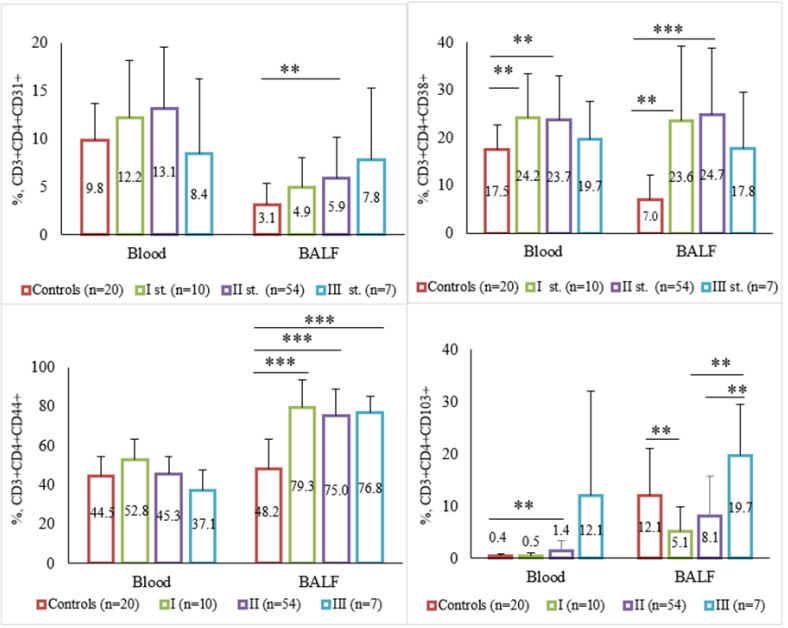


Figure 4. Blood vs. BALF CD4+ T-lymphocyte subsets in different radiographic stages of sarcoidosis and controls groups. Data are shown as the mean ± standard deviation. Statistical significance was denoted by a p-value as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

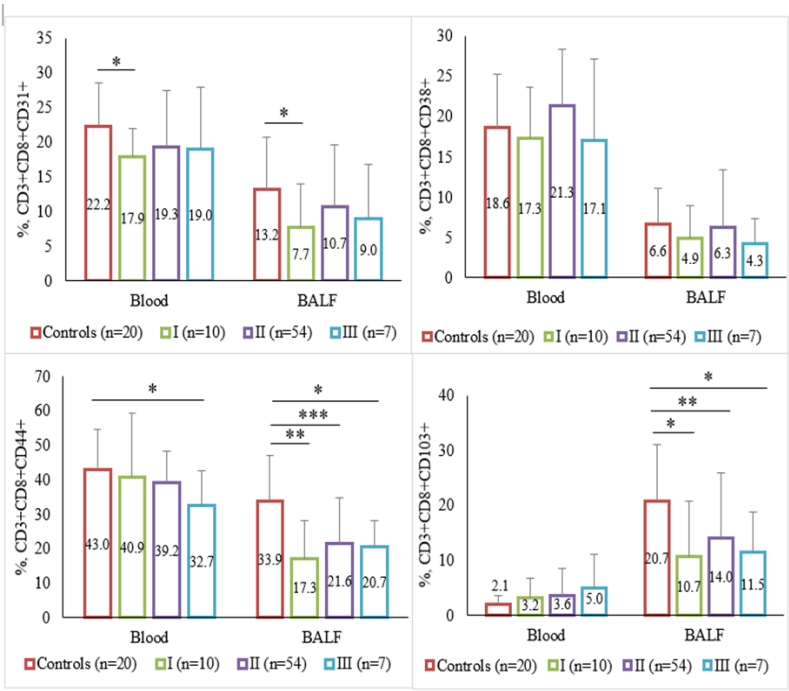


Figure 5. Blood vs. BALF CD8+ T-lymphocyte subsets in different radiographic stages of the sarcoidosis and control groups. Data are shown as the mean ± standard deviation. Statistical significance was denoted by a p-value as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

We also performed a separate analysis of blood and BALF lymphocyte subsets in acute sarcoidosis (Löfgren syndrome) vs. non-Löfgren syndrome patients and non-smoker patients (presented in Figure 6) vs. smoker patients (Figure 7).

CD4+CD38+ expression on blood T cells seems to be the only parameter that depends on smoking status. Comparison of the control groups showed significantly more positive lymphocytes in non-smokers (19.2%±4.5% vs. 12.6%±3.4%, $p<0.008$). In BALF, the non-smoker control group had a significantly higher CD8+CD38+ lymphocyte percentage than smokers (7.6%±4.5% vs. 3.4%±2.7%, $p<0.03$).

3.2 Blood vs. BALF lymphocyte subsets in the non-Löfgren syndrome and control groups compared with non-smokers and smokers

We compared non-smoking and non-Löfgren syndrome patients with controls, and only the percentage of CD4+CD103+ cells was found to be significantly higher in the non-Löfgren group compared with the controls, 3.6%±10.5% vs. 0.3%±0.4%, p=0.03. In the relevant groups of smokers, significantly more CD4+CD38+ marker positive cells were found in sarcoid patients, 23.8%±10.5% vs. 12.6%±3.4% (p=0.025); and CD8+CD103+, 6.1%±7.7% vs. 1.2%±1.6% (p=0.033). In BALF, differences between the non-Löfgren syndrome and control groups were significantly more pronounced. For non-smokers, CD4+CD31+ was 6.8±4.6% in the non-Löfgren group and 3.2±2.3% in the control group (p=0.007), CD4+CD38+ was 17.9±12.6% vs. 7.8%±5.6%, (p=0.001) and CD4+CD44+, 74.2%±13.7% vs. 48.8%±14.8%, respectively (p< 0.0001). The only percentage with no significant difference was CD4+CD103+. In BALF CD8+ T cells, on the contrary, the expression of the CD103+ marker was considerably higher in the non-smoking control group (20.9%±10.4%) compared with the non-Löfgren non-smoking group (13.3%±8.9%, p=0.011).

The non-Löfgren group of smokers had a significantly higher CD4+CD38+ percentage in BALF, reaching 22.5%±11.9% vs. 4.6%±2.99% in the control group of smokers (p=0.004), as well as CD4+CD44+, 68.9%±16.0% vs. 46.4%±18.1%, p=0.011.

3.3 Blood vs. BALF lymphocyte subsets in the Löfgren syndrome and control groups compared with non-smokers and smokers

Independent of smoking status, patients with Löfgren syndrome had a higher percentage of CD4+CD31+ T cells in blood (14.7%±6.0% for non-smokers and 12.0%±6.8% for smokers) versus the controls (10.4%±4.1% (p=0.045) for non-smokers and 8.2%±2.8% (p=0.06) for smokers). The difference was more pronounced for the

peripheral blood percentage of CD4+CD38+: the marker was expressed on 26.8%±6.7% T cells in non-smoker Löfgren syndrome patients versus 19.2%±4.5% (p=0.001) for non-smoking controls, and on 26.0%±8.2% lymphocytes in smoker Löfgren syndrome patients versus 12.6%±3.4% (p=0.001) for smoking controls. Expression of CD44+ (50.8%±8.7% in the Löfgren syndrome group vs. 42.8%±7.4% in the control group, p=0.009) and of CD103+ (1.4%±2.1% in the Löfgren syndrome group vs. 0.3%±0.4% in the control group, p=0.001) on CD4+ blood T lymphocytes differed in non-smokers only.

In BALF, differences were also much more pronounced in the non-smoker Löfgren syndrome group. CD38+ on CD4+ T cells was 30.6%±15.2% in the Löfgren syndrome group and 7.8%±5.6% in the control group (p=0.002), CD44+ was 83.5%±8.3% in the Löfgren syndrome group vs. 48.8%±14.8% in the control group (p=0.011), and CD103+ was 4.8%±5.4% in the Löfgren syndrome patients vs. 12.9%±9.2% in controls (p=0.002). On BALF CD8+ T cells, all the activity markers studied, with the exception of CD8+CD38+, had a significantly higher expression in the control non-smoker group compared with the non-smoker Löfgren syndrome group.

For smoker Löfgren group patients, the number of BALF CD4+CD38+ lymphocytes significantly exceeded the same population in smoking controls (45.0%±11.4% vs. 4.6%±2.9%, p=0.001). Also, the BALF CD4+CD44+ T-cell count was found to be significantly higher in smoker Löfgren group patients compared with smoking controls (87.0%±9.5% vs. 46.4%±18.1%, p=0.001).

3.4 Blood vs. BALF lymphocyte subsets in the non-Löfgren and the Löfgren groups compared with non-smokers and smokers

We compared patients in the non-Löfgren group and the Löfgren group, and the impact of smoking status on the percentage of the markers studied was clearly seen both in blood and BALF. In the blood of the non-smoking groups, most of the activity markers on CD4+ T

cells differed significantly in the Löfgren syndrome group vs. non-Löfgren syndrome patients (p=0.01 for CD4+CD31+, p=0.002 for CD4+CD38+, p=0.002 for CD4+CD44+). In BALF, some indices were higher in non-smoker Löfgren group patients, specifically CD4+CD38+ (30.6%±15.2% vs. 17.9%±12.6%, p=0.002) and CD4+CD44+ (83.5%±8.3% vs. 74.2%±13.7%, p=0.011). Nevertheless, in most cases the percentages in the Löfgren group were significantly lower than in non-Löfgren syndrome patients: CD4+CD103+ (4.8%±5.4% vs. 10.5%±8.8%, p=0.002), CD8+CD31+ (5.7%±3.6% vs. 11.6%±8.4%, p=0.007), CD8+CD44+ (13.8%±7.1% vs. 22.3%±11.9%, p=0.006), and CD8+CD103+ (13.3%±8.9% vs. 3.9%±2.4%, p=0.004).

Although smoking masked the differences mentioned above, but CD4+CD38+ and CD4+CD44+ were also higher for smokers in the Löfgren group compared with non-Löfgren patients, while other markers (CD31+ and CD103+) were lower. A significant difference was found only for CD4+CD103+ T cells (3.0%±4.4% vs. 12.7%±9.1%, p=0.01).

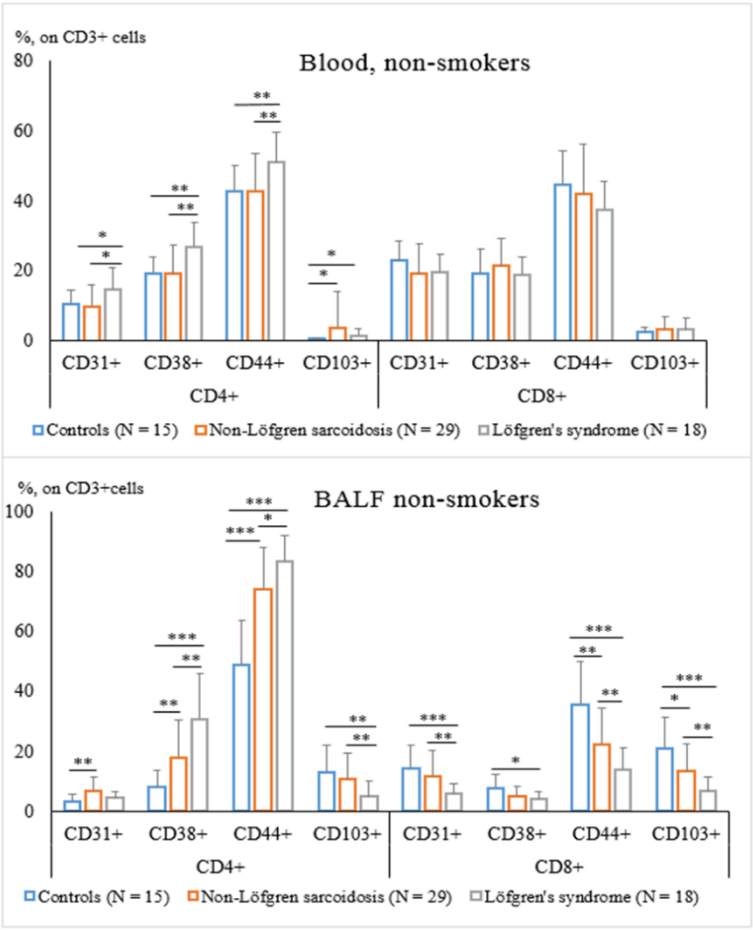


Figure 6. BALF and blood lymphocyte subsets in non-Löfgren sarcoidosis patients and Löfgren syndrome non-smoking patients. Data are shown as the mean ± standard deviation. Statistical significance was denoted by a p-value as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

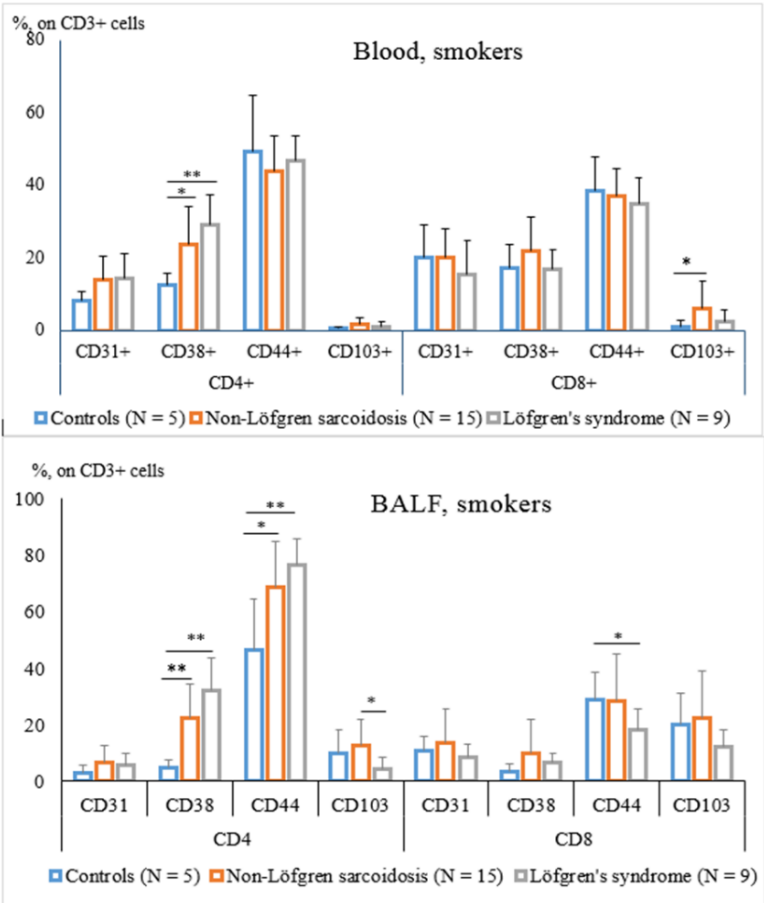


Figure 7. BALF and blood lymphocyte subsets in non-Löfgren sarcoidosis patients and Löfgren syndrome smoking patients. Data are shown as the mean ± standard deviation. Statistical significance was denoted by a p-value as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

3.5 Lung tissue lymphocyte subsets

Surprisingly, few lymphocyte subsets were found to be significantly different in the sarcoidosis group compared with controls. Specifically, in lung tissue, the percentage of CD4+ and CD103+ cells was found to be significantly higher and the percentage of CD44+ cells was lower (patients with Löfgren syndrome were excluded from this analysis).

We have not found a clear relationship between lymphocyte subsets and the sarcoidosis stage. Moreover, analysis of the impact of smoking on lung tissues surprisingly revealed that only the percentage of collagen was decreased in smoking sarcoid patients compared with non-smokers (17.9%±8.5% vs. 22.6%±5.3%, p=0.015).

3.6 Correlations between blood, BALF and lung tissue lymphocyte subsets

Several significant correlations between blood, BALF and lung tissue lymphocyte subsets and other indices were found. Only the most important ones are presented here. Positive correlations between the percentage of blood CD4+CD38+ and BALF CD4+CD38+ (r=0.510, p=0.0001) and between the percentage of blood CD4+CD44+ and BALF CD4+CD44+ (r=0.362, p=0.002) were found. The percentage of blood CD8+CD103+ positively correlated with the tissue total CD44+ cell count (r=0.632, p=0.0001).

Surprisingly, while comparing the corresponding BALF and lung tissue lymphocyte subsets, only BALF CD4+CD103+ correlated with the tissue total CD103+ cell count (r=0.473, p=0.020), CD103+ (%) cells (r=0.514, p=0.010), and CD103+ density (r=0.408, p=0.048).

Furthermore, we found weak but significant negative correlations between the percentage of blood CD4+CD103+ and the DLCO (r=−0.259, p=0.259) value and between BALF CD4+CD31+ and total lung capacity (TLC) (r=−0.261, p=0.029) and vital capacity (VC) (r=−0.242, p=0.043). Moreover, we found significant negative

correlations between tissue CD44+ and CD103+ cells and several pulmonary function test indices: CD44+ total and forced vital capacity (FVC) (r=−0.406, p=0.044), TLC (r=−0.503, p=0.011) and VC (r=−0.406, p=0.044); CD44+ (%) and DLCO (r=−0.414, p=0.040); CD103+ total and FVC (r=−0.459, p=0.021), TLC (r=−0.415, p=0.039) and VC (r=−0.481, p=0.015).

3.7 Relationships between CT patterns, BALF and PFT values

In the first phase of the study, 64 patients (80%) had typical lymphadenopathies and 36 patients (20%) had atypical lymph node localisation. There were 14 patients (17.5%) with calcinosis of lymph nodes. Micronodules (77.5%), macronodules (53.8%) and linear opacities (45%) were the most frequently seen CT patterns in patients with pulmonary sarcoidosis. Notwithstanding this, the number of micronodules was significantly higher (p = 0.004) in stage III as compared with other stages.

Significant correlations between the consolidation scores on CT and FVC (r =−0.227, p=0.043), FEV1 (r=−0.299, p=0.007), FEV1/FVC (r =−0.245, p=0.029), as well as between the ground glass opacity score and DLCO (r=−0.267, p=0.017), were established. Surprisingly, we did not find any significant correlations between the micronodule or macronodule scores and PFT indices.

There were significant differences (p=0.021) between the percentage of BALF neutrophils in radiographic stages I and III. We observed a significant increase in the proportion of CD8+ T lymphocytes (p=0.005), as well as a decrease in the proportion of both CD4+ T lymphocytes (p=0.035) and the CD4+/CD8+ ratio (p=0.011) in smokers (40%, 53% and 2.8, respectively) as compared with non-smokers (22%, 68% and 5.1, respectively) in stage II. As there was only one smoking patient in stage I and stage III, we did not compare smokers versus non-smokers in these groups.

The percentage of BALF lymphocytes and macrophages correlated with TLC values: the percentage of lymphocytes was negatively

correlated (r =−0.27, p=0.02) and the percentage of macrophages was positively correlated (r=0.27, p=0.02). Furthermore, BALF cells correlated with the presence of typical lymphadenopathy: the percentage of neutrophils correlated negatively (r =−0.41, p=0.015) and the percentage of CD4+ cells and the CD4+/CD8+ ratio both correlated positively (r=0.38, p=0.026; r=0.3, p= 0.078, respectively).

In the second phase of the study, the stage of pulmonary sarcoidosis was determined by chest radiography and CT. Computed tomography examination provided a more accurate indication of the stage of pulmonary sarcoidosis, as it was possible to detect some lesions that were invisible (or not clearly visible) on chest radiographs (miliary nodules, micronodules, ground glass opacity, etc.). In 22 cases, the first stage of sarcoidosis according to chest radiographs was evaluated as the second on CT. Chest CT allowed for the identification of nodules. In addition, we compared nodules in the lungs between stages and found that the number of miliary and micronodules increased with increasing stage of the disease.

In our study, we evaluated the relationship between nodules and blood, BALF and PFT values. Patients with pulmonary sarcoidosis with nodules on CT images had decreased levels of CD4+CD44+ T lymphocytes (p=0.005) in blood compared with patients without nodules on CT. These data were consistent with the changes we found in the blood: CD4+CD44+ T lymphocytes decreased with increasing disease stage. We also found decreased levels of blood CD4+CD44+ T lymphocytes (p=0.004), but increased BAL CD4+CD103+ T lymphocytes (p=0.018) in patients with CT lesions characteristic of higher stages of sarcoidosis (septal thickening, typical and atypical fibrous patterns, consolidation) compared with patients without such changes on CT. These data were also consistent with changes in BAL between stages of the disease: CD4+CD44+ T lymphocytes decreased and CD4+CD103+ increased with increasing disease stage. This was confirmed by the correlations found. We found negative correlations between miliary nodules (r =−0.237, p=0.047) and micronodules (r=−0.373, p=0.001) and blood CD4+CD44+ lymphocytes and

positive correlations between miliary nodules and BAL fluid CD4+CD103+ (r=0.410, p=0.001) and CD8+CD103+ (r=0.385, p=0.001) T lymphocytes.

No statistically significant correlations were found between nodules on CT and PFT values.

3.8 Models for predicting the course of sarcoidosis

We evaluated the results of PFT and CT after two years.

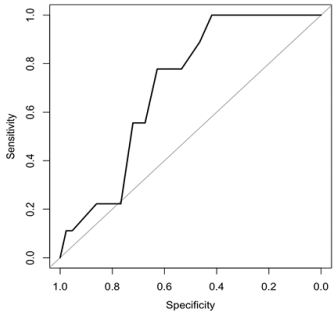
10% or more FVC (%) increased in 13 (25.00%), decreased in 5 (9.62%), and remained unchanged in 34 patients (65.38%); FEV1 (%) increased in 13 (25.00%), decreased in 3 (5.77%), and unchanged in 36 (69.23%) patients; TLC (%) increased in 17 (32.69%), decreased in 8 (15.38%), and unchanged in 27 (41.93%) patients; RV (%) increased in 27 (51.92%) patients, decreased in 12 (23.08%) patients, and remained unchanged in 13 (25.00%) patients; DLCO (%) increased in 21 (40.38%), decreased in 9 (17.31%), and unchanged in 22 (42.31%) patients.

Lymph nodes shown by CT disappeared in 22 (50%) patients, decreased in 11 (25%) and remained unchanged in 11 (25%). Nodules in the lungs disappeared in 17 (41.5%) patients, decreased in 4 (9.8%), increased in 2 (4.9%), and remained unchanged in 18 (43.9%) patients. Ground-glass opacity seen on CT disappeared in 3 (75%) patients and remained present in 1 (25%) patient. Consolidation disappeared in 5 (55.6%) patients, remained unchanged in 2 (22.2%), decreased in 1 (11.1%), and also increased in 1 (11.1%) patient.

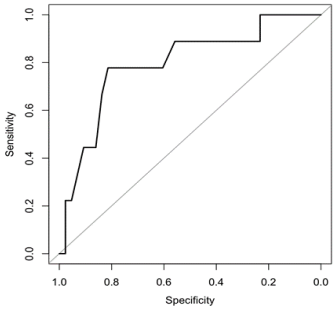
The aim of the study was to determine what factors influence the course of sarcoidosis (progression of the disease). Blood, BAL fluid, lung biopsy tissue indices and CT-detected foci in the lungs were analysed for this purpose. Using a logistic regression model, we found the following: if blood CD4+CD31+ T lymphocytes were ≤14.5, the odds ratio for disease progression was 13.78 (p=0.02); if blood CD4+CD44+ T lymphocytes were ≤37.5, the probability of disease progression was 15.31 (p<0.001); when BAL CD8+CD31+

T lymphocytes were ≥13.5, the odds ratio for disease progression was 10.00 (p=0.01); when BAL CD8+CD103+ T lymphocytes were ≥15.5, the odds ratio for disease progression was 8.75 (p=0.01). We also found that the odds ratio for sarcoidosis progression was 18.46 at ≥15.0 miliary and microfocal lungs (p<0.001). The graphical expression of the results is shown in Figure 8.

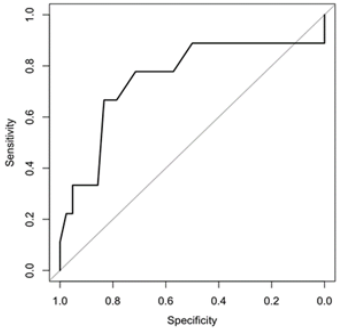
Blood CD4+CD31+



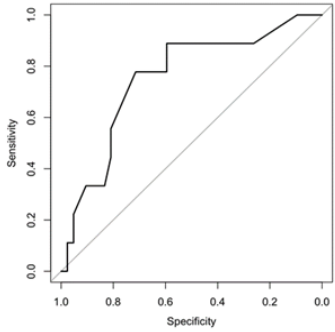
Blood CD4+CD44+



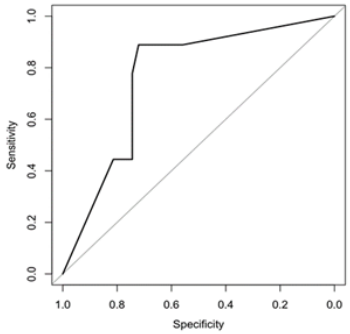
BALF CD8+CD31+



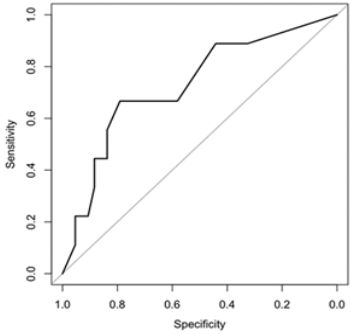
BALF CD8+CD103+



Miliary nodules



Micronodules



Miliary nodules and Micronodules

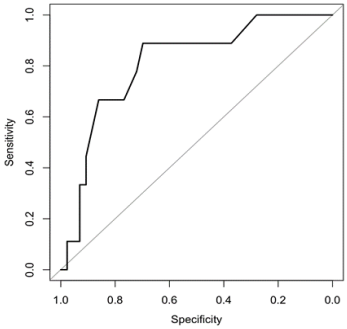


Figure 8. ROC curves

DISCUSSION

The most characteristic signs of sarcoidosis are granulomas, and the essential component of the immune response for granuloma formation is T-cell activation and preferential homing of activated T cells to the tissue. In spite of long-lasting efforts of the scientific community, research in the determination of the causative agents of sarcoidosis is still lacking. Studies of the key cellular players are essential for the understanding the etiopathogenesis of this disease. The mechanisms driving the transition from inflammation to fibrosis are also poorly understood; although, two phases are apparent. The first is chronic inflammation and the second is fibrotic transformation. It is likely that chronic sarcoidosis may not simply be the persistence of acute sarcoidosis, but a fundamentally distinct form of remitting disease from the very beginning.

We investigated 71 patients with newly diagnosed sarcoidosis. For the analysis, study data were grouped by radiologic disease stages, clinical manifestation, and smoking status.

In general, the majority of the investigated T-lymphocyte subsets and activation marker expression profiles differed significantly between sarcoidosis patients and controls, especially in BALF. A marked difference in the percentage of lymphocyte subsets (in blood and in BALF) was found when comparing patients with and without Löfgren syndrome. Additionally, the percentage of lymphocyte subsets differed significantly in patients with different radiological stages of sarcoidosis (both in blood and in BALF). Smoking status also had a considerable impact on the lymphocyte subset profiles of sarcoidosis patients.

The most interesting and promising results were obtained for the cell markers represented by CD31+, CD38+, CD44+, and CD103+ expression on T lymphocytes and especially on CD4+ T cells. These results are: (1) increase in the percentage of CD3+CD4+CD38+ both in BALF and blood, and increase in the percentage of CD3+CD4+CD44+ in BALF in Löfgren syndrome patients compared

with patients without Löfgren syndrome (probable reflection of acute immune response); (2) increase in the percentage of CD3+CD4+103+ T cells in BALF and blood in patients without Löfgren syndrome (compared with Löfgren syndrome patients) and increase in the percentage of CD3+CD4+103+ T cells both in BALF and blood with increasing (i.e. more advanced) sarcoidosis stage (probable reflection of ongoing immune response); (3) percentage of BALF CD3+CD4+CD31+ is increased in sarcoidosis patients when compared with controls independently of the presence of Löfgren syndrome, smoking status or stage of the disease (possible additional diagnostic marker).

CD31 is expressed in naïve recent thymic emigrants, but is downregulated after T-cell activation events and is absent from memory cells. In CD8+ T cells, CD31+ expression is dynamically regulated, e.g., strongly downregulated during acute infection, but re-expressed to intermediate levels in memory cells. CD31+ is implicated in the development of atherosclerosis and its clinical complications. Nevertheless, papers describing CD31+ cells in sarcoidosis or expression on BALF cells in other respiratory diseases are still lacking. To our knowledge, only Ziora et al. have analysed the serum concentrations of the soluble PECAM-1 molecule in the blood of sarcoidosis patients, reporting that PECAM-1 concentrations were similar in sarcoidosis patients and controls. In our study, we observed a very similar phenomenon, e.g., CD31+ expression on the peripheral blood T-cell surface in sarcoid patients and controls was comparable.

While analysing CD31+ expression on the immune cell surface, we discovered that a much higher proportion of blood CD4+ and CD8+ T cells express this receptor compared with BALF. This difference is possible due to a higher number of BALF memory T lymphocytes which lack the CD31 molecule on their surface. Moreover, the expression of CD31+ on CD4+ T cells in BALF was found to increase with sarcoidosis stage ($p=0.002$ for stage II vs. controls), whereas stage I sarcoid patients, on the contrary, were found to have

significantly less CD31+ positive cells on CD8+ T lymphocytes (p=0.035).

Guedes et al. demonstrated the involvement of the CD38 molecule in the early phases of lymphocyte binding to the endothelium through a direct interaction with CD31. CD38–CD31 interactions upregulate integrin expression and promote the ensuing steps in the adhesion cascade. The finding of selectin-like behaviour of the CD38 molecule resulted in the identification of CD31 as an endothelial cell surface ligand. It was shown that CD38 regulates inflammation by modulating leukocyte responses and migration to the sites of inflammation. It was proposed as an early immune marker that reflects T-cell activation in allergies and some infectious diseases. CD38 is defined as both a cell surface enzyme (i.e., ectoenzyme) and as a receptor. The possible effect of the CD38 receptor on fibrosis formation was indicated by El-Chemaly et al., showing that the blood CD38+ memory B cell count was significantly higher in patients with pulmonary fibrosis compared with unaffected controls. Furthermore, Lee et al. revealed a significantly increased proportion of activated CD38+ cells within the naïve B-cell compartment of severe chronic sarcoidosis patients in comparison with healthy controls. Nevertheless, no data on CD38+ T-cell expression in sarcoidosis have been reported to date.

The impact of this molecule on mucosal immunity was studied by Deaglio et al. on intestinal lamina propria colonising T lymphocytes. This research showed that virtually all CD31+ cells co-expressed CD38+, whereas only ~50% of CD38+ cells were CD31+. When comparing the values of CD31+ and CD38+ marker expression observed in our study, it can be assumed that the same is valid for BALF. Unfortunately, as well as we applied three-colour cytometry, e.g. FITC conjugated monoclonal antibodies were used for both CD31 and CD38 molecules, it could not be directly proven. However, we noted many similarities in CD31+ and CD38+ expression both on the peripheral blood and BALF T-cell surface. On peripheral blood CD4+ T cells, CD38+ was also more pronounced in stage II sarcoid patients compared with controls (p=0.004). Unlike CD31+, the effect of

smoking was not seen on blood or BALF cells. For both smoking and non-smoking sarcoidosis patients, and especially the Löfgren syndrome group, patients' CD4+ T cells had significantly elevated CD38+ expressions compared with controls. Therefore, the increase in CD38+ expression on CD4+ T lymphocytes may be associated with an acute inflammatory response in sarcoidosis.

M. Culty et al. have shown that CD44+ expression is higher in the areas of granuloma formation and fibrosis. We did not find significant differences when comparing blood CD44+ expression on CD4+ T cells between the control group and the sarcoidosis group, only a tendency for the expression to increase ($p=0.056$) between stage I sarcoid patients and controls. Meanwhile, CD44+ expression on CD8+ T cells was found to be significantly lower ($p=0.035$) when comparing stage III and control groups. When analysing blood lymphocyte subsets, grouped by sarcoidosis activity, significant activation of CD4+CD44+ expression was revealed in non-smoking Löfgren syndrome patients ($p=0.007$) compared with controls. However, no difference was found in smokers or in the non-Löfgren group. Kasuga et al. evaluated soluble CD44 in the serum (sCD44) of 13 sarcoidosis patients and 56 controls, as well as in the BALF of 11 sarcoidosis patients and 10 controls. In patients with sarcoidosis, the serum CD44 level was significantly higher than that of controls. BALF sCD44 levels tended to be higher in sarcoidosis patients than in controls. Nevertheless, no statistically significant difference was recognised. We investigated the percentage of CD44+ cells and, in our study, the difference in the percentages of BALF lymphocyte subsets was found to be much more pronounced than in blood. We detected significantly more BALF CD4+CD44+ T cells in non-smoker Löfgren syndrome patients, compared with non-Löfgren syndrome ones. Smoking masked this difference, but the trends remained the same. Kaiser et al. reported contrary findings on CD44+ expression. In this BALF study of four Löfgren syndrome patients (all HLA-DRB1*03 allele positive) and four non-Löfgren syndrome patients (all HLA-DRB1*03 allele negative), 33 markers, including CD44, were

analysed by mass cytometry. Expression of the adhesion molecule CD44 was found to be significantly reduced in Löfgren syndrome CD4+ T cells compared with the background. Conversely, non-Löfgren syndrome cells showed a shift in CD44+ expression of similar magnitude but opposite direction. The reasons for the discrepancy between the work by Kaiser et al. and our findings are not clear. The association between HLA and genetic background was not analysed in our study. Besides, our study used a different BALF preparation and cell enumeration method. Lately, knowledge on the genetic architecture of sarcoidosis has been evolving, showing genetic differences between Löfgren syndrome and non-Löfgren syndrome patients, but the epigenetic events that determine the course of the disease are not sufficiently known. Our findings on CD4+CD38+ and CD4+CD44+ T cells suggest that further investigations are required to assess these markers as indicators of inflammatory activity in sarcoidosis patients. Analysis of these markers during active episodes of sarcoidosis seems to be most promising.

Integrin CD103 was the only cell marker we chose that had possible diagnostic value in terms of its expression as an additional biomarker of sarcoidosis. It is known that this molecule can promote T-cell migration into the epithelium and is involved in the retention of lymphocytes in the mucosa. Constant CD103 expression can reflect antigen(-s) persistence in lung tissue.

CD103+ expression on control group peripheral blood T cells was very low compared with other markers studied by us. CD103+ was the only marker which expression in BALF was significantly higher compared with peripheral blood ($p<0.00001$ for both CD4+ and CD8+ T lymphocytes). The percentage of the CD103 molecule on blood cells was found to be sarcoidosis-stage-dependent: the percentage of CD4+CD103 T cells in controls was 12.6%±8.8%, i.e. significantly higher than in stage I sarcoid patients (5.1%±4.8%, $p=0.006$), and increased with stages (8.1%±7.7% for stage II, 19.7%±9.7% for stage III). In BALF, this molecule expression was also dependent on sarcoidosis activity, being significantly lower in Löfgren syndrome

patients compared with non-Löfgren syndrome patients. In line with these findings, the percentage and density of CD103+ positive cells in lung tissue were significantly higher in sarcoidosis patients (notably, non-Löfgren syndrome patients) compared with controls. Additionally, we found a negative correlation between the percentage of blood CD4+CD103+ T cells and the diffusing capacity for carbon monoxide (DLCO) value.

Our results on CD4+CD103+ T cells in BALF are in line with the study by Lohmeyer et al. These authors found that CD103+ expression on BALF CD4+ T cells displayed subgroup dependency: proportions were found to be significantly lower than normal in chest radiographic stage I, but increased in stages II and III. The lowest expression was found in Löfgren syndrome patients. Other authors have also revealed that BALF CD4+ T lymphocytes in sarcoidosis patients have reduced CD103+ expression compared with other interstitial diseases.

Several explanations were proposed (discussed in Kolopp-Sarda et al., 2000). In brief, it is suggested that CD4+ BALF lymphocytes in sarcoidosis originate not from the mucosal inductive sites, but from the peripheral blood. In other lung diseases, a mucosal origin for BALF lymphocytes is suspected, with CD103+ lymphocytes relocating from mucosal areas into the lung and proliferating in response to local stimuli.

Braun et al. suggested that in patients with inflammatory lung diseases CD103-expressing CD4+ T cells in the lung are continuously activated, long-living cells. However, Braun et al. found no differences between the CD103+ and CD103– populations with respect to pro-inflammatory parameters. Based on their data, Heron et al. proposed that a higher proportion of CD4+CD103+ T cells might be associated with fibrosis formation in pulmonary sarcoidosis. Bretagne et al. found a significant negative correlation between the BALF CD103+CD4+/CD4+ ratio and FVC, TLC, and DLCO at last study visit. They suggested that the BALF CD103+CD4+/CD4+ ratio may be of interest as a prognostic marker in sarcoidosis.

Taking the data from this study and the reports of other authors together, it is reasonable to consider that CD4+CD103+ T cells in BALF and blood might be potential prognostic markers in sarcoidosis. At present, we speculate two possible scenarios: a relative deficit of CD4+CD103+ T cells in BALF reflects acute inflammation (immune response), or relative augmentation of CD4+CD103+ T cells in BALF reflects persistent inflammation (immune response) in the lungs. It seems reasonable to address this issue in future studies.

Like our previous preliminary findings and the results of other authors, clinical manifestation, radiological sarcoidosis stage, and smoking status should always be taken into account when analysing lymphocyte subsets and activation marker expression. It is still for future studies to resolve which lymphocyte subpopulations are most important as prognostic factors and, maybe, as additional diagnostic markers for sarcoidosis.

Our study has strengths and limitations. The strength of our study is that it was carried out prospectively, with the primary aim of investigating several biomarkers in newly diagnosed sarcoidosis patients. Secondly, we investigated lymphocyte subsets simultaneously in blood, BALF, and lung tissue. Thirdly, we applied DIA for the enumeration of lung tissue cells. Moreover, the study population was fairly large, and the study was performed in a specialised tertiary healthcare facility.

The limitations of the study are related to the unavailability of lung biopsy data in patients with Löfgren syndrome (due to the study design). It was not possible to analyse lymphocyte subsets in lung tissue for this group of patients. Also, lung tissue cells were analysed by single immunohistochemistry, without the possibility to obtain multiplex data on the lymphocyte subpopulations and other lung tissue cells which should be removed from final analysis. In contrast, in blood and BALF, we used triple staining with monoclonal antibodies. Another limitation is that this was a single-centre study (reflecting the patient population of our country, since our centre is the primary

centre for interstitial lung disease). Therefore, these results may reflect the manifestation of sarcoidosis in our patient population.

CONCLUSIONS

1. Chest computed tomography is significantly superior to X-ray in detecting micronodules in the lungs that are important in predicting the course of sarcoidosis. Chest CT analysis is recommended in all cases of sarcoidosis. About 30% of cases of sarcoidosis categorised as stage I on chest radiographs actually turned to be stage II sarcoidosis on CT, i.e. the disease affected not only the lymph nodes, but also the lung parenchyma. This is predictably important.

2. Advanced lung function testing is more accurate than spirometry for assessing lung function impairment in lung sarcoidosis, because it allows the assessment of lung diffusion capacity which is usually reduced in lung sarcoidosis. DLCO is decreased in more than half (55%) of patients with sarcoidosis, although spirometry shows normal data.

3. Examination of BAL fluid and bronchoscopic lung biopsy are significantly complementary, although both reflect changes in lung tissue. Pulmonary sarcoidosis can be confirmed by both BAL fluid testing and bronchoscopic lung biopsy. However, the results obtained with each of these two test methods may not be sufficient to confirm sarcoidosis. The results of BAL fluid immunostaining may be valuable not only in diagnosing sarcoidosis, but also in predicting its course. The study revealed a potential additional marker for the diagnosis of sarcoidosis, BAL fluid CD4+CD31+. It was detected in BAL fluid of significantly more patients with sarcoidosis compared with healthy subjects, and its diagnostic significance increases with increasing stage of the disease.

4. The results of blood immunostaining may be valuable in assessing acute and persistent immune responses in sarcoidosis and in predicting the course of the disease. Increased levels of CD4+CD38+ T lymphocyte in BAL fluid and blood and of CD4+CD44+ in BAL fluid may be markers of an acute immune response in sarcoidosis. Elevated levels of CD4+CD103+ T cells in BAL fluid and blood are

markers of a persistent immune response in sarcoidosis patients and are a probable prognostic factor of the chronic course of the disease.

5. Lymphocyte subtype profiles in blood and BAL fluid were different in patients without Löfgren syndrome comparing with Löfgren syndrome patients. The majority of markers of blood and BAL fluid CD4 + T cells in sarcoid Löfgren syndrome patients were significantly increased compared with patients without Löfgren syndrome.

6. The immune response in BAL fluid was different in sarcoidosis patients and depended on smoking status. The smoker group had a significantly lower percentage of BAL CD4+CD44+ and a higher percentage of CD8+CD38+, CD8+CD44+, CD8+CD103+ T lymphocytes compared with the non-smoker group.

7. Significant correlations have been found between CT patterns and PFT and BAL fluid immune markers in newly diagnosed pulmonary sarcoidosis. PFT are significantly decreased when consolidation or ground glass is detected on CT. Thickening, fibrotic lesions and consolidation on CT are accompanied by increased CD4+CD103+ T lymphocytes and decreased CD4+CD44+ T lymphocytes in BAL fluid. CD4 + CD103 + and CD8 + CD103 + T lymphocytes in BAL fluid have been found to increase with the increasing number of miliary nodules.

8. In sarcoidosis, PFT values and CT patterns may regress, remain unchanged or progress. DLCO (%) increased in 21 (40.38%), decreased in 9 (17.31%) and remained unchanged in 22 (42.31%) patients. Lymph nodes detected on CT disappeared in 22 (50%) patients, decreased in 11 (25%) and remained unchanged in 11 (25%). Nodules in the lungs disappeared in 17 (41.5%) patients, decreased in 4 (9.8%), increased in 2 (4.9%), and remained unchanged in 18 (43.9%).

9. Some markers of blood and BAL fluid immune response and CT patterns have been associated with a different course of sarcoidosis. When the blood CD4+CD31+ T lymphocyte count is less than 14.5, the CD4+CD44+ T lymphocyte count is less than 37.5, the BAL fluid

CD8+CD31+ T lymphocyte count is above 13.5 and/or CD8+ CD103+ exceeds 15.5, there is an increased likelihood that sarcoidosis will progress. The likelihood of sarcoidosis progression is also increased if more than 15.0 miliary and micronodules are detected on CT.

THE LIST OF PUBLICATIONS

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SHORT COMMUNICATION ABOUT THE AUTHOR

Regina Aleksonienė was born in Druskininkai and studied at Druskininkai Secondary School No. 1. In 1990, she graduated from the Faculty of Medicine of Vilnius University. She continued her residency studies and was recognised as a pulmonology specialist. Since 1993, she has been working as a pulmonologist at the Pulmonology and Allergology Centre of Vilnius University Hospital Santaros Clinics. Regina Aleksonienė also worked as an assistant at the Faculty of Medicine of Vilnius University for many years. Together with co-authors, she has published scientific articles in Lithuanian journals and publications cited in the international database *Analytics Web of Science (CA WoS)* with a citation index as well as presented reports at conferences in Lithuania and abroad (congresses of the European Respiratory Society).

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