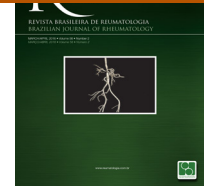




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Original article

Serum Clara cell 16-kDa protein levels and lung impairment in systemic sclerosis patients



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ABSTRACT

Objective: To assess clinical utility of serum Clara cell 16-kDa protein measurements in relation with staging system for systemic sclerosis associated interstitial lung disease.

Materials and methods: Serum levels of Clara cell 16-kDa protein were determined by ELISA in 28 systemic sclerosis patients and 30 healthy controls, and correlated with staging system for systemic sclerosis associated interstitial lung disease in systemic sclerosis patients. Lung involvement was assessed functionally (body plethysmography, diffusing capacity of the lung for carbon monoxide) and radiologically (an average disease extent on high resolution computed tomography of the lungs) in SSc patients.

Results: We observed statistically significant differences in serum Clara cell 16-kDa protein levels between systemic sclerosis patients and healthy controls only in non-smokers. However, serum Clara cell 16-kDa protein concentrations were significantly elevated in patients with high resolution computed tomography extent >20% in comparison to patients with high resolution computed tomography extent <20% ($p=0.01$). They correlated positively with average disease extent on high resolution computed tomography ($p=0.04$), an extent of a reticular pattern on high resolution computed tomography ($p<0.01$), and negatively with a total lung capacity ($p=0.03$) and the results of the 6-min walk test ($p<0.01$).

Conclusions: Clara cell 16-kDa protein levels can be considered as a supplemental serum biomarker for systemic sclerosis associated interstitial lung disease.

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Níveis séricos de proteína de células de Clara de 16 kDa e comprometimento pulmonar em pacientes com esclerose sistêmica

R E S U M O

Palavras-chave:

CC16

Doença intersticial pulmonar

Esclerose sistêmica

Objetivo: Avaliar a utilidade clínica das medições séricas da proteína de células de Clara de 16-kDa em relação ao sistema de estadiamento para doença pulmonar intersticial associada a esclerose sistêmica.

Materiais e métodos: Foram determinados os níveis séricos de proteína de células de Clara de 16-kDa por ELISA em 28 pacientes com esclerose sistêmica e 30 controles saudáveis, e correlacionados com o sistema de estadiamento para doença pulmonar intersticial associada a esclerose sistêmica em pacientes com esclerose sistêmica. O envolvimento pulmonar foi avaliado funcionalmente (pletismografia corporal, capacidade de difusão de monóxido de carbono) e radiologicamente (extensão média da doença na tomografia computadorizada de alta resolução dos pulmões) em pacientes com esclerose sistêmica.

Resultados: Foram encontradas diferenças estatisticamente significativas nos níveis séricos de proteína de células de Clara de 16-kDa entre pacientes com esclerose sistêmica e controles saudáveis apenas em não tabagistas. No entanto, as concentrações séricas de proteína de células de Clara de 16-kDa eram significativamente elevadas em pacientes com extensão >20% na tomografia computadorizada de alta resolução em comparação a pacientes com extensão <20% na tomografia computadorizada de alta resolução ($p=0,01$). Os níveis séricos de proteína de células de Clara de 16-kDa se correlacionaram positivamente com a extensão média da doença na tomografia computadorizada de alta resolução ($p=0,04$) e com a extensão de padrão reticular na tomografia computadorizada de alta resolução ($p<0,01$), e negativamente com a capacidade pulmonar total ($p=0,03$) e com os resultados do teste de caminhada de 6 min ($p<0,01$).

Conclusões: Os níveis de proteína de células de Clara de 16-kDa podem ser considerados como biomarcadores séricos suplementares para a doença pulmonar intersticial associada a esclerose sistêmica.

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Introduction

Systemic sclerosis (SSc) is a chronic, autoimmune connective tissue disease characterised by vasculopathy, inflammation and progressive fibrosis of the skin and internal organs. The leading causes of morbidity and mortality in patients with SSc are two SSc-related pulmonary syndromes: pulmonary arterial hypertension (SSc-PAH) and interstitial lung disease (SSc-ILD).¹ To assess SSc-ILD, high-resolution computed tomography (HRCT) and pulmonary function tests are performed. In addition, a few lung-specific serological markers of SSc-ILD have been described.²⁻⁴ This group includes Clara cell 16-kDa protein (CC16). It is one of the major proteins secreted by Clara cells and there is growing evidence on its protective role against pulmonary inflammatory response.⁵

CC16 is a 15.8-kDa homodimeric protein encoded by a gene localised to chromosome 11.⁶ It has potent natural immunosuppressive and anti-inflammatory properties. CC16 has been shown to modulate inflammatory response, including inhibition of cytosolic phospholipase A2 activity and interferon- γ in vitro.⁷ Changes in serum CC16 concentrations were observed in patients with different disorders affecting the lungs, including decreased CC16 levels in bronchial asthma and increased in sarcoidosis and idiopathic pulmonary fibrosis.^{8,9} They were also noted after exposure to lung irritants.¹⁰ Moreover, the

serum level of CC16 has been found to be an indicator of active pulmonary fibrosis in SSc patients.¹¹ In regard to this finding, in the present study we evaluated serum CC16 levels and examined their association with SSc-ILD assessment in patients with SSc.

Materials and methods

We recruited for the study 28 consecutive patients with SSc (25 females and 3 males, aged between 24 and 70 years) according to the American College of Rheumatology classification criteria.¹² All patients were also analysed with the use of 2013 classification criteria for SSc.¹³ Patients were grouped according to the 2-cutaneous subset classification as having diffuse cutaneous (dcSSc) or limited cutaneous (lcSSc) form of the disease on the basis of the extent of their skin involvement.¹⁴ Healthy controls ($n=30$) were non-smoking volunteers and were statistically matched by gender and age (18-29, 30-44, 45-54 and 55-70 years of age). The study was approved by the Institutional Review Board at Poznan University of Medical Sciences and written informed consent was obtained from every participant. A protocol of the conducted research conforms to the principles of the World Medical Association's Declaration of Helsinki.

Clinical assessment comprised the complete medical histories and physical examinations, which included ulcer

assessment and evaluation of skin involvement using modified Rodnan skin thickness score.¹⁵ European League Against Rheumatism (EULAR) Scleroderma Trials and Research (EUSTAR) Systemic Sclerosis Activity Score was calculated for all SSc patients. Disease duration was measured from the onset of the first symptom, other than Raynaud's phenomenon, consistent with SSc. Pulmonary involvement was assessed functionally (body plethysmography, diffusing capacity of the lung for carbon monoxide [DLCO] and the 6-min walk test) and radiologically (high resolution computed tomography of the lungs, HRCT) as proposed by Goh et al.¹⁶ Moreover, HRCT images were also assessed for the following findings: the average extent of the disease, the extent of a reticular pattern, the extent of ground glass appearance and the presence of honeycombing. All HRCT examinations were evaluated by a radiology expert on HRCT ILD. To describe SSc patients as 'without pulmonary function impairment', three functional parameters: total lung capacity (TLC), vital capacity (VC) and DLCO were defined as >80% predicted. Blood samples from patients were collected at the time of the clinical examination on fasting conditions and obtained sera were stored at -70 °C before the assays were performed. The inflammatory activity was determined by the erythrocyte sedimentation rate (ESR, Westergren), high-sensitivity C-reactive protein concentration (CRP, enzyme-linked immunosorbent assay (ELISA), BioCheck, USA) and complement components C3 and C4 levels (radial immunoelectrophoresis). The titres of antinuclear antibodies (ANAs) were assessed by an indirect immunofluorescence assay with two BIOCHIPS per field containing HEp-20-10 cells and monkey liver tissue (Euroimmun, Germany). Antibodies to extractable nuclear antigens (ENA) were determined using blot type test ANA Profile 3 (Euroimmun, Germany). Serum concentrations of CC16 were measured using commercially available ELISA kits (BioVendor, the Czech Republic) according to the manufacturer's protocol.

Patients' demographic data were analysed using descriptive statistics and the data were tested for normal distribution using the Kolmogorov-Smirnov test. The results are presented throughout the article as the mean \pm standard deviation (SD) for normally distributed data or as the median (interquartile range, IQR) for non-normally distributed data. Associations between different values were examined using Spearman's rank-order correlation analysis. Differences between the groups were calculated using the Mann-Whitney U test. A *p*-value less than 0.05 was considered statistically significant. All statistical analyses were performed with STATISTICA data analysis software system (StatSoft, Inc., 2011, version 10, www.statsoft.com).

Results

Out of 28 SSc patients, 11 had dcSSc (9 females and 2 males) and 17 had lcSSc (16 females and 1 male). Ten patients (35.7%) had a modified Rodnan skin thickness score of more than 14 and 7 (25%) had active disease according to EUSTAR activity score. One patient (3.6%) had SSc-PAH and none of the patients had impaired renal function. In 27 patients (96.4%) lung-function tests showed decreased DLCO, VC was diminished in 4 patients (14.3%) and TLC in 1 patient (3.6%). There

Table 1 – Clinical, laboratory and radiological characteristics of systemic sclerosis patients.

Disease duration (months)	51.5 \pm 12.1
Modified Rodnan skin score	13 (17)
Antinuclear antibodies	25 (89.3)
Anti-topoisomerase antibodies	10 (35.7)
Anti-centromere antibodies	15 (53.6)
Erythrocyte sedimentation rate (mm/h)	25 (22)
C-reactive protein (mg/L)	0 (5.4)
C3 complement component (g/L)	1162 (236)
C4 complement component (g/L)	191 (66)
EUSTAR systemic sclerosis activity score	2 (3)
HRCT	15 (25)
Extent of disease, %	10 (15)
Extent of a reticular pattern, %	5 (10)
Extent of ground glass, %	9 (32.1)
Presence of honeycombing	58.4 (16.5)
Total lung capacity (p%)	108.2 (21)
Vital capacity (p%)	98.3 (19.7)
Six-minute walk test, distance (m)	375 (90)
Therapy with cyclophosphamide	9 (32.1)
Therapy with methotrexate	5 (17.9)
Therapy with azathioprine	2 (7.1)
Current smokers	6 (21.4)

HRCT, high resolution computed tomography.

Data presented as the median (interquartile range), mean \pm standard deviation or as *n* (%).

was no case of concomitant bronchial asthma or sarcoidosis in the investigated group. Comparison between dcSSc and lcSSc revealed no statistically significant differences in pulmonary function tests with regard to percentage of predicted TLC, VC and DLCO. Significant abnormalities on HRCT (ILD extent of >5%) were observed in 53.6% of the patients and ILD extent >20% was present in 32.1% of the patients with SSc. There were no patients with the indeterminate extent of the disease on HRCT. Further characteristics of the patient group at the time of examination is shown in Table 1.

We did not observe statistically significant differences in serum CC16 levels between SSc patients (median 9.9 (7.3)ng/mL) and healthy controls (median 8.4 (3.7)ng/mL) (Fig. 1), or between dcSSc and lcSSc subgroups. However,

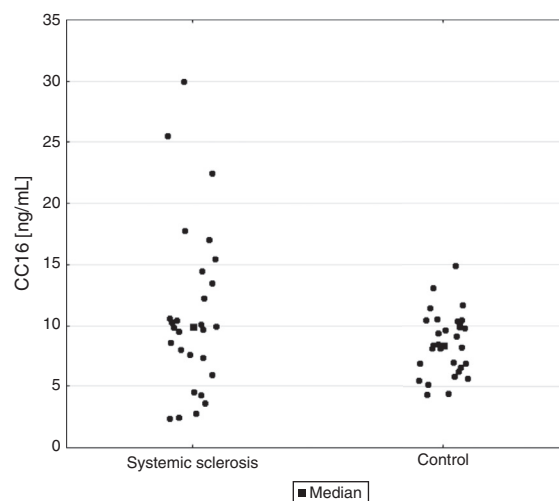


Fig. 1 – Serum CC16 (ng/mL) concentrations in healthy controls (*n* = 30) and patients with systemic sclerosis (*n* = 28).

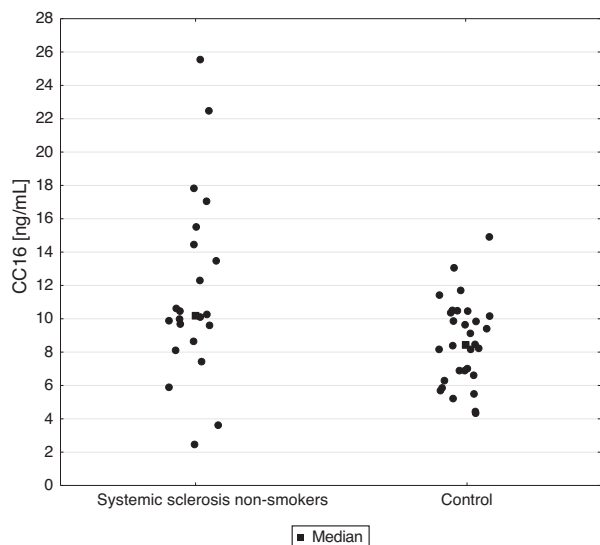


Fig. 2 – Serum CC16 (ng/mL) concentrations in non-smoker patients with systemic sclerosis (n = 22) and in healthy controls (n = 30).

exclusion of smokers from this analysis revealed significantly increased serum CC16 concentrations in non-smoker SSc patients in comparison to the controls (median 10.2 (5.8) vs. 8.4 (3.7) ng/mL, $p=0.03$) (Fig. 2). In the whole investigated SSc group CC16 concentrations were also associated with patients' age ($r=0.41$, $p=0.03$) and were significantly decreased in smokers ($p=0.03$). Serum levels of CC16 were significantly elevated in patients with HRCT extent $>20\%$ in comparison to patients with HRCT extent $<20\%$ ($p=0.01$) (Fig. 3). Moreover, CC16 levels significantly correlated with HRCT extent when presented as a continuous variable ($r=0.44$, $p=0.04$) and with the extent of reticular pattern ($r=0.61$, $p<0.01$). With regard to pulmonary function tests CC16 concentrations were

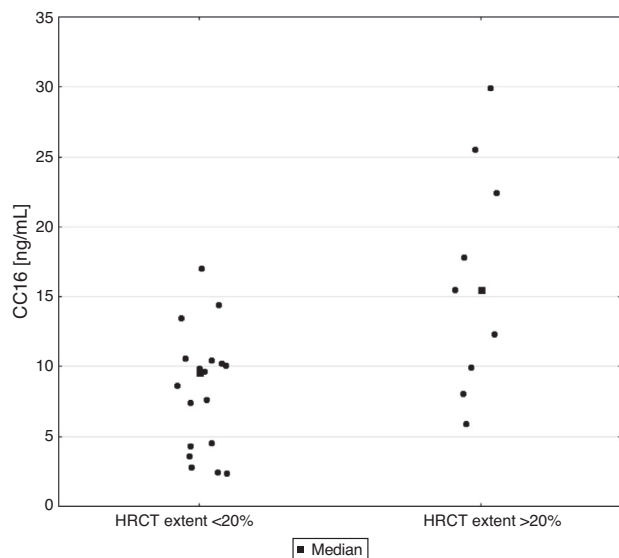


Fig. 3 – Serum CC16 (ng/mL) concentrations in systemic sclerosis patients with HRCT extent $<20\%$ and HRCT extent $>20\%$.

associated negatively with TLC ($r = -0.41$, $p = 0.03$) and with the result of the 6-min walk test ($r = -0.55$, $p < 0.01$). No other statistically significant associations were detected between serum CC16 levels and clinical or laboratory findings, as shown in Table 1.

Discussion

The current report focuses on serum levels of CC16 in SSc patients in relation to staging system of SSc-ILD.

We found statistically significant difference in serum CC16 levels only between non-smoker SSc patients and healthy controls, but not when smoker and non-smoker SSc patients were analysed together as a whole group. It is consistent with the results from the previous report, where SSc patients had higher, but not statistically significant, serum levels of CC16 compared with the levels of healthy controls.¹¹ However, we cannot exclude a bias caused by a small sample number of smokers and a relatively low number of all individuals included to the study.

Further, we did not observe a relationship between CC16 levels and VC, as it was reported by the others.¹¹ The most probable explanation for these discrepancies is the difference in treatment (no treatment vs. 57.1% of patients treated with immunosuppressive drugs in our study). These divergent results can also be influenced by a small sample size in the present study. However, we found that CC16 levels were significantly elevated in SSc patients with average disease extent on HRCT $>20\%$ compared to patients with average disease extent $<20\%$, and that serum CC16 significantly correlated with the average extent of the disease on HRCT when analysed as continuous variable. These observations are in agreement with the finding that serum CC16 levels in SSc patients with pulmonary fibrosis were remarkably higher in active vs. inactive lung disease. In the study by Hasegawa et al., diagnosis of active pulmonary fibrosis was based on the presence of ground-glass appearance or reticular pattern on HRCT of the chest and $>10\%$ change in VC or $>15\%$ change in DLCO within 1 year. As our study was based on the case-control approach, we could not judge the SSc-ILD activity basing on the same criteria, but our results also indicate CC16 as a marker of SSc-ILD, as we showed an association of CC16 serum levels with the extent of reticular pattern on HRCT. This finding is of particular clinical importance as in the study by Goh et al.¹⁶ mortality in SSc patients was strongly linked not only to the extent of disease on HRCT, but also to the extent of the reticular pattern. Therefore, elevated serum CC16 level can be a candidate for a prognostic marker in SSc patients, especially in non-smokers.

Additionally, in this study we observed decreased CC16 serum concentrations in smoking SSc patients. It is concordant with the previous results, which showed approximately 30% reduction of CC16 levels in smokers.¹⁷

To sum up, our data indicate that elevated CC16 concentrations reflect the degree of lung damage in the course of SSc as scored with staging system by Goh et al.¹⁶ Further, CC16 level could be considered as a potential serum prognostic marker in this group of patients, but to confirm this issue prospective and longitudinal studies are needed.

Conflicts of interest

The authors declare no conflicts of interest.

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