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ANTIBODIES TO CARBONIC ANHYDRASE IN PATIENTS WITH CONNECTIVE TISSUE DISEASES: RELATIONSHIP WITH LUNG INVOLVEMENT

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The aim of this study is to evaluate the presence of antibodies to carbonic anhydrase I and/or II (ACAI and ACAII) in patients affected by connective tissue diseases (CTD) and to investigate their association with lung involvement evaluated by High resolution CT scan (HRCT). Ninety-six patients affected by CTD were studied, i.e. 33 rheumatoid arthritis (RA), 8 psoriatic arthritis (PA), 8 ankylosing spondilitis (AS), 23 Systemic Lupus Erythematosus (SLE), 10 Sjogren Syndrome (SS), and 14 Systemic Sclerosis (SSc). ACA were detected by ELISA. The lung involvement was evaluated by means of a previously described HRCT score. According to a receiver operator characteristic curve, patients were divided into those with HRCT score ≥ 10 and those with HRCT score < 10, where HRCT score ≥ 10 was predictive of interstitial lung disease. ACAI and/or ACAII were detected in 30/96 patients (31.2%) (P<0.0001 in comparison with controls). In particular, the prevalence of ACAI and/or ACAII was significantly higher in patients with RA (P = 0.002), PA (P < 0.0001), SLE (P = 0.0003) and SSc (P< 0.0001). A positive correlation was found between HRCT scores and CRP or ACAI levels (P=<0.0001 and P=0.004, respectively). Thirty-nine of 96 patients (40.6%) showed a HRCT score ≥ 10 and both their CRP and ACAI levels were significantly higher when compared with patients showing a HRCT score < 10 (P<0.0006 and P = 0.0009, respectively). Moreover, C3 and C4 complement fractions inversely correlated with HRCT scores (P = 0.0004 and P<0.0001, respectively) and lower values of C3 and C4 complement fractions were found in patients with HRCT score ≥ 10 than in those with HRCT score < 10 (P = 0.014 and P = 0.007, respectively). Due to the lower levels of complement fractions detected in patients with HRCT score ≥ 10, a possible immune-complex-mediated pathogenic mechanism of lung involvement could be suggested.

Carbonic anhydrase (CA) is a metalloenzyme which catalyzes the reversible hydration of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O = HCO_3^- + H^+$). In humans several CA isoforms (i.e. cytoplasmic, membrane-bound, mitochondrial, and secretory) with different immune specificity are present (1-3).

Autoantibodies against CAI and/or CAII (cytoplasmic isoenzymes) have been described in different immune-mediated diseases (4-11),

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however, there is no conclusive evidence indicating their pathogenic role and it is not clear whether anti-CA (ACA) may represent a disease-specific marker.

Hosoda et al. have reported that purchased CAII antigen can be contaminated by IgG-like material responsible for pseudopositive reactions or high background (12) and they suggested appropriate assay conditions to be used in ACAII determination. Accordingly, caution should be used in interpreting previous data on the prevalence and clinical relevance of these autoantibodies. As to the putative pathogenic role of ACA, significantly higher levels of ACAII in patients affected with Systemic Sclerosis (SSc) with restrictive lung disease (RLD) were found in comparison to SSc patients without lung involvement and healthy controls (6). Since CAII isoform is abundantly present in alveolar epithelial cells and bronchial glands (13-16), a possible pathogenic role of ACAII in the development of lung damage in SSc was suggested. Besides SSc, all connective tissue diseases (CTD) may be complicated, to a varying extent, by pulmonary abnormalities, including lymphocytic interstitial pneumonitis, pleuritis, and interstitial lung disease (ILD).

High resolution CT scan (HRCT) represents a non-invasive technique showing high sensitivity and specificity in ILD diagnosis as well as providing prognostic indications. In this setting, HRCT results should be taken as predictive of ILD (17-18).

Therefore, the aim of our study is to investigate in patients affected by different rheumatic diseases: (i) the levels and prevalence of ACAI and ACAII in appropriate assay conditions; (ii) the relationship between ACA and lung involvement, evaluated by HRCT.

MATERIALS AND METHODS

Ninety-six patients were studied: 33 rheumatoid arthritis (RA), 8 psoriatic arthritis (PA), 8 ankylosing spondilitis (AS), 23 Systemic Lupus Erythematosus (SLE), 10 Sjogren Syndrome (SS), and 14 SSc. Salient demographic and clinical characteristics of the study population are reported in Table I.

Criteria of exclusion were: i) secondary forms of ILD; ii) history or clinical findings of respiratory infection at the time of study entry.

Laboratory evaluations included Westergreen erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), haemoglobin, white blood cell (WBC) and platelet count, rheumatoid factor, and antinuclear antibodies assessed with indirect immunofluorescence on Hep-2 cell line. Samples were stored at –80C° until assayed. Patient samples were coded and investigators performing ACA quantification were unaware of the diagnosis, and clinical and laboratory findings. As control group (CTR), sera were obtained from 35 apparently healthy gender-matched subjects. Since significant difference between SLE and RA groups regarding age was found, CTR were matched with the mean age of the total cohort of patients.

Reagents

Human CAI and CAII (purified from human erythrocytes, lot 073K9304 and 043K9294, respectively), bovine serum albumin (BSA), *o*-phenylenediamine (OPD), and Tween-20 were purchased from Sigma Chemical Co, St Louis, MO, USA; Protein-G Sepharose was obtained from Pharmacia Fine Chemicals, Hounslow, UK, and peroxidase (HRP)-conjugated affinity purified goat antibodies to the Fc portion of human IgG were obtained from Jackson Immunoresearch, Avondale, PA, USA.

Enzyme linked immunosorbent assay (ELISA)

Phosphate–buffered saline (PBS, pH 7.4) containing 1% (w/v) of BSA, PBS/0.1% BSA/0.05% (v/v) Tween-20, and PBS/0.05% Tween-20 were used as blocking, diluent and washing buffer, respectively.

Evaluation of CAI and CAII reactivity with anti-human IgG

One hundred µl/well of CAI and CAII (5 µg/ml) in 0.05 M carbonate/bicarbonate buffer (pH 9.6, coating buffer) were dispensed in triplicate in 96-well ELISA plates (Falcon, ProBind, Becton Dickinson, Oxnard, CA, USA) and left overnight at 4°C. Plates were washed three times and blocked with 100 µL/well of PBS/1% BSA for 2 hours. After three washes, 100 µL/well of HRP-conjugated anti-human IgG diluted 1:10,000 were added for 30 min. After washings, 100 µL of freshly prepared solution of OPD (0.4 mg/ml) and hydrogen peroxide 30% (0.4 µL/ml) in citrate buffer (pH 5) were added. The reaction was stopped with 50 µL/well of 2M H_2SO_4 and the optical density (OD) was read at 492 nm with a plate reader (DAS srl, Rome, Italy).

Purification of CAI and CAII antigens

IgG-like material was removed from CAI and CAII antigens by Protein-G Sepharose chromatography. One ml of CAI or CAII at the concentration of 2.5 mg/mL, in PBS, was stirred with 1 mL Protein-G Sepharose and incubated overnight at 4°C. The purified reagents

were stored at -20°C until used. Antigen purification was checked by ELISA coating the plates with CAI and CAII antigens before and after Protein-G Sepharose chromatography.

Detection of ACAI and ACAII

ACAI and ACAII were detected by ELISA. Briefly, after antigen purification with Protein-G Sepharose, half of the wells of each plate (alternate rows) were coated with 500ng/100 μL/well of CAI or CAII in coating buffer, whilst the other half with 100 μL of coating buffer alone. Coating and blocking steps were carried out as described above. Serum specimens, diluted 1:100 were added (100 μL/well) in duplicate in antigen-coated wells as well as in non-coated wells, the latter to assess the non-specific binding. Serum from a patient with endometriosis with high levels of both ACAI and ACAII was included in each plate as positive control. The assay blank value was obtained by using 100 μL of diluent buffer in place of serum. After 30 min incubation, the plates were washed, HRP-conjugated anti-human IgG was added and the ELISA was continued as described. Sample non-specific binding was subtracted from the mean OD and net-OD was computed for each sample. Results were expressed as percent of the positive control.

Sera for ACAI and ACAII were assessed on the same day and the same batch of reagent was used. The intra-assay coefficient of variation was always < 10%.

HRCT

All patients underwent standard chest X-ray with latest generation digital radiography (Digital Phosphor Storage System - Konica-Minolta) and HRCT scan of the chest (CT Scan Somatom 16; Siemens). Scans were performed in the prone position when supine views were suspected of having artefacts due to gravity-dependent perfusion. A high spatial frequency reconstruction algorithm (sharp or bone) was used. HRCT scans were reviewed separately by two radiologists who were blinded to clinical data.

The parenchymal abnormalities identified on HRCT were coded and a score was defined according to a method previously used in SSc patients (19). Every single segment was evaluated and a point value was assigned to each abnormality (ground glass appearance, 1; irregular pleural margins, 2; septal/subpleural lines, 3; honeycombing, 4; subpleural cysts 5). For each patient a "severity of disease" score was determined by adding point values. An "extent of disease" score was obtained by counting the number of bronchopulmonary segments involved for each abnormality: 1 to 3 segments scored 1; 4 to 9 segments scored 2; more than 9 segments scored 3. Finally, severity of disease and extent of disease scores were added to form a total HRCT score, with a possible range from 0 to 30. For example, a patient with a ground glass appearance (severity of disease score: 1) in three segments (extent of disease score: 1) and septal/subpleural lines (severity of disease score 3) in five segments (extent of disease score: 2) would have a total HRCT score of 1+1+3+2=8.

As previously reported (20), the score of 10 points, as indicated by receiver operator characteristic (ROC) curve, represented the best compromise between sensitivity and specificity in predicting functional impairment. Accordingly, patients were divided into those with HRCT ≥ 10 and those with HRCT < 10.

Statistical analysis

Statistical analysis was performed with version 3.0 of GraphPad prism software (GraphPad, San Diego, CA, USA). Continuous variables are reported either as mean ± SD or median and 25th-75th percentiles. Numbers are presented as counts (percentage) for dichotomous data. According to the Gaussian or non-Gaussian distribution continuous data were compared using Student's t-test or Mann Whitney U test, respectively, while correlations were analyzed by Pearson correlation and Spearman rank correlation coefficient, when appropriate. Categorical variables were compared with Fisher's exact test. P values < 0.05 were taken as statistically significant. Sensitivity and specificity of the HRCT score was evaluated with ROC curve.

RESULTS

Anti-human IgG reacted with CAI and CAII in the absence of human serum showing an O.D. of 0.222±0.013 and 0.810±0.035, respectively. After removing contaminating material with Protein-G Sepharose, OD fell to 0.018±0.003 and 0.023±0.004, respectively.

Scattergrams of ACAI and ACAII values obtained with purified CAI and CAII as coating antigens are reported in Fig. 1. Since ACAII levels were not normally distributed in CTR, cut-offs were set at 99° percentile and for ACAI and ACAII were 38.3% and 19.9% of the positive control, respectively.

The prevalence of ACAI and/or ACAII was reported in Table II. ACAI were detected in 16/96 (16.7%) as well as ACAII in 16/96 (16.7%). The prevalence was significantly higher (P=0.006) when compared with CTR. Two patients were both ACAI and ACAII positive, thus 30/96 patients (31.2%) were cumulatively ACAI and/or ACAII positive, this prevalence being significantly higher when compared with CTR (P<0.0001). ACAI prevalence

Table I. *Selected clinical and demographic features of study population.*

	RA (n = 33)	SA (n = 8)	PA (n = 8)	SLE (n = 23)	SS (n = 10)	SSc (n = 14)	Controls (n = 35)
Sex (F/M)	29/4	7/1	7/1	22/1	10/0	13/1	30/5
Age; mean±SD (range)	62.9 ± 8.4 (44-76)	52 ± 16.7 (24-72)	56 ± 10.5 (38-71)	44.6 ± 13.4 (21-66)	58.3 ± 9.9 (39-72)	55.4 ± 10.3 (39-71)	53.5 ± 13.2 (21-71)
Disease duration years; mean±SD	10.4±7.7	9.5±7	5.7±4.9	11.7±8.5	7±5.3	13.2±9	nd [a]
Smokers; n (%)	9 (27.3)	4 (50)	5 (62.5)	4 (17.4)	0 (0)	2 (14.3)	8 (23)
ESR; mean±SD	33.2±14.7	29.1±30	27.2±22	35±20.4	22.6±9	24±20.1	nd
CRP (mg/L) [b], median (range)	14.4 (0.8-116)	6 (1-25)	10 (2-26)	15 (0-178)	2 (0-10)	10.5 (1.5-107)	nd
C3 (mg/dL) [c]; mean±SD	94.6±20	109.6±30.3	89.1±17.8	85.1±22.1	110.6±26.7	93.1±20.45	nd
C4 (mg/dL) [d]; mean±SD	17±9.8	24.1±10.4	23.4±12.8	12±6.7	18.7±7.9	17.2±9.9	nd

RA= rheumatoid arthritis; SA= psoriatic arthritis; PA= ankylosing spondilitis; SLE= Systemic Lupus Erythematosus; SS= Sjogren Syndrome; SSc = Systemic Sclerosis
[a] *not determined*
[b] *normal values < 5 mg/L*
[c] *normal values : 90-180 mg/dL*
[d] *normal values : 10-40 mg/dL*

was significantly higher in SLE (P=0.02) and SSc (P=0.005), while ACAII prevalence was significantly higher in PA (P=0.0006) and SLE (P=0.02). When cumulative ACAI and/or ACAII positivity was considered in each group, the prevalence was significantly increased in patients with RA (P=0.002), PA (P<0.0001), SLE (P=0.0003), and SSc (P<0.0001) in comparison with CTR. The highest percentage of ACA-positive patients was found in patients with PA (62.5%) or SSc (50%) (Table II). Neither ACAI nor ACAII were found in CTR. No correlation between ACAI or ACAII levels and age or disease duration was detected.

Median levels of HRCT score were not significantly different (P=0.33) (Table II) between each patient group. According to the ROC curve, patients were subdivided into those with HRCT score ≥ 10 and those with HRCT score < 10. Thirty-nine out of 96 patients (40.6%) showed a HRCT score ≥ 10. The highest percentage of patients with HRCT score ≥ 10 (57%) was detected in the SSc group.

Twenty-five patients (26%) were smokers at study entry (Table I) but the mean HRCT score found (7.4±5.7) was not significantly different in comparison with the score detected in non-smokers (8.3±5.1) (P=0.48).

In the whole cohort of patients a positive correlation was documented between CRP and HRCT scores (Spearman r= 0.41, P<0.0001; Fig. 2, panel A), and patients with HRCT score ≥ 10 showed significantly higher levels of CRP (median 18.7; 25th-75th percentiles 9-30.1) than those with HRCT score <10 (6.9; 2-13.1) (P=0.0006; Fig. 2, panel B).

HRCT scores correlated with ACAI levels (r=0.29; P=0.004) (Fig. 3) and patients with HRCT ≥ 10 had higher ACAI levels (median 22.8; 25th-75th percentiles 11.6-40.1) than patients with HRCT

Table II. *High resolution CT scan (HRCT) scores and anti-carbonic anhydrase (ACA) prevalence in patients with Connective Tissue Diseases.*

	RA (n = 33)	SA (n = 8)	PA (n = 8)	SLE (n = 23)	SS (n = 10)	SSc (n = 14)	Controls (n = 35)
HRCT score ; median (range)	9 (0-19)	7.5(0-17)	5 (0-11)	8 (0-16)	8.5(0-13)	12(0-20)	nd [a]
HRCT score ≥ 10 [b]; n (%)	14 (42)	2(25)	2 (25)	9 (39)	4 (40)	8 (57)	nd
ACAI only ; n (%)	4 (12.1)	0(0)	1 (12.5)	4 (17.4) [d]	1 (10)	4(28.6) [e]	0(0)
ACAII only ; n (%)	3(9)	1 (12.5)	4 (50) [f]	4 (17.4) [d]	0 (0)	2(14.3)	0(0)
ACAI and II ; n (%)	1 (3)	0 (0)	0 (0)	0 (0)	0(0)	1 (7)	0 (0)
Cumulative ACA[c]; n (%)	8(24.2) [g]	1(12.5)	5(62.5) [h]	8(34.8) [i]	1(10)	7(50) [h]	0(0)

[a] *not determined*
[b] *according to a receiver operator characteristic curve, HRCT score ≥ 10 was predictive of interstitial lung disease*
[c] *Patients showing ACAI and/or ACAII*
Comparison of ACAI, ACAII or cumulative ACA prevalence between single patient groups and controls was evaluated by Fisher's exact test. Significant differences found as follows:
[d] *P = 0.02;* [e] *P = 0.005;* [f] *P = 0.0006;* [g] *P = 0.002;* [h] *P< 0.0001;* [i] *P = 0.0003*

score < 10 (8.9; 3.8-17.3) (P=0.0009). ACAI levels correlated with CRP (Spearman r = 0.34; P = 0.0006) (data not shown) and 10/16 (62.5%) ACAI positive patients had a HRCT ≥ 10.

Due to the scanty number in each patient group, a systematic analysis of relationship between ACAI and/or ACA II with other autoantibodies was not performed. However, in patients with SSc no association was found between ACAI and/or ACAII and anti-centromer antibodies.

There was no significant correlation between HRCT scores and carbon monoxide (CO) diffusing capacity (DLCO) values, nor between DLCO and ACAI or ACAII levels, even though a clear tendency to inverse correlation, near to statistical significance (P= 0.051), was found between HRCT and DLCO.

C3 and C4 complement fractions showed inverse correlation with HRCT scores (Spearman r – 0.35; P = 0.0004 and – 0.39; P<0.0001, respectively). In each group, patients with HRCT score ≥ 10 had serum levels of C3 and C4 lower in comparison with C3 and C4 found in patients with HRCT score < 10 (Fig. 4, panel A and panel B, respectively). When analysed in the whole cohort this difference reached statistical significance (C3: 87.7 ±22.4 *vs* 99.3 ± 22.1, P = 0.014, Fig. 4, panel A; C4 13.9 ± 8.5 *vs* 19.3 ± 10.1, P = 0.007, Fig. 4, panel B).

DISCUSSION

Despite CA, in particular CAII, having been regarded as a possible target antigen in autoimmune phenomena, the pathogenic role of ACA remains to be conclusively demonstrated.

In the first step of our study we evaluated whether CAI and CAII antigens purchased for ACA detection reacted with HRP-conjugated Fc-specific anti-human IgG. Our data confirm the previous findings reported by Hosoda et al. (12). Thus, although an antigen control should be routinely determined in all ELISA, the accurate check of a possible contamination with IgG-like material in commercially available CAI and CAII antigens is mandatory.

The prevalence of ACAI and ACAII observed in our CTD patients is similar to the prevalence found by Itoh and Reichlin (4) and Ono et al. (5). However, in Ito and Reichlin's study patients with PA or SA were not included while Ono et al. investigated only the levels and the prevalence of ACAII, not including in their research patients with RA or SA.

Itoh and Reichlin did not report any relationship between ACA and clinical findings, and also in Ono's

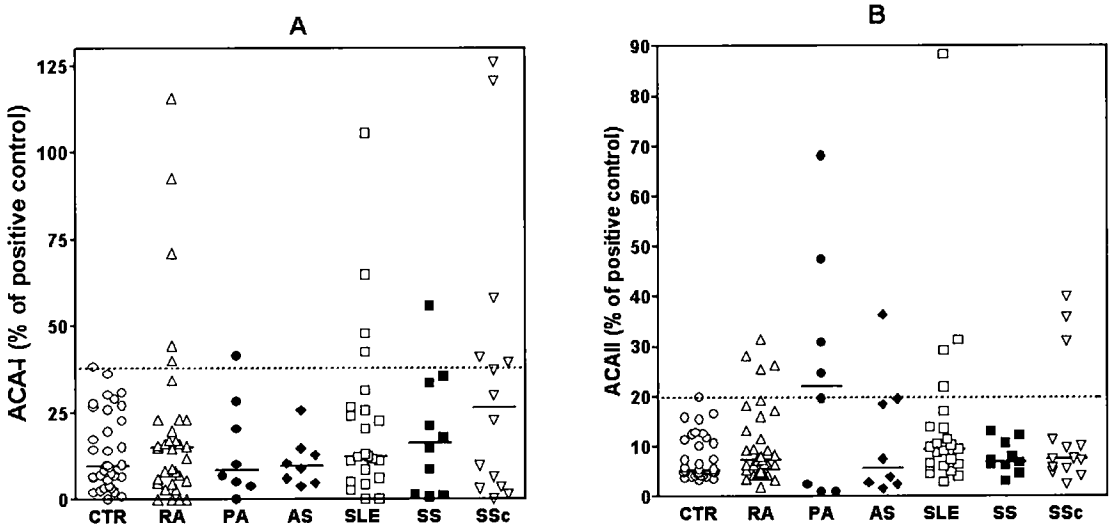

Fig. 1. *ACAI (panel A) and ACAII (panel B) levels in different connective tissue disease (for abbreviations see text) and control group (CTR). The dotted lines represent the cut-off values, i.e. 99° percentiles of the positive control.*

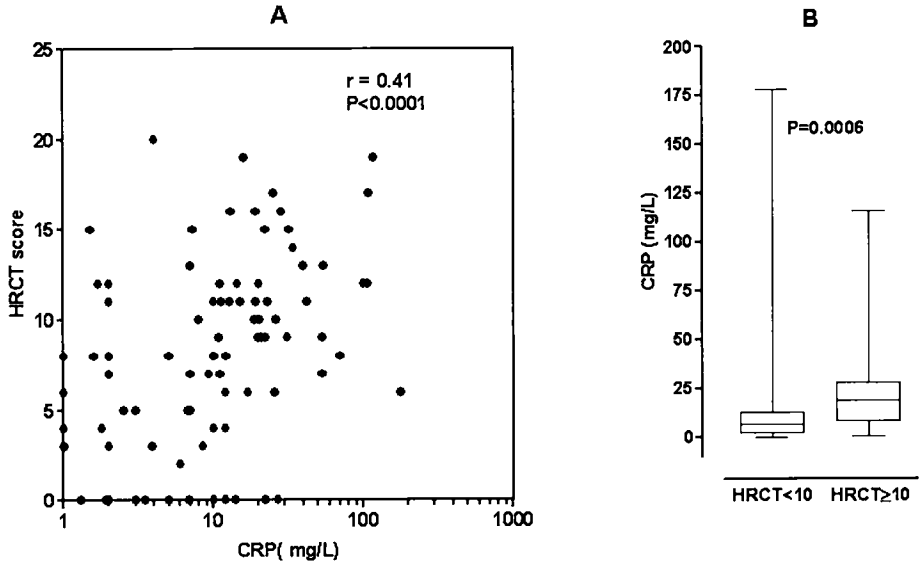

Fig. 2. *Panel A: Positive correlation between CRP and HRCT scores in the entire cohort of patients. Panel B: Difference of CRP between patients with HRCT scores < 10 (n = 57) and patients with HRCT scores ≥ 10 (n = 39). The data are depicted as box-plot diagrams, with the box encompassing the range values from the 25th percentile (lower bar) to the 75th percentile (upper bar). The horizontal line within the box represents the median, and the vertical lines represent maximum and minimum values.*

study the presence of ACAII was not correlated with clinical conditions.

In patients affected by CTD, autoantibodies against various antigens have been reported and, in this context, ACA may simply represent additional autoantibodies, whose pathogenic and clinical relevance remains to be elucidated. Nevertheless, since in our cohort ACAI significantly correlated with CRP, they could be taken as an additional marker of inflammation and/or disease activity.

Concerning the relationship between ACA and lung involvement, Alessandri et al. recently investigated the presence of ACAII in patients with SSc (6). They found significantly higher levels of

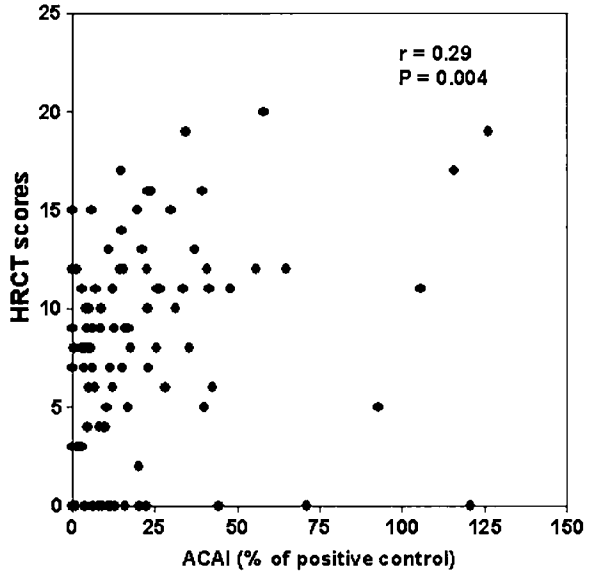

Fig. 3. *Positive correlation between ACAI levels and HRCT scores in the entire cohort of patients.*

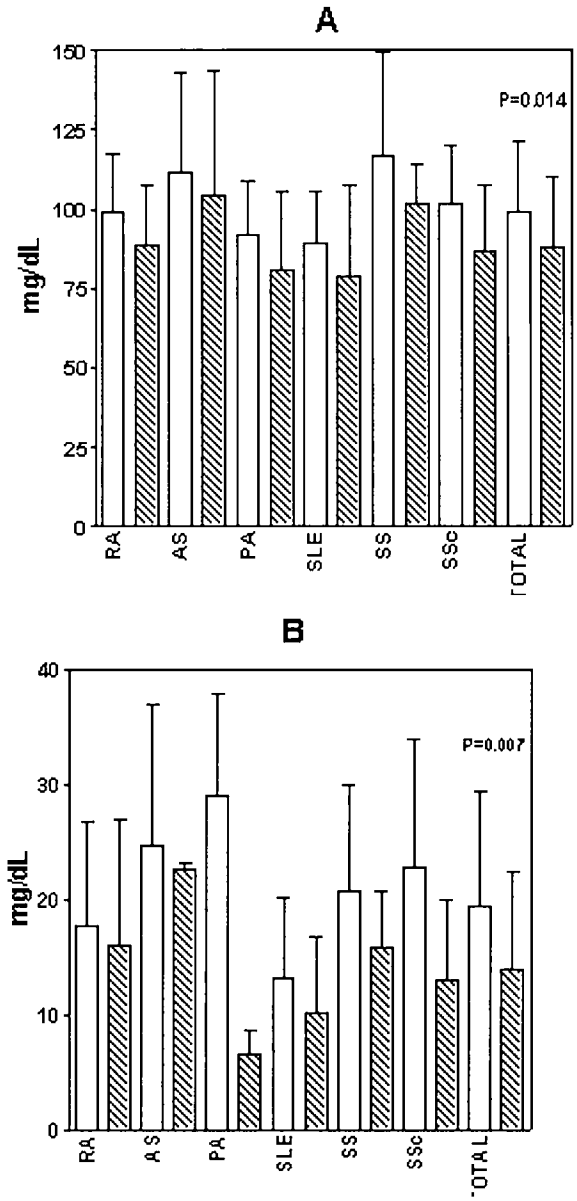

Fig. 4. *Differences of C3 (panel A) and C4 (panel B) complement fractions between patients with HRCT scores < 10 (clear bars) and patients with HRCT scores ≥ 10 (hatched bars). Each bar represents the mean ± SD of values.*

ACAII in patients with RDL, diagnosed according to criteria published by Steen et al. (21), as well as in patients with pulmonary hypertension. Their study was focused only on ACAII, since CAII and not CAI is expressed on alveolar epithelium where it is involved in CO_2 elimination, fluid secretion, and post-capillary pH regulation (13). Accordingly, a putative pathogenic role of ACAII in lung dysfunction was hypothesized.

In our study, we evaluated the presence of ACAI and ACAII in various CTD since, besides in SSc, lung involvement may occur in almost all CTD.

We have documented a positive correlation between ACAI but not ACAII and HRCT scores, as well as between ACAI and CRP. However, since in Alessandri's work ACAI were not evaluated, a comparison with our results cannot be made. Among our SSc patients, 7/14 (50%) had an HRCT score ≥ 10 and ACAI or ACAII were detected in 36% and 21.4% of them, respectively, and the percentage of ACAII-positive patients with SSc (3/14, 21.4%) was similar to the percentage found by Alessandri et al. (6/34, 17.6%).

The finding that in our study ACAI levels showed significant correlation with HRCT but not with DLCO deserves some comments. Since HRCT results may be predictive of ILD, thus anticipating overt impairment of lung function, it is possible that the presence of ACAI, regardless of any putative pathogenic mechanism, could precede the DLCO reduction.

Botrè et al. recently reported the effect of affinity chromatography purified antibodies to human CAI and CAII on the catalytic activity of both isoforms

(22). The *in vitro* incubation of CAI and CAII with specific antibodies partially reduced CO_2 diffusion, since the decrease rate was 31% and 16% following the CAI-ACAI and the CAII-ACAII interaction, respectively. Based on these results it can be speculated that the inhibition of CAI and /or CAII functions is not the only ACA-mediated pathogenic mechanism.

The production and deposition of specific immune complexes may be another possible ACA-mediated pathogenic mechanism. In this respect, it is worth mentioning the observation reported by Itoh and Reichlin who measured ACA-CA immune complexes in serum samples obtained at different time points from a patient who developed renal failure when maximum amount immune complexes were present (4).

Recently, in a large cohort of patients affected by mixed CTD, assessed with chest HRCT scanning, it has been shown that sera from patients with active ILD contained significantly higher amounts of immune complexes, as well as decreased complement levels than patients without ILD (23).

Although in our study detection of immune-complexes was not performed, a possible immune complex-mediated pathogenic mechanism of ACA should be taken into account due to the inverse correlation between C3 and C4 levels and HRCT scores and the significant reduction of serum levels of C3 and C4 complement fractions found in patients with HRCT score ≥ 10.

This hypothesis raises the question why only ACAI showed a positive correlation with CRP and HRCT, since an identical prevalence of ACAI and ACAII was found in our patients.

The molecular weight and the number of aminoacid residues of CAI and CAII are superimposable, but the amount of low activity isoform CAI is six-fold compared to CAII, thus giving a higher number of molecules putatively involved in immune complex formation.

In conclusion, our results show that autoantibodies directed against CAI and/or CAII are frequently detectable in patients with CTD, particularly in patients with PA, SSc, and SLE. The correlation of ACAI with CRP and HRCT suggests that ACAI could have a clinical relevance, provided that they do not merely represent an additional disease activity marker without a specific pathogenic role. Therefore, additional investigations on a larger cohort of CTD patients, as well as a systematic investigation of ACA association with other clinical and laboratory parameters are warranted to address this issue.

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