

ANTI-TISSUE ANTIBODIES ARE RELATED TO LUNG FUNCTION IMPAIRMENT IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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Running title: Circulating autoantibodies in COPD

At a glance commentary:

Scientific Knowledge on the Subject

Auto-immunity can contribute to the pathogenesis of COPD. The prevalence of circulating antinuclear (ANA) and anti-tissue (AT) antibodies in COPD, as well as their potential relationship with other domains of the disease, are unknown.

What This Study Adds to the Field

Our results show that between a third and a quarter of patients with clinically stable COPD present abnormal levels of circulating ANA and AT, the latter being related to lung function impairment. These observations provide further support to the hypothesis that the pathogenesis of COPD involves an auto-immune component.

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ABSTRACT

Background. Chronic obstructive pulmonary disease (COPD) is a multi-component disease. Auto-immunity can contribute to the pathogenesis of COPD. This study investigates the prevalence of circulating antinuclear (ANA) and antitissue (AT) antibodies, two common markers of autoimmunity, in COPD and their relationship with several components of the disease.

Methods. We determined lung function, the serum titers of ANA and AT by immunofluorescence and the serum levels of C - reactive protein (CRP) by high sensitivity nephelometry in 328 patients with clinically stable COPD and in 67 healthy controls recruited in the PAC-COPD study. Multiple linear and logistic regression analysis were used to analyze results.

Results. The prevalence of abnormal ANA and AT titers was 34% and 26% in patients and 3% and 6% in controls, respectively. Levels of AT \geq 1:320 were seen in 21% of COPD patients and were independently associated with the severity of airflow limitation and gas transfer impairment ($p < 0.05$). Neither ANA or AT titers were related to the body mass index, current smoking status, use of inhaled steroids, the Charlson index or serum CRP values.

Conclusions. Between a quarter and a third of patients with clinically stable COPD present abnormal titers of circulating ANA and AT. The observed relationship between AT and lung function supports a role for auto-immunity in the pathogenesis of COPD.

Abstract word count: 217

Keywords: auto-immunity; bronchitis; emphysema; immune system; tobacco; smoking

INTRODUCTION

An enhanced and persistent inflammatory response to the inhalation of particles and gases, mostly tobacco smoking, is considered a key pathogenic mechanism of chronic obstructive pulmonary disease (COPD). Recent evidence indicates that auto-immunity can play a significant role in this response (1). In this context, circulating antibodies against elastin (2), a key component of the matrix of the lung, and the pulmonary epithelium (3), have been recently identified in COPD patients, and an animal model of auto-immune emphysema has been described (4).

Antinuclear (ANA) and anti-tissue (AT) antibodies are two markers of autoimmunity commonly used in clinical practice (5) that have not been specifically investigated in patients with COPD. Our study sought to further explore the participation of an abnormal immune response in COPD by: (1) determining the distribution of circulating ANA and AT titers in a large and well characterized sample of COPD patients (6) and healthy controls; and, (2) exploring their relationship with relevant disease domains, such as smoking history, degree of airflow obstruction, nutritional status, the BODE index, presence of emphysema, comorbidities or systemic inflammation or use of inhaled steroid therapy. Preliminary results of this investigation have been previously reported in abstract form (7).

METHODS

Study design

This is a pre-specified, cross-sectional analysis of the baseline data of the PAC-COPD study, whose design and methodological details have been described in detail elsewhere (6). Briefly, patients were recruited during their first hospitalization episode due to an exacerbation of COPD, and they were studied, when clinically stable, three months after discharge.

Population and ethics

From January 2004 to March 2006, all subjects hospitalized for the first time because of an exacerbation of COPD in the nine participating hospitals in Spain were approached by the investigators. The criteria of first admission was based on the exclusion of patients with previous admissions as assessed by a questionnaire and information contained in the clinical records of the patient in the hospital, if available. The diagnosis of COPD was established when clinically stable according to the ATS/ERS recommendations (8). Patients younger than 45 years of age, with cancer, residual extensive tuberculous lesions of more than 1/3 of the pulmonary parenchyma, pneumonectomy and/or pneumoconiosis did not entry the study. A total of 342 individuals were originally included in the cohort (6). We report here results of the 328 patients with blood samples available for the measurement of ANA and AT (96%). As a control population we studied 67 healthy volunteers (31 smokers and 36 non smokers) recruited from primary care clinics, blood donors and hospital workers. All of them signed the informed consent, which had been previously approved by the Ethics Committee of all participating institutions.

Clinical characterization

Anthropometric data and information regarding relevant clinical aspects of the medical history of the patient were obtained using structured questionnaires (6). Particular attention was given to the cumulative dose of tobacco smoked before recruitment (pack-yrs), current or former smoking habits, presence of co-morbidities (Charlson index) (9) and therapy prescribed at the time of recruitment. Nutritional status was assessed by the body mass index (BMI). Dyspnea was assessed using the mMRC questionnaire (10). A six minute walking test was performed according to international guidelines (11). The BODE index was calculated according to Celli *et al* (12).

Lung function

Forced spirometry (before and after bronchodilation) (13) and the carbon monoxide diffusing capacity (DL_{CO}) (14), a surrogate for the presence of emphysema (15), were measured according to international guidelines. Reference values were those of a Mediterranean population (16,17). COPD severity was categorized according to the ATS-ERS classification (8). The partial pressure of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) were measured in an arterial blood sample according to international standards (18). DL_{CO} and arterial blood gases were not measured in controls.

Blood sampling

A venous blood sample (10 ml) was obtained by peripheral venipuncture in the early morning after fasting overnight. Active smokers were asked to refrain

smoking 8 hours before. Blood was centrifuged at 2000 rpm for 10 min immediately after sampling, and serum was stored frozen at – 80°C until analysis. All analyses were performed in the same centre (Hospital Univ. Son Dureta, Palma Mallorca, Spain) by specialized technicians.

Circulating autoantibodies

The serum titers of antinuclear antibodies (ANA) were quantified by indirect immunofluorescence on Hep2 lines (INOVA. San Diego, USA). Anti-ENA antibodies were investigated by semiquantitative ELISA (INOVA. San Diego, USA). In ANA positive samples with a homogeneous pattern, we tested the presence of anti-double-stranded DNA (dsDNA) antibodies by IFI on *Crithidia lucilliae* slides.

Antitissue autoantibodies (AT), including mitochondrial (AMA), liver-kidney microsomal (LKM), smooth muscle (SMA) and parietal gastric cell (PGC) autoantibodies, were determined by immunofluorescence on composite block of rodent liver, kidney and stomach sections (Immunofluor ANA-AMA-SMA-APCA. MT Promedt Consulting. GmbH. St Ingbert. Germany). In anti-SMA positive samples (>90% of AT total positive cases), we determined the presence of anti-F-actin, the main SMA reactivity found in Type 1 autoimmune hepatitis (19), by ELISA (Quantalite Actin IgG ELISA. INOVA. San Diego. USA).

All tests were blindly performed by a technician and reviewed by an immunologist (MRJ). Both for ANA and AT, titers < 1:160 were considered

negative whereas those 1:160, 1:320 and >1:320 were considered increasingly positive (20,21) .

C-reactive protein

Serum levels of C reactive protein (CRP) were determined by high sensitivity immunonephelometry (Dade Behring, Murgburg, Germany). Assays were performed in duplicate with a variation coefficient lower than 5%. The lower level of detection was 0.1 mg/L. CRP values below 3 mg/L were considered normal (22,23).

Statistical analysis

Results are expressed as mean (SD) for quantitative variables, or as frequencies and percentages for qualitative variables. Sample size calculations showed that, accepting an α risk of 0.05 and a β risk of 0.20 in a two-sided test, 45 control subjects and 45 patients were necessary to recognize as statistically significant a difference greater than or equal to 20 percent units when the proportion of autoantibodies in the control group was estimated in 5% (according to previous literature (20,24)). Comparisons between patients and controls, and across controls were performed by means of Students t and Chi squared tests. Comparisons across stages of disease severity and variables between patients were performed by means of ANOVA and Chi squared tests, for quantitative and qualitative variables, respectively. To identify subjects characteristics and COPD components (including BODE index) related to ANA and AT levels, ANOVA and Chi squared tests were used in the bivariate

approach. Multivariate linear or logistic regression models (depending on the distribution of the outcome variables) were built for each COPD component using ANA or AT as categorical exposures. Other patients' characteristics and COPD components were included as covariates only if they were both related to autoantibodies and each specific outcome variables. As sensitivity analysis, we repeated all calculations excluding women. A p value lower than 0.05 was considered significant. Data analysis was conducted using Stata 10.1 (StataCorp, College Station, TX, USA).

RESULTS

Clinical data

Table 1 presents the main anthropometric, clinical and lung function characteristics of the 328 patients studied, grouped according to the ATS/ERS classification of disease severity (8). Age was similar between groups. Most patients had moderate (stage 2) or severe (stage 3) COPD and were male. The BMI decreased and BODE increased significantly in proportion to disease severity. Cumulative smoking exposure (pack-yr) was intense and similar in all groups. The percentage of patients using inhaled steroids increased in proportion to disease severity. As expected, gas exchange deteriorated with increasing airflow limitation severity (Table 1). Mean CRP values tended to be higher than normal (3 mg/L) (22,23) but there were not significantly different between ATS/ERS stages of disease severity (Table 1). Table 2 shows the main anthropometric and functional characteristics of controls as compared to the entire population of COPD patients. Because we did not find any significant difference in antibody titers according to the smoking status of controls (never,

former or current smokers) (data not shown), they were analysed as a single group.

Prevalence of positive ANA and AT titers

Figure 1 presents the frequency distribution of ANA and AT titers in the patients and controls studied. Overall, 34% of patients had an abnormally high ANA titer ($\geq 1:160$) (20), a prevalence eleven times higher than that seen in our control group (Figure 1, panel a), and seven times higher than that reported in healthy subjects (5%) (20). Eleven percent of COPD patients had ANA titers $\geq 1:320$, a figure that is much higher than that observed in healthy subjects (Figure 1, panel a). Among patients with positive ANA titers, less than 1 % of patients had anti-dsDNA or anti-ENA positive results. The pattern of ANA positivity was speckled (n=57), mixed (n=39), cytoplasmic (n=8), nucleolar (n=5), centrilolar (n=1) or homogeneous (n=1).

We also found that 26% of the patients studied showed AT positivity ($\geq 1:160$), a prevalence 4.5 times higher than determined in our controls (Figure 1, panel b), and four times higher than that reported in healthy subjects (6%) (24). Of note, 21% of patients had AT titers $\geq 1:320$ whereas this was not the case in any single healthy subject (Figure 1, panel b). Most (n=80) AT positive patients were SMA positive, whereas only occasionally we observed individuals with CPG positive (n=6), reticuline-like pattern (n=3), endomysial (n=1) and mitochondrial (distinct than the M2 CBP-associated pattern) (n=1). Among SMA positive patients, we detected reactivity against F-actin in only 10% of them, and in these cases, this was always at low or moderate levels. In 20% of cases

abnormal AT and ANA titers occurred in the same patient. Results did not change when females were excluded from analysis (data not shown).

Relationship between circulating antibodies and patients characteristics

ANA positivity was more prevalent in women but their limited number (n= 21) limit the generalizability of this observation. On the other hand AT positive patients were younger and more likely to be active smokers (Table 3). As shown in Figure 2, ANA titers were not related to the severity of airflow limitation (panel a) or gas transfer (DL_{CO}) deficit (panel b). By contrast, AT titers increased significantly with increasing airflow limitation (panel c) and gas transfer limitation (panel d). Multivariate models adjusted for age and smoking (identified as potential confounders in Table 3) showed that having AT positive titer ($\geq 1:160$) was associated with: (i) a reduction of 3.7 percentual units of FEV1 (p=0.142); and, (ii) an increased risk (OR=2.0, CI 1.21-3.51) of moderate-severe DL_{CO} impairment (<60%) (p=0.016). There was no relationship between autoantibodies titres (ANA and AT) and any other of the COPD variables shown in Table 1. Specifically, the prevalence of positive ANA and/or AT titers was not related to BODE quartiles (Table 4).

DISCUSSION

Our results show that between a quarter and a third of patients with clinically stable COPD have abnormal titers of circulating ANA (34%) and AT (26%), a prevalence much higher than determined in healthy controls (3% and 6% respectively) and also higher than that reported in previous studies in the general population (20,24). Besides, AT titers were clearly elevated ($\geq 1:320$)

among patients with abnormal levels ($\geq 1:160$), and AT (but not ANA) titers were related to lung function impairment (airflow limitation and gas transfer defects).

Previous studies

ANA and AT are two non-specific markers of autoimmunity (5). In 1976 Hodson and Turner-Warwick reported that 28% of 50 patients with “severe chronic bronchitis”, most likely what we would have called COPD today, had increased titers of circulating ANA, a figure that was much higher than that determined simultaneously in age and sex matched “non-bronchitic” controls (4%) (25). This study, however, has gone mostly unnoticed to date. Our results confirm these previous findings in a larger and better characterized cohort by showing a remarkably similar prevalence of circulating ANA (34%) in patients with COPD. Besides, our study extends these findings by quantifying the prevalence of AT autoantibodies. We found that a relatively high proportion of patients with COPD (26%) also had increased titers of AT and, at variance with ANA, 70% of AT positive patients have very high levels ($>1/320$). These observations are in keeping with recent reports of circulating antibodies directed against components of the lung matrix (2) and epithelium (3) in patients with COPD, although it is worth noting that not all studies found such evidence (26-28). Differences in the types of patients studied (COPD, cystic fibrosis, A1AT deficiency and lung fibrosis), specific antibodies quantified, as well as in sample size of previous studies can explain this discrepancy. It is also possible, as in fact our results show, that not all patients with COPD have evidence of autoimmunity and that only a subset of them develop it, as previously suggested (1).

Interpretation of findings

Because this is an observational study, we can only speculate with the mechanisms underlying the observed associations. ANA and AT can either be non-specific markers of an ongoing autoimmune response (5) or, alternatively, they may be directly involved in the pathogenesis of the disease. That ANA titers were not related to lung function (or current smoking or comorbidity), and that the most frequent antigen specificities of ANA in other autoimmune diseases (ENA, dsDNA) were negative, support the former possibility (29,30). By contrast, the fact that AT were inversely related to airflow limitation and DL_{CO} impairment (Figure 2) supports the latter one. In this context, it is of interest that more than 90% of AT positive patients were SMA positive. This may be relevant for airway remodeling in COPD because airway smooth muscle cells can synthesize extracellular matrix proteins, downregulate matrix metalloproteinases and upregulate their tissue inhibitors (31). In any case, both alternatives are not mutually exclusive and provide further support to the involvement of an abnormal autoimmune response in the pathogenesis of COPD (1,32).

The BODE index is a multicomponent score with prognostic value in COPD that combines pulmonary and extra-pulmonary dimensions of the disease (12). We specifically explored if patients with higher BODE scores (quartiles) present a higher prevalence of positive ANA and/or AT antibodies titers, but found this not to be the case (Table 4). This observation suggests that the presence of autoantibodies is likely more related to the pulmonary components the disease.

Systemic inflammation is normally considered an important pathogenic mechanism underlying many of the extra-pulmonary effects of COPD (33). Yet, in keeping with the above discussion on the BODE index, we did not find a clear relationship between ANA or AT titres and CRP values, a marker of systemic inflammation. Similarly, we did not find any relationship between the presence of autoantibodies and the use of inhaled steroids.

Strengths and limitations

The large sample size of our cohort (one order of magnitude higher than that of previous reports investigating circulating antibodies in patients with COPD (23,25)), the wide range of COPD severity included in the analysis (Table 1), the careful phenotypic characterization and the analysis of the relationship between circulating autoantibodies and several important domains of the disease are strengths of our study. Yet, our study also has some limitations. First, in keeping with previous studies (34), we found that ANA titres were higher in female patients (Table 3) but this was not the case in controls. Yet, due to the relatively low number of female patients studied (6.5%), we can not be confident that females with COPD have higher ANA titers than male patients. In any case, their inclusion in the study did not influence its results because they were unchanged when females were excluded from the analysis. Second, AT levels were associated with age and smoking status in COPD patients in the bivariate analysis. It is well known that autoantibody titers increase with age as well as with smoking (29,35). In fact, anti-elastin antibodies have been considered a marker of aging in animal models (36). Yet, we think that this does not

invalidate our results because FEV1 and DLCO were expressed as percentage of the reference value and were, therefore, age-corrected, and smoking status was included in the multivariate analysis specifically. However, we cannot exclude that increased AT antibodies contribute mechanistically to the accelerated lung aging process that may characterize COPD (37). Finally, it is possible that the exclusion of patients with multiple hospitalizations, that has recently been demonstrated as a stable phenotype (38) prior to study enrolment can bias the results against finding an association and, conversely, the inclusion of COPD patients who haven't been hospitalized before has the potential to skew the data in the opposite direction. Acknowledging these limitations, however, we found it remarkable that a substantial percentage of the patients studied show abnormal auto-antibody titers.

Conclusions

The results of this study show that between a third and a quarter of patients with clinically stable COPD present abnormal levels of circulating ANA and AT, and that the latter are related to impairment of lung function. These observations provide further support to the hypothesis that the pathogenesis of COPD involves an auto-immune component (1).

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FIGURE LEGENDS

Figure 1. Frequency distribution of ANA (panel a) and AT titers (panel b) in patients and controls.

Figure 2. Frequency distribution of ANA and AT titers according to the severity of airflow limitation (a, c) and gas transfer (DLCO) impairment (b, d). For further explanations, see text.

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Table 1. Anthropometric, clinical and functional data of 328 COPD patients, by the ATS/ERS classification of disease severity (see below):

	ATS/ERS stage of disease severity				<i>p</i> value
	<i>I</i> <i>Mild</i>	<i>II</i> <i>Moderate</i>	<i>III</i> <i>Severe</i>	<i>IV</i> <i>Very severe</i>	
<i>N</i>	19	159	124	26	
Age (yrs), m (SD)	67.8 (8.5)	68.2 (9.0)	68.8 (7.7)	64.0 (8.7)	0.074
Males, n (%)	15 (79)	145 (91)	122 (98)	25 (96)	0.004
BMI (Kg/m ²), m (SD)	29.1 (5.2)	29.2 (4.4)	28.0 (4.3)	23.9 (4.3)	<0.001
Inhaled steroids* users, n (%)	9 (47)	90 (57)	91 (73)	24 (92)	<0.001
Current smokers, n (%)	4 (22)	52 (33)	36 (30)	12 (48)	0.245
Pack-yr, m (SD)	67.7 (50.0)	68.8 (39.2)	69.4 (40.4)	63.6 (28.6)	0.924
Charlson index, m (SD)	2.2 (1.8)	2.1 (1.3)	2.2 (1.5)	2.2 (1.5)	0.931
PostBD FEV ₁ (% ref), m (SD)	87.4 (7.4)	61.7 (7.9)	41.5 (5.0)	24.3 (4.1)	<0.001
PostBD FEV ₁ /FVC (%), m (SD)	64.8 (4.3)	59.7 (8.5)	48.2 (10.2)	34.5 (7.2)	<0.001
DL _{CO} (% ref), m (SD)	90.7 (18.4)	70.2 (18.1)	59.9 (18.5)	42.5 (21.2)	<0.001
PaO ₂ (mmHg), m (SD)	82.1 (10.9)	76.6 (10.9)	72.1 (9.5)	67.1 (7.7)	<0.001
PaCO ₂ (mmHg), m (SD)	39.8 (4.3)	40.5 (4.9)	42.9 (5.2)	46.1 (5.3)	<0.001
BODE index	0.5 (0.9)	1.3 (1.2)	3.4 (1.5)	5.5 (2.1)	<0.001
C-reactive protein (mg/L), m (SD)	3.4 (2.2)	7.3 (15.8)	10.6 (22.0)	6.8 (10.5)	0.257

* Alone or in combination. For abbreviations, see text. Some variables had missing data: 7 in smoking, 7 in pack-yr, 1 in Charlson index, 45 in DL_{CO}, 11 in PaO₂, 10 in PaCO₂, and 7 in CRP.

ATS/ERS stages: I) Mild, FEV1/FVC < 0.7 and FEV1 \geq 80% ref; II) Moderate
FEV1/FVC < 0.7 and FEV1 <80% and \geq 50 % ref; III) Severe, FEV1/FVC < 0.7
and FEV1 <50% and \geq 30 % ref; IV) Very severe, FEV1 <50% and \geq 30 % ref.

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Table 2. Anthropometric and functional characteristics (mean \pm SD) in controls and COPD patients.

	Controls n=67	COPD patients n=328	p value
Males, n (%)	62 (92.5)	307 (93.6)	0.75
Age (yrs)	66.7 \pm 5.9	68.0 \pm 8.5	0.22
Pack-yrs	40.0 \pm 22.1	65.5 \pm 39.5	<0.01
FEV ₁ (% ref)	101.8 \pm 18.8	52.6 \pm 16.3	<0.01
FEV1/FVC (%)	79.3 \pm 6.5	53.6 \pm 12.0	<0.01

Table 3. Patients characteristics by autoantibodies levels.

	ANA					AT				
	<1:160	1:160	1:320	>1:320	<i>p value</i>	<1:160	1:160	1:320	>1:320	<i>p value</i>
	n=216 (66%)	n=75 (23%)	n=25 (7%)	n=12 (4%)		n=242 (74%)	n=16 (5%)	n=13 (4%)	n=54 (17%)	P
Age (yrs), m (SD)	68.0 (8.6)	67.6 (8.3)	70.5 (7.2)	66.3 (10.8)	0.430	68.8 (8.1)	66.5 (8.8)	64.0 (8.3)	66.1 (10.0)	0.042
Males, n (%)	207 (96)	69 (92)	22 (88)	9 (75)	0.016	224 (93)	15 (94)	13 (100)	52 (96)	0.581
BMI (Kg/m ²), m (SD)	28.2 (4.8)	28.5 (4.4)	28.1 (3.4)	29.3 (4.4)	0.838	28.1 (4.6)	28.5 (4.8)	29.1 (4.4)	28.8 (5.0)	0.739
Inhaled steroids users, n (%)	143 (66)	49 (65)	13 (52)	9 (75)	0.471	159 (66)	14 (88)	7 (54)	33 (61)	0.197
Current smokers, n (%)	66 (31)	29 (39)	5 (20)	4 (36)	0.319	65 (27)	5 (31)	6 (46)	27 (52)	0.005
Pack-yr, m (SD)	70.8 (41.3)	67.1 (34.9)	58.8 (38.8)	57.2 (32.2)	0.360	67.0 (40.1)	64.8 (39.0)	85.6 (33.7)	71.0 (37.8)	0.377
Charlson index, m (SD)	2.1 (1.4)	2.1 (1.4)	2.6 (1.3)	1.4 (0.7)	0.083	2.1 (1.3)	2.1 (1.5)	2.2 (1.5)	2.3 (1.7)	0.644

Table 4. Number (and percentage) of patients with different titers of anti-nuclear (ANA) and anti-tissue (AT) antibodies by BODE quartiles.

		BODE 0-1 n=71 (24%)	BODE 2 n=75 (25%)	BODE 3 n=76 (25%)	BODE 4-10 n=79 (26%)
ANA p=0.760	<1/160	46 (65%)	48 (64%)	50 (66%)	56 (71%)
	1/160	16 (22%)	15 (20%)	21 (28%)	16 (20%)
	1/320	5 (7%)	8 (11%)	4 (5%)	5 (6%)
	>1/320	4 (6%)	4 (5%)	1 (1%)	2 (3%)
AT p=0.167	<1/160	56 (80%)	57 (77%)	55 (72%)	52 (67%)
	1/160	3 (4%)	4 (5%)	5 (7%)	2 (3%)
	1/320	5 (7%)	3 (4%)	2 (3%)	2 (3%)
	>1/320	6 (9%)	10 (14%)	14 (18%)	21 (27%)

Figure 1

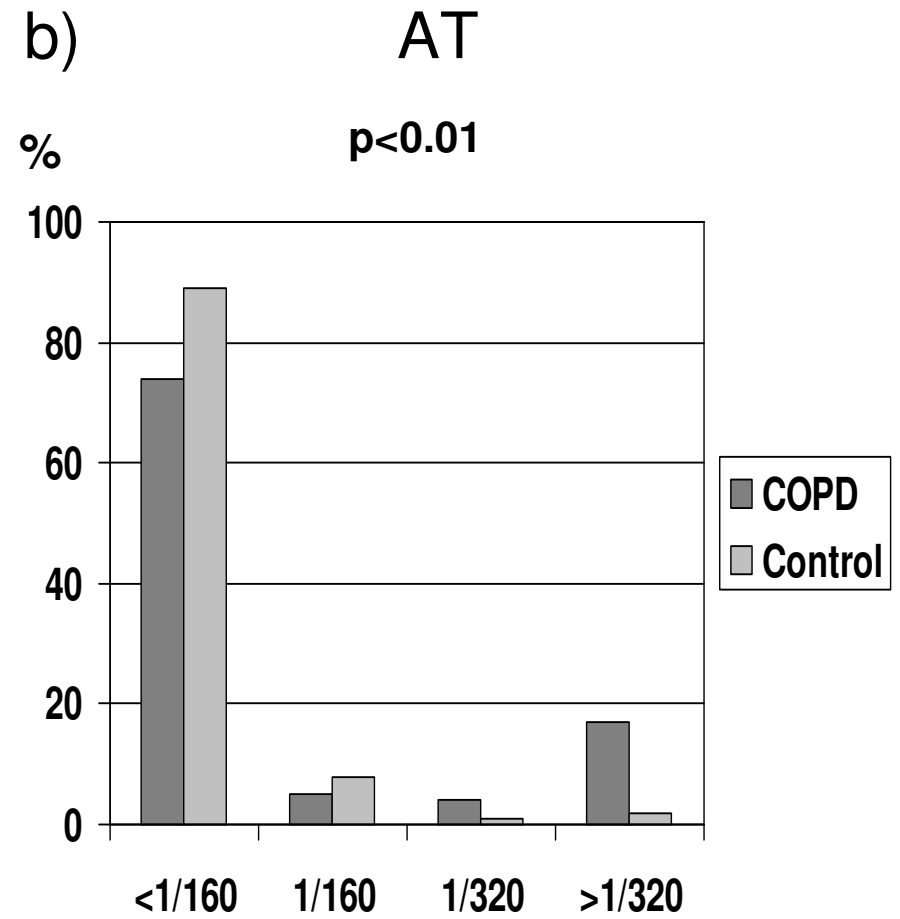
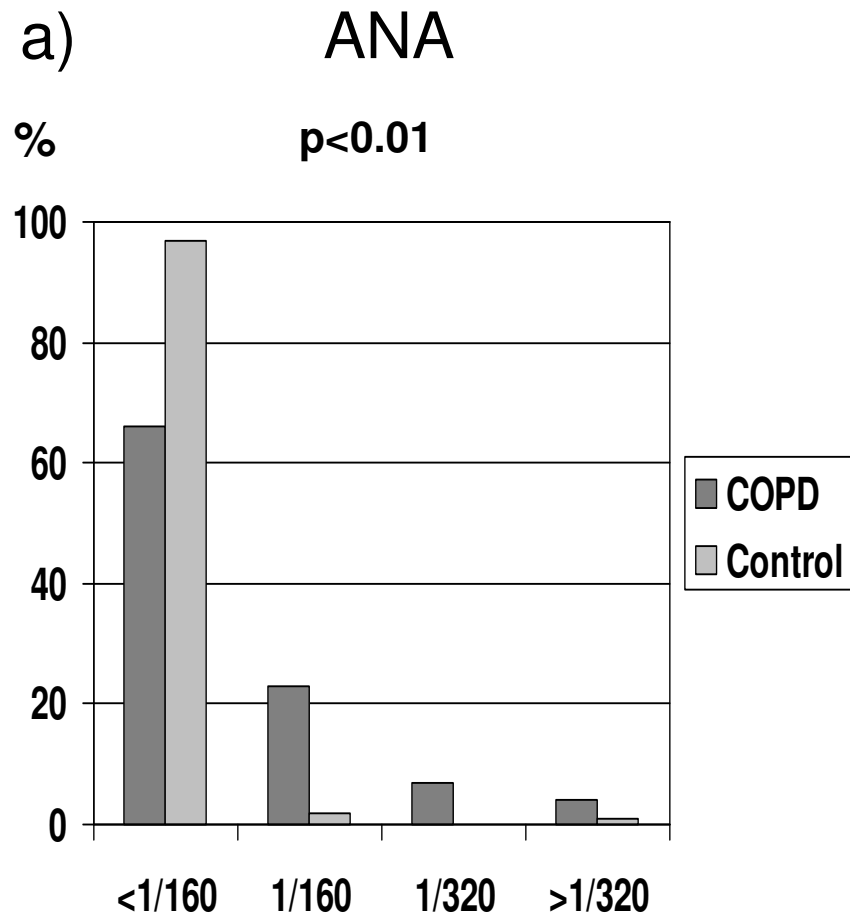


Figure 2.

