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SMOKE EXPOSURE AS A DETERMINANT OF AUTO-ANTIBODY TITRE IN AATD AND COPD

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ABSTRACT

Background

Liberation of elastin peptides from damaged lung may be a mechanism of autoimmune lung disease. Citrullination, and anti-citrullinated protein antibody formation occurs in smokers, but the role of smoking in autoantibody generation relevant to pulmonary disease is unclear.

Methods

Anti-elastin, anti CCP and anti-MCV antibodies were measured in 257 subjects with alpha 1 antitrypsin deficiency (AATD) , 113 subjects with usual COPD and 22 healthy non-smokers. Levels were compared between groups, against phenotypic features, and against smoke exposure.

Results

Anti-elastin antibodies were higher in controls relative to AATD ($p=0.008$) and usual COPD ($p<1x10^{-5}$), and in AATD relative to usual COPD ($p<1x10^{-5}$). Anti-elastin levels showed a threshold at 10 pack years, being higher in those who had smoked less ($p=0.004$). No relationships between antibody levels and clinical phenotype were seen after adjustment for smoke exposure. Anti-CCP antibodies were higher in usual COPD than AATD ($p=0.002$) but the relationship to smoke exposure was less clear.

Conclusions

Smoke exposure is the main determinant of anti-elastin antibody levels, which fall after 10 pack years. Local antibody complexes may be a better measure of elastin directed autoimmunity than circulating levels.

INTRODUCTION

The protease-antiprotease hypothesis of the pathogenesis of chronic obstructive pulmonary disease (COPD) concerns imbalance between proteases that digest elastin and other components of the extra-cellular matrix in the lung parenchyma, and anti-proteases that protect[1-2]. The origin of this theory comes from the observation that patients with Alpha 1 antitrypsin deficiency (AATD) develop early onset emphysema[3]. Alpha one antitrypsin (AAT) is an anti-protease, which acts predominantly to block the action of neutrophil elastase (NE), a protease released by neutrophils. Neutrophil elastase is a serine protease, the first of three classes of protease important in COPD. The remaining two classes are the cysteine proteases, such as cathepsin-B, and the matrix metalloproteases (MMP's) [4]. In general the serine and cysteine proteases are capable of degrading elastin and some forms of collagen [4], whilst MMP's have more of an effect on collagen, gelatin and laminin [2], all of which are components of the extracellular matrix of the lung.

Breakdown of the extracellular matrix, particularly elastin, in the lung in the lung is a key feature of both COPD and AATD, as shown by the presence of high levels of elastin breakdown products. A desmosine cross-link is unique to elastin and may be used as a marker of its degradation[5]. Desmosine and elastin peptides are elevated in the plasma, urine and sputum of COPD patients[6], whilst urinary levels positively correlate with the annual rate of decline in FEV1 in smokers[7]. In general elastin breakdown is greater in subjects with AATD at a given level of smoking because of a relative excess of NE activity in the lung[8], confirmed by elevated desmosine levels compared to usual COPD subjects[6]. Elastin breakdown has been proposed as a mechanism for generation of auto-antibodies directed against elastin, which could in theory perpetuate and/or aggravate lung destruction[9]. In both AATD and COPD the main stimulus to elastin breakdown is cigarette smoking, but studies of anti-elastin antibody prevalence in these conditions to date have been too small to assess

smoke effects on antibody levels adequately [9-11]. Although the original report of circulating anti-elastin antibodies in COPD was able to show antibody generation by lung tissue and a correlation between peripheral blood responses to elastin peptides and severity of emphysema on CT scan[9] the effect of anti-elastin antibodies in the lung remained uncertain.

It is also interesting to note that smoking in and of itself is now of interest as a risk factor for autoimmunity, as discussed in a recent review[12]. In some autoimmune diseases this has been noted at epidemiological level, and there is now biological evidence for direct smoke effects on antibodies diagnostic of rheumatoid arthritis, known as anti citrullinated protein antibodies (ACPA). Citrullination is a post-translational modification of proteins which occurs in inflammatory disease[13]. It is recognized to occur in the lung of smokers, and is thought to influence occurrence of antibodies directed against citrullinated proteins (ACPA) [14]. Two main ACPAs are recognized – anti-cyclic citrullinated peptide (anti-CCP) and anti-mutated citrullinated vimentin (anti-MCV) antibodies. Both are markers of disease severity in rheumatoid arthritis[15-16] and there is some suggestion that they occur more in extra-articular disease[17], particularly lung disease[18]. We reasoned that pulmonary citrullination might be a relevant process generating auto-antibodies in COPD, alongside elastin liberation.

We hypothesized that anti-elastin and anti-citrullinated protein antibodies would be higher in subjects who had smoked more, and that anti-elastin antibodies would be higher in AATD than COPD due to excessive elastin breakdown.

METHODS

Subjects and clinical phenotyping

AATD

257 PiZZ subjects from the UK national registry for AATD were studied, this being the number with a baseline serum sample available at the time of the study. The project was

approved by the local research ethics committee and all subjects gave informed consent. All AATD subjects had a full clinical history (including smoke exposure), a serum alpha-1-antitrypsin (AAT) level of <11μM, PiZZ genotype confirmed by specific PCR (Heredilab, Salt Lake City, UT, USA) and underwent full pulmonary function testing, as described previously[19]. None had ever received AAT replacement therapy.

COPD

The West Midlands COPD Collection (WMCC) comprises subjects with COPD, excluding those with AATD, defined as FEV1/FVC<0.7 and post-bronchodilator FEV1 <80% predicted, recruited in the central region of the UK. Studies of clinical phenotype and genetic risk in the WMCC have been approved by the local ethics committee. All subjects in the WMCC have undergone pulmonary function testing and clinical history gathering in the same manner as the AATD group. At the time of data collection there were 116 subjects registered with the WMCC, all of whom had given informed consent.

Healthy non-smokers

The control group consisted of22 healthy non-smokers with normal pulmonary function tests, in whom AATD had been excluded, with a stored serum sample available and who had consented to participate in research.

Antibody levels

Anti-elastin antibodies were measured in duplicate in serum using methods published by Lee *et al*[9] on all AATD and COPD subjects. The original publication measured antibodies in plasma; thus the validity of the assay in serum was confirmed by measurement of antibodies in both serum and plasma samples taken at the same time point from 10 individuals; titres were no different between the 2 sample types (p=0.841). Serial dilutions of a pooled serum sample were used to make a standard curve and IU/ml for subject samples calculated from this. The inter assay coefficient of variation over the working range was 8.30% (n=20 repeats in a single subject). The spike recovery was 87.34%, and assay

specificity for anti-human IgG was confirmed by a zero reading when control antibody was used.

ACPA antibodies were measured in serum or plasma using commercial ELISA kits (valid for use in both sample types) for anti-CCP and anti-MCV antibodies (Phadia UniCAP system, UK and Orgentec Diagnostika, Germany respectively). These are clinically validated tests, with a threshold for positivity of 10IU/ml and 20IU/ml respectively.

Statistical analysis

Data were analysed using SPSS version 16.0 (Chicago, USA). Comparisons of antibody levels between groups were carried out using the Mann-Whitney and Kruskal Wallis tests. Comparisons of antibody levels within each group were first analysed as univariate models, using Pearson and Spearmans rank correlations, as dictated by the distribution of the data, for continuous variables, the student's t-test and Mann-Whitney tests for binary outcomes, and the Chi square test for frequency variables. Adjustments for smoke exposure were made using coefficients from disease group regression models seeking predictors of antibody levels.

RESULTS

Characteristics of the subjects

Characteristics of the 3 groups are shown in table 1. COPD subjects were generally older and had smoked more than those with AATD (both $p<0.001$), consistent with known features of the conditions. There were more current smokers ($p<0.001$), gas transfer was lower ($p=0.02$), and chronic bronchitis more common ($p<0.001$) in the usual COPD group than those with AATD. Tests of significance regarding the difference in emphysema frequency between AATD and COPD were considered invalid in view of the differences in proportion scanned.

	AATD n=257	COPD n=116	Healthy n=22
Age	50.71 (0.70)	68.83 (0.93)	47.07 (1.64)
Male gender	146 (56.8)	64 (55.17)	7 (31.82)
Pack years smoked	15.30 (0.91)	46.31 (2.69)	0
Current smoker	13 (5.06)	32 (27.58)	0
Never smoked	64 (24.90)	0	22
FEV1 post BD (l)	1.93 (0.07)	1.89 (0.86)	4.56 (0.33)
FEV1 % predicted	57.67 (2.11)	43.43 (1.66)	101.40 (7.59)
FEV1/FVC post BD	0.46 (0.01)	0.37 (0.20)	0.74 (0.01)
KCO (mmol/min/kPa.l)	1.12 (0.03)	1.09 (0.86)	1.74 (0.09)
KCO % predicted	71.35 (1.34)	56.58 (4.21)	97.50 (0.71)
COPD	208 (80.93)	116 (100)	0
Emphysema	158 (61.47)	38 (32.76)*	Not scanned
Chronic bronchitis	80 (31.50)	68 (58.62)	0
Anti-elastin antibody (IU/ml)	31.18 (5.39)	5.90 (0.88)	68.04 (11.98)
Anti-CCPantibody (IU/ml)	2.68 (0.49)	18.01 (8.13)	1.34 (0.76)
Anti-MCV antibody (IU/ml)	23.57 (5.04)	39.12 (12.09)	15.87 (6.92)
Positive anti-CCP (>10IU/ml)	8 (3.2)	6 (5.2)	0
Positive anti-MCV (>20IU/ml)	34 (13.4)	20 (17.7)	2 (9.1)

Table 1: Characteristics of the disease cohorts
The table shows the mean (SE) for all variables, except gender, current and never smoked status, COPD, emphysema and chronic bronchitis, which are shown as N (%).
* Proportionally fewer subjects in the usual COPD group had undergone a CT scan – when the percentage is calculated as a proportion of those scanned it rises to 88.40%.

Anti-elastin antibody levels and associations

Anti-elastin antibodies were detected in all 257 AATD samples, all 22 healthy subjects and 113/116 COPD subjects. The mean level was significantly higher in healthy subjects than those with COPD ($p=1x10^{-5}$), and those with AATD (p=0.008). Levels in AATD were higher than in usual COPD ($p<1x10^{-5}$), illustrated in figure 1. Levels in AATD were higher in those within this group who did not have COPD (p=0.032, Figure 2).

Within AATD anti-elastin antibodies showed a negative correlation with pack years smoked (r=-0.199, p=0.001), although visually the relationship did not appear linear (Figure 3). We therefore stratified into categories by pack years smoked, and showed a clear threshold at 10 pack years, above which antibodies were lower (Figure 3, p=0.004). Consistent with this, the mean level in never smokers was significantly higher than the smoke

exposed (51.14 (13.05) v 27.04 (5.21), p=0.04). A concomitant positive correlation with FEV1 was seen (r=0.189, p=0.005) which was lost after adjustment for pack years smoked (r=0.084, p=0.251). A trend towards higher levels in those without emphysema (51.12 (17.33) v 29.30 (6.06); p=0.074), was also lost after similar adjustments (p=0.622). The unadjusted correlations in AATD appeared to be driven by never smokers, and indeed in usual COPD, where all individuals had smoked, no relationship between antibody level and pack years or FEV1 was seen (both p>0.25). Emphysema analyses were not performed in usual COPD because of the low proportion of CT scans available.

Anti-elastin antibody levels were then compared between groups after adjustment for pack years smoked; the difference between AATD and usual COPD was lost (p=0.258). This lack of relationship remained when including only AATD individuals who had spirometrically defined COPD (p=0.131). All other between group comparisons of antibody levels became non-significant after adjustment for smoke exposure (all p>0.6). Never smokers with AATD and no evidence of COPD (n=24) were then compared to healthy never smokers without AATD (n=22) in order to ascertain if AATD itself were a significant determinant of antibody levels after control for smoke exposure and the presence of COPD; there was no difference in their anti-elastin antibody levels (p=0.295).

Current versus past smoking did not show a significant difference in antibody levels in either group (both p>0.5). Years since last cigarette exposure did not relate to antibody levels, nor did age at commencement of smoking (both p>0.5). No gender or age relationships to antibody levels were seen in AATD or usual COPD, and sub-stratification by gender did not change the relationship with pack years seen (r=-0.208, p=0.014 in males and r=-0.255, p=0.018 in females).

ACPA associations

Anti-CCP antibodies were higher in usual COPD than AATD (p=0.002, Figure 4), and exceeded the conventional cut-off for a positive level in 8 AATD subjects and 6 of those with usual COPD. Anti-MCV antibodies were positive in 34 AATD subjects and 20 with usual COPD; the level did not differ between groups (p=0.876, Figure 4). There was no difference in either ACPA between AATD and controls (p=0.412). In AATD neither ACPA showed any relationship to FEV1 (anti-CCP: p=0.608 for high levels, p=0.868 as a continuous variable; anti-MCV: p=0.710 and p=0.864 respectively). Equally, in usual COPD neither ACPA was independently associated with FEV1 (anti-CCP: p=0.424 and 0.969; anti-MCV: p=0.957 and 0.406). No age or gender associations were seen in either group (all p>0.5). In gender sub-stratified analyses the lack of relationship with FEV1 persisted (all p>0.6).

Neither anti-CCP nor anti-MCV antibodies differed between those who had never smoked and those who had been smoke exposed in AATD (p=0.903), a test which could not be performed in usual COPD since all subjects had smoked. No linear dose response relationship (pack years v ACPA) existed in AATD (anti-CCP: r=0.002, p=0.979; anti-MCV: r=-0.069, p=0.301) or usual COPD (anti-CCP: r=-0.158, p=0.161; anti-MCV: r=-0.024, p=0.864). However, when split into pack year categories in AATD, as before, a threshold was apparent at 10 pack years, although less clear than that seen for anti-elastin antibodies (Figure 5). However, the difference in titre between those either side of the 10 pack year threshold was not significant (anti-CCP, p=0.661; anti-MCV, p=0.110), and the incidence of positive ACPA did not differ above and below this threshold (p=0.761 and 0.503 respectively). Given that no relationship with smoke was observed further inter-group comparisons after adjustment for smoke exposure were not performed.

Since ACPA are diagnostic of rheumatoid arthritis, the incidence of this, and other autoimmune diseases, was determined in all those with a positive ACPA. Of the 36 AATD subjects with at least one positive ACPA none had RA either at baseline or during follow up

(mean duration of follow up since serum collection was 9 years). However, 4 had autoimmune thyroid disease at baseline, 1 had ulcerative colitis (UC) at baseline and a further individual developed UC during follow up. Amongst the 22 ACPA positive individuals with COPD 3 had RA. None have completed 1 year of follow up to date.

DISCUSSION

The data presented here shows a clear relationship between circulating anti-elastin antibody levels and smoke exposure, and no relationship between these antibodies and clinical phenotype after smoking is taken into account. Conversely ACPA, which theoretically are generated after smoke exposure, did not show any significant relationship to smoke exposure in our cohorts, and less marked differences between groups.

One study has reported high anti-elastin antibody levels in COPD previously[9], which we were unable to confirm. This study concerned a group with emphysema, thus it could be argued that our analyses should have been restricted to those with CT proven emphysema if we wished to confirm their findings. We did not exclude subjects who had not been scanned or who did not have emphysema as our primary objective was to compare levels between groups whose elastin breakdown might be expected to differ[6], and against smoke exposure, not to reproduce the work of Lee *et al*. However, secondary analyses comparing antibody titre between individuals with and without emphysema in AATD did not reveal a significant difference. It should be noted that differences in titre seen by Lee *et al* between groups were small, and that this observation was a small part of their study, which examined anti-elastin autoimmunity more specifically, using lung tissue to assess anti-elastin antibody complexes, and T cell responses to elastin peptides[9]. Our results, in direct contrast to theirs, showed anti-elastin antibodies were higher in those with better lung function and in healthy individuals. This implies that the presence and level of circulating anti-elastin antibody is not

a key feature of COPD, an observation recently reported in another study[11], and may even fall as disease progresses. We hypothesise that this might be due to ongoing, or increasing, antibody consumption by binding in the lung. This is consistent with the increased antibodies seen in lung tissue by Lee *et al[9]*, although longitudinal studies involving both lung tissue and circulating samples would be required to definitively answer this question. Antibody levels do not rise with cessation of smoke exposure, consistent with the perpetuation of inflammation and lung damage in COPD seen after this time, with measureable disease progression in many subjects [20].

None of the studies of anti-elastin antibodies to date have taken effects of smoking into consideration in the analyses pertaining to antibody levels, despite the theory that anti-elastin antibody generation occurs secondary to smoke induced elastin liberation in the lung [9]. The data presented here shows a clear difference in anti-elastin antibody levels between smokers and those who have never smoked in AATD, and a relationship between pack years smoked and antibody levels. There was also a threshold level of smoke exposure apparent, a feature identified in another large cohort of AATD subjects recently, which contributed to their predictive model for FEV1[21]. Smoke exposure was common in AATD, higher in usual COPD, and absent in controls thus between group comparisons of antibody levels, which appeared highly significant initially, required adjustment for this exposure. After such adjustment not only was the relationship with FEV1 lost within AATD, but all inter-group comparisons ceased to be significant. This, together with the recent suggestion that smoking may be a risk factor for autoimmunity [12], is important to note in future studies of the prevalence of other auto-antibodies in COPD and control populations. Subjects with AATD may be more sensitive to other environmental influences on lung function, as shown by relationships between outdoor air pollution and lung function in this group [22]. We surmised that differences in elastin breakdown and subsequent anti-elastin generation might still occur

between non-smokers with and without AATD for this reason. This was not confirmed, perhaps because the effect sizes of agents other than cigarette smoke are small in both groups [22-23].

Although differences in ACPA titre were seen between COPD, AATD and controls, the differences were less marked than with anti-elastin antibodies and less clearly related to smoke exposure. This is the first study, to our knowledge, of these antibodies in lung disease, and our findings will require further validation, nevertheless it is interesting to consider their significance. The pathogenic role of ACPA in RA is thought to involve complement activation[24] and alteration of the T cell repertoire in the joint[25], with reaction to self as a result. Our data would suggest that this may be organ specific, occurring just in the joint despite the fact that any protein containing citrulline could be a target for ACPA. Alternatively the effect on synovium could depend upon a further genetic or environmental influence, given that ACPA positive individuals here did not always exhibit RA (or indeed other autoimmune diseases). Shared epitope alleles, contained within class II of the human leukocyte antigen system *(HLA-DRB1*04, *01 and *10)*, are important in determining risk of RA[26] and ACPA status[27]. Recent data suggests that smoking is a key feature of determining ACPA positive disease risk in the presence of these alleles[27-28], consistent with this hypothesis. ACPA may occur up to 10 years before clinical features of RA appear [29], the titre relating inversely to the time until diagnosis[30], such that highly positive individuals are likely to be diagnosed soon. It will therefore be of importance to follow up positive individuals in the cohort, particularly since the incidence of positive anti-CCP antibodies exceeded that reported in healthy controls[29] fivefold in AATD and eightfold in usual COPD. The majority of AATD subjects have, however, been followed up for over 8 years since their baseline assessment and blood sampling, making it unlikely that many new cases will be found in this group. In the usual COPD cohort high ACPA levels were more

common than in AATD, and more common in both groups than in the general population. Intuitively the role of smoking in determining pulmonary citrullination[14], and subsequent antibody formation, might have been thought responsible. Again, however, our data suggested that in AATD ACPA levels were lower in heavier smokers, albeit not statistically different. Since there was no significant dose response effect adjustments of ACPA level for lifetime lack of smoke exposure analyses could not be carried out to determine if differences between AATD, COPD and healthy individuals persisted after adjusting for this feature. Larger cohorts may be required for further clarification of this issue. Further study of citrullination in smokers, is also warranted to clarify the mechanism of antibody generation and relationship to ACPA suggested here.

Autoimmunity is a current topic of debate in COPD pathogenesis, since some characteristic features of autoimmunity are present. Firstly, a lymphocytic infiltrate in the lung occurs, with increasing pulmonary CD4+ and CD8+ T cell numbers relating to worsening disease[31-32]. Auto-antibodies to pulmonary epithelium have also been demonstrated [33]. Furthermore, oligoclonal T cell populations have been demonstrated in the lung, suggesting accumulation due to antigenic stimulation[34]. Evidence from animal models also supports a role for autoimmunity in COPD. Specifically, the transfer of pathogenic CD4+ T cells from rats with emphysema and anti-endothelial cell antibodies results in the development of emphysema in healthy animals[35] and mice injected with elastin peptides develop both anti-elastin antibodies and a bronchaoalveolar lavage (BAL) fluid profile similar to that seen in COPD[36-37]. The initial report of elevated anti-elastin antibodies by Lee *et al* [9] has now been refuted by two small studies (in addition to the data presented here) – the first in a mixed disease cohort[10], and the second in pure disease cohorts, but with small numbers in each[11]. Indeed in the latter study the authors noted that antibody levels were higher in healthy subjects, consistent with the data we report here.

Autoimmune processes are generally thought to be more common in female subjects, hence we undertook some gender specific analyses – again no differences were seen. Taken together the results suggest that anti-elastin antibodies are unlikely to be a useful disease marker in COPD, and if autoimmunity exists directed against elastin circulating anti-elastin antibody titres may be of less importance than local levels in lung tissue.

Our study is weakened by the absence of a healthy smoking group for comparison to the usual COPD group, although comparisons within AATD of healthy individuals and those with lung disease, controlled for smoking, act as a suitable surrogate for this. We also have not tested for IgM, IgA and other antibody subtypes for anti-elastin. This feature was investigated by a previous group, whose findings concurred with ours in terms of between group comparisons, such that we felt it was unnecessary to repeat their experiments[11].

In summary our data show a clear relationship between smoke exposure and anti-elastin antibody levels, and no difference between AATD, COPD and healthy individuals after adjustment for this feature. This suggests that circulating anti-elastin levels cannot be used as a disease marker and that future study of the autoimmunity hypothesis may need to be directed to antibody complexes in lung tissue rather than circulating antibodies.

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Competing interests

The authors declare they have no competing interests.

Figure legends

Figure 1: Mean anti-elastin antibody levels stratified by group

The chart shows the mean (SE) of circulating antibody comcentration in each group. Healthy subjects exhibited significantly higher conentrations than those with COPD ($p=1x10^{-5}$), and AATD (p=0.008). AATD subjects also exhibited higher concentrations than usual COPD subjects ($p<1x10^{-5}$). The values shown are unadjusted for smoke exposure.

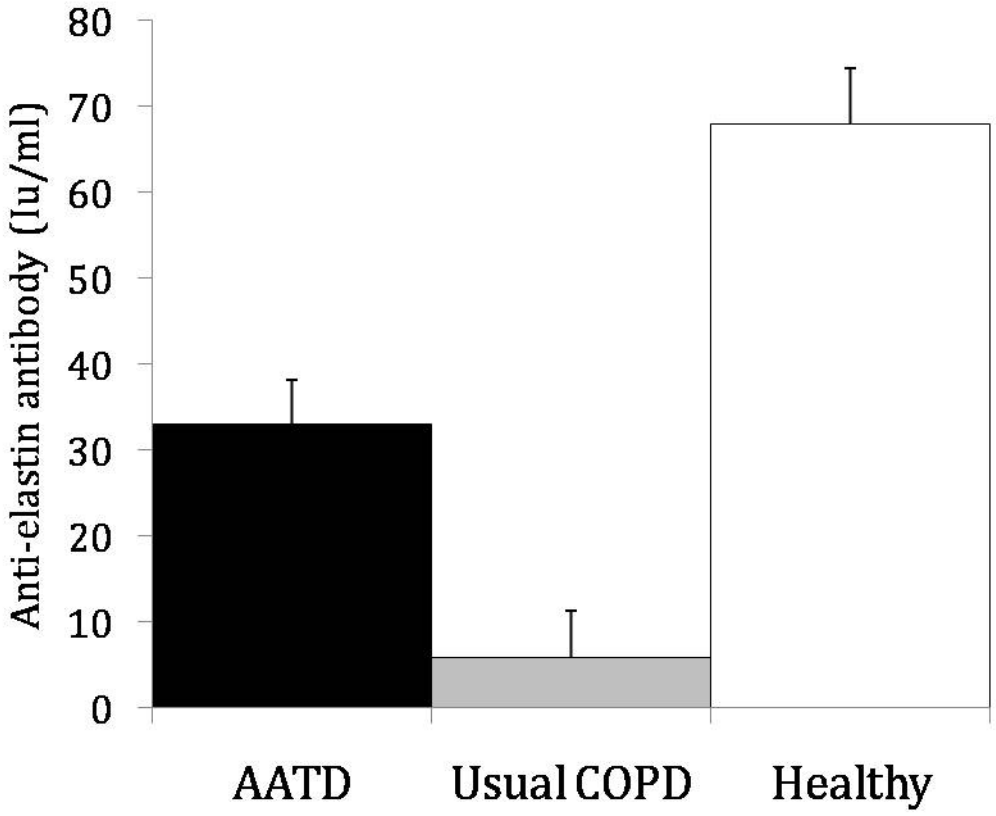


Figure 2: Anti-elastin antibody concentrations are higher in those with normal lung function in AATD

The chart shows the relationship between FEV1and anti-elastin levels in AATD which is significant; those with FEV1>80% predicted and no evidence of airflow obstruction had higher levels (p=0.032)

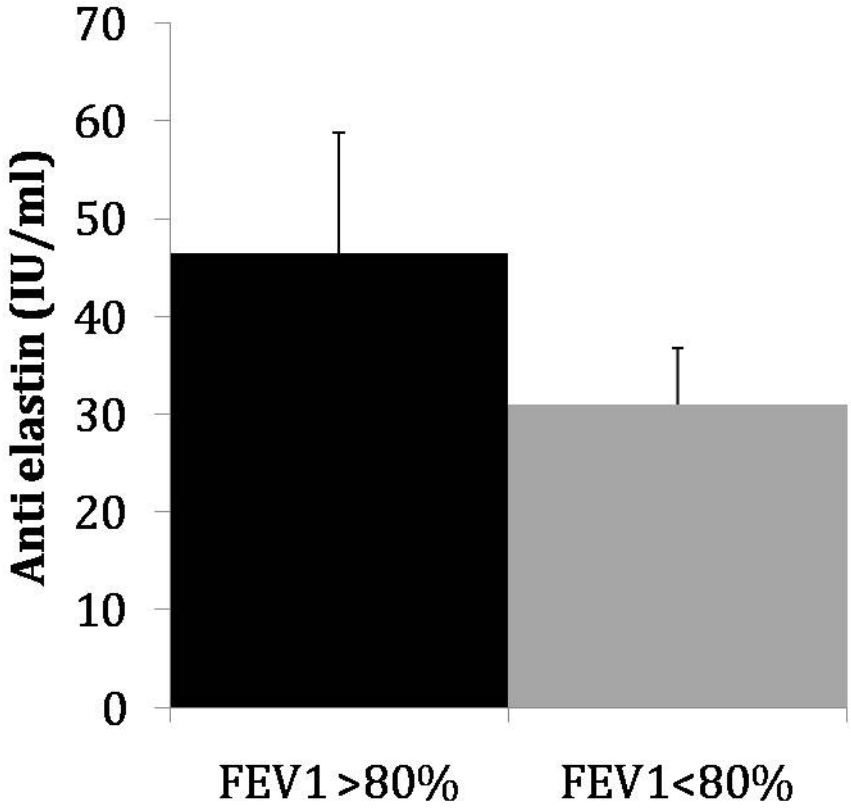


Figure 3: Anti-elastin antibody concentrations relate to smoke exposure in AATD

(a) The scatterplot shows the relationship between smoke exposure and anti-elastin antibody titre. Whilst it is statistically significant as a linear relationship (r=-0.199, p=0.001), visually it appeared to be driven by low level smoking. (b) Smoke exposure was divided in categories according to pack years smoked. Anti-elastin antibodies then appeared to exhibit a threshold level at 10 pack years. (c) Comparisons of antibody levels were made using 10 pack years to stratify the population. Antibody levels were higher in those who have smoked less than 10 pack years (p=0.004).

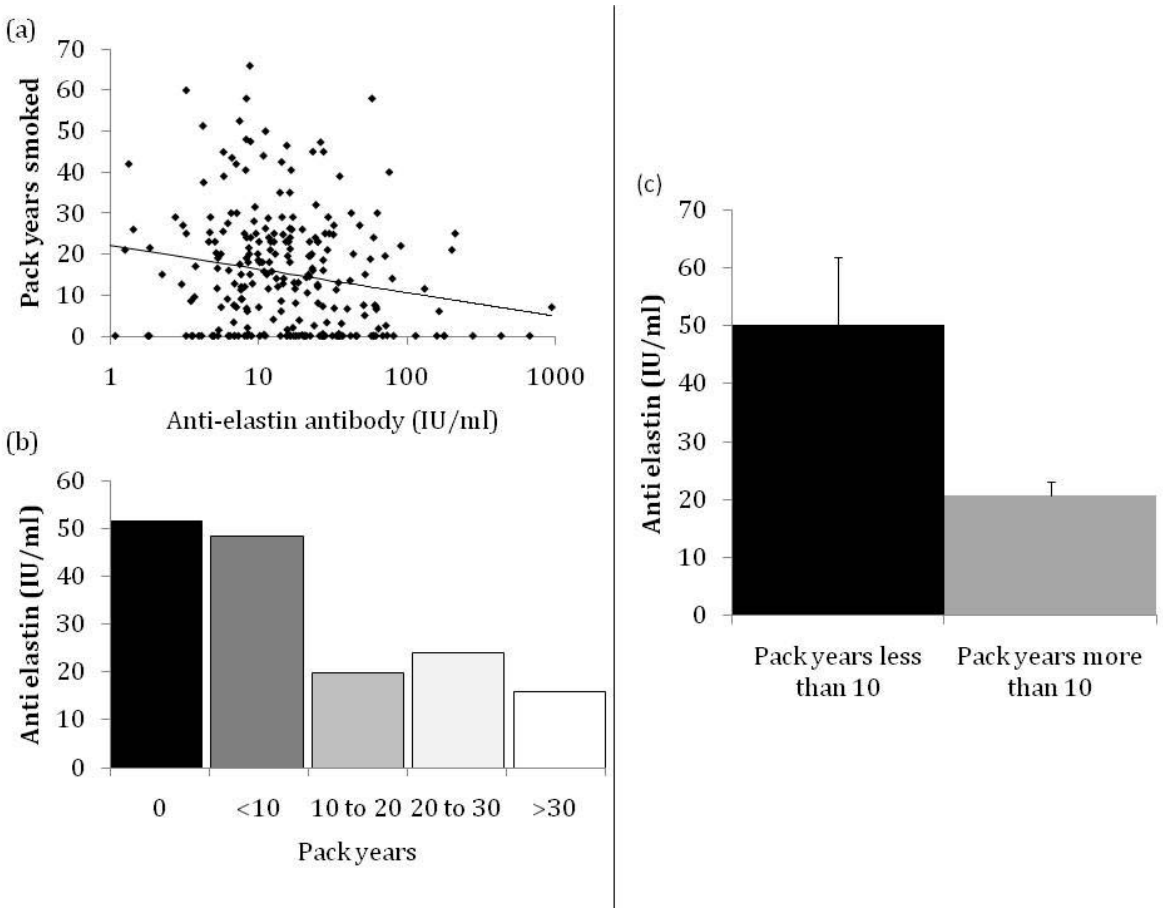


Figure 4: ACPA concentrations stratified by group

The chart shows the mean anti-CCP and anti-MCV concentration in the 3 groups studied. Anti-CCP titre was significantly higher in usual COPD than AATD (p=0.002) but no other significant differences were seen.

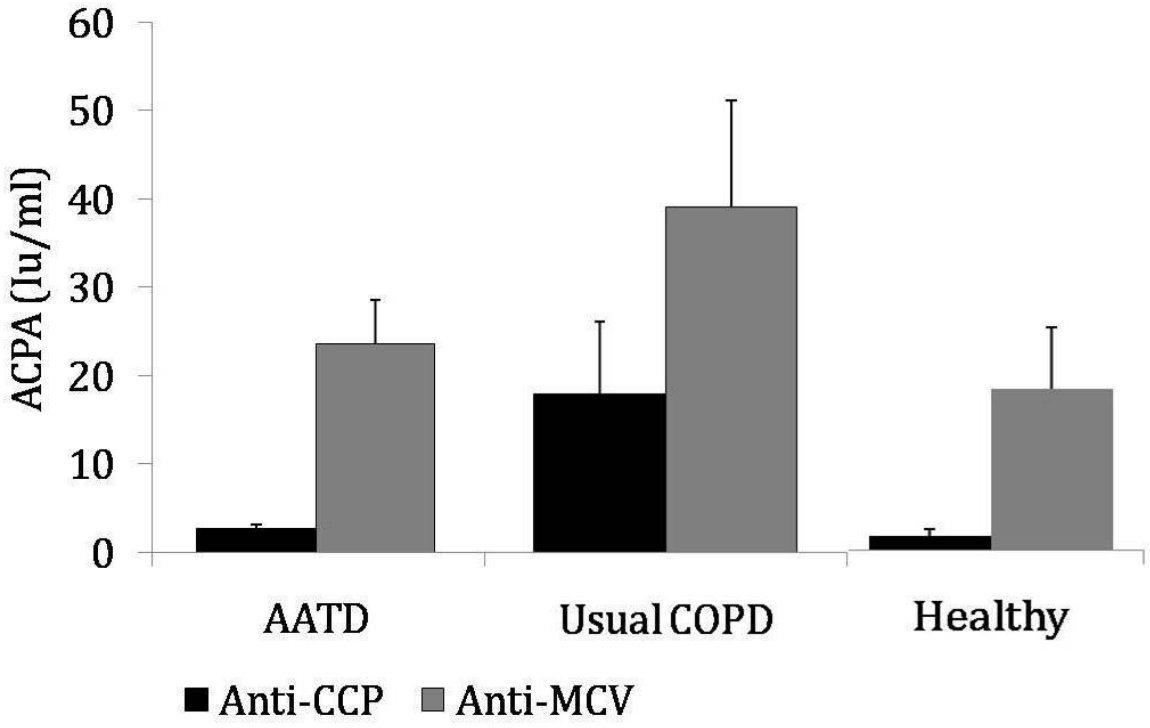
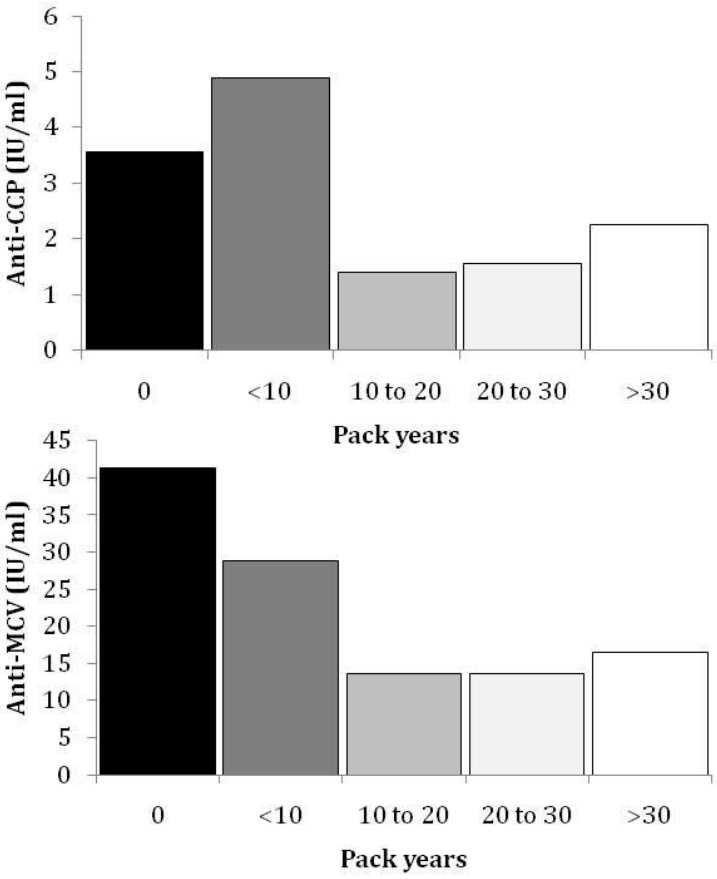


Figure 5: ACPA relationships to smoke exposure in AATD

The two charts show ACPA levels stratified by smoke exposure categories. Although a threshold was suggested visually at 10 pack years this was not significant (anti-CCP, p=0.661 and anti-MCV, p=0.110).


Anti-CCP (IU/ml)
6
5
4
3
2
1
0
0
<10
10 to 20
20 to 30
>30
Pack years

Anti-MCV (IU/ml)
45
40
35
30
25
20
15
10
5
0
0
<10
10 to 20
20 to 30
>30
Pack years

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