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Changes in elastic fibres in the small airways and alveoli in COPD.

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Abstract

Small airways are the major site of airflow obstruction in COPD. This is attributed to loss of elastin in alveoli and fibrosis in small airways. We hypothesized that changes to elastic fibres in alveoli might be paralleled by a similar reduction in elastic fibres in small airways.

We studied tissue blocks from patients who had lobectomy for bronchial carcinoma. Patients were classified as COPD (FEV_1 <80% predicted, $FEV_{1/}VC < 0.7$) or controls ($FEV_1 \geq 80\%$ predicted, $FEV_{1/}FVC \geq 0.7$). Elastic fibres were visualised using Elastic van Gieson staining and the volume/fraction (v/f) of elastic fibres determined as percentage of tissue volume using point counting. Elastic fibre networks were also visualised by confocal microscopy.

The v/f for elastic fibres in alveoli was 18.6% for COPD and 32.8% in controls. In the airways v/f was 14.6% for COPD and 25.5% in controls. FEV_1% predicted was correlated with v/f in both alveoli (r=0.66) and small airways (r=0.73).

The volume fraction of elastic fibres is reduced to a similar extent in small airways and alveoli in COPD and both are correlated with the extent of airflow obstruction. Loss of elastic fibres in small airways may contribute to the development of airflow obstruction in COPD.

Introduction

The small airways are the major site of airflow obstruction in Chronic Obstructive Pulmonary Disease[1]. Emphysema is thought to contribute to this airflow obstruction through the loss of the alveolar attachments to the small airways which in turn leads to the loss of elastic recoil and increased narrowing of the airways[2]. This view has been challenged because some morphometric studies on post-mortem tissue and on tissues obtained at surgery have only shown a weak correlation between the degree of emphysema and measures of airflow obstruction such as FEV_1[3,4]. This has led to the suggestion that remodelling of the airway wall is more important as a cause of airflow obstruction in COPD. A study that used a semi-quantitative score to rate changes in the small airways including goblet cell hyperplasia, squamous cell metaplasia, inflammatory infiltrate in the airway and the amount of fibrosis and muscle in the airway wall did correlate with lung function[5]. A more recent study that studied a larger number of subjects, who had surgical resection of lung tissue, found that volume of tissue in the wall of small airways increased progressively as lung function declined[6]. In these studies there has been no comment on changes in elastin in the small airways.

In COPD the inflammation that occurs is characterised by an increase in $CD8^+$ T lymphocytes and in more severe disease there is also an increase in neutrophils[7]. A similar pattern of inflammation is seen in both the small airways and the alveoli[8]. This led us to wonder if the loss of elastin that has been described in the lung parenchyma[9-11] could also occur in the small airways. If this was the case it could contribute to the narrowing of the small airways in COPD. To test the hypothesis that there is a reduction in elastic fibres in the small airways as well as in the alveoli in COPD we

have examined changes in the volume/fraction of elastic fibres in both the small airways and the alveoli in lung tissue from subjects with COPD and from smokers with normal lung function.

Methods

The study was conducted using archived formalin fixed, paraffin embedded tissues from patients who had one or more lobes resected for bronchial carcinoma. The specimens were identified using the computerized records of the Department of Pathology, Green Lane Hospital. The operations were performed between January 1992 and September 1996. Only blocks of tissue from a site remote from the tumour were used. Many but not all of these tissue blocks were used in a previous study[12]. Further information including smoking history, past medical history, medication and preoperative lung function were obtained from the patient's hospital notes. The patients were classified as control subjects or COPD on the basis of their lung function. The control subjects had $FEV_1 \geq 80\%$ predicted and $FEV_1/FVC \geq 0.7$. The patients with $FEV_1 < 80\%$ and FEV1/FVC < 0.7 were classified as COPD. Patients with a diagnosis of asthma, bronchiectasis or interstitial lung disease were excluded and there were no changes seen in the tissue sections from the included subjects to suggest these diagnoses. Samples were obtained from 26 control and 17 COPD subjects ranging in age from 58 to 90 and 61 to 84 years respectively. Approval was obtained from the Auckland Ethics Committee to conduct the study.

The samples had been fixed in neutral buffered 10% formalin and embedded in paraffin. Staining was performed on 4 μm sections mounted on glass slides. Slides were dewaxed and rehydrated through a xylene and graded alcohol series. Elastic fibres were visualised by Elastic van Gieson staining. The slides were incubated in a solution containing 0.5g hematoxylin powder, 10 ml 95% ethanol, 4 ml 10% ferric chloride and 4 ml Verhoeff's iodine for 15 minutes. Following incubation, sections were rinsed briefly in tap water, differentiated in 2% ferric chloride, then rinsed

thoroughly in tap water before rapid incubation (5 seconds) in van Gieson mixture (360 ml picric acid, 40 ml 1% acid fuchsin, 400 ml distilled water). The slides were then rapidly dehydrated through a graded series of ethanol and yxlene, mounted and coverslipped. With the Elastic van Gieson stain elastic fibres appear black. In order to standardise elastin staining for comparative morphometric analysis, the elastic laminae of arteries were used as an internal control for each slide.

The volume fraction (v/f) of elastic fibres was determined as a percentage of total tissue volume by point counting[13]. The analysis was performed by an investigator (PC) who was blinded to the patient's lung function. The sections were examined under a light microscope at 40x magnification linked by a video camera to a computer screen. The on-screen magnification was 400x. Alveoli, alveolar rims and small airways (<2 mm diameter) were studied. A 100 point grid (covering 2,500 sq microns) was overlaid on each area of interest on the computer screen. The volume fraction percent was calculated from the number of times a darkly stained elastic fibre registered as a hit (i.e. fell on the grid). This was expressed as a percentage of the total number of times that alveoli walls, alveolar rims or airway walls registered as a hit on the grid. For each patient 10 sites were randomly sampled for alveoli and alveolar rims and 4 sites for the airway wall. For each patient the mean number of alveolar tissue points sampled was 420 ± 118 (SD) and for alveolar rim regions 520 ± 98 (SD). For airway wall the four sites were randomly sampled according clock face positions of 12, 3, 6 and 9. For the analysis the airway wall was separated into inner and outer layers. The inner layer was the area between the basal lamina of the epithelial cells and the smooth muscle. The outer layer was the area between the smooth muscle and the outer perimeter of the adventitia. The mean number of tissue points sampled for

each airway wall was 625 ± 120 (SD). Airway wall thickness was measured from the basal lamina to the outer margin of the adventitia at the four random sites. Luminal diameters of the airways were determined on a MacIntosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb/info.gov.nih-image/.). Minimum and maximum diameters were averaged for each airway to avoid overestimating diameters of airways cut slightly tangentially. The limited number of tissue blocks available for each patient meant that suitable airways were not found for every individual. A total of 27 airways were identified from 13 of the control subjects and 30 airways from 11 of the COPD subjects.

Thick sections (~150μm) of lung tissue from three COPD patients and two control subjects were also analysed under a Leica TCS SP2 confocal microscope to visualise elastin fibres and elastic fibre networks in three dimensions. Sections, dewaxed, and rehydrated, were mounted in Dako fluorescent mounting medium (S3023) and optical sections (70 for alveoli0; 25 for airway wall) acquired with a 515 nm wavelength source to detect the autofluorescence of elastin. Stereoscopic and projection views were constructed from the optical slices. Areas of control and COPD lung tissue sampled were chosen by overlaying sections with a 9 x 9 grid and selecting grid points using the last two numbers of random numbers from a random number table.

Results are expressed as mean ± standard deviation. Data were analysed by Students T test (between groups) and by least squares linear regression with FEV_1 % predicted, FVC % predicted or FEV_1/FVC as the dependent variable. P values of <0.05 was taken as significant.

Results

The characteristics of the subjects are shown in Table 1. The subjects with COPD were similar to the controls with respect to age, sex and smoking history but as one would anticipate had lower lung function. FEV_1 was 62 ±8% (mean ± SD) of predicted in the subjects with COPD compared with 94 ±11% for the controls. Only 5 of the subjects with COPD and none of the controls were on treatment with inhaled bronchodilators and/or inhaled steroids.

Figure 1 shows elastic fibres in sections of alveoli and airway wall, stained with Elastic van Gieson (Fig 1a-d), and visualised by fluorescent confocal microscopy (Fige-h). Elastic fibres were more evident in alveoli and airway wall from control subjects than in individuals with COPD. The confocal images were constructed from serial images of thick (150 μm) sections and show the elastic fibre networks, which display autofluorescence, and the loss of elastin in both alveoli and airway wall in COPD. The concentration of elastin around the entrance to or the mouths of alveoli (alveolar rim region) of control lung was noticeably diminished in COPD lung. The confocal images also showed punctate autofluorescence of erythroctes stacked within the capillaries. Red-green off-set images were also constructed to show the network in three dimensions (data not shown).

The volume fraction (v/f) for elastic fibres, determined by point counting, was reduced in the COPD patients compared with the control patients in the alveolar walls, alveolar rims and airway walls. The mean v/f for elastic fibres in the alveolar walls was 18.6% ± 5.55 (SD) in COPD compared with 32.8% ± 7.66 in controls

($p<0.001$). Despite differences in elastic fibres there was no difference between the COPD and control samples in the volume fraction of the total alveolar wall tissue.

Similar findings were observed in the alveolar rims and the airway walls. In the alveolar rims the v/f for elastic fibres was 31.5% ± 6.25 in the COPD samples and 39.0% ± 7.93 in the control samples ($p<0.002$). For the airway walls, the results were analysed for the inner and outer layers. For inner layer the v/f for elastic fibres was 17.0% ± 4.09 for COPD and 27.8% ± 7.13 for controls ($p<0.001$). In the outer layer the corresponding values were 12.3% ± 6.58 for COPD and 22.7% ± 5.77 for controls ($p<0.001$). When the two layers were combined the v/f for elastic fibres was 14.6% ± 4.7 for COPD and 25.5% ± 5.23 for controls ($p<0.001$. Wall thicknesses were not significantly different between the two groups (control = 98.9 μm SD 28.0; COPD = 103.6 μm SD 16.8; $p<0.63$) and neither were luminal diameters (control = 0.70mm SD 0.46, range 0.19 – 1.99mm; COPD = 0.64mm SD 0.36, range 0.26 – 1.79mm; $p<0.59$). No difference was seen in the v/f for elastic fibres in airways with a diameter of <0.5mm compared with those with a diameter ≥ 0.5mm.

Figure 2 shows the relationship between FEV_1% predicted and the v/f for elastic fibres in the alveoli and airways respectively. Figure 3 shows the relationship between FVC% predicted and v/f for elastic fibres while Figure 4 shows the relationship between FEV_1/FVC and v/f for elastic fibres. The FEV_1% predicted ($r=0.66$, $p<0.001$), FVC% predicted ($r=0.41$, $p<0.001$) and FEV_1/FVC ($r=.056$, $p<0.001$) were all related to the v/f for elastic fibres in the alveoli. In the airway walls (Figure 3) there was also a significant relationship between FEV_1% predicted, FVC% predicted, FEV_1/FVC and v/f for elastic fibres regardless of whether the analysis was for the

inner or outer layer or the combination of both layers. For the combination of layers the correlation coefficient for FEV_1% was r=0.73 ($p<0.001$), for FVC% predicted it was r=0.56 ($p<0.001$) and for FEV_1/VC it was r=0.51 ($p<0.001$). There was however no correlation between the number of pack years smoked and v/f for elastic fibres in either the alveoli or the small airways.

Figure 5 shows the relationship between the v/f for elastic fibres in the alveoli and the airway walls. The two were associated (r=0.6, $p<0.01$). Subjects with lower with a lower v/f for elastic fibres in the alveolar wall tended to have a lower v/f for elastic fibres in the small airways.

Discussion

The elastic fibres in the alveoli of patients with emphysema are abnormal and morphological changes are seen that include fragmentation of elastic fibres[9, 14-16]. Despite this early studies that tried to quantitate the amount of elastin in lung tissue from patients from emphysema using biochemical assays did not find a reduction in the amount of elastin[17,18]. These studies used gravimetric assays and the reliability of these approaches has been questioned[9]. Subsequent studies that have measured desmosine and isodesmosine, amino acids that are specific to elastin, as a proportion of the total connective tissue in the lung and these studies have found that the amount of elastin is reduced in emphysema[9-11]. There are, however, few studies that have used morphometric measurements to quantify elastic fibres in pulmonary tissue from patients with COPD. Using histochemistry and point counting we were able to confirm that there is a reduction of elastic fibres in the lung parenchyma with a decrease in the volume fraction of elastic fibres from 32.8% to 18.6%. Vlahovic and colleagues also used a morphometric approach[19]. They studied surgically resected lobes from 7 individuals, with mean FEV_1 of 77% predicted and FVC of 94% predicted, and found an increase in the volume of the alveolar septum with a parallel increase in elastic fibres. The difference with our study may be because their subjects only had very mild impairment of lung function while our subjects had more severe COPD with a mean FEV_1 of 62% of predicted and FVC of 74% predicted.

In our study we found that elastic fibres were reduced not only in the alveoli but also in the small airways in COPD with a reduction in the volume fraction of elastic fibres from 25.5% to 14.6%. This is similar in magnitude to the changes in elastic fibres that we observed in the alveolar walls in COPD.

A potential weakness of our study is that the specimens were not inflated in a standard fashion before fixation and this meant that it was not possible to calculate the average distance between the alveolar walls or alveolar surface area to volume. Nonetheless there was a clear difference in lung function between the two groups and we are confident that we are comparing a group with mild to moderate COPD (GOLD Stages 1 and 2) with a group with normal lung function (GOLD Stage 0). We demonstrated a reduction in elastic fibres using point counting. At the same time we found that there was no difference between the subjects with COPD and the control subjects in the thickness of the airway wall or in the volume fraction of the alveolar wall tissue indicating that the reduction in elastic fibres was not an artefact caused by an increase in the thickness of the alveolar or airway walls. In addition the reduction in elastic fibres was also seen by confocal microscopy where the elastin networks were visualised in three dimensions. Ideally we would also have liked to study a group of non-smoking controls but tissue from such a group of individuals was not available to us. However the patients with COPD and the control subjects in our study were very well matched not only for age but also for smoking history which makes us confident that the differences that we observed between the two groups were a consequence of COPD and did not simply reflect different smoking exposures. We do have to acknowledge that the specimens of tissue were not chosen by a method that ensured that this was a truly random sample and we cannot exclude the possibility that this may have influenced the results.

We saw changes in the volume/fraction of elastic fibres even though there was no difference between COPD and control samples in the volume fraction of the total alveolar wall tissue. This suggests that changes in the elastic fibres may occur

relatively early before evidence of emphysema is marked. Our study does not address the question of why there is a reduction in elastic fibres in the airways and alveoli of patients with COPD but it may be due to increased formation of elastolytic enzymes such as matrix metalloproteinase-9 and and matrix metalloproteinase-12 in patients with COPD compared with healthy smokers and there are a number of studies that provide support for this idea[20,21].

Descriptions of the pathology of COPD often contrast the loss of elastin and destruction of the alveolar walls in the lung parenchyma with the fibrosis in the small airways. Our finding that there is a reduction in elastic fibres in both the small airways and the alveoli suggests that similar pathological changes are occurring in the airways and in the lung parenchyma. This would not be entirely surprising because the inflammatory changes are similar, with increases in CD8 T lymphocytes and macrophages, in both the airways and the alveolar wall[7,8]. Parallel changes in the airways and alveoli may occur not only with elastin but also with collagen. In our study we did not assess changes in collagen but other studies have reported that increases in collagen occur in the lung parenchyma[11,22]. A number of studies have reported an increase in fibrosis in the small airways in COPD[5,23]. There has been less quantitative research on the changes in collagen in the small airways in COPD but a recent report found that there was an increase in collagen deposition in the small airways of patients with GOLD Stage 2 disease compared with controls[24]. In contrast there was less collagen in the small airways of patients with GOLD Stage 4 disease compared with subjects with normal lung function[24].

We found a correlation between the volume fraction of elastic fibres in the alveoli and FEV_1 % predicted and FEV_1/FVC. These findings are consistent with the idea that in patients with COPD the loss of elastic tissue in the parenchyma leads to airflow obstruction. The decrease in expiratory flow rates in COPD are attributed to a reduction in alveolar driving pressure because of loss of elastic recoil and to increases in airway resistance because of loss of elastic airway support[25,26]. A decrease in elastic fibres in the alveoli will contribute to the reduction in elastic recoil while a loss of alveolar attachments to the airways will mean loss of support for the small airways and greater narrowing of the small airways in expiration.. Interestingly we saw a similar relationship between the volume fraction of elastic fibres in the small airways and both FEV_1 % predicted and FEV_1/FVC. This may have just been due to the correlation between the changes in elastic fibres in the small airways and in the alveoli ($r=0.6$, $P<0.1$). Another explanation is that loss of elastic fibres in the small airways has a direct effect on the physical properties of the airways and can cause them to narrow more readily on expiration in the same way as the loss of alveolar attachments does. Parallel observations have been made in the airways in severe asthma. . Mauad and her colleagues performed morphometric studies on the central airways of subjects with fatal asthma and found fragmentation of elastic fibres and a reduction in the content of elastic fibres in the subepithelial portion of the airway wall[27]. In a subsequent study of fatal asthma they reported a reduction in the content of elastic fibres in the adventitial layer of the small airways although they also noted a reduction in alveolar attachments to the small airways but without any evidence of changes in the elastic fibres elsewhere in the alveoli[28]. Although changes in the alveolar attachments could contribute to the loss of elastic recoil observed in patients with severe asthma, Mauad and co-workers speculated that damage to and loss of

elastic fibres in the airway wall also contributes to early airway closure in expiration. While it is possible that loss of elastic fibres in the small airways in asthma and COPD leads to excessive narrowing of the airways and early closure of airways on expiration a word of caution is necessary because our study did not look directly at the elastic properties of the small airways..

Our observation that similar changes to the elastic fibres occur in both the small airways and the alveoli provides further evidence that there are similar pathological changes occur in the airways and in the lung parenchyma in COPD. The only intervention that has so far been shown to slow the progression of COPD is smoking cessation but there is interest in the development of treatments to promote repair in the lungs of patients with COPD[29]. Our findings make it more likely that a treatment that promotes repair in the alveoli will also have beneficial effects in the airways.

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Table 1

Characteristics of Subjects Who Provided Archival Tissue.

	COPD (n=17)	**Controls (n=26)**	***P***
Gender (M:F)	14:3	20:6	NS
Age (years)	65.8 (6.1)	64.9 (9.7)	0.73
Pack years	42.9 (21.9)	44.3 (22.6)	0.83
FEV_1 % predicted	62 (8)	94 (11)	<0.0001
FVC % predicted	74 (11)	92 (14)	<0.0001
FEV_1/FVC %	58 (8)	76 (6)	<0.0001

Values are shown as mean ± S.D.
All of the subjects had a lobectomy for bronchial carcinoma.
Subjects with COPD had an $FEV_1 < 80\%$ predicted and $FEV_1/FVC < 70\%$.
Control subjects had an $FEV_1 \geq 80\%$ predicted and $FEV_1/FVC \geq 70\%$.

Legends

Figure 1

Control (A,C,E,G) and COPD (B,D,F,H) lung sections showing elastic fibres in alveolar (A,B,E,F) and airway walls (C,D,G,H) visualised by Elastic van Gieson stain (A-D) (black fibres) and by fluorescence confocal microscopy (E-H). Alveolar rims are indicated by the arrows in A and B. The confocal images are projected images constructed from serial optical slices from 150μm sections and show autofluorescent elastic fibres (white fibres indicated by white arrow heads) and, in E and F, punctuate autofluorescence erythrocytes in capillaries of the alveolar wall (white arrowhead with black border). Thick fibres in control lung parenchyma (E) mark the rims of the alveoli. COPD lung contains fewer and generally thinner elastic fibres compared to control lung. Magnifications: A,B x150 (inserts of fibres in alveolar walls x300); C,D x300; E,F 130; G,H x200.

Figures 2A and 2B

Relationship between elastic fibre volume fraction (v/f) and FEV_1 % predicted in the alveolar walls (A) ($r=0.66$, $p<0.001$) and the airway walls (B) ($r=0.73$, $p<0.001$). Controls are shown as triangles and subjects with COPD as circles.

Figures 3A and 3B

Relationship between elastic fibre volume fraction (v/f) and FVC % predicted in the alveolar walls (A) ($r=0.41$, $p<0.001$) and the airway walls (B) ($r=0.56$, $p<0.001$). Controls are shown as triangles and subjects with COPD as circles.

Figures 4A and 4B

Relationship between elastic fibre volume fraction (v/f) and FEV_1/FVC % in the alveolar walls (A) ($r=0.56$, $p<0.001$) and the airway walls (B) ($r=0.51$, $p<0.001$). Controls are shown as triangles and subjects with COPD as circles.

Figure 5

Relationship between elastic fibre volume fraction (v/f) in the alveolar walls and the elastic fibre volume (v/f) in the airway walls ($r=0.6$, $p<0.01$).

Figure 1

Figure 2A

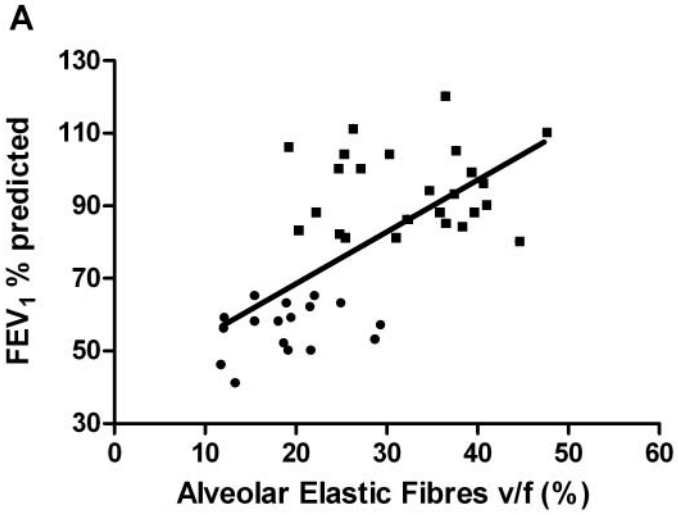


Figure 2B

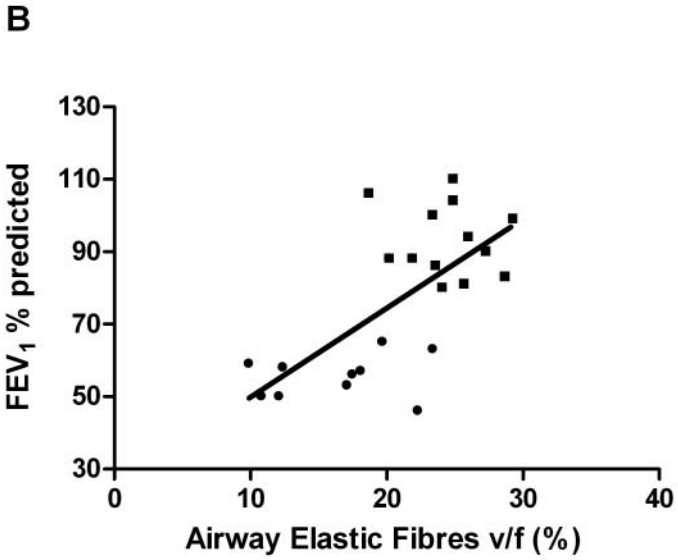


Figure 3A

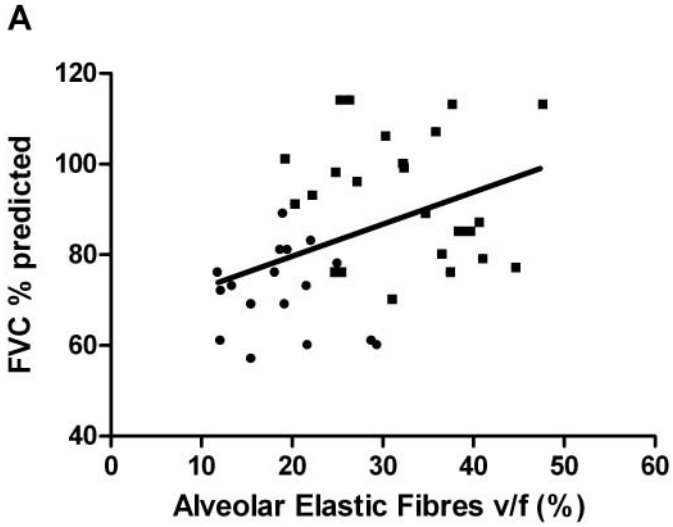


Figure 3B

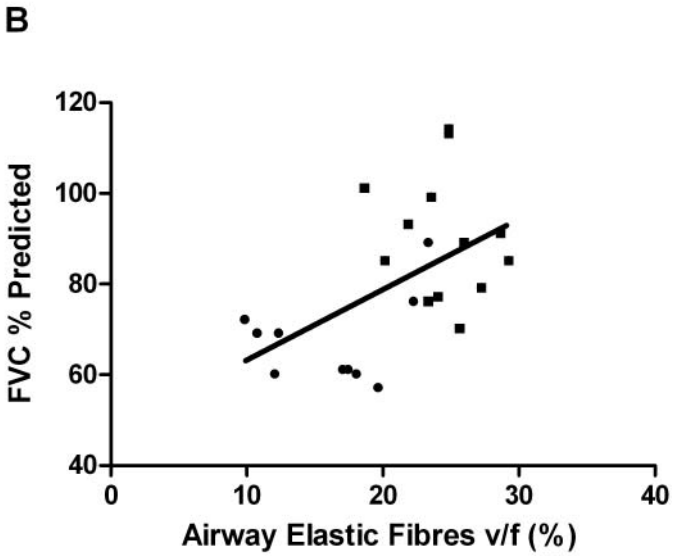


Figure 4A

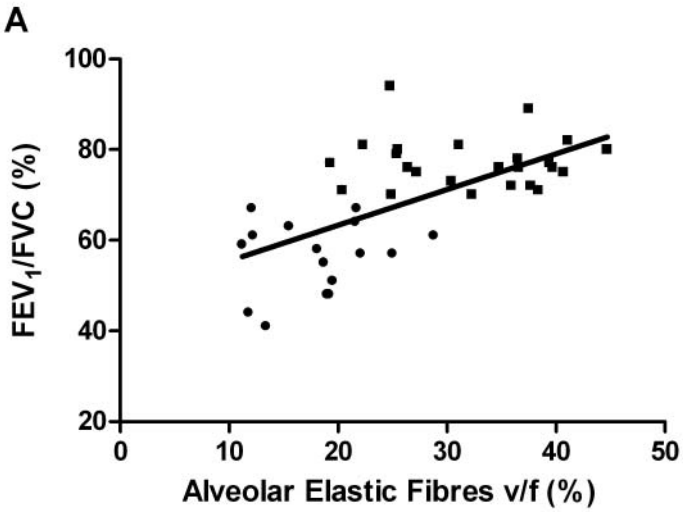


Figure 4B

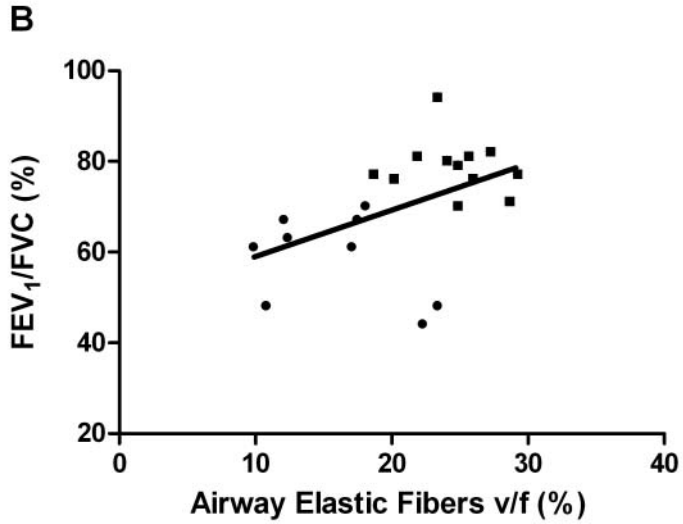
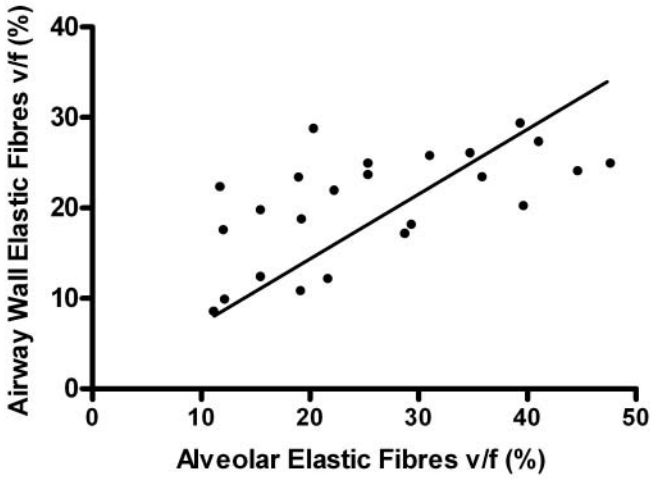


Figure 5


Airway Wall Elastic Fibres v/f (%)
40
30
20
10
0
0
10
20
30
40
50
Alveolar Elastic Fibres v/f (%)