Ultrastructure of the reticular basement membrane in asthmatic adults children and infants

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Abstract (200 words)

Background: Reticular basement membrane (RBM) thickening in asthma is considered the result of subepithelial fibrosis. Thus the RBM in asthma should contain an excess of fibrils identified as interstitial collagen and the ratio of fibril to matrix should be increased above normal.

Methods: Electron micrographs of the RBM were compared with those of interstitial collagen deeper in the bronchial wall using endobronchial biopsies from adult asthmatics (n=10; 18-41years), children with difficult asthma (n=10; 6-16 years), wheezy infants with reversible airflow limitation (n=10; 0.3-2 years) and age-matched, non-asthma controls: 10 adults, 9 children and 9 symptomatic infants with normal lung function.

Results: Fibrils in the RBM were significantly thinner (median width 0.039 [0.03-0.052]μm vs 0.059 [0.048-0.073]μm, $p<0.001$) and fewer fibrils were 'banded' than in the interstitial collagen (median ratio of banded to non-banded fibrils 0.08 [0-0.17] vs 0.22 [0-1.3] $p<0.001$). The ratio of fibrils to matrix in the thickened RBM of asthmatics did not differ from that of their respective controls (median 1.34 [0.63-2.49] vs 1.18 [0.31-2.6] NS).

Conclusion: The ratio of fibril to matrix in the thickened RBM of asthma is as normal and, contrary to what is expected in fibrosis, the fibrils do not resemble those of interstitial collagen.

Introduction

Morphologically, the subepithelial basement membrane in human airways is composed of a basal lamina (sometimes referred to as the "true" basement membrane), to which the epithelium is attached. Extending externally from the basal lamina is a lamina reticularis, (henceforth referred to as the reticular basement membrane). Immunohistochemistry demonstrates that type IV collagen is a major component of the basal lamina. [1] However, without immunostaining, the basal lamina can be visualised only by electron microscopy because its width (~80nm) is below the resolution of the light microscope. By contrast the reticular basement membrane (RBM) is considerably thicker (~ 4 μm in healthy adults) and can be seen by light microscopy. The RBM is characteristic of normal humans and other primates, such as monkeys. [2] Histological sections stained with haematoxylin and eosin demonstrate that the RBM is present in all normal healthy individuals, but also that it is homogenously thickened and hyaline in appearance in asthma. [3,4]

The abnormal thickening of the RBM begins early in asthma, as early as 4 years of age, and it is already thickened and maximally so in children aged 6-16 years with severe asthma. [5,6,7]

As immunohistochemistry demonstrates the presence of epitopes for collagen sub-types I, III and V in the RBM, the abnormal thickening of this layer in asthma has been thought to result from a fibrotic process sometimes described as "subepithelial fibrosis". [8] However, these epitopes are part of a morphologically heterogeneous family of glycoproteins that include both amorphous and fibrillar forms, collectively termed collagen. Fibrosis, as is seen in fibrotic lung diseases, is defined on the basis of the accumulation of abnormal amounts of interstitial collagen, or scar tissue. Whilst the process of interstitial pulmonary fibrosis (IPF), in the lung parenchyma, is considered irreversible, thickening of the epithelial RBM in asthma can be reversed, at least partially, following anti-inflammatory treatment [9] or following removal of the asthmatic from an occupational cause. [10] Until now, there has been no objective quantitative examination of this key aspect of remodelling in asthma.

Interstitial collagen, and that associated with pulmonary fibrosis, is composed of fibrils of defined width, each with an ultrastructurally characteristic pattern of periodic banding. [11] We have used these criteria to test the hypothesis that RBM thickening in asthma, in adults and children, is the result of fibrosis and would therefore have an increased ratio of fibril to matrix in which the fibrils would have the ultrastructural characteristics of interstitial collagen. The aim of our study was to compare the ultrastructural appearance and width of RBM fibrils to those of interstitial collagen, deeper in the bronchial wall. The ultrastructural features and the ratio of fibrils to matrix were compared in adult and paediatric asthmatics and wheezy infants with reversible airflow obstruction, to age-matched controls. In order to confirm the proposition that interstitial collagen in the bronchial wall and collagen in

IPF have a similar ultrastructure, we also looked at the fibril width and banding in lung biopsies from 5 adult cases with cryptogenic fibrosing alveolitis (CFA).

Methods

Subjects

Adults: Ten non-smoking adults (median age 27 [range 18-41] years) with mild, steroid naive asthma were compared to 10 healthy, non-atopic, non-smoking controls, (median age 33 [range 21-42] years). The details of these patients have been described previously, [3] and are summarised in table 1. Collagen ultrastructure in open lung biopsies from 5 adults with histologically diagnosed CFA was also assessed: clinical details are summarised in table 2.

Children: Ten children (median age 10 [range 6-16] years) with difficult asthma and 9 non-asthmatic paediatric controls (median age 11 [range 7-16] years) undergoing bronchoscopy for other respiratory indications, as previously described. [5] Difficult asthma was defined as persistent symptoms requiring rescue bronchodilator therapy > 3 days per week, despite > 1600 micrograms per day of inhaled budesonide (or equivalent), and long acting β_2 agonists, and/or regular oral steroids.

Infants: Asthmatic infants, as previously described. [12] Asthma in this group was defined as persistent symptoms of wheeze and/or cough and reduced airways conductance with bronchodilator reversibility. Lung function was measured by total body plethysmography. The infant controls were also symptomatic, but had normal lung function. Ten asthmatic infants (median age 12 [range 4-24] months) and 9 control infants (median age 11.5 [range 3-24] months) were included.

Bronchoscopy and endobronchial biopsies

Flexible bronchoscopy was performed in the adults and children, and endobronchial biopsies (EB) were taken from the sub-carinae of the right lower lobe. Details of the procedures have been described previously for both the children [5] and adults. [3] Rigid bronchoscopy was performed in the infants under general anaesthetic as previously described. [12] Informed consent was obtained from the adults and the parents of the infants and children to use the EB for research purposes.

Biopsy processing

Biopsies were fixed in 2.5% glutaraldehyde in 0.05-M sodium cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide (in the same buffer), and dehydrated and embedded in epoxy resin (Araldite). Plastic sections (1μm thick) were stained with alkaline toluidine blue. An area of the section that contained epithelium, reticular basement membrane and subepithelium, was selected. Ultra-thin sections (70nm) were cut, placed on high transmission, 200 mesh thin-bar copper grids and stained with uranyl acetate and lead citrate. Micrographs were obtained in a Hitachi H7000 (Nissei Sanyo, Tokyo, Japan) transmission electron microscope.

Quantification

From each ultra-thin section, 2 micrographs were taken of areas of RBM immediately below the basal lamina and 2 were taken at random from subepithelial areas deep to the RBM and at least 100μm deep to the basal lamina, the latter to represent areas of interstitial collagen. Each micrograph was taken at a magnification of x15000. Micrographs were coded and assessed by a single observer who was blind to the patient details and the area of the biopsy that had been photographed. The technique

of point-counting morphometry was used to assess fibril banding and ratio of fibril:matrix. A sampling grid of points, arranged 1mm apart in a triangular array was used. [13] The hexagonal area associated with each point representing approximately $0.75\mu m^2$. Four randomly selected fields with at least 200 points in each field were counted on each micrograph. [14] A count was made of whether each point landed on a fibril or matrix. Furthermore, if the point was on a fibril, a count of whether the fibril appeared banded or not at any position along its length was made (figure 1). Points landing on the border of fibril and matrix were counted separately, and were divided equally between fibril and matrix counts in the final analysis. To assess collagen fibril diameter, four fields each of an area representative of RBM and interstitial collagen were photographed for each patient (x45000). All micrographs were developed in a similar manner to ensure the contrast remained similar. Five collagen fibrils per field were selected randomly and the width of each fibril was measured using Image Pro 4 software with a threshold autotracer. Twenty fibrils were measured in areas representing RBM and interstitial collagen, and the results expressed as a mean.

In the CFA patients, collagenous areas of lung parenchyma with preserved alveolar architecture were selected, and electron micrographs and measurements were made using the same methods as described above.

Statistical Analyses

A ratio of banded:non-banded fibrils was calculated for the RBM and interstitial collagen, and a ratio of fibrils:matrix was calculated for the RBM in each age group. Nonparametric tests were applied to test for inter-group differences. Comparison between all groups was made using the Kruskal-Wallis test, followed by a Mann-

Whitney U test, if a significant difference (p<0.05) was found. The Bonferroni correction was introduced for multiple comparisons. Variability of counts was calculated using the percentage coefficient of variation (%CV). Data were analysed using Statistical Package for the Social Sciences (SPSS version 11.5).

Variability

The intra-observer repeatability of performing 3 separate counts of fibril banding on the same micrographs on different occasions was <5%. Intra section variability, assessed by taking up to 6 micrographs from a region, and by calculating the cumulative mean of counts of the micrographs, revealed that 2 micrographs provided data representative of the fibril banding. The %CV for ratio of fibril:matrix measured on the same micrograph on separate occasions was 2.4% for RBM and 2.7% for interstitial collagen.

Results

Two features of fibril ultrastructure were evaluated: fibril width and banding (figure 1). These features in RBM and interstitial collagen were compared in all age groups.

Fibril width in the RBM and interstitial collagen

Values for fibril width were significantly less in the RBM compared to interstitial collagen in all age groups in both asthma and controls. Infants: RBM fibril width (median [range] μm) 0.047 [0.037-0.05] μm vs interstitial collagen fibril width 0.059 [0.048-0.069] μm, p < 0.001. Children: RBM fibril width 0.036 [0.031-0.042] μm vs 0.054 [0.048-0.069] μm, p < 0.001. Adults: RBM fibril width 0.036 [0.03-0.049] μm vs 0.061[0.048-0.074] μm, p < 0.001 (figure 2).

Ratio of banded:non-banded fibrils

There were significantly fewer banded fibrils in the RBM compared to interstitial collagen in all age groups in both asthma and controls (figure 3).

Ratio of fibrils to matrix in RBM:asthma vs controls

The ratio of fibrils:matrix in the RBM of the adult, paediatric and infant asthmatics was similar to that in the age-matched controls. Ratio in adult asthma vs controls: (median [range]) 0.94 [0.60-1.46] vs 0.64 [0.47-1.1], p = 0.09. Paediatric asthma vs controls: 1.24 [0.63-2.49] vs 1.31 [0.5-2.57], p = 0.96. Infant asthmatics vs controls: 1.67 [0.68-3.08] vs 1.29 [0.31-2.96], p = 0.74 (figure 4).

Ratio of fibrils to matrix in bronchial interstitial collagen: adult asthmatics vs controls

In order to assess whether a pattern of a more classical interstitial fibrosis was present within the submucosa of asthmatic tissue, the ratio of fibrils:matrix in the deeper interstital collagen was compared in the adult asthmatics and controls. There was no difference in ratio of fibril to matrix in the deeper interstitial collagen between adult asthmatics and controls. Ratio in adult asthma vs controls: (median [range]) 1.21 [0.71-1.78] vs 1.18 [0.81-2.15], NS).

Comparison of fibril width and banding in bronchial interstitial collagen and parenchymal collagen in CFA

At all ages, interstitial collagen fibril width was similar to that of the parenchymal collagen of CFA patients. Median [range] collagen width in infants 0.059 [0.048-

0.069] μm, vs children 0.054 [0.048-0.069] μm, vs adults 0.061 [0.048-0.074] μm vs CFA 0.055 [0.043-0.063] μm, p = NS (figure 5). All collagen fibrils in CFA patients were banded.

Comparison of RBM fibril width in asthma vs parenchymal collagen fibril width in CFA

The RBM fibril width was significantly thinner in both the adult and paediatric asthmatics compared to the parenchymal collagen fibril width of CFA patients (median [range] fibril width adult asthma vs child asthma vs CFA; 0.036 [0.03-0.043] μm vs 0.037 [0.031-0.041] μm vs 0.055 [0.043-0.063] μm, p<0.01 adult asthma and child asthma vs CFA). The RBM fibril width was also thinner in the infants compared to parenchymal collagen fibril width of CFA patients, but the difference in width was not significant, median [range] width infants vs CFA; 0.048 [0.043-0.052] μm vs 0.055 [0.043-0.063] μm, NS (figure 6).

Discussion

We have shown that the ultrastructure of the RBM is distinct to that of bronchial interstitial collagen, the latter being similar to the alveolar wall collagen of pulmonary fibrosis. Even when the RBM is thickened, as in asthma, the fibrils are thinner and less obviously banded than those of airway wall interstitial collagen. Moreover, the ratio of fibrils to matrix in asthma, at all ages, remains unchanged from that of their respective non-asthma controls. Our ultrastructural data do not support the hypothesis that thickening of the RBM in asthma results from the same fibrotic process as that seen in fibrotic lung disease.

Collagen is a universal term applied to a family of glycoproteins and includes distinct structural forms such as interstitial collagen, reticulin and constituents of basal

laminae. [1] In mammals, the fibrils of interstitial collagen are thicker with a diameter between 10 – 500 nm (mean diameter 60 nm) than those of reticulin and they usually follow a wavy course without branching. [11] Additionally, interstitial collagen has fibrils that each have characteristic transverse bands termed D-periodicity with a repetitive band frequency of approximately 64-70nm. [15] In contrast, reticulin is composed of fibrils that form a relatively tangled, loosely packed, network of fine fibrils with a diameter of about 20-40 nm and the D-periodicity is absent or, at least, much less obvious. [16] The term "subepithelial fibrosis" has been suggested and is variably applied by different investigators to describe the characteristic thickening of the RBM layer in asthma. [8] To many, the term implies that it is the addition of interstitial collagen that contributes to the RBM thickening, suggesting a fibrotic process similar to that described in IPF.

A previous quantitative study has assessed the RBM by electron microscopy but only to determine its thickness in asthma as compared to controls: [17] none has assessed its ultrastructural composition. We acknowledge that the description "reticular" basement membrane per se already implies a distinction to interstitial collagen. However, we have demonstrated for the first time using quantitative and ultrastructural techniques, that the composition of the bronchial RBM in the normal, as well as that which is thickened in asthma, is ultrastructurally different from the banded, thicker fibrils of interstitial collagen characteristic of fibrotic scar and that seen in parenchymal collagen from IPF patients. This ultrastructural distinction, together with the lack of a relative increase of the fibrillary component, and the non-progressive nature of the thickening, which we have previously shown is already

maximal in asthmatic children, [5] indicates that the process of RBM thickening in asthma is different to the fibrosis characteristic of IPF.

Experimental studies provide support for our interpretation. In allergen challenged mice, silver stains (designed to highlight reticulin) demonstrate that subepithelial reticulin in the airways increases 3-fold that of sham-challenged mice and that it is this component that contributes to increased RBM thickness in this animal model of asthma. [18] Moreover, we have additionally shown that both fibrils and matrix increase in equal proportion in human asthma so that the thickened RBM in asthma, in the child or adult, has the same ultrastructural appearance as that of age-matched controls. We, and others, have shown previously that the RBM is present and thickened in both adults [3] and children with asthma, [5,6] and we have recently reported that the RBM is developed, but not yet thickened, in infants below the age of 24 months. [12] In the present paper, we have shown that age does not influence the ultrastructural composition of the RBM, either in asthmatics or non-asthmatic controls. Also, the ultrastructural appearance of the RBM might differ in patients with more severe disease, defined by the presence of persistent airflow limitation (PAL). [19] In the current study we addressed this in the children, who all had difficult asthma. Although numbers were small (6 with PAL and 4 without PAL), there were no differences in RBM ultrastructure between the subjects with or without PAL.

One potential criticism of our study is that the RBM has been assessed using only transmission electron microscopy without the application of immunohistochemical and silver staining techniques to identify reticulin. To apply these in repetition of previous studies [20] would have required the taking of additional biopsies processed

using different techniques to those used for electron microscopy. We did not have sufficiently large numbers of biopsies, particularly in the children, or the ethical approval to do this. Neither did we consider this as a requirement for our conclusions, as electron microscopy so clearly distinguishes reticulin from fibrils of interstitial collagen without the need of special stains. [21] Many studies have used immunohistochemical staining techniques to assess which collagen sub-types are increased in the RBM in asthma. [17] These researchers have shown that the main components of the RBM are collagen sub-types III [8,22] and V, [8,17] which is in keeping with increased reticulin, as immunohistochemically, reticulin fibrils do express the epitopes for collagen type III [21,23] and type V [24] whereas interstitial collagen fibrils most closely resemble collagen type I. [1,23] A recent study has reported increased collagen sub- type III in both adult and paediatric asthmatics.[25] This is not likely due to an increased density of type III collagen, but rather the result of an increase in total thickness of the RBM. Moreover, such a light microscopic study in which immunostaining is performed does not allow assessment of the ratio of fibrils to matrix in the thickened asthmatic RBM: for this electron microscopy is required. As performed previously, but using snap-frozen material [8], we performed immunostaining of paraffin wax-embedded biopsies for collagen sub-types I, III & V where tissue was available, this was in addition to our ultrastructural assessment of all plastic-embedded biopsies. The clear discrimination that was observed between RBM reticulin and deeper interstitial collagen by electron microscopy was not reflected by similarly distinct distributions of collagen types III. Also, the variability associated with staining for collagen types I and V in the biopsies that had been processed to paraffin wax made their interpretation unconvincing.

Thus, while we acknowledge that the epithelial RBM of asthma does stain for the epitopes that mark collagen, we consider that electron microscopy is a technique that offers an additional and novel way to examine and compare the structural components of the RBM and interstitial collagen in asthma, and that there are marked differences of structure between the composition of the RBM and deeper bronchial or parenchymal interstitial collagen, the last taken from patients with fibrosing lung disease.

Finally, myelofibrosis is a condition involving increased bone marrow deposition of collagen and reticulin. [26] In bone marrow disease, the terms "reticulin fibrosis" or "collagen fibrosis" are used, [27] and a variable response of reticulin fibrosis to corticosteroids has been reported. [27] Even in patients with CFA, variable amounts of both type I and III collagen are present, and patients with a larger proportion of type III collagen show a better response to steroid therapy. [28] This is consistent with evidence that long term steroid therapy can result in a reduction in RBM thickness in asthma. [29] We suggest that a distinction between reticulin fibrosis and collagen fibrosis be recognised in asthma and that that the former term would be more appropriate and specific than the term "subepithelial fibrosis". Alternatively, the term reticular basement membrane thickening, while less specific, is accurate and is less likely to confuse and to imply an as yet equivocal mechanism to explain its thickening in asthma.

In summary, we have shown, by examination of its ultrastructure, that RBM thickening in asthma represents not interstitial collagen but rather increased deposition of reticulin and matrix in equal and normal proportion. These findings in
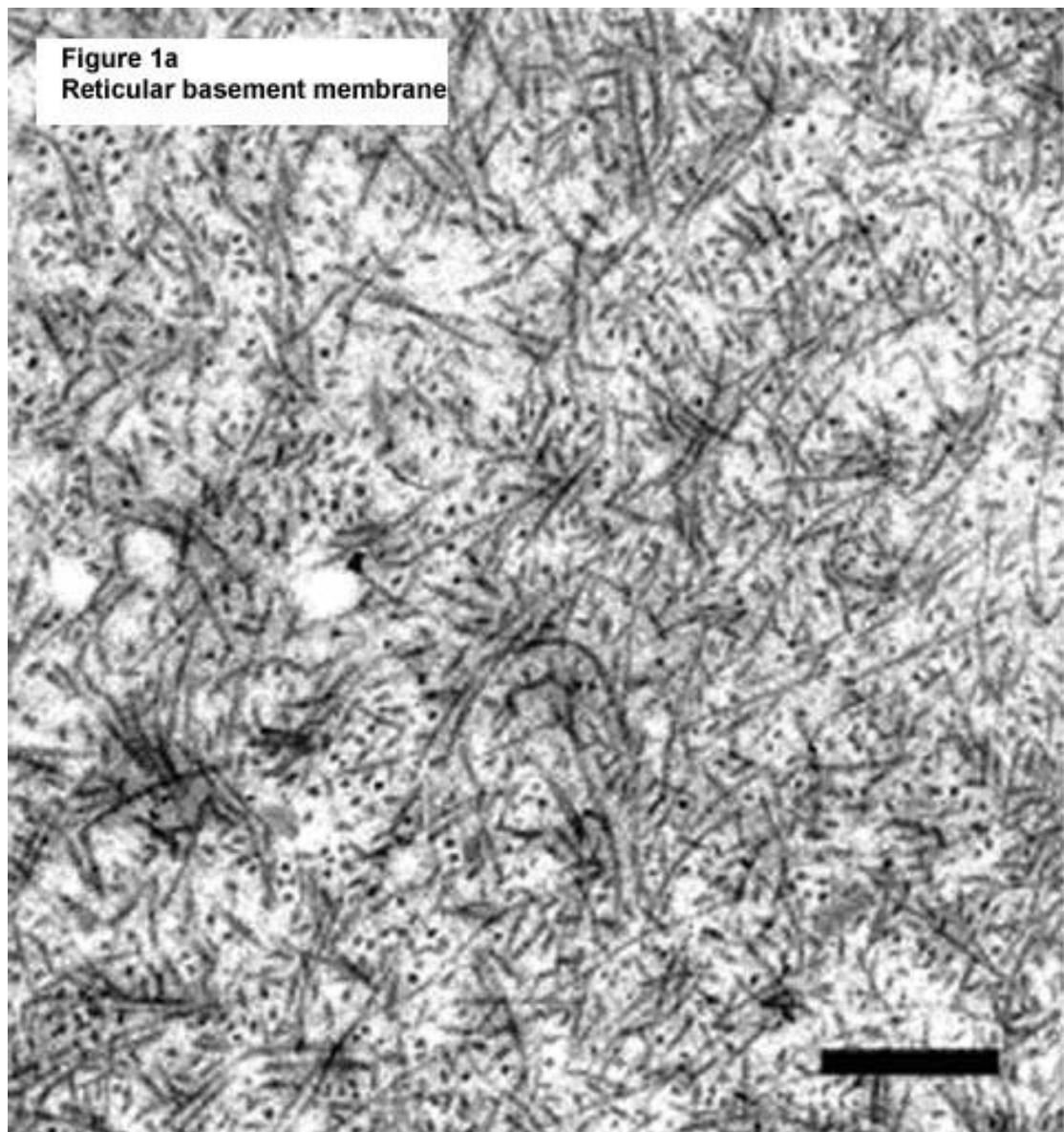
children and adults, lead us to the conclusion that the RBM thickening in asthma represents an increased quantity of lamina reticularis of the same composition as that found in healthy individuals. Whilst immunostaining identifies epitopes usually associated with collagen, our ultrastructural data demonstrate distinctions from the interstitial collagen associated with fibrosis and challenge the notion that the RBM and its thickening in asthma are the result of a fibrotic process of the kind seen in fibrotic lung disease.

Legends for figures

Figure 1. Electron micrographs of the ultrastructural appearance of the bronchial a) reticular basement membrane (RBM) and b) deeper interstitial collagen (IC). Fibrils in 1b are more obviously banded and thicker than those in 1a. Pictures taken at the same magnification (x15 000), scale bar represents 0.5μm
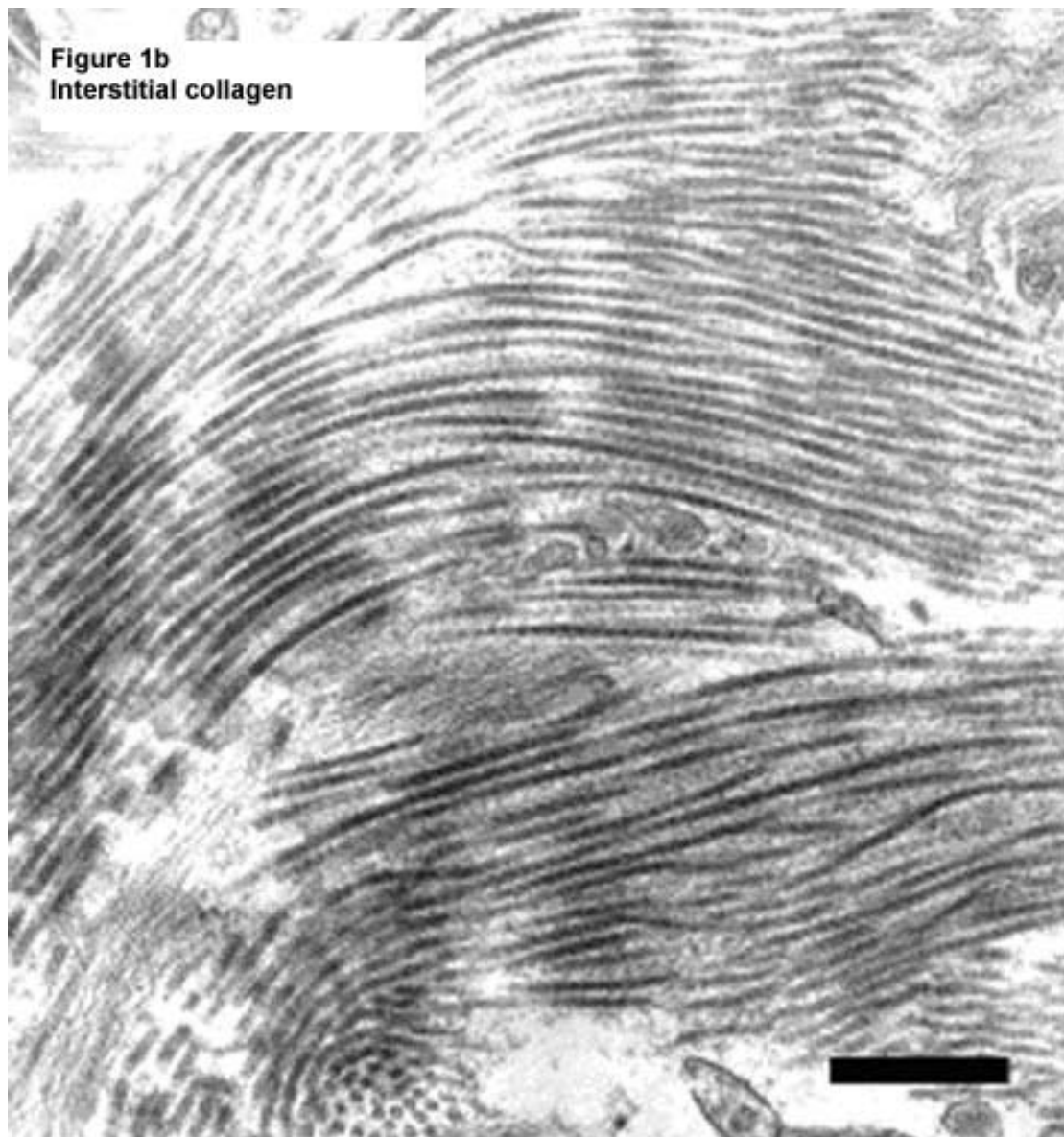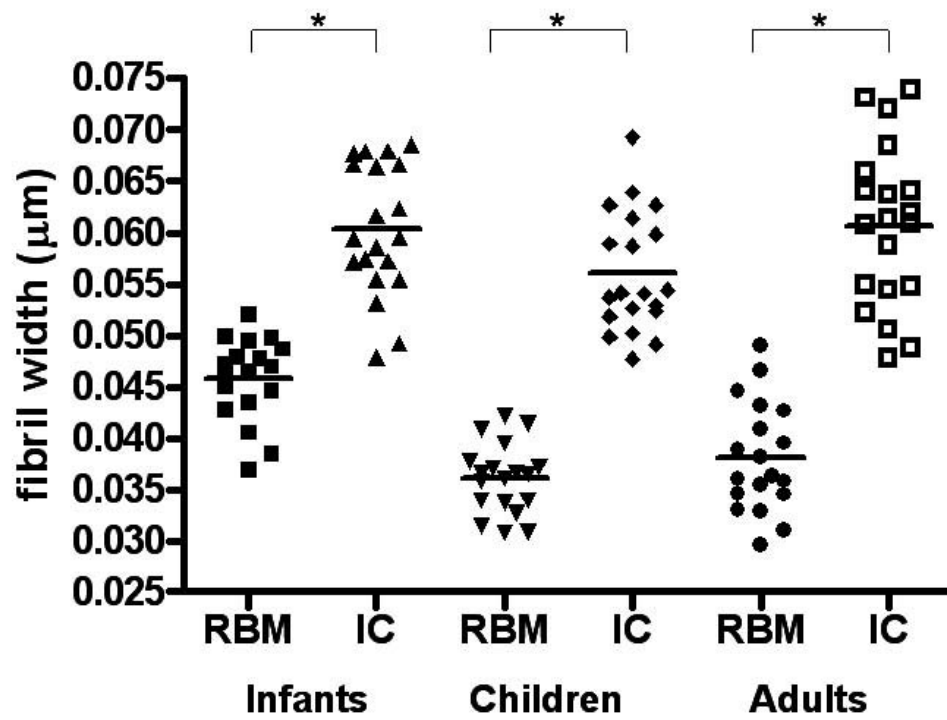
Figure 1a
Reticular basement membrane

Figure 2. Fibril width in reticular basement membrane compared to interstitial

collagen in infants, children and adults

Figure 2

* p < 0.001

RBM: reticular basement membrane, IC: interstitial collagen

Figure 3. Ratio of banded:non-banded fibrils in the reticular basement membrane and interstitial collagen in infants, children and adults
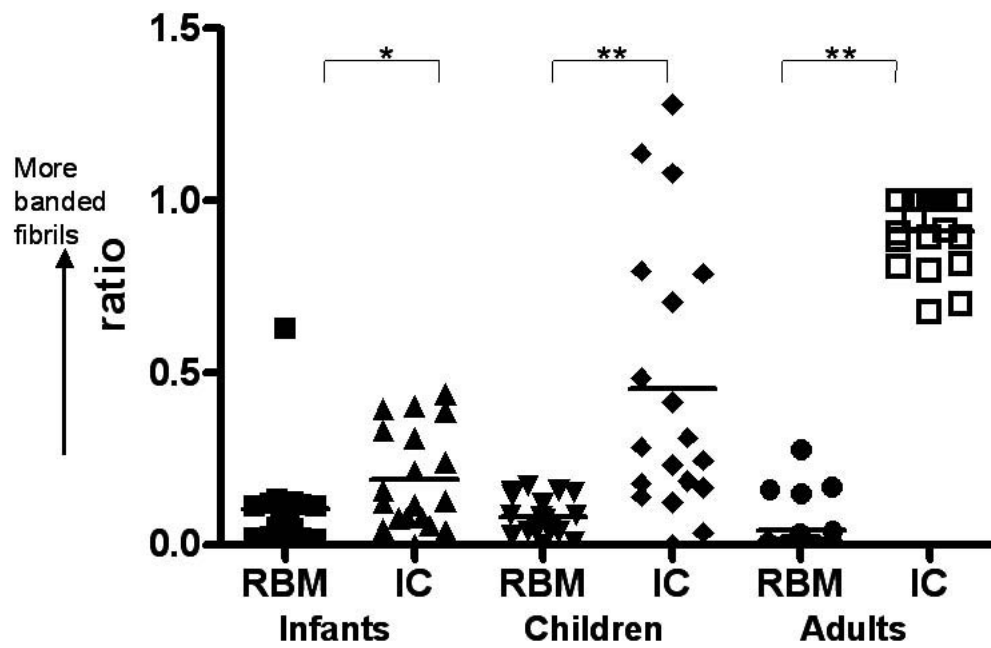
Figure 3

* p < 0.03, ** p < 0.001

RBM: reticular basement membrane, IC: interstitial collagen

Figure 4. Ratio of fibrils:matrix in the reticular basement membrane in asthmatics compared to age matched controls (all ages together)

Figure 4

NS

More banded fibrils

ratio

3.5
3.0
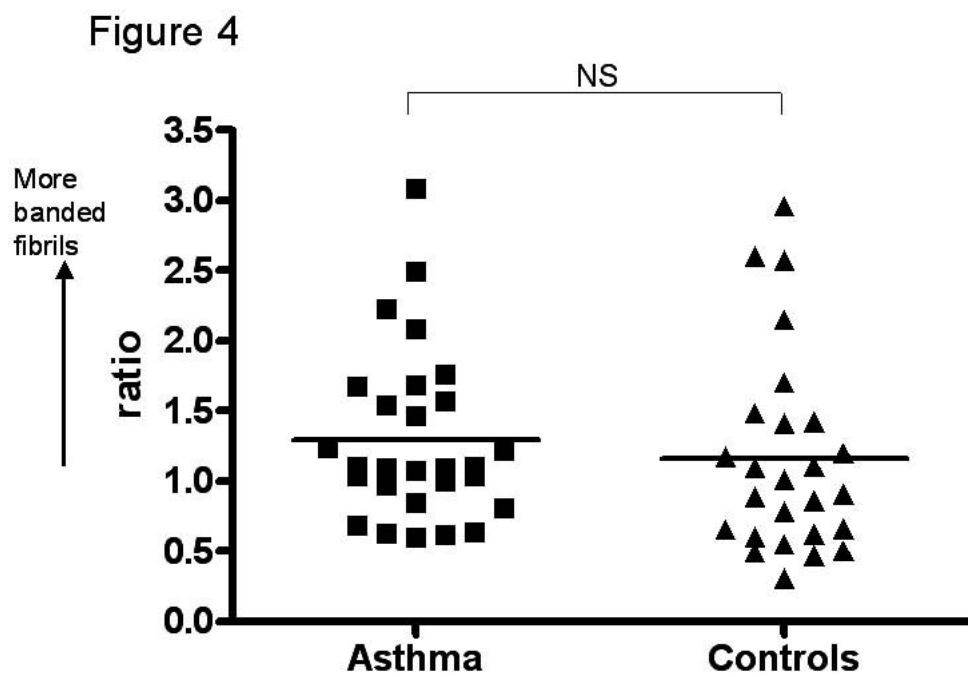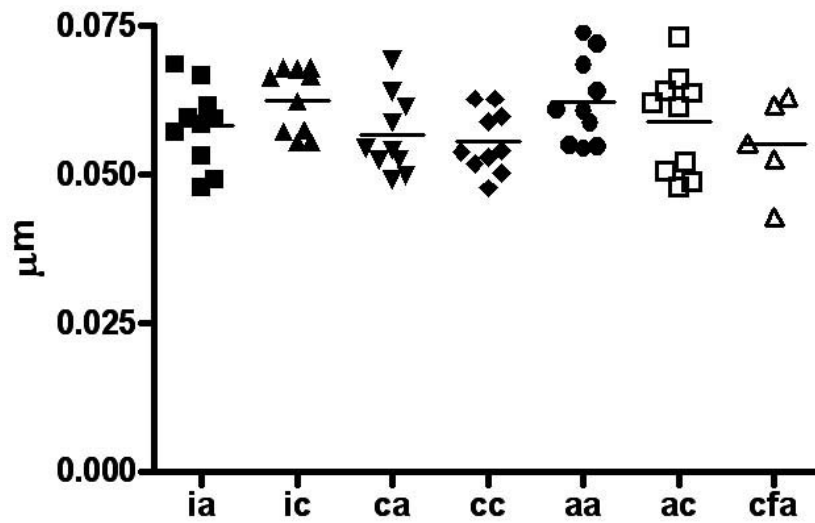2.5
2.0
1.5
1.0
0.5
0.0

Asthma   Controls

Figure 5. Interstitial collagen fibril width from infants, children and adults compared to adults with cryptogenic fibrosing alveolitis

Figure 5

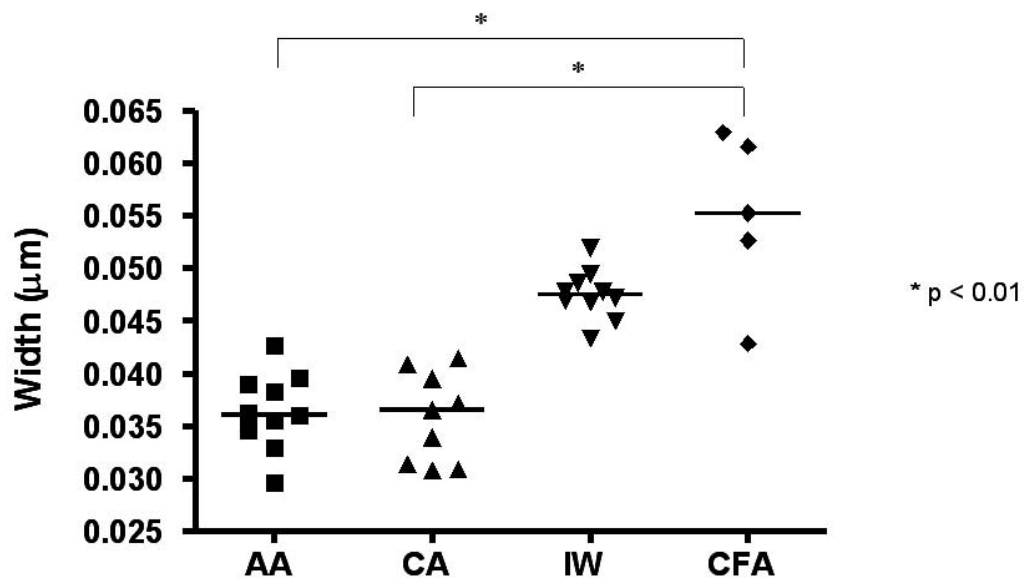ia: infant asthma, ic: infant controls, ca: child asthma, cc: child controls, aa: adult asthma, ac: adult controls, cfa: cryptogenic fibrosing alveolitis

Figure 6. RBM fibril width in asthmatics of all ages compared to parenchymal collagen fibril width in adults with cryptogenic fibrosing alveolitis

Figure 6

* p < 0.01

AA: adult asthma, CA: child asthma, IW: infant wheeze, CFA: cryptogenic fibrosing alveolitis

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Table 1. Clinical details of adults, children and infants

	AA	AC	CA	CC	IW	IC
Number	10	10	10	10	10	9
Male	9	4	6	2	3	2
Age* (years)	28.5 (18–41)	38 (21–45)	12.5 (7–16)	10.5 (7-16)	0.77 (0.4-1.4)	0.96 (0.3-2.0)
FEV$_1$* (% predicted)	103.5 (89–116)	110.5 (92–121)	62 (38–103)	86(69–108)	Not done	Not done
PC20* (mg/ml)	3.5 (0.5 – 7.0)	> 16	Not done	Not done	Not done	Not done
sGaW § (s^{-1}kPa^{-1})	Not done	Not done	Not done	Not done	1.4 (0.6–2.3)	2.7 (2.0–3.2)
Atopic	10 / 10	0 / 10	10 / 10	2 / 10	4 / 10	2 / 9
ICS dose* (budesonide)	N/A	N/A	800 (400-2000) mcg	N/A	N/A	N/A

AA - adult asthma; AC - adult controls; CA – child asthma; CC – child controls; IW – infant wheeze; IC – infant controls

* median (range)

§ specific airways conductance - mean (range)

ICS – inhaled corticosteroids

Table 2 Clinical details of patients with cryptogenic fibrosing alveolitis

Subject	Sex	Age (yrs)	Clinical details	Lung Function	Pathology
1	M	64	Fibrosing alveolitis diagnosed aged 49 years, following history of increasing breathlessness. On long- term systemic steroids. Presented in respiratory failure. Non-smoker.	FEV_1: 1.76L (57%) FVC: 2.09L (53%)	Diffuse, severe interstitial fibrosis with superimposed acute inflammation.
2	F	52	Bilateral hilar lymphadenopathy on chest x-ray, giving an initial clinical diagnosis of sarcoidosis. But increasing dyspnoea on minimal exertion, with hypoxia. On steroids. Never smoked	FEV_1: 1.08L (51%) FVC: 1.18L (47%)	Marked interstitial fibrosis and smooth muscle hypertrophy. Loss of air spaces.
3	F	42	Progressive dyspnoea over	Unable to	Interstitial fibrosis,

			2 years. Oxygen dependent. On systemic steroids. Never smoked	perform lung function.	heavy chronic inflammatory infiltrate and honeycombing
4	M	53	6 months of increasing breathlessness on exertion. No cough. Smoked until 18 months previously	FEV$_1$: 2.82L (79%) FVC: 3.40L (76%)	Severe interstitial fibrosis with honeycombing. Focal inflammation
5	M	59	I year of persistent breathlessness. Intermittent productive cough. Smoker.	FEV$_1$: 3.32L (93%) FVC: 4.01L (89%)	Marked interstitial fibrosis and honeycombing. Mild chronic inflammation