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Airway remodeling in a mouse asthma model assessed by in-vivo respiratory-gated micro-computed tomography

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Abstract The aim of our study was to evaluate the feasibility of non-invasive respiratory-gated micro-computed tomography (micro-CT) for assessment of airway remodelling in a mouse asthma model. Six female BALB/c mice were challenged intranasally with ovalbumin. A control group of six mice received saline inhalation. All mice underwent plethysmographic study and micro-CT. For each mouse, peribronchial attenuation values of 12 bronchi were

measured, from which a peribronchial density index (PBDI) was computed. Mice were then sacrificed and lungs examined histologically. Final analysis involved 10 out of 12 mice. Agreement of measurements across observers and over time was very good (intraclass correlation coefficients: 0.94–0.98). There was a significant difference in PBDI between asthmatic and control mice (–210 vs. –338.9 HU, $P=0.008$). PBDI values were correlated to bronchial muscle area ($r=0.72$, $P=0.018$). This study shows that respiratory-gated micro-CT may allow non-invasive monitoring of bronchial remodelling in asthmatic mice and evaluation of innovative treatment effects.

Keywords Asthma · Remodelling · Small animal imaging · Micro-CT · In vivo

Introduction

Asthma is an increasingly prevalent disease characterised by bronchial hyperresponsiveness (BHR), chronic bronchial inflammation, and remodelling [1]. This latter is thought to be an excessive repair process of the airways that has been characterised histologically by several features including an increase in smooth muscle mass [2]. Such remodelling has been associated with a decrease in lung function and partly reversible airway obstruction leading to a more severe asthma phenotype [3]. Both inflammation and remodelling of the asthmatic airways increase the wall thickness, which can be assessed in vivo using thin-section computed tomography (CT) [4–7].

For pathophysiological purposes, several asthmatic models have been developed in animals, mainly in mice [8, 9]. However, evaluation of bronchial wall thickness requires histological studies and thus sacrificing of animals. To date, there is no means for assessing bronchial wall thickness in animal asthmatic models in vivo and such a method would be highly useful for preclinical studies.

In the past decade, micro-computed tomography (micro-CT) has become a powerful technique in laboratory investigations, enabling sufficient spatial resolution to generate images of small animals. In the field of lung diseases, micro-CT has been used for imaging excised lung specimens of rodents [10] or humans [11]. Using

respiratory gating and micro-CT, in vivo imaging of rodents has become possible in various pathological conditions including lung tumours, lung fibrosis or emphysema [12–15]. However, to the best of our knowledge, there are no studies reporting the use of micro-CT for the assessment of bronchial remodelling in rodent asthma.

Hypothesizing that bronchial wall inflammation and remodelling could modify wall attenuation values, the aims of our study were (1) to compare in vivo respiratory-gated micro-CT bronchial wall density between asthmatic and control mice and (2) to correlate this parameter with plethysmographic and histological data.

Materials and methods

Animals

Twelve female BALB/c mice (5 weeks old) were purchased from Elevage Janvier (Le Genest, St Isle, France) and acclimatised for 1 week before the experiment started. All animal use procedures were approved by our local Animal Care Committee.

Chronic model of asthma

Six mice were sensitised by two intraperitoneal injections of 100 µg of ovalbumin on days 0 and 14 in the absence of aluminium hydroxide. Mice were anaesthetised using an intraperitoneal injection of both 50 µg/g ketamine (Panpharma, Fougères, France) and 5 µg/g xylazine (Sigma-Aldrich, Saint-Quentin-Fallavier, France). They were then challenged intranasally with 500 µg of ovalbumin on days 14, 27, 28, 29, 47, 61, 73, 74 and 75 as described by Henderson et al. [9]. The control group ($n=6$) received normal saline intraperitoneally or intranasally on the same days.

Bronchial hyperresponsiveness measurement

BHR to methacholine (Sigma-Aldrich, Saint-Quentin Fallavier, France) was determined in unrestrained conscious mice by single-chamber plethysmography (Emka Technologies, Paris, France). Enhanced pause parameter (Penh) was measured and averaged for 3 min, 30 s after each successive inhalation of an increasing dose of aerosolised methacholine (1–32 mg/ml) [16, 17]. These measurements were performed on days 0 and 76 for all mice and expressed as a ratio of Penh measured in response to 8 mg/ml methacholine to that with normal saline.

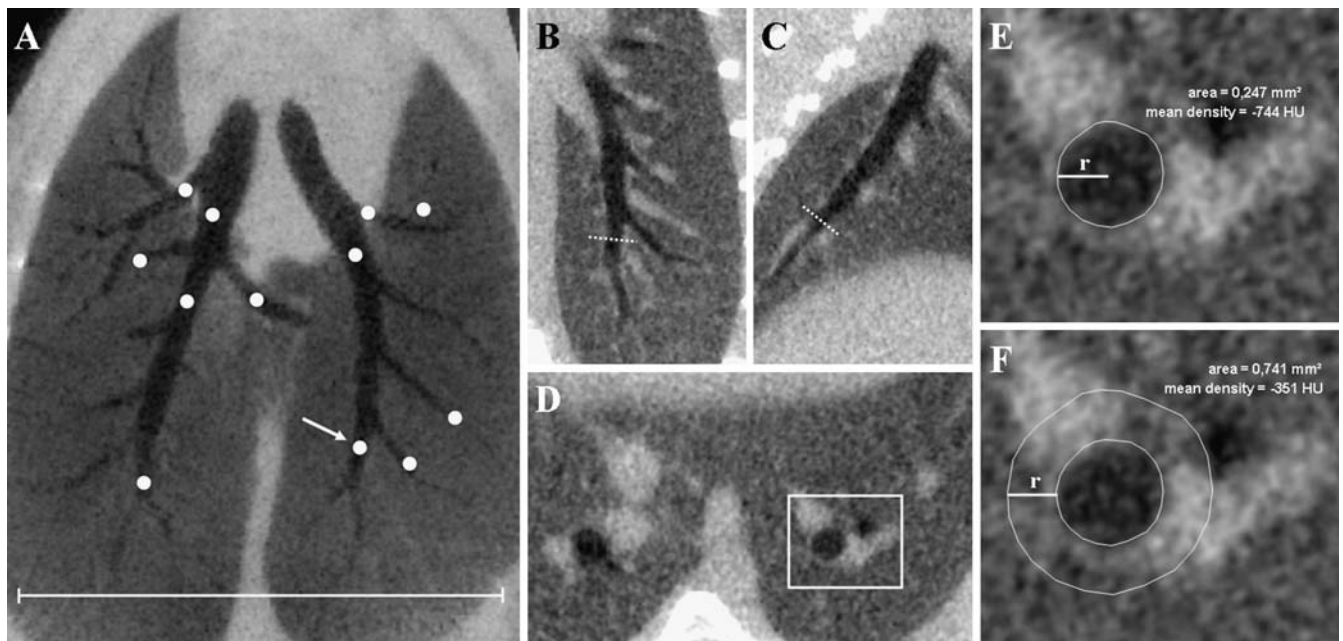


Fig. 1 Typical in vivo micro-CT images from a control mouse (voxel dimensions = $46 \mu\text{m}^3$). **a** Thick coronal minIP reformation of the entire bronchial tree showing the 12 reconstructed bronchi (dots). Scale bar indicates 1 cm. Arrow indicates the bronchus of interest. **b** Coronal and **c** sagittal thin-section reformations of the left bronchial tree. The dotted lines placed perpendicular to the main axis of the studied bronchus indicate the plane for the orthogonal

reformation. **d** Thin-section reformation of the studied bronchus orthogonal to its long axis, as defined on **b** and **c**. The outlined area indicates the studied bronchus. **e** Magnification of **d** showing the lumen delineation for lumen area and lumen density measurement. r Radius of the lumen. **f** A hollow ring-shaped ROI is drawn around the lumen area with an ROI thickness equal to r in order to measure the mean peribronchial density

Micro-CT imaging

On day 81, mice were anaesthetised using the protocol described above and tracheal intubation was performed with a 22-Gauge Teflon intravenous catheter that was immediately connected to a dedicated ventilator Alphalab (Minerve, Esternay, France). Mice were ventilated using 100% FIO₂, 40/min breathing frequency, and a 20 cm H₂O inspiratory pressure peak. They were then positioned supine on a dedicated polycarbonate bed and placed within a thermostatic chamber, CTI-120 (Minerve, Esternay, France). Water, bone and air standards were placed in the chamber, in order to normalise the Hounsfield Units (HU) scale for each dataset acquisition. Mice were then placed within the micro-CT system (GE Healthcare eXplore Locus, GE Healthcare, London, ON, Canada) for in vivo data acquisition. The output signal of the ventilator allowed data acquisition to be triggered at the end of expiration. Images were obtained in the absence of any contrast agent at 80 kV, 0.45 mA. A total of 720 projections were acquired with a 0.5° angular increment during a 360° circular orbit. A single frame was acquired at each angle, with a 400-ms nominal exposure time per projection. The entire acquisition lasted 17 min and the X-ray entrance dose was 0.26 Gy per acquisition. Contiguous axial images in DICOM format were reconstructed using a 23-mm field of view, a 512 ×

512 matrix and a high-resolution reconstruction algorithm. The resulting voxel size was 46 × 46 × 46 µm. A mouse thorax image data set contained a mean of 300 images, typically with the lungs included in a mean of 180 images.

Image post-processing

Volume datasets were exported to a commercial workstation (DXMM, Medasys, Gif-sur-Yvette, France) in DICOM format, and information about the group was deleted. All micro-CT images were analysed in random order. Commercially available software (Myrian, Intrase, Montpellier, France) using a volume-growing algorithm from bi-thresholded voxels (−1000 to −100 HU) was used for total lung volume and mean density measurement. Furthermore, three bronchial parameters were measured from the same 12 bronchi in each mouse (Fig. 1). Multiplanar reformations and anatomical landmarks helped to identify the selected bronchi. For each bronchus, a cross-sectional reformation was first performed at a fixed distance from its origin. On each orthogonal reformation, the bronchial lumen was manually delineated, and lumen area (LA) and mean lumen density (LD) were measured. Because the outer limit of the bronchial wall was not clearly identifiable in asthmatic mice, a ring-shaped region

Table 1 Mice characteristics

	Asthmatic mice (n=6)	Control mice (n=6)	P value
Weight at day 76 (g)	20.8±0.8	21.2±1.3	0.579
Plethysmographic data			
Penh ratio at day 0	1.81±1	1.92±1.24	0.937
Penh ratio at day 76	10.51±2.35	2.23±1.67	0.002
Histological data			
Peribronchial space area (µm ²)	52,778±27,977	15,518±3,861	0.002
Number of nucleated cells within the peribronchial space area (cell/µm ²)	599±317	232±57	0.026
Wall area (µm ² /bronchus)	34,977±11,023	19,201±2,289	0.041
Wall area/basement membranous length (µm)	8.17±1.98	5.29±0.63	0.009
Bronchial muscle area (µm ² /bronchus)	13,400±4,994	6,280±3,293	0.041
Bronchial muscle area/basement membranous length (µm)	14.54±4.56	7.86±3.34	0.016
Vascular muscle area (µm ² /vessel)	8,700±2,907	5,872±1,481	0.065
Micro-CT data	(n=5)	(n=5)	
Total lung volume (cm ³)	0.67±0.19	0.63±0.09	0.0675
Total lung density (HU)	−467±65	−472±29	0.859
TLA (mm ²)	3.73±1.46	3.14±0.41	0.690
LDI (HU)	−658.2±61	−714.4±27.9	0.222
PBDI (HU)	−210±40	−338.9±55	0.008

Data are means ± standard deviation

TLA Total lumen area, LDI lumen density index, PBDI peribronchial density index

Table 2 Measurement agreement of micro-CT bronchial lumen areas and peribronchial densities

	Over-time agreement	Across-observer agreement
Bronchial lumen areas		
Pearson correlation coefficient (<i>P</i> value)	0.977 (<0.001)	0.968 (<0.001)
Difference ^a	0.01±0.06	0.03±0.09
Intra-class correlation coefficient	0.976	0.943
Sw	0.04	0.07
Spearman rank correlation coefficient ^b (<i>P</i> value)	0.31 (<0.001)	0.53 (<0.001)
Peribronchial densities		
Pearson correlation coefficient (<i>P</i> value)	0.976 (<0.001)	0.947 (<0.001)
Difference ^a	7.92±26.88	4.96±40.29
Intra-class correlation coefficient	0.97	0.95
Sw	19.74	28.59
Spearman rank correlation coefficient ^b (<i>P</i> value)	0.057 (0.053)	−0.16 (0.08)

Note: agreements were evaluated using data from 120 reconstructed bronchi

Sw Error measurement

^aData are mean ± standard deviation

^bData are correlation coefficients between mean measurements and their standard deviations

of interest (ROI) was then drawn around the lumen in order to measure the peribronchial density (PBD). The thickness of the ROI was chosen equal to the radius of the bronchial lumen since peribronchial infiltration was shown to grossly scatter just beyond this limit in a previous histological study using the same asthma model [9]. From LD and PBD, we calculated a lumen density index (LDI) and a peribronchial density index (PBDI) by assigning to each of the 12 studied bronchi a weighting depending on its LA. LDI was then calculated as $LDI = \sum (LA \times LD) / \sum LA$ and PBDI as $PBDI = \sum (LA \times PBD) / \sum LA$. Lastly, we computed the total lumen area (TLA) as the sum of the 12 bronchial LA. Bronchial measurements were performed by two observers, one of them repeating the analysis after 1 month.

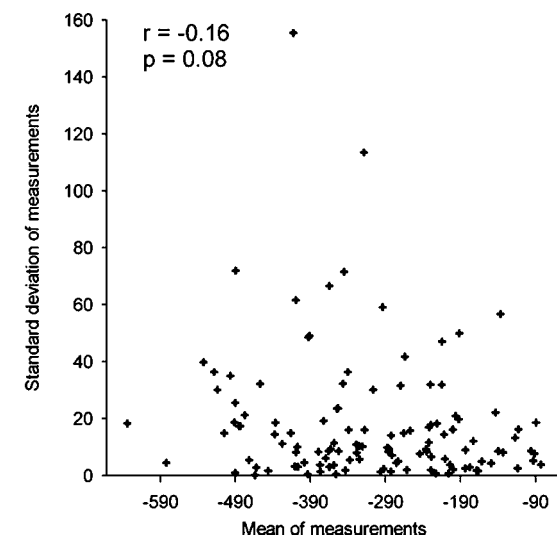
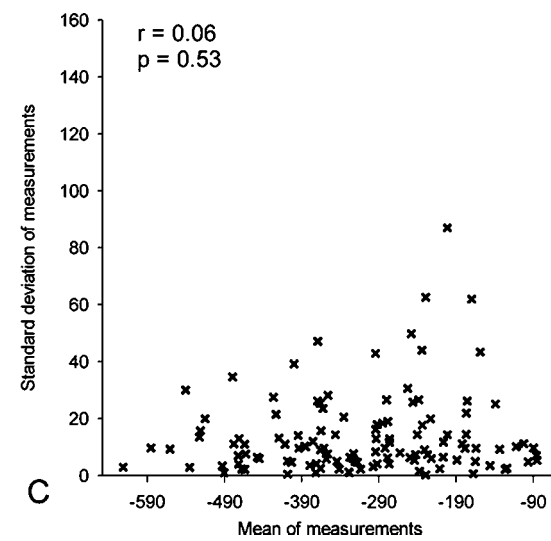
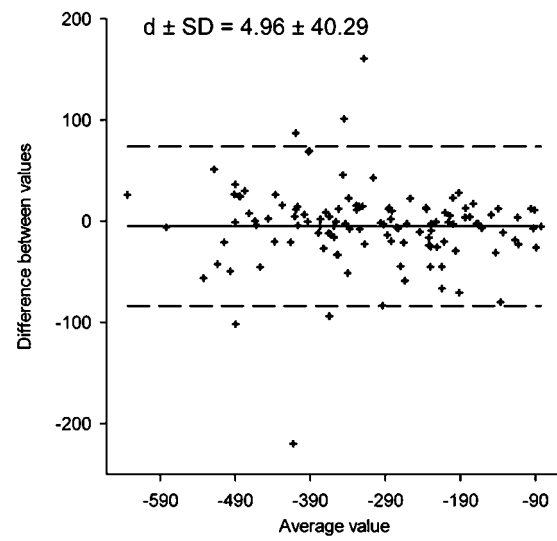
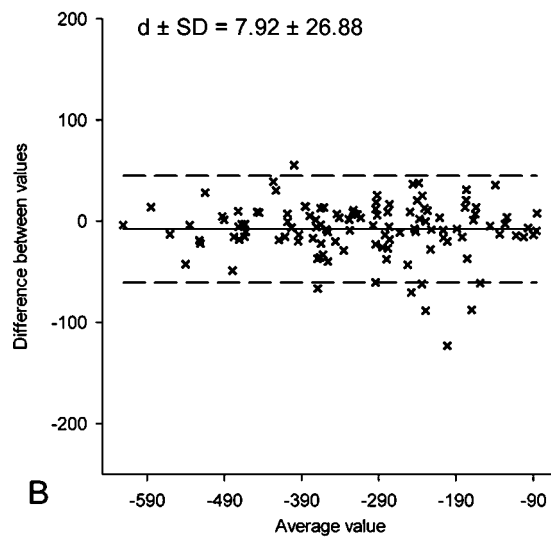
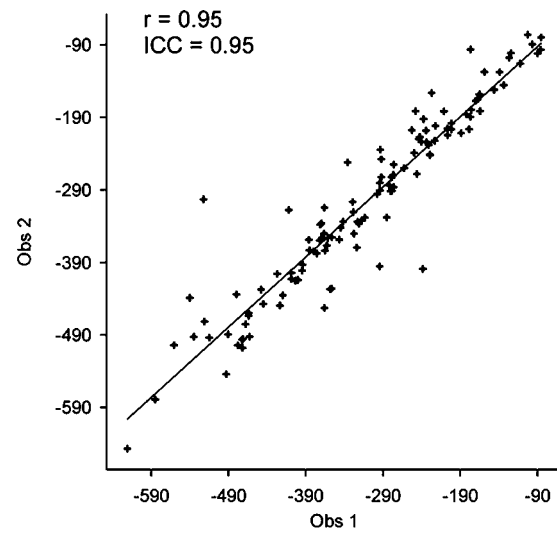
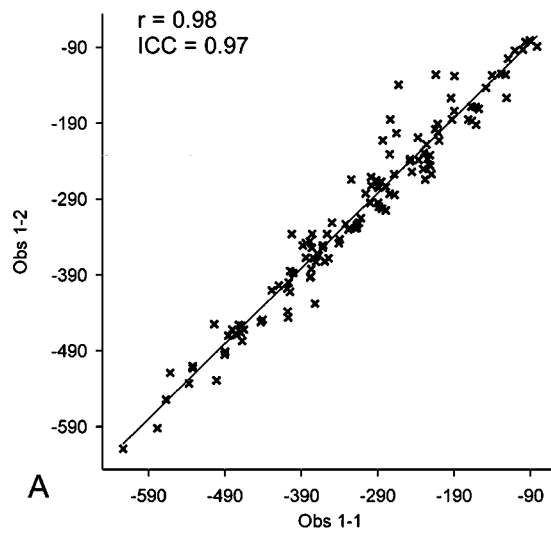
Lung histology and immunohistochemistry

On day 81, anaesthetised mice were exsanguinated by cardiac puncture within 6 h of micro-CT. Right and left lung tissue were dissected out, fixed in formaldehyde and embedded in paraffin. Histological analysis was performed on 4-μm-thick lung slices stained with haematoxylin-eosin-safran and Masson's trichrome.

Immunohistochemistry was performed after deparaffinisation and rehydration through graded alcohols [18]. Endogenous peroxidase was then inhibited using a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 30 min, followed by two rinses in phosphate-buffered saline (Invitrogen, Cergy Pontoise, France). Non-specific staining was blocked using 10% bovine serum albumin

(Dako, Trappes, France) for 30 min. Sections were incubated for 2 h at room temperature with an anti-mouse alpha smooth muscle actin or the relevant antibody (both from Sigma-Aldrich). After rinsing in phosphate-buffered saline, biotinylated rabbit secondary antibody (Dako) was applied to the sections for 2 h and followed by the streptavidin-biotin horseradish-peroxidase complex (Dako) for a further 2-h period. After rinsing in phosphate-buffered saline, 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Dako) in the presence of 0.02% hydrogen peroxide was incubated for 6 min and used as a substrate to develop a peroxide-dependent brown color reaction at room temperature. Finally, the sections were rinsed and counterstained with Mayer's haematoxylin. Smooth muscle was identified by a pathologist in a blinded fashion using both morphological characteristics and α-smooth muscle actin staining as described previously [19]. Various quantitative parameters were assessed using Quancoul (Quant'Image, Bordeaux, France) software at magnifications of 200× to 400× [20]. The peribronchial space and the basal membrane length were manually delineated. The number of

Fig. 2 Bland-Altman analysis for peribronchial density. **a** Correlation over time (*left*) or across observers (*right*) of peribronchial density measurements. The *diagonal lines* correspond to the lines of equality. **b** Means of measurements over time (*left*) or across observers (*right*) are plotted against their differences. *Solid lines* correspond to the mean difference. *Dashed lines* correspond to the mean difference ± 2 standard deviations. **c** Means of measurements over time (*left*) or across observers (*right*) are plotted against their standard deviations. *ICC* Intraclass correlation coefficient, *Obs1-1* first measurement obtained by observer 1, *Obs1-2* second measurement obtained by observer 1, *Obs1* average value obtained by observer 1, *Obs2* measurement obtained by observer 2, *r*Spearman correlation coefficient



nucleated cells was automatically counted within the peribronchial space. The wall area (defined by the sum of epithelial, submucosal and smooth-muscle areas) and the bronchial smooth muscle area were automatically delineated from a mean of five bronchi per mouse. Similarly, the vascular smooth muscle area was automatically delineated from a mean of five arterial pulmonary vessels per mouse.

Statistical analysis

According to Bland-Altman analysis, intra- and inter-observer agreement of LA and PBD were evaluated by (1) Pearson's correlation coefficient, (2) lack of agreement (i.e. bias estimated by the mean difference and the standard deviation from the mean), (3) intra-class correlation coefficient (ICC), and (4) means of both measurements plotted against their differences [21–23]. We also analysed the measurement error graphically by plotting the standard deviation of measurements against their mean and analytically by Spearman's correlation coefficient of data and the within-subject standard deviation.

Comparison of functional data, micro-CT measurements (total lung volume, total lung mean density, PBDI, LDI and TLA), and histological data between groups was achieved using Wilcoxon's signed-rank test. Correlations between PBDI on the one hand, and functional or histological data on the other hand were achieved using Spearman's correlation test. For the purpose of comparisons and correlations, we used values of PBDI averaged across observers and over time. Results were considered statistically significant when $P < 0.05$. All analyses were performed using NCSS software (NCSS 2001, Kaysville, UT, USA).

Results

Relevance of the asthmatic mouse model

Chronic ovalbumin sensitisation of BALB/c mice did reproduce the main characteristics of asthma. Before sensitisation, the Penh ratio between the groups was not statistically different. Conversely, after sensitisation, asthmatic mice presented a bronchial hyperresponsiveness to methacholine with a Penh ratio significantly increased at day 76 compared with control mice and with the baseline level (Table 1). Histological data showed a significantly higher bronchial wall area in asthmatic mice than in controls. The number of nucleated cells within the peribronchial space, which defines bronchial inflammation, and the bronchial smooth-muscle area, which defines bronchial remodelling, were also significantly higher in asthmatic mice than in controls (Table 1).

Micro-CT analysis

Micro-CT was successfully performed in 10 out of 12 mice. One mouse from the asthmatic group did not recover from anaesthesia, and one from the control group was not sufficiently anaesthetised, resulting in a poorly synchronised acquisition that led to motion artefacts. Micro-CT analysis was performed from the 3rd to 7th bronchial generations. Lumen diameters ranged from 292 to 923 μm .

LA and PBD measurement agreement across observers and over time was very good (Table 2, Fig. 2). The error measurement found was minimal (Table 2). Standard deviations of measurements correlated with mean values for LA only ($P < 0.001$).

Comparisons of micro-CT parameters between asthmatic and control mice

Total lung volume, total lung density, TLA and LDI were not significantly different between asthmatic and control mice (Table 1). By contrast, PBDI was significantly higher in asthmatic mice than in control mice (-210 vs -338.9 HU, $P = 0.008$) (Table 1, Fig. 3).

Correlations between micro-CT and plethysmographic or histological parameters

PBDI was positively and significantly correlated to the Penh ratio and various histological parameters including peribronchial space, wall area and bronchial muscle area (Table 3, Fig. 4). Conversely, PBDI correlated neither with the number of nucleated cells infiltrating the peribronchial space nor with the vascular muscle area (Table 3).

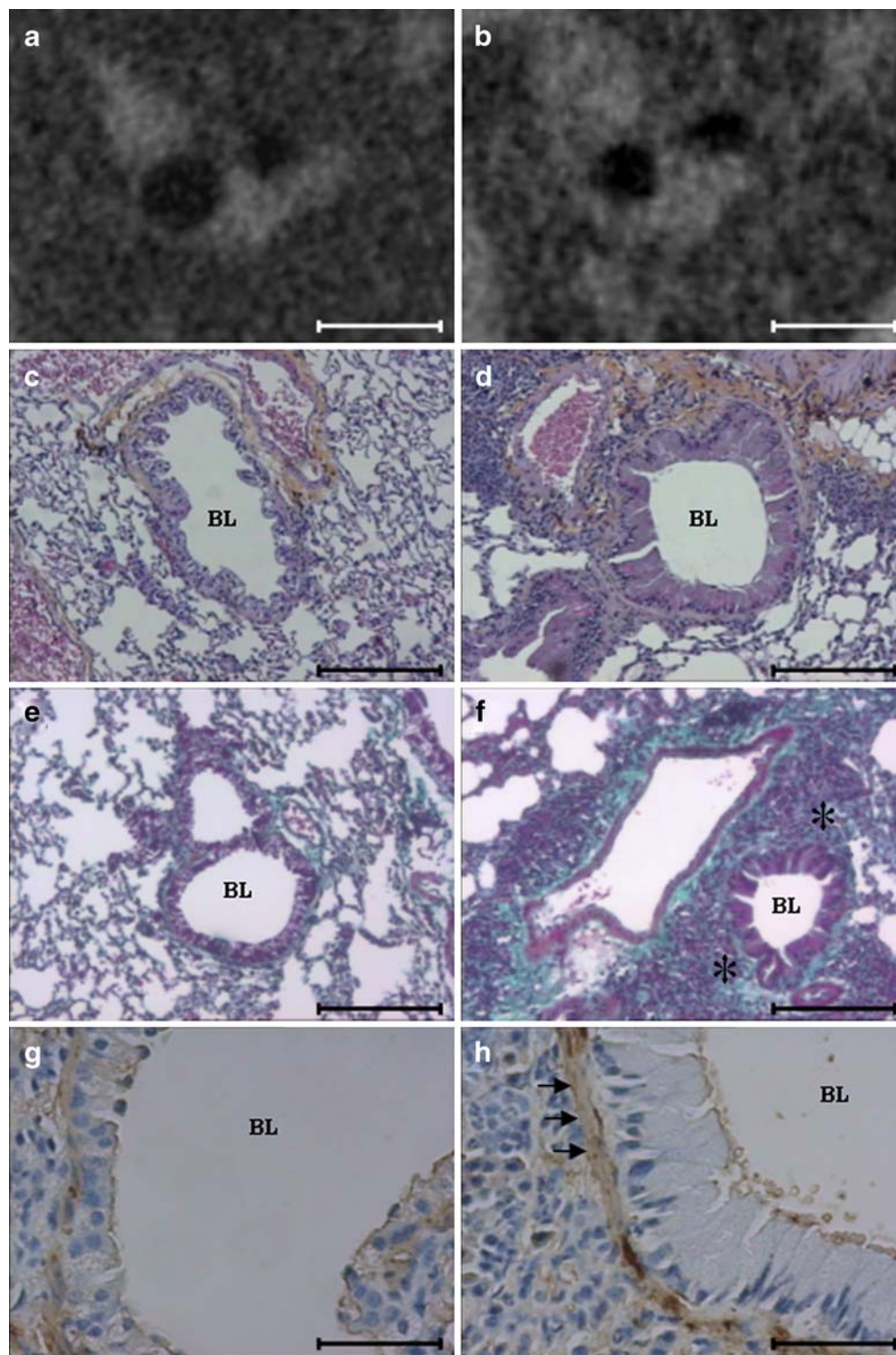
Discussion

This study demonstrates that, in a validated model of murine asthma, *in vivo* measurement of PBDI using respiratory-gated micro-CT discriminates between asthmatic and control mice and correlates with functional and histological data. To the best of our knowledge, this is the first *in vivo* CT study assessing bronchial measurement in asthmatic mice.

We used a model of asthma developed in BALB/c mice and exhibiting the three characteristics of the disease, i.e. bronchial hyperresponsiveness, inflammation and remodelling [9]. This model resulted in asthma-related peribronchial modifications only, as attested in our mice by the absence of difference in lumen area, lumen density, total lung volume and total lung density between groups.

One of the major findings of our study was the strong correlation between PBDI and bronchial smooth muscle size, which reflects bronchial remodelling. Hypertrophy

Fig. 3 Reformatted thin-section micro-CT of the left postero-basal bronchus from a control (**a**) and an asthmatic mouse (**b**). The PBDI (definer) was -351 and -232 HU in **a** and **b** respectively. Representative optic microscopic images from bronchial sections stained with HES (**c, d**), Masson's trichrome (**e, f**) or α -smooth muscle actin (**g, h**) obtained from a control (**c, e, g**) and an asthmatic mouse (**d, f, h**). Note the diffuse cell infiltration of the peribronchial space (*asterisks*) and the increased smooth-muscle mass (*arrowheads*) in the asthmatic mouse compared with the control. BL Bronchial lumen. Scale bars represent 1 mm (**a, b**), 200 μ m (**c-f**, 100 $\times$ magnification) or 50 μ m (**g, h**, 400 $\times$ magnification)



and/or hyperplasia of smooth muscle cells are major components of bronchial remodelling [3] that constitute potential targets for new treatments [19, 24]. As a consequence, micro-CT could be a reliable non-invasive

tool to monitor treatment-induced evolution of bronchial remodelling in preclinical studies.

Another finding of our study was the positive correlation between hyperresponsiveness and PBDI. Because in the

Table 3 Correlation between PBDI values and functional or histological parameters

Parameters	PBDI (all mice)
Penh ratio	0.64 (0.048)
Peribronchovascular space area	0.69 (0.025)
Number of nucleated cells within the peribronchial space	0.47 (0.174)
Wall area	0.67 (0.033)
Wall area/basement membranous length	0.81 (0.005)
Bronchial muscle area	0.72 (0.018)
Bronchial muscle area/basement membranous length	0.81 (0.005)
Vascular muscle area	0.55 (0.098)

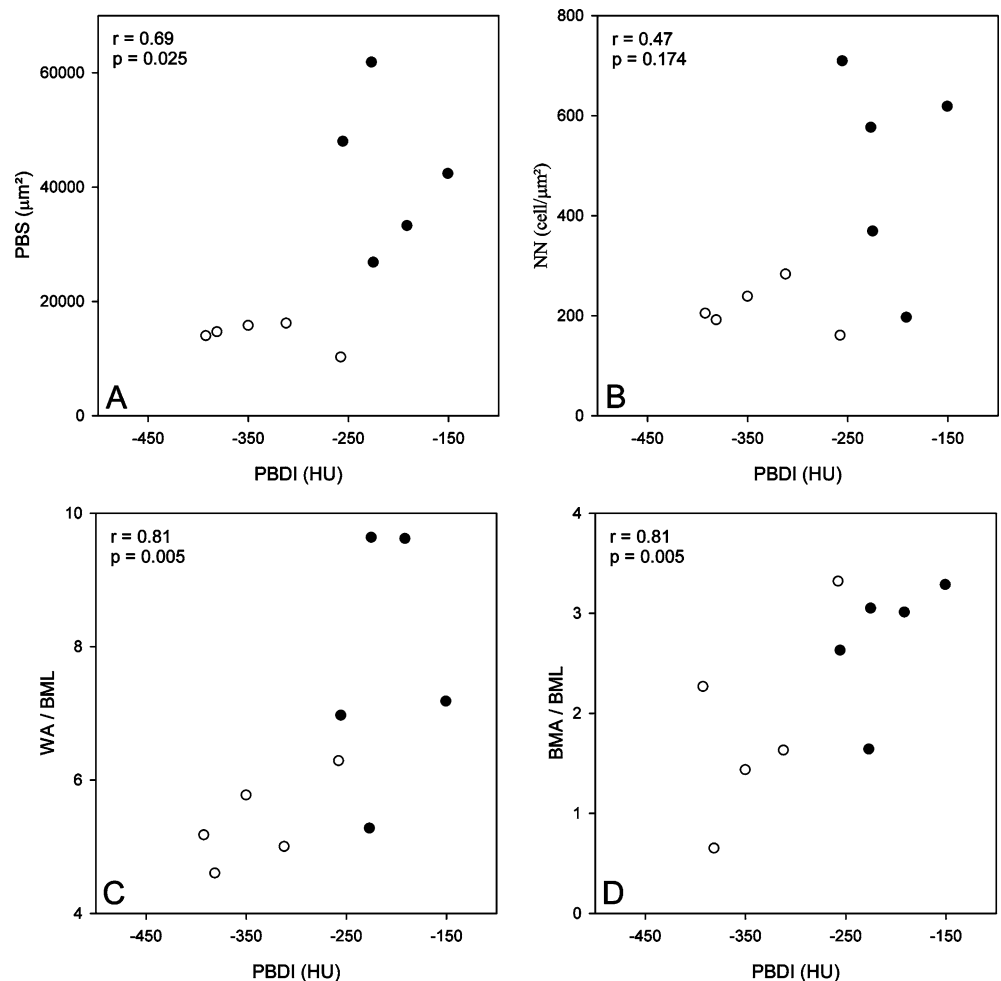
Data are Spearman rank correlation coefficients. Data *in parentheses* are *P* values

BALB/c strain bronchial hyperresponsiveness has been related to eosinophil accumulation [25], this result suggests that PBDI could reflect peribronchial inflammation. Nevertheless, we did not find any correlation between the number of inflammatory cells and PBDI despite histology showing an intense peribronchial cell inflammation in the

asthmatic mice. However, we did not specifically count eosinophils, and this issue requires further study.

The choice of the PBDI parameter in asthma deserves further comment. Using a 100- μm^3 spatial resolution, Johnson had already proposed attenuation value measurement from a ring-shape ROI drawn around a single bronchus

Fig. 4 Scatterplots show correlation between peribronchial density index (PBDI) with various histological parameters: **a** peribronchial space (PBS), **b** number of nucleated cells within the PBS (NN), **c** wall area/basement membranous length ratio (WA/BML), **d** bronchial muscle area/basement membranous length ratio (BMA/BML). Spearman correlation coefficients (*r*) and *P* values are indicated



in a mouse model of lung fibrosis [13]. In humans, asthma-related histological changes are limited to the bronchial wall [26]. Consequently, the analysis of bronchial wall thickness and cross-sectional surfaces appears to be relevant when studying human asthma using CT [5–7]. Conversely, in mice, a previous histological study using this asthma model showed that cell infiltration was largely scattered in the peribronchial space [9]. This pattern was also displayed in our mice (Fig. 3f) and likely explained the ill-defined outer limit of the bronchial wall on micro-CT images (Fig. 3b). As a consequence, measurements of wall thickness/area in mice could be inaccurate; therefore, we decided to measure the mean attenuation value of a peribronchial ring-shaped ROI, the thickness of which was equal to the bronchial radius. By doing so, most of remodelling elements were expected to be included in the ROI [9]. Finally, delineating the inner limit of the bronchial wall was the only operator-dependent key step. Using micro-CT, both high spatial resolution and contrast between bronchial wall and lumen allowed an accurate delineation of the inner bronchial limit, as shown by the very good agreements when measuring LA.

The micro-CT technique used in this study also deserves further discussion. Mice were imaged at end-expiration, which is the period of minimal motion. This respiratory-gated technique is commonly used [12, 13]. However, it lasts 17 min and requires endotracheal intubation and mechanical ventilation, which can induce lung injury [27], especially in animals with impaired respiratory function. Some authors have developed free-breathing techniques without intubation [15, 28, 29] that permit both anatomical

and functional assessments. Nevertheless, mechanical ventilation results in a slower and more regular breath than free-breathing techniques. As a consequence, exposure time was longer leading to a better image quality. Flat-panel volumetric CT is another interesting method that can be used in small animals and allows acquisition of a large volume with reduced imaging times and exposure dose [30]. However, despite imaging times approaching 8–10 s, respiratory gating remains necessary.

Furthermore, assessment of small bronchi requires high spatial resolution and consequently long exposure times. To reach a spatial resolution of 46 μm , we used a 400-ms nominal frame exposure. In order to increase the expiratory phase duration and avoid motion artefacts, the respiratory rate was set at 40 cycles/min, which is markedly different from the physiological breathing rate (163/min) as assessed by the Mouse Phenome Database [29].

Finally, whereas the radiation dose delivered to the animal in the present study can be considered adequate and safe [31], repeated micro-CT, especially with short-time intervals, may induce radiation pneumonitis and alter the experimental outcomes [32, 33]. Further technical improvements are thus required.

In conclusion, we showed that peribronchial attenuation values measured using in vivo respiratory-gated micro-CT discriminate between normal and asthmatic mice and correlate with bronchial hyperresponsiveness and remodelling. The peribronchial density index may be used to evaluate the effects of new drugs on bronchial remodelling in asthma.

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