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Pathology of idiopathic pulmonary fibrosis assessed by a combination of micro-computed tomography, histology, and immunohistochemistry

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Abstract (Unstructured, 218 /220 words)

Idiopathic pulmonary fibrosis (IPF) is a fibrotic disease showing the histology of usual interstitial pneumonia (UIP). While the pathologist's visual inspection is central in histological assessments, three-dimensional microCT assessment may complement pathologist's scoring. This study examined associations between the histopathological features of UIP/IPF in explanted lungs and quantitative microCT measurements including alveolar surface density, total lung volume taken up by tissue (tissue%), and terminal bronchiolar number. Sixty frozen samples from 10 air-inflated explanted lungs with severe IPF and 36 samples from 6 donor control lungs were scanned with microCT and processed for histology. An experienced pathologist scored 3 major UIP criteria (patchy fibrosis, honeycomb, and fibroblastic foci), 5 additional pathological changes such as emphysema, and immunohistochemical staining for CD68, CD4, CD8, and CD79a positive cells, graded on a 0-3+ scale. The alveolar surface density and terminal bronchiolar number decreased and the tissue% increased in IPF compared to controls. In lungs with IPF, lower alveolar surface density and higher tissue% were correlated with greater scores of patchy fibrosis, fibroblastic foci, honeycomb, CD79a-positive cells, and lymphoid follicles. A decreased number of terminal bronchioles was correlated with honeycomb score, but not with the other scores. The three-dimensional microCT measurements reflect the pathological UIP/IPF criteria and further suggest that the reduction in the terminal bronchioles may be associated with honeycomb cyst formation.

Keywords: MicroCT, Interstitial lung disease, lung, airway, pulmonary fibrosis

List of abbreviation:

IPF = Idiopathic pulmonary fibrosis

Lm = Mean linear intercept

Tissue% = total lung volume taken up by tissue

UIP = Usual interstitial pneumonia

This paper includes an online supplemental video.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic fibrotic disease characterized by a rapid decline in lung function and poor prognosis[1]. The ATS/JRS/and Latin american (ALAT) guidelines all recommended for the diagnosis and management[2] based on an integrative multidisciplinary assessment of clinical information, radiological assessment, histopathologic diagnosis, but while diagnosis can be confirmed without histology in cases that present features of IPF including honeycomb cysts on high resolution computed tomography (HRCT)[2], histological assessment remains important especially for diagnosing the early stage of IPF and also for improving understanding of the pathogenesis of the disease.

The histopathological changes in IPF are characterized by patchy dense fibrosis that is often accompanied by honeycomb cyst formation[2]. Proliferating fibroblasts and myofibroblasts produce collagen in the active regions of fibroplasia, termed fibroblastic foci[3-5]. In contrast, the infiltration of inflammatory immune cells into fibrotic regions is generally considered to be mild[2]. However, recent histology and gene expression analyses have suggested that a B cell–mediated immune response and lymphoid follicles formations may be associated with fibrosis[6-9].

The pathologist's visual scoring of UIP/IPF features on lung samples from surgical lung biopsy is essential in histological assessment[1, 2]. Although the morphometric approach has been less used in the examination of IPF lungs compared to other lung diseases such as COPD[10-13], studies have suggested that alveolar collapse onto the alveolar duct mainly contributes to a reduction in alveolar surface area and impairs diffusion capacity in patients with IPF[14-16]. Nonetheless, little remains known regarding direct associations between histologic features of UIP/IPF and quantitative morphometric indices, including the mean linear intercept (Lm), total lung volume taken up by tissue (tissue%), alveolar surface density defined as alveolar surface area per lung volume[17].

The introduction of micro-computed tomography (microCT) has enabled three-dimensional (3D) morphological assessments of lung tissues that are very difficult to achieve with conventional histology[12, 13, 18, 19]. In addtion, microCT scans of frozen air-inflated tissue enable quantitative assessment without shrinkage and physical cutting of tissues[20]. Mai et al.[16] combined CT, microCT, and histological assessments, and showed that fibrosis and honeycomb cysts formation in IPF extends from the peripheral to the central region of the pulmonary lobules. Further, McDonough et al.[21] presented preliminary microCT based analysis of the complex relationship between honecomb cysts and conducting airways at the American Thoracic Society International Conference which suggested that the honeycomb cyst formation could be a result of airway remodeling. Very recently, Verleden et al.[22] proposed the importance of the small airway disease in IPF by showing that the numbers of the terminal bronchioles (defined as the last generation of the conducting airways) were reduced in lungs with end stage IPF compared to control lungs. Collectively, these findings have demonstrated that quantitative morphometric microCT measurements complement the pathologist's visual inspections of lungs with IPF.

The aim of this study was to extend the understanding of the pathology of UIP/IPF lungs by investigating the relationship between the experienced pathologist's scorings of histological features such as honeycomb cysts and microCT measurements of alveolar surface density, tissue%, Lm, and the number of the terminal bronchioles.

Materials and Methods

Informed consent: was obtained either directly from the patient or from the next of kin of the donors that served as controls under conditions approved by the ethical (S52174) and biosafety (MS20101571) committees at the Katholieke Universiteit Leuven and accepted by all the other participating institutions.

Protocol: A diagnosis of IPF was based on the current ATS/ERS/JRS/ATLT guidelines that include dominant airway-centered changes as an exclusion criterion[1, 2]. The major features of this protocol have been described in detail elsewhere[9, 22, 23]. Briefly intact lung specimens donated by patients with very severe IPF treated by lung transplantation and unused donor lungs that served as controls were inflated with air and frozen solid with liquid nitrogen vapor. The specimen was kept frozen while cutting it into 2 cm thick transverse slices. Two lung tissue samples were obtained from either the upper, middle, and lower part of the lung (n=6 per lung) to compare samples with different severity of the disease[9, 22, 23].

MicroCT-based morphometric quantification: The tissue samples were kept frozen while scanned at 9.98 μm voxel resolution with a SkyScan 1172 scanner (Kontich, Belgium)[22]. As previously described, image thresholding was applied to separate tissue and airspaces. The tissue segmentation was used to compute tissue% and alveolar surface density (defined as alveolar surface area per volume of lung[9, 23]. The airspace segmentation was used to compute the mean airspace size (mean linear intercept, Lm) by measuring and averaging interalveolar wall distances[22]. The terminal bronchioles were defined as the last generation of conducting bronchioles and counted manually in the microCT scans of each sample. The number of terminal bronchioles per ml of lung was calculated by dividing the number per sample by the sample volume[13, 20].

Histology: The pathologist's scoring was performed in the present study using histological sections obtained in the previous IPF study[22]. Following microCT imaging, portions of the frozen samples were fixed in alcohol-based formalin at -20°C overnight, warmed to room temperature, and then processed into paraffin blocks from which histological sections were cut and stained with H&E and Movat Pentachrome stains. As shown in Figure 1, these histological sections were examined by an experienced pulmonary pathologist (TVC) who scored 8 pathological features that consisted of the 3 major UIP criteria including patchy fibrosis,

honeycomb cyst formation, and fibroblastic foci, as well as 5 additional pathological changes including emphysema, degree of inflammation, hyaline membrane formation, lymphoid follicles, and respiratory bronchiolitis, all on a 0-3+ scale. In addition, other portions of the frozen samples were briefly warmed to -1°C, vacuum embedded in the optimum cutting temperature compound (OCT, SAKURA FINETEK), immediately returned to -80°C, and cut into serial frozen sections (8 μm thick) for immunohistochemistry. These sections were stained with primary antibodies for CD68 (DAKO, M0876, 1:200 dilution), CD4 (DAKO, M7310, 1:200 dilution), CD8 (DAKO, M7103, 1:400 dilution), and CD79a (DAKO, M7050, 1:200 dilution) as previously reported[22]. These sections were also scored by the same pathologist on a 0-3+ scale.

Statistical Analysis: Data are expressed as mean ± standard deviation. Statistical analysis was performed with the R statistical program (R Core Team: R: A Language and Environment for Statistical Computing. URL http://www.R-project.org/. accessed 2019 Nov 1. version 3.4.1). The Spearman correlation tests and Mann Whitney comparison were used for correlation tests and group comparisons, respectively. Multiple comparisons were performed with Wilcoxon tests with Holm correction.

Results

Table 1 shows that there is no difference in age, sex, height, or weight between the patients with IPF and the control subjects. Table 2 summarizes microCT and histological scores. Alveolar surface density and the number of terminal bronchioles /ml lung were lower, while tissue% and Lm were higher in IPF compared to controls. In addition, CD68, CD4, CD8, and CD79a positive cells were greater in IPF than controls. Supplemental figure 1 demonstrates that the significant difference between IPF and controls was also present when comparing microCT indices in the upper, middle, and lower regions separately.

Figure 2A and B show examples of histological regions with and without honeycomb formation (score 0 and 1, respectively) that were registered to the microCT scans. Figure 2C and D show that the alveolar surface density and number of terminal bronchioles were lower in the honeycomb regions (Score≥1) than in the non-honeycomb regions (Score=0). This finding was visualized on Figure 2E and a video that shows a microCT stack of the same sample as used in Figure 2B and E (Supplemental video 1). The video shows that many branches of the small airway tree (pink) were located in the “normal” appearing regions, but not in the honeycomb region, and that the conducting airway leading into the honeycomb region was directly connected to those severely distorted airspaces (orange). Further, Figure 3 shows that the decreases in the alveolar surface density and number of terminal bronchioles in the honeycomb region were also confirmed in a subanalysis that included non-emphysematous IPF samples (histological emphysema score =0) and controls.

Table 3 shows Spearman correlation coefficients between microCT indices and pathological scores in IPF samples (n=59). Decreased alveolar surface density and increased tissue% and Lm on microCT were correlated with the histological scores of patchy fibrosis, fibroblastic foci, and honeycomb. In contrast, decreased number of the terminal bronchioles was correlated with an increased score of honeycomb, but not with patchy fibrosis and fibroblastic foci.

Table 4 shows Spearman correlation coefficients between microCT indices and scores of lymphoid cells in IPF samples (n=60). Increased tissue% was correlated with increased scores for CD68, CD4, CD8, and CD79a positive cells and lymphoid follicles, whereas Lm and the number of terminal bronchioles were not associated with any of the scores of immune cells and lymphoid follicles.

Discussion

This study compared standard histopathological criteria of UIP/IPF and quantitative morphological measures obtained from microCT. The microCT findings of decreased alveolar surface density and increased tissue% were positively associated with the pathologist's scoring of patchy fibrosis, fibroblastic foci, honeycomb formation, infiltration of CD79a-positive lymphocytes, and lymphoid follicle formation. Furthermore, a combination of histological assessment and the three-dimensional microCT information revealed that a reduction in the number of terminal bronchioles was associated with honeycomb formation, but not with patchy fibrosis or fibroblastic foci. These findings indicate that three-dimensional morphometric assessment via microCT can be used to complement the pathologist's visual inspection to by showing the pathological relationship between the peripheral airways and parenchyma in lungs with IPF.

From a histopathological perspective, UIP/IPF lungs are characterized by spatially heterogeneous fibrosis with fibroblastic foci and honeycomb lesion[1-3], and from a physiological perspective, IPF lungs are characterized by impaired diffusion capacity which affects the mortality[24]. This structure-function relationship has been explained by multiple morphometric studies showing that a collapse of alveoli onto the alveolar ducts in IPF lung leads to a reduction of alveolar surface area[14, 15, 25]. However, to the best of knowledge, no prior report has tested the direct relationship between the pathological UIP/IPF criteria and morphometric assessment of IPF lung. Therefore, the close correlations between alveolar surface density, tissue%, and the histopathological scores of UIP/IPF presented here provide an explanation for the clinically relevant impairment in diffusion capacity present in IPF patients.

The widely accepted hypothesis that IPF is generally a parenchymal disease in which the airways are spared was recently challenged by a microCT-based study that showed a loss of the terminal bronchiole number already occurs in minimal fibrotic regions in lungs with

IPF compared to controls[22]. Since the terminal bronchioles are located in the centre of the secondary lobules, these recent microCT findings have raised the question if there is an interaction between small airway disease, such as loss of the terminal bronchioles, and parenchymal pathology, such as alveolar collapse. The present study sheds light on this issue by showing that the loss of terminal bronchioles is associated with honeycomb formation, but not with patchy fibrosis, which leads to the conclusion that these might be two somewhat separate process. This finding is consistent with the hypothesis proposed by Evans et al.[26] that the peripheral airway injury is associated with honeycomb cyst formation independent of fibroproliferation in the parenchyma. In addition, although COPD studies have shown a close association between emphysema and a reduction in the terminal bronchioles[13, 27, 28], the reduced number of terminal bronchioles in the honeycomb regions was confirmed even in the subanalysis that excluded IPF samples with emphysema (histological score ≥1).

Furthermore, the supplemental video 1 provided portrays the 3D spatial relationship between the small conducting airways and microscopic honeycomb regions, which on the conventional 2D histological sections would be difficult to detect. The 3D visualization demonstrates that the conducting airways are directly connected to the air spaces within the honeycomb regions. This finding suggests that the potential airways present within the honeycomb region are remodeled beyond recognition, and the small airways might be an origin of honeycomb cysts in IPF. This concept requires further detailed investigation which is beyond the scope of the present study.

Staats et al.[29] showed that a histologic finding of bronchiolectasis is associated with honeycomb score on high-resolution CT (HRCT), and Walsh et al.[30] showed that traction bronchiectasis on HRCT is closely associated with fibroblastic foci profusion on histology. These suggest that traction bronchiectasis and honeycombing are part of a “continuous spectrum of lung remodeling” as noted in clinical observations[31], and are in line with the

present microCT findings that demonstrate a direct communication between the small airway tree and honeycomb regions in lungs with IPF.

Together with previous findings that the polymorphism in the promoter region of the MUC5B gene which regulates mucin production from bronchiolar epithelium is associated with the pathogenesis of IPF[26, 32] and the honeycomb regions are lined with bronchiolar-like epithelium[33], we speculate that the terminal bronchiole remodeling might be involved in the honeycomb formation.

This study used a single histological section for each tissue sample. Although microscopic pathologies could vary throughout the tissue sample, it is speculated that within-sample variation is smaller than the inter-samples variation because the cylindrical tissue samples used in this study are relatively small (20 mm high and 14 mm in diameter). Moreover, the close correlation between the pathologist's score of patchy fibrosis and the tissue % on microCT that was obtained from the entire microCT stack, suggests that the single histological section is sufficiently representative of the pathology of the sample core.

Extensive research has shown that a transition of fibroblasts into synthetic myofibroblast, and subsequent deposition of collagen, plays an important role in the progressive fibrotic processafter repeated injuries in IPF[34, 35], however, the role of inflammatory immune cells is not established. Histological studies have found infiltration of inflammatory immune cells such as B cell aggregates[8, 36, 37] while clinical trials using immunosuppressive therapy have consistently failed to show the effectiveness in patients with IPF[38]. A recent study by Verleden et al. showed that the CD79a positive cell infiltration and lymphoid follicle formation are present even in minimal fibrotic regions of IPF lungs[22]. The present finding extends it by demonstrating an association of lymphoid follicle formation score with increased tissue% and decreased alveolar surface density and further supports the

notion that the persistent adaptive immune response contributes to a fibrotic remodeling process in IPF.

Moreover, an increase in CD68-positive cells was associated with the increased tissue% in IPF lungs. This finding is consistent with the hypothesis that macrophages are mainly involved in the pathogenesis of IPF[39, 40] but could also in part reflect the smoking history in all the patients. Macrophages are subcategorized into functional phenotypes such as M1 and M2, and play various roles in the lung, including host defense to external insults and wound healing after injury[41]. Therefore, in addition to staining with CD68 antibody, different approach such as gene expression profiling and flowcytometry should be integrated in a future study to explore the pathogenic roles of each macrophage phenotype in IPF.

There are limitations in the present study worth noting. First, all cases with IPF were from former smokers. Since smoking is a major cause of emphysema that is closely assocaited with the loss of the terminal bronchioles[13, 27, 28], the present finding of reduced number of terminal bronchioles in IPF might have been affected by smoking. However, there was no correlation between the pathologist's score for emphysema and the number of terminal bronchioles, suggesting that the influence of smoking-related emphysematous destruction is minimal in this study. Second, the sample number is small and all cases were very-severe IPF that required lung transplantation. In order to broaden insights into disease phenotypes, it would be beneficial if the design of future studies would include lung specimen from subjects with different stages of IPF as well as different smoking status (i.e. both smokers and non-smokers). Third, the static cross-sectional nature of the study limits causal inferences. Therefore, the present study was not able to test whether patchy fibrosis and honeycomb cysts formation induce infiltration of immune cells in IPF lungs or if specific immune cells induce a fibrotic process in IPF lungs.

In conclusion, this is the first study to show that quantitative morphometric microCT measurements of alveolar surface density, tissue%, and Lm are closely associated with the general histo-pathological scoring of patchy fibrosis, fibroblastic foci and honeycomb lesions in IPF. These data suggest that microCT measurements provide a reliable structural assessment of IPF lungs especially since the established major criteria of UIP/IPF are associated with reduced alveolar surface area which potentially impairs diffusion capacity in IPF patients. Furthermore, the three-dimensional microCT evaluation revealed that honeycomb formation, but not patchy fibrosis, is associated with a greater reduction in the number of terminal bronchioles in IPF. Volumetric microCT based quantification of the lung structure complements histological assessment of cellular composition of IPF lungs, and by combining these methods with subsequent gene expression analysis or single cell sequencing it may be possible to identify a novel therapeutic target for this devastating lung disease.

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470

471

Figure legend

Figure 1. Examples of pathological scores on IPF tissue section

H&E staining. (A) Mild patchy fibrosis (score=1) without honeycomb cysts formation (score=0) or emphysema (score=0). (B) Severe patchy fibrosis (score=3) without honeycomb cysts formation (score=0) or emphysema (score=0). (C) Mild patchy fibrosis (score=1) and emphysema (score=1) without honeycomb cysts formation (score=0). (D) Severe patchy fibrosis (score=3) and honeycomb (score=1) without emphysema (score=0). Scale bar indicates 2mm.

Figure 2. Comparisons of microCT measures between regions with and without honeycomb cysts formation in IPF samples.

H&E staining. (A) Patchy fibrosis (score =2) without honeycomb cysts formation (score=0). (B) Patchy fibrosis (score =2) with honeycomb cysts formation (score = 1). Arrow indicates honeycomb region. The histological sections were matched with microCT images. (C and D) The alveolar surface density and number of terminal bronchioles per ml lung volume on microCT were decreased in honeycomb regions (n=12) compared to non-honeycomb regions (n=47). * indicates $p<0.05$. (E) Three-dimensional rendering of the small airway tree (pink) overlaid onto microCT images from the same stack as used in panel B (see also online supplemental video 1). The small airway was connected with airspace in the honeycomb region (orange).

Figure 3. Comparisons of alveolar surface density and number of terminal bronchioles between non-emphysematous regions with and without honeycomb cysts formation.

(A) The alveolar surface density and (B) Number of terminal bronchioles were compared between control, non-emphysematous IPF samples with and without honeycomb regions (n=36, 10, and 23). The absence of emphysema was determined based on the histological emphysema score of 0. * indicates $p<0.05$ compared to controls. † indicates $p<0.05$ compared to non-emphysematous IPF samples without honeycomb regions.

Tables

Table1. Demographic data of subjects

	Control (N=6)	IPF (N=10)
Age	58 ± 10	57 ± 5
Height (cm)	175 ± 6	173 ± 7
Weight (kg)	80 ± 15	73 ± 10
Sex	M : F = 6 : 0	M : F = 10 : 0
Smoking history	F : N = 2 : 4	F : N = 10 : 0
FEV_1 (% predicted)	NA	61 ± 15
FVC (% predicted)	NA	59 ± 20
D_{LCO} (% predicted)	NA	28 ± 8

IPF = idiopathic pulmonary fibrosis. M : F = Male : Female. F : N = Former smoker : Never smoker. FEV_1 = forced expiratory volume in one second, FVC= Forced vital capacity, D_{LCO}= diffusing capacity for carbon monoxide.

513 **Table2. Comparisons of microCT and histological findings between control and IPF**

	Control	IPF
No. tissue cores	36	60
MicroCT		
Alveolar surface density (/mm)	15.5 ± 2.0	8.9 ± 3.6**
Tissue% (%)	28.4 ± 3.7	50.5 ± 14.2**
Lm (um)	360 ± 53	529 ± 298**
No. terminal bronchioles/ml lung	4.1 ± 1.6	1.8 ± 1.3**
Histology scoring		
Patchy fibrosis	NA	1.3 ± 1.0
Fibroblastic Foci	NA	0.8 ± 0.8
Honeycomb	NA	0.2 ± 0.5
CD68	0.7 ± 0.5	1.6 ± 0.6**
CD4	0.4 ± 0.6	1.6 ± 0.7**
CD8	1.1 ± 0.4	1.6 ± 0.6**
CD79a	0.1 ± 0.3	1.2 ± 0.8**
Lymphoid follicle	0 ± 0	0.8 ± 0.8**

514 Tissue%=total lung volume taken up by tissue, Lm =the mean linear intercept. All scores range
515 from 0 to 3. NA = not available. **<p<0.005.

516

Table 3. Spearman correlation coefficients between microCT indices and pathological scores in IPF samples

	Alveolar surface density	Tissue%	Lm	No. terminal bronchioles
Major criteria for UIP				
Patchy fibrosis	-0.66**	0.69**	0.41**	-0.02
Fibroblastic foci	-0.52**	0.58**	0.39**	0.05
Honeycomb	-0.53**	0.38**	0.40**	-0.34**
Other scores				
Emphysema	0.01	-0.56**	0.19	-0.09
Inflammation	-0.25	0.31*	-0.03	0.09
Hyaline membrane	0.01	0.06	-0.13	0.00
Respiratory bronchiolitis	0.18	-0.17	-0.14	0.00

Tissue%=total lung volume taken up by tissue, Lm =the mean linear intercept.

*p<0.05, **<p<0.005.

Table 4. Spearman correlation coefficients between microCT indices and scores of infiltrated inflammatory immune cells in IPF samples

	Alveolar surface density	Tissue%	Lm	No. terminal bronchioles
CD68	-0.20	0.49**	0.02	0.20
CD4	-0.11	0.30*	0.09	0.19
CD8	-0.17	0.41**	0.05	0.09
CD79a	-0.44**	0.53**	0.19	0.03
Lymphoid follicle	-0.41**	0.44**	0.14	-0.06

Tissue%=total lung volume taken up by tissue, Lm =the mean linear intercept.

*p<0.05, **<p<0.005.

Figure 1

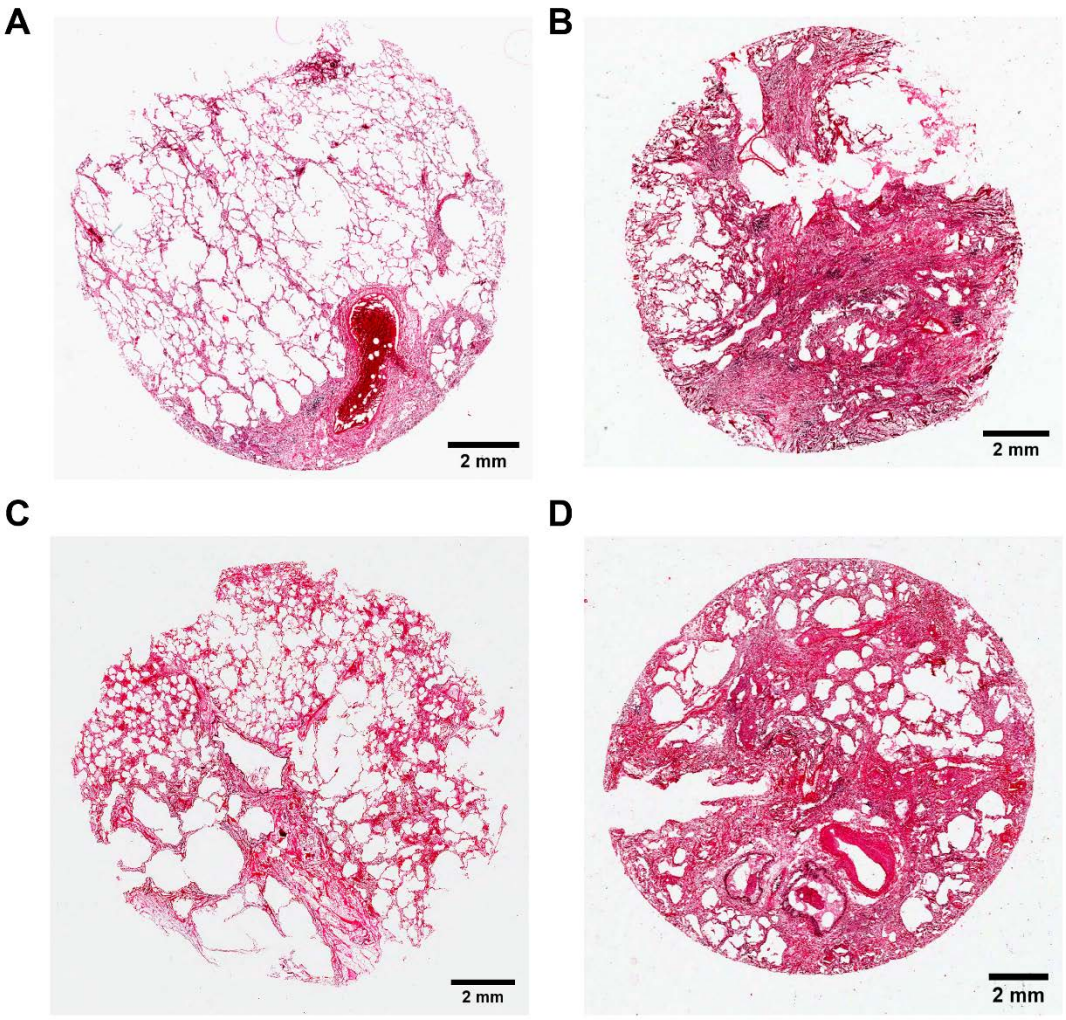

Figure 2

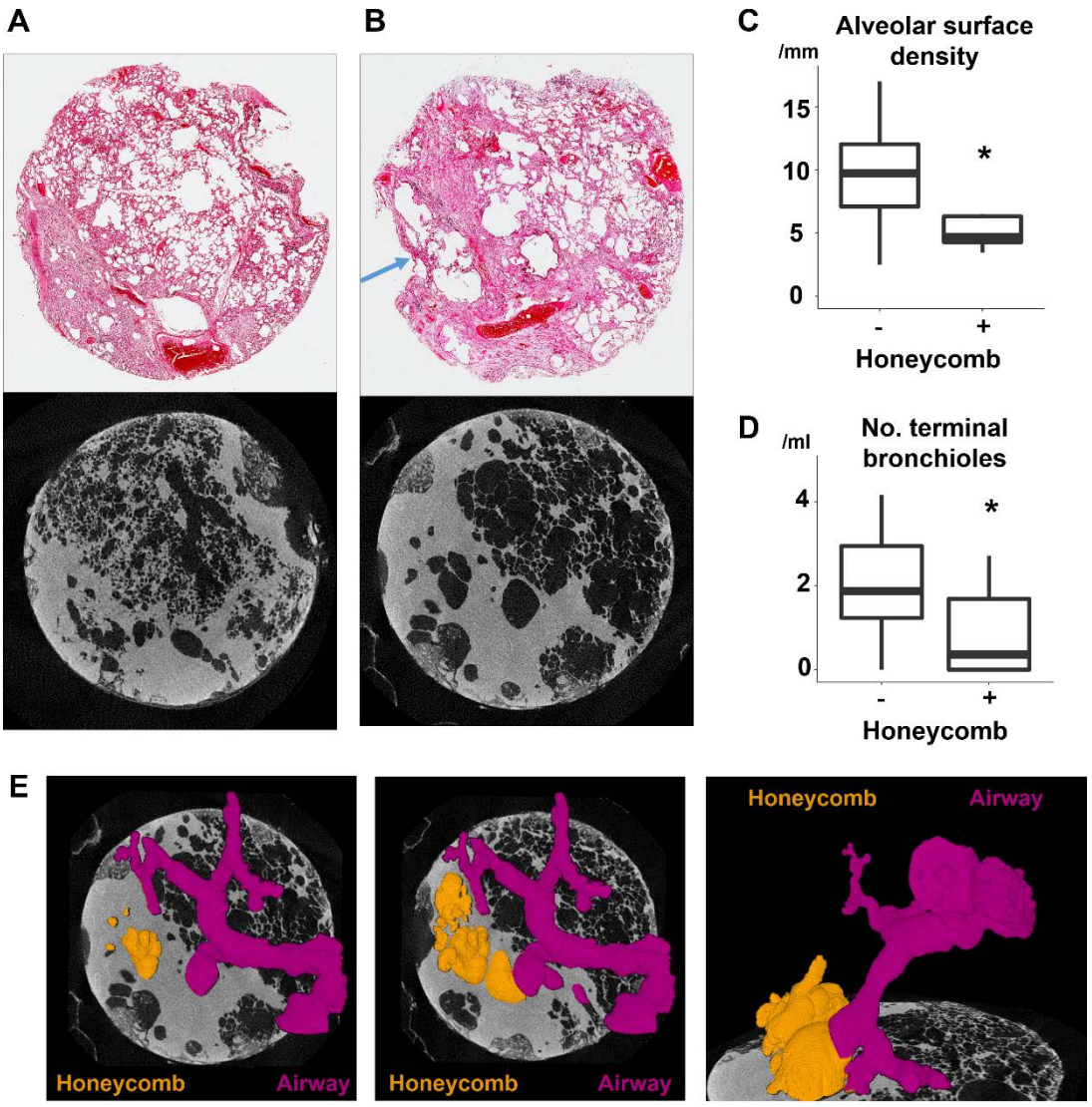

Figure 3