MACHINE LEARNING, COMPUTATIONAL PATHOLOGY, AND BIOPHYSICAL IMAGING

Pathology of Idiopathic Pulmonary Fibrosis Assessed by a Combination of Microcomputed Tomography, Histology, and Immunohistochemistry

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Idiopathic pulmonary fibrosis (IPF) is a fibrotic disease with the histology of usual interstitial pneumonia (UIP). Although the pathologist's visual inspection is central in histologic assessments, three-dimensional microcomputed tomography (microCT) assessment may complement the pathologist's scoring. We examined associations between the histopathologic features of UIP and IPF in explanted lungs and quantitative microCT measurements, including alveolar surface density, total lung volume taken up by 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 histologic analysis. An experienced pathologist scored three major UIP criteria (patchy fibrosis, honeycomb, and fibroblastic foci), five additional pathologic changes, and immunohistochemical staining for CD68-, CD4-, CD8-, and CD79a-positive cells, graded on a 0 to 3+ scale. The alveolar surface density and terminal bronchiolar number decreased and the tissue percentage increased in lungs with IPF compared with controls. In lungs with IPF, lower alveolar surface density and higher tissue percentage 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 and IPF criteria and suggest that the reduction in the terminal bronchioles may be associated with honeycomb cyst formation. (*Am J Pathol* 2020; 190: 2427–2435; <https://doi.org/10.1016/j.ajpath.2020.09.001>)

Idiopathic pulmonary fibrosis (IPF) is a chronic fibrotic disease characterized by a rapid decline in lung function and poor prognosis.¹ The American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Latin American Thoracic Association guidelines all recommend that the diagnosis and management² be based on an integrative multidisciplinary assessment of clinical information, radiologic assessment, and histopathologic diagnosis, but although diagnosis can be confirmed without histologic assessment in cases that present features of IPF, including honeycomb cysts on high-resolution computed tomography (HRCT),² histologic assessment remains

important especially for diagnosing the early stage of IPF and for improving understanding of the pathogenesis of the disease.

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The histopathologic 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 histologic and gene expression analyses have suggested that a B-cell–mediated immune response and lymphoid follicle formation may be associated with fibrosis.^{6–9}

The pathologist's visual scoring of UIP and IPF features on lung samples from surgical lung biopsy is essential in histologic assessment.^{1,2} Although the morphometric approach has been less used in the examination of lungs with IPF compared with other lung diseases, such as chronic obstructive pulmonary disease (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 and IPF and quantitative morphometric indexes, including the mean linear intercept (Lm), total lung volume taken up by tissue (%), and alveolar surface density defined as alveolar surface area per lung volume.¹⁷

The introduction of microcomputed tomography (microCT) has enabled three-dimensional (3D) morphologic assessments of lung tissues that are very difficult to

achieve with conventional histologic analysis.^{12,13,18,19} In addition, microCT of frozen air-inflated tissue enables quantitative assessment without shrinkage and physical cutting of tissues.²⁰ Mai et al¹⁶ combined CT, microCT, and histologic assessment, and found that fibrosis and honeycomb cyst formation in IPF extend from the peripheral to the central region of the pulmonary lobules. Furthermore, McDonough et al²¹ presented preliminary microCT-based analysis of the complex relationship between honeycomb 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. Recently, Verleden et al²² proposed the importance of the small airway disease in IPF by reporting 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 with control lungs. Collectively, these findings indicate 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 lungs with UIP and IPF by investigating the relationship between the experienced pathologist's scorings of histologic features, such as honeycomb cysts, and microCT measurements of alveolar surface density, tissue percentage, Lm, and the number of the terminal bronchioles.

Figure 1 Examples of pathological scores on idiopathic pulmonary fibrosis (IPF) tissue section (hematoxylin and eosin 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 bars = 2 mm.

Table 1 Demographic Characteristics of Study Participants

Characteristic	Control	IPF
Age, y	58 ± 10	57 ± 5
Height, cm	175 ± 6	173 ± 7
Weight, kg	80 ± 15	73 ± 10
Sex, n M:F	6:0	10:0
Smoking history, n former:never	2:4	10:0
FEV ₁ , % predicted	NA	61 ± 15
FVC, % predicted	NA	59 ± 20
D _{LCO} , % predicted	NA	28 ± 8

Data are expressed as means ± SD unless otherwise indicated. *n* = 6 control; *n* = 10 IPF.

D_{LCO}, diffusing capacity for carbon monoxide; FEV₁, forced expiratory volume in one second; FVC, forced vital capacity; IPF, idiopathic pulmonary fibrosis; NA, not applicable.

Materials and Methods

Study Protocol

A diagnosis of IPF was based on the current American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Latin American Thoracic Association 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 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 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} Informed consent was obtained directly from the patient or from the next of kin of the donors who 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.

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,^{9,23} image thresholding was applied to separate tissue and airspaces. The tissue segmentation was used to compute tissue percentage and alveolar surface density (defined as alveolar surface area per volume of lung). The airspace segmentation was used to compute the mean airspace size (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 milliliter of lung was calculated

by dividing the number per sample by the sample volume.^{13,20}

Histologic Analysis

The pathologist's scoring was performed in the present study using histologic sections obtained in the previous IPF study.²² After microCT, 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 histologic sections were cut and stained with hematoxylin and eosin and Movat Pentachrome stains. These histologic sections were examined by an experienced pulmonary pathologist (T.V.C.), who scored eight pathological features that consisted of the three major UIP criteria, including patchy fibrosis, honeycomb cyst formation, and fibroblastic foci, as well as five additional pathologic changes, including emphysema, degree of inflammation, hyaline membrane formation, lymphoid follicles, and respiratory bronchiolitis, all on a 0 to 3+ scale (Figure 1). In addition, other portions of the frozen samples were briefly warmed to −1°C, vacuum embedded in the OCT compound (Sakura Finetek, Torrance, CA), 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 (M0876; 1:200 dilution; Dako Cytomation, Carpinteria, CA), CD4 (M7310; 1:200 dilution; Dako Cytomation), CD8 (M7103; 1:400 dilution; Dako Cytomation), and CD79a (M7050; 1:200 dilution; Dako Cytomation) as previously reported.²² These sections were also scored by the same pathologist on a 0 to 3+ scale.

Table 2 Comparisons of microCT and Histologic Findings between the Control and IPF Groups

Variable	Control	IPF
Tissue cores, <i>n</i>	36	60
microCT		
Alveolar surface density, mm ² /mm ³	15.5 ± 2.0	8.9 ± 3.6*
Tissue, %	28.4 ± 3.7	50.5 ± 14.2*
Lm, μm	360 ± 53	529 ± 298*
Terminal bronchioles in lung, n/mL	4.1 ± 1.6	1.8 ± 1.3*
Histologic 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*

Data are expressed as means ± SD unless otherwise indicated. All scores range from 0 to 3.

**P* < 0.005.

Lm, the mean linear intercept; IPF, idiopathic pulmonary fibrosis; microCT, microcomputed tomography; NA, not available; tissue, %, total lung volume taken up by tissue.

Statistical Analysis

Data are expressed as means $\pm$ SD. Statistical analysis was performed with the R statistical program version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org>). The Spearman correlation test

and *U*-test were used for correlation tests and group comparisons, respectively. Multiple comparisons were performed with Wilcoxon tests with Holm correction.

Results

There is no significant difference in age, sex, height, or weight between the patients with IPF and the controls (Table 1). Table 2 summarizes microCT and histologic scores. Alveolar surface density and the number of terminal bronchioles per milliliter of lung were lower, whereas tissue percentage and Lm were higher in patients with IPF compared with controls. In addition, CD68-, CD4-, CD8-, and CD79a-positive cells were greater in patients with IPF than controls. The significant difference between the patients with IPF and controls was also present when comparing microCT indexes in the upper, middle, and lower regions separately (Supplemental Figure S1).

Figure 2, A and B gives examples of histologic regions with and without honeycomb formation (score 0 and 1, respectively) that were registered to the microCTs. The alveolar surface density and number of terminal bronchioles were lower in the honeycomb regions (score ≥ 1) than in the nonhoneycomb regions (score = 0) (Figure 2, C and D). This finding was visualized (Figure 2E) and a video that shows a microCT stack of the same sample (Figure 2, B and E) (Supplemental Video S1). 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). Furthermore, the decreases in the alveolar surface density and number of terminal bronchioles in the honeycomb region were also confirmed in a subanalysis that included nonemphysematous IPF samples (histologic emphysema score = 0) and controls (Figure 3).

Table 3 shows Spearman correlation coefficients between microCT indexes and pathologic scores in IPF samples ($n = 59$). Decreased alveolar surface density and increased tissue percentage and Lm on microCT were correlated with the histologic scores of patchy fibrosis, fibroblastic foci, and honeycomb. In contrast, a decreased number of the terminal

Figure 2 Comparisons of microcomputed tomographic (microCT) measures between regions with and without honeycomb cysts formation in idiopathic pulmonary fibrosis (IPF) samples (hematoxylin and eosin 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 histologic sections were matched with microCT images. **C and D:** The alveolar surface density and number of terminal bronchioles per milliliter of lung volume on microCT were decreased in honeycomb regions compared with non-honeycomb regions. **E:** Three-dimensional rendering of the small airway tree (pink) overlaid onto microCT images from the same stack as used in **B** (Supplemental Video S1). The small airway was connected with airspace in the honeycomb region (orange). $n = 12$ honeycomb regions; $n = 47$ nonhoneycomb regions. * $P < 0.05$ versus nonhoneycomb regions. Original magnification, $\times 1$ (**A**, **B**, and **E**).

Figure 3 Comparisons of alveolar surface density and number of terminal bronchioles between nonemphysematous regions with and without honeycomb cysts formation. The alveolar surface density (**A**) and number of terminal bronchioles (**B**) were compared between control, non-emphysematous idiopathic pulmonary fibrosis (IPF) samples with and without honeycomb regions. The absence of emphysema was determined based on the histologic emphysema score of 0. $n = 36$ control; $n = 10$ nonemphysematous idiopathic pulmonary fibrosis (IPF) samples with honeycomb regions; $n = 23$ nonemphysematous idiopathic pulmonary fibrosis (IPF) samples without honeycomb regions. * $P < 0.05$ versus controls; † $P < 0.05$ versus nonemphysematous IPF samples without honeycomb regions.

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 indexes and scores of lymphoid cells in IPF samples ($n = 60$). Increased tissue percentage 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 histopathologic criteria of UIP and IPF and quantitative morphologic measures

obtained from microCT. The microCT findings of decreased alveolar surface density and increased tissue percentage 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 histologic assessment and the 3D 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 3D morphometric assessment via microCT can be used to complement the pathologist's visual inspection by showing the pathological relationship between the peripheral airways and parenchyma in lungs with IPF.

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

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 found that a loss of the terminal bronchiole number already occurs in minimal fibrotic regions in lungs with IPF compared with controls.²² Because the terminal bronchioles are located in the center of the secondary lobules, these recent microCT findings have raised the question of whether there is an interaction between small airway disease, such as loss of the terminal bronchioles, and parenchymal pathologic findings, such as alveolar collapse. The present study sheds light on this issue with the finding 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 separate processes. This finding is consistent with the hypothesis proposed by Evans et al²⁶ that the peripheral airway injury is associated with honeycomb cyst formation independent of fibroproliferation in the parenchyma. In addition, although COPD studies have found 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).

Table 3 Spearman Correlation Coefficients between Microcomputed Tomography Indexes and Pathological Scores in Idiopathic Pulmonary Fibrosis Samples

Variable	Alveolar surface density, mm ² /mm ³	Tissue, %	Lm	Terminal bronchioles, n/mL
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

P* < 0.05, *P* < 0.005.

Lm, mean linear intercept; tissue, %, total lung volume taken up by tissue; UIP, usual interstitial pneumonia.

Furthermore, [Supplemental Video S1](#) portrays the 3D spatial relationship between the small conducting airways and microscopic honeycomb regions, which on the conventional 2D histologic sections would be difficult to detect. The 3D visualization indicates 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 that is beyond the scope of the present study.

Staats et al²⁹ found that a histologic finding of bronchiolectasis is associated with honeycomb score on HRCT, and Walsh et al³⁰ found that traction bronchiectasis on HRCT is closely associated with fibroblastic foci profusion on histologic analysis. These findings suggest that traction bronchiectasis and honeycombing are part of a continuous spectrum of lung remodeling as noted in clinical observations³¹ and are in line with the present microCT findings that indicate 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 histologic section for each tissue sample. Although microscopic pathologic findings could vary throughout the tissue sample, it is speculated that within-sample variation is smaller than the intersample 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 percentage on microCT that was obtained from the entire microCT stack suggests that the single histologic section is sufficiently representative of the pathologic findings of the sample core.

Extensive research indicates that a transition of fibroblasts into synthetic myofibroblast and subsequent deposition of collagen play an important role in the progressive fibrotic process after repeated injuries in IPF^{34,35}; however, the role of inflammatory immune cells is not established. Histologic studies have found infiltration of inflammatory immune cells, such as B-cell aggregates,^{8,36,37} whereas clinical trials using immunosuppressive therapy have consistently failed to find the effectiveness in patients with IPF.³⁸ A recent study by Verleden et al²² found that the CD79a-positive cell infiltration and lymphoid follicle formation are present even in minimal fibrotic regions of lungs with IPF. The present finding extends this observation by finding an association of lymphoid follicle formation score with increased tissue

Table 4 Spearman Correlation Coefficients between Microcomputed Tomography Indexes and Scores of Infiltrated Inflammatory Immune Cells in Idiopathic Pulmonary Fibrosis Samples

Variable	Alveolar surface density, mm ² /mm ³	Tissue, %	Lm	Terminal bronchioles, n/mL
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

P* < 0.05, *P* < 0.005.

Lm, mean linear intercept; tissue, %, total lung volume taken up by tissue.

percentage 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 percentage in lungs with IPF. 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 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 to the present study worth noting. First, all cases with IPF were from former smokers. Because smoking is a major cause of emphysema that is closely associated with the loss of the terminal bronchioles,^{13,27,28} the present finding of a reduced number of terminal bronchioles in IPF might have been affected by smoking. However, no correlation was found 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 of IPF were very severe and required lung transplantation. To broaden insights into disease phenotypes, it would be beneficial if the design of future studies would include lung specimen from individuals with different stages of IPF as well as different smoking status (ie, both smokers and nonsmokers). 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 cyst formation induce infiltration of immune cells in lungs with IPF or if specific immune cells induce a fibrotic process in lungs with IPF.

In conclusion, this is the first study, to our knowledge, to find that quantitative morphometric microCT measurements of alveolar surface density, tissue percentage, and Lm are closely associated with the general histopathologic scoring of patchy fibrosis, fibroblastic foci, and honeycomb lesions in IPF. These data suggest that microCT measurements provide a reliable structural assessment of lungs with IPF, especially since because established major criteria of UIP and IPF are associated with a reduced alveolar surface area, which potentially impairs diffusion capacity in patients with IPF. Furthermore, the 3D 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 histologic assessment of cellular composition of lungs with IPF, 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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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.09.001>.

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