Micro-CT analysis of paraffin embedded lung tissue: Is small airway obstruction an early feature of COPD?

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ABSTRACT

Rationale: Airflow obstruction, the hallmark characteristic of Chronic Obstructive Pulmonary Disease (COPD) has long been attributed to a combination of small airways disease and emphysematous destruction, however, the relative role of each pathological feature is not well understood. McDonough et al. (*NEJM*, 2011), recently reported a significant reduction in terminal bronchiolar number in end-stage COPD compared to controls with normal lung function. Further, the study reported that the loss of terminal bronchioles occurred in regions of lung with and without emphysema, leading to our hypothesis that *'The obstruction and obliteration of small airways occurs early in COPD and precedes the development of emphysema'.*

Methods: Lung samples were obtained from patients with known pulmonary function undergoing surgical resection for lung cancer or lung transplant. Lungs were inflated, sliced and sampled prior to formalin fixation and paraffin embedding (FFPE). Eight FFPE cores per patient were randomly sampled throughout the lung and scanned using a Nikon Metrology micro-CT scanner, and volumetric data sets were examined to determine mean linear intercept (Lm), number of terminal (TB) and respiratory bronchioles (RB) per ml of tissue. Image registration was used to precisely locate regions of interest, enabling efficient sectioning and staining with Movat's Pentachrome for a more comprehensive analysis of terminal bronchiole morphology.

Results: Our study demonstrates that micro-CT scans of FFPE cores provide adequate resolution of fine lung architecture when compared to mean linear intercept measurements obtained from matched histological sections (Bland-Altman). We report that the total number of terminal bronchioles is significantly decreased from 6.2 ± 1.1 TB/ml in smokers with normal lung function, to 4.6 ± 1.0 TB/ml in mild/moderate COPD (P<0.05), and 3.1 ± 1.8 TB/ml in severe COPD. The number of obstructed terminal bronchioles (with complete occlusion of the lumen) is significantly increased from 0.3 ± 0.4 TB/ml in our smoker control group to 2.0 ± 1.9 TB/ml in mild/moderate disease (P<0.05). Further, we demonstrate a significant decrease in respiratory bronchiolar number from 16.7 ± 7.2 RB/ml in smokers with normal lung function to 10.0 ± 6.0 RB/ml in mild/moderate COPD, and 2.1 ± 2.7 RB/ml in severe COPD (P<0.001). When correlating the number of terminal and respiratory bronchioles to Lm, we find that the bronchioles are destroyed in tissues where no emphysema is present. Lesions of interest were characterized by histology and multi-photon microscopy to further understand the pathological process.

Conclusions: Clinical trials and the treatment of COPD have traditionally focused on patients with severe disease (GOLD 3+4), and no current pharmacological therapies have been shown to affect long term, lung function decline. Our findings suggest that irreversible pathological events occur in the early stages of COPD, emphasizing the importance of early diagnosis and intervention to modify the progression of this debilitating respiratory disorder.

INTRODUCTION and HYPOTHESIS

- The persistent and progressive airflow limitation which defines chronic obstructive pulmonary disease (COPD) is caused by small airways disease and / or emphysematous destruction.
- Using retrograde catherization, it has been shown that small airways (<2mm in luminal diameter) are the major site of airflow obstruction in COPD (Hogg et al. *NEJM*, 1968).
- McDonough et al. (*NEJM*, 2011), using a combination of MDCT and micro-CT demonstrated a significant reduction in the number of terminal bronchioles in regions of the lung with and without emphysema in end-stage COPD. This led to our hypothesis that:

  'The obstruction and obliteration of small airways occurs early in mild / moderate COPD and precedes the development of emphysema'
- Micro-xray computed tomography (micro-CT), in contrast to clinical multi-detector computed tomography (MDCT) scanners provide the resolution required to visualize the distal small airways and fine lung parenchyma. Further, micro-CT has a significant advantage over traditional histology as it offers non-destructive 3D imaging at the microscopic level.
- The acquisition of 3D imaging enables virtual sectioning of the sample in any orientation for a better understanding of the intricate lung architecture. In addition, structures of interest can be localized within the volumetric micro-CT image for histological staining, decreasing the blinded, laborious and costly sectioning of samples.
- Formalin fixed, paraffin embedded (FFPE) tissue, the gold standard for routine histological examination has previously been precluded from micro-CT imaging due to the similar densities of lung tissue and paraffin wax. Our innovative micro-CT imaging protocol enables non-destructive imaging of archival FFPE lung tissue for the assessment of terminal bronchiolar number and emphysema to further understand the inter-relationship of these pathological events.

SAMPLE COHORT

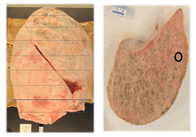
	Controls: Smokers with normal lung function n = 10	Mild/Moderate COPD		Severe COPD
		GOLD 1 n=10	GOLD 2 n=10	GOLD 4 n=6
Gender (Female: Male)	6:4	3:7	4:6	1:5
Age (years)	64 ± 10	67 ± 6	61 ±15	59 ± 2
Smoking history (pack years)	33 ± 7	38 ± 10	37 ±10	41 ± 28
FEV_1 (% predicted)	96.0 ± 6.8	88.0 ± 8.1	63.4 ± 7.0	20.5 ± 8.1
FEV1/FVC (% of FVC)	77.0 ± 5.0	66.0 ± 4.0	61.4 ± 4.2	30.0 ± 8.5

Table 1. Lung samples from donors with normal lung function and mild/moderate COPD (GOLD 1+2) were donated from patients undergoing surgical resection for lung cancer treatment. Lung samples from donors with severe COPD (GOLD 4) were donated from patients undergoing lung transplantation. All specimens were donated with consent to the James Hogg Research Lung Registry.

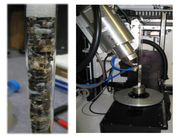
METHODS

Figure 1. Methods of tissue collection, image acquisition and analysis

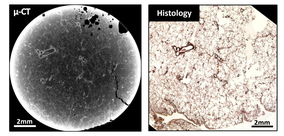
The pre-operative MDCT scan prior to lung surgery is used to determine whole lung volumes and lung densities.



- Lungs are inflated and sliced from apex to base.
- Eight random samples are extracted uniformly over lung height, formalin fixed and embedded in paraffin for micro-CT scanning.



- Imaging protocol conducted at University of Southampton, UK
- Nikon micro-CT scanner
- X-ray energy: 50-70kV
- Current: 110-150μA
- 3D reconstruction with voxel dimension of 6.7μm, allowing analysis of fine lung morphometry.



- Air space size is measured using mean linear intercept (Lm).
- 3D visualization of the branching airway network allows terminal and respiratory bronchioles to be identified and counted.
- Lesions of interest are located using image and spatial registration, and sectioned for further histological analysis.

RESULTS

Figure 2. Micro-CT imaging of FFPE lung tissue samples provides comparable measurements of mean linear intercept (Lm) to those performed on matched histological sections

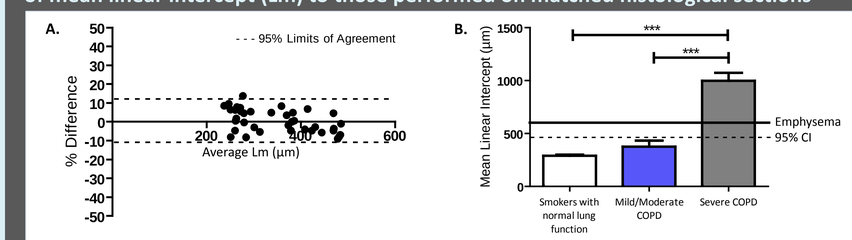


Figure 2A. Bland-Altman comparison of mean linear intercept (Lm) analyzed on micro-CT and matched histological sections (n=40), validating such measurements provided by micro-CT imaging of FFPE lung tissue samples

Figure 2B. Comparison of mean Lm per case in smokers with normal lung function (white bar, n=5), mild/moderate COPD (blue bar, n=5) and severe COPD (grey bar, n=6). Emphysema is determined as Lm >600 μm, the absolute limit of frequency distribution of Lm in normal lung tissue. Lm value of <489 μm depicts the upper 95% confidence interval in normal lung tissue (McDonough et al. *NEJM*, 2011).

Figure 3. Comparison of the number of non-diseased and obstructed terminal bronchioles per ml of lung tissue and the relationship with mean linear intercept

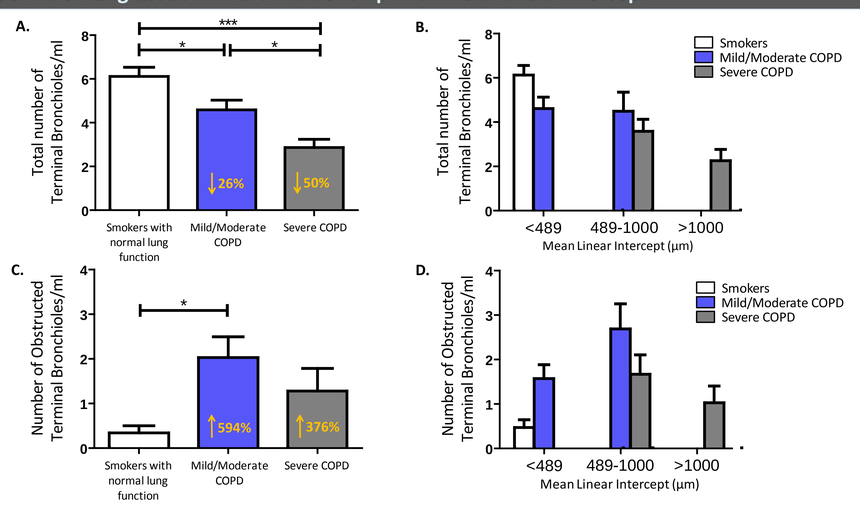


Figure 3A. The mean number of terminal bronchioles per milliliter (TB/ml) is decreased in mild/moderate COPD (P<0.5, n=5) and severe COPD (P<0.05, n=6) compared to smokers with normal lung function (n=5). Orange indicates the percentage difference from the smoker control group.

Figure 3B. The distribution of the mean number of terminal bronchioles per milliliter segregated by the mean linear intercept (Lm). Lm <489 μm depicts the upper 95% confidence interval of normal lung tissue, >600 μm depicts the absolute value for emphysema.

Figure 3C. The mean number of obstructed terminal bronchioles per milliliter is significantly increased in mild/moderate COPD (P<0.05, n=5) compared to smokers with normal lung function (n=5). There is no significant difference in the severe COPD group (n=6).

Figure 3D. The distribution of the mean number of obstructed terminal bronchioles per milliliter segregated by the mean linear intercept (Lm). Lm <489 μm depicts the upper 95% confidence interval of normal lung tissue, >600 μm depicts the absolute value for emphysema.

Data are analysed using a multi-variant ANOVA with Tukey's pairwise comparison.

RESULTS

Figure 4. Comparison of the number of respiratory bronchioles per ml of lung tissue and the relationship with mean linear intercept

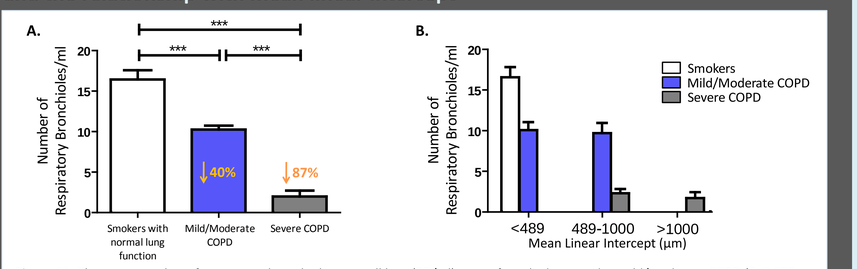
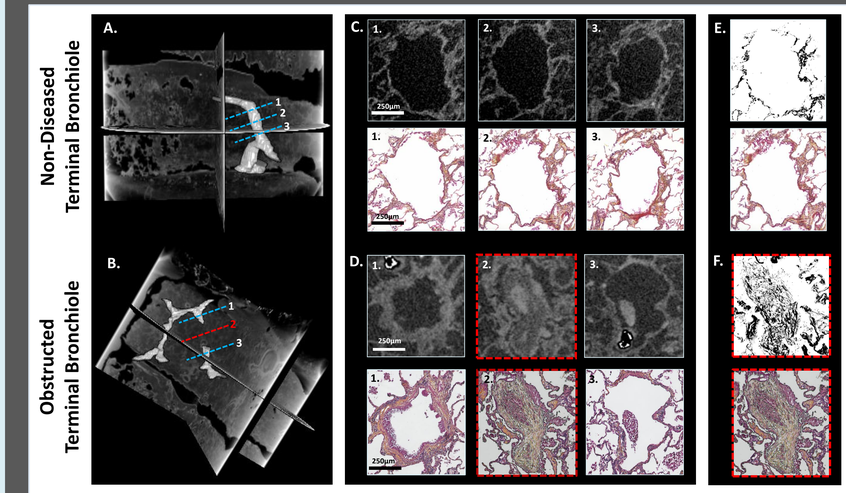


Figure 4A. The mean number of respiratory bronchioles per milliliter (RB/ml) is significantly decreased in mild/moderate COPD (P<0.005, n=5) and severe COPD (P<0.005, n=6) compared to controls (n=5).

Figure 4B. The distribution of the mean number of respiratory bronchioles per milliliter segregated by mean linear intercept. Lm <489 μm depicts the upper 95% confidence interval of normal lung tissue, >600 μm depicts the absolute value for emphysema.

Figure 5. Characterization of obstructed terminal bronchioles by histology and multi-photon microscopy using micro-CT as a scouting tool for efficient sampling



Left panel: 3D segmentation enables pathological lesions of interest to be observed in perspective to the branching airway network and surrounding structures such as vessels and parenchyma. Figure 5A demonstrates the luminal 3D rendering of a non-diseased terminal bronchiole generated from semi-automatic tracing. In comparison, Figure 5B demonstrates an obstructed terminal bronchiole from a GOLD 2 COPD patient with complete luminal obstruction.

Middle panel: Micro-CT imaging provides precise locations of airway lesions enabling efficient serial sectioning. The numbered lines indicate where corresponding sections were taken. Histological sections are stained with Movat's pentachrome to identify structural cells and matrix components (Figures 5C+D).

Right panel: Further characterization of the lesion of interest using multi-photon microscopy shows the presence of fibrillar collagen (shown in black) in the area of luminal obstruction (Figure 5F).

CONCLUSIONS and CLINICAL SIGNIFICANCE

- Micro-CT scans of FFPE lung tissue enables the visualization of small airways <2mm in luminal diameter and parenchymal structures, not amenable by clinical MDCT.
- We demonstrate that there is a decrease in the number of terminal and respiratory bronchioles in mild to moderate COPD (GOLD 1+2) lungs.
- These findings demonstrate that irreversible structural damage in the small distal airways has already occurred despite a preserved FEV_1, indicating a need for a more sensitive diagnostic tool for early detection.
- Our data shows that the obstruction and obliteration of terminal and respiratory bronchioles begins in the presence of no emphysematous destruction.
- Clinical trials of severe COPD patients have shown that current pharmacological therapies do not slow or reverse the rate of decline in lung function in COPD patients. Our data indicate that earlier therapeutic intervention and targeting of the distal small airways may provide a more successful outcome in modifying the progression of this debilitating respiratory disorder.

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