Loss of small airways occurs in mild and moderate Chronic Obstructive Pulmonary Disease

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ABSTRACT (Word count = 298)

BACKGROUND
The concept that the small conducting airways <2mm in diameter become the major site of airflow obstruction in chronic obstructive pulmonary disease (COPD) is well established in the literature. It has also been shown that the last generation of small conducting airways, terminal bronchioles, are significantly destroyed in patients with very-severe COPD. What is not known is at what stage in the development of COPD the loss of small airways occurs, or how loss of terminal and transitional (first generation of respiratory airways) bronchioles - relates to the loss alveolar surface area that characterizes emphysema.

METHODS
A novel multi-resolution computed tomography (CT) imaging protocol was applied to systematically, randomly sampled whole lungs or lobes of smokers with normal lung function (n=10), mild (n=10), moderate (n=8), and very-severe COPD (n=6). The 34 lung specimens provided 262 lung tissue samples for stereological assessment of the number and morphology of terminal and transitional bronchioles, airspace size (Lm), alveolar surface area.

FINDINGS
The new data demonstrate that 41% of terminal bronchioles, 57% of transitional bronchioles, and 37% of the alveolar surface area is lost in patients with mild and moderate COPD compared to control smokers, before any emphysematous changes can be detected by CT. We also show these pathological changes correlate with lung function decline. Importantly, we demonstrate that loss of terminal and transitional bronchioles occurs in regions of the lung that have no loss of alveolar surface area. Further, we validated using histology, that the surviving small airways have thickened walls and narrowed lumens which become more obstructed as the disease progresses.

INTERPRETATION
These data demonstrate that small airways disease is an early pathological feature in mild and moderate COPD. Importantly, this study emphasises that early intervention in mild and moderate COPD patients is most likely required for disease modification.

RESEARCH IN CONTEXT (Word count = 344)

Evidence before this study
Results from several physiological studies have demonstrated that the small conducting airways less than 2mm in internal diameter are the major site of airflow obstruction in patients who have very-severe chronic obstructive pulmonary disease (COPD). To date, the pathology of small airway disease, and its relationship to the development of emphysema has not been studied in mild and moderate COPD due to the limitations in the spatial resolution of clinical computed tomography (CT) imaging.

Added value of this study
This study uses a novel approach that combines stereology, multi-resolution CT imaging and histology that for the first time provides direct evidence that significant loss of small airways (41% of terminal and 57% of transitional bronchioles) occurs in patients with mild and moderate COPD, prior to the detection of emphysema by clinical CT. Further, these new data demonstrate that destruction of terminal and transitional bronchioles can occur in regions of the lung with normal alveolar surface area - a robust measure of internal lung structure measured using micro-CT. We further validated using histology that the surviving small airways have thickened walls and narrowed lumens which become more obstructed as the disease progresses. These data indicate that small airways disease is an early feature of mild and moderate COPD.

Implications of all of the available evidence
Although these results must be considered preliminary based on the small number of cases that have been studied to date, they strongly support the concept that small airways disease is well established by the time the diagnosis of mild or moderate COPD is made. Further, the data suggest that the reason most clinical trials investigating COPD treatments in severe COPD, may have failed is because they were initiated after a substantial number of terminal and transitional bronchioles were already destroyed, and that early intervention in mild and moderate COPD patients is most likely required for disease modification. We expect that the findings from this research will prompt discussion of guidelines and policies to improve early diagnosis, and effective management of patients with mild and moderate COPD.

INTRODUCTION

It is well established that the small conducting airways less than 2mm in internal diameter that offer less than 10% of the total resistance to airflow in the normal lung,[1-3] become the major site of airflow obstruction in chronic obstructive pulmonary disease (COPD).[4-6] To develop effective treatments for COPD it is therefore vital to understand disease pathogenesis within these small airways. Current computed tomography (CT) imaging protocols applied in longitudinal COPD studies with 1-2mm in-plane spatial resolution and low dose radiation exposure (1.5mSv) can only resolve airways larger than 2·5mm in internal diameter.[7, 8] A recent study using ultra-high resolution CT and high dose radiation exposure (11.2mSv), has shown that it is possible to visualize small airways 0.8 mm in diameter.[9] However, to date only micro-CT has the spatial resolution required to resolve the alveolar structure and reliably identify and characterize the smallest generation of conducting airways, the terminal bronchioles, with an average lumen diameter of 424μm.[10] McDonough et al.,[10] using micro-CT on fixed and dried lung samples showed that compared to controls (n=4), terminal bronchioles are reduced by 72% in very-severe GOLD4 (Global Initiative for Obstructive Lung Disease Guidelines)[11] COPD patients with panlobular emphysema (n=7) and by 89% in GOLD4 COPD patients with centrilobular emphysema (n=4). In addition, they showed that terminal bronchiole numbers were reduced even in tissue samples that had no detectable emphysema as measured by mean linear intercept (Lm). These data suggested that terminal bronchiole obliteration may precede emphysematous tissue destruction in COPD.[10]

It is currently not possible to test the hypothesis that terminal bronchioles are lost prior to emphysematous destruction in a longitudinal study of disease progression within the same subject. In the present study, we performed a cross-sectional analysis of lungs donated following pneumonectomy and lobectomy surgery by smokers with normal lung function (n=10), mild (GOLD1, n=10), moderate (GOLD2, n=8) and very-severe (GOLD4, n=6) COPD patients with centrilobular emphysema. The purpose of this study was to determine if destruction of the terminal and for the first time transitional bronchioles (the first generation of respiratory airways)[12, 13] occurs prior to, or in parallel with, emphysematous tissue destruction. Further, in this study emphysematous tissue destruction was assessed using the measurement of alveolar surface area which: 1) provides information on the alveolar tissue present for functional gas exchange, and 2) compared to Lm is a robust measure of internal lung structure that is not affected by changes in lung tissue compliance,[14, 15] an important physiological change in the COPD lung. Since lungs resected for lung cancer are invariably fixed prior to study we also utilized a novel micro-CT imaging protocol for formalin-fixed, paraffin embedded lung tissue samples,[16] which enabled a matched histological investigation of the "surviving" small airways.

METHODS

Subjects

Informed consent was obtained from 28 patients treated for small peripheral lung cancers (primary tumours, no metastasis) by lobectomy and pneumonectomy surgery at St. Paul's Hospital with approval by the Providence Health Care Research Ethics Board (PHCREB, Vancouver, Canada); and from 6 patients treated by lung transplantation for GOLD4 COPD at the University of Pennsylvania Hospital, with the approval of the University of Pennsylvania Hospital Institutional Review Board (Philadelphia, USA). All specimens were stored in a dedicated archival Lung Registry with approval of the PHCREB (#H13-02173). The 34 subjects included: ten controls (smokers with normal lung function), ten patients with mild (GOLD1) COPD, eight with moderate (GOLD2) COPD and six with very-severe (GOLD4) COPD with centrilobular emphysema. The demographic and clinical characteristics including post-bronchodilator spirometric data of all donors are presented in Table 1.

CT analysis

Preoperative inspiratory thoracic CT scans were available for 30 of the 34 patients because four subjects with mild and moderate COPD did not have preoperative scans. All 30 of the CT scans were acquired at suspended full inspiration by coaching the subject to inhale to total lung capacity in the supine position, as previously described.[17] The CT image acquisition parameters were; 120-140kV, 80-345mA, 5-10mm slice thickness, 0-10mm space between slices. Semi-automatic lung segmentation was performed on the CT scans using a custom software package EmphylXLJ[18] to isolate the specific resected lung or lobe (table 2) and calculate lung volumes. To quantify emphysema on the 10mm thick slice CT scans a density threshold of -910 Hounsfield Units (HU) (% low-attenuation area (LAA) < -910) was used, as this pathology validate threshold defined by Muller et al.[19] has been shown to yield the best correlation with emphysema when using 10mm thick slice CT datasets.[19]

Lung tissue preparation
Briefly, resected lobes and whole lungs were inflated, frozen and sliced into contiguous 2cm thick slices in the trans-axial plane as previously described.[10,20] Excluding the tumour and normal margin, tissue samples measuring 1.5cm in diameter were extracted from the lung slices using a systematic uniform sampling method to obtain representative unbiased samples of the lobe or lung. For this study, one tissue sample per slice was obtained resulting in 6 to 11 samples per subject given the varying size of lobes and lungs, which yielded a total of 262 lung samples. All samples were fixed in pre-cooled alcohol based formalin overnight, then infiltrated with low melting point paraffin using a tissue processor (Leica, Model ASP6025). The diameter, cross-sectional area and volume of each tissue sample were measured pre- and post-processing to assess tissue shrinkage, which was applied as a correction factor to the quantitative measurements.

Micro-CT imaging
The paraffin embedded tissue samples were scanned with a micro-CT scanner (HMX225, Nikon Metrology, Michigan, USA), using a previously developed imaging protocol[16] (50kV, 180µA, reconstructed with an isotropic voxel size of 6·7µm). Image processing was performed using ImageJ software (Version 1.47q, National Institutes of Health, USA) and data collection and analysis were performed in a blinded manner by three observers. To ensure measurements obtained by micro-CT imaging of formalin fixed, paraffin embedded tissue were comparable to the methodology of glutaraldehyde fixation and critical point drying previous used by McDonough et al.,[10] adjacent lung tissue samples from the GOLD4 donor lungs were processed using both methods, and we found no difference between the two methods when measuring alveolar surface area (appendix pp 4).

Stereology
Following the American Thoracic Society and European Respiratory Society (ATS/ERS) guidelines for stereology, ten systematic uniform randomly sampled image slices in each micro-CT scan were extracted and used for measurement of mean linear intercept (Lm) using a line-grid[15, 21] and alveolar surface area using a point counting grid[15, 22] in Image-Pro Plus (Version 5·1, Media Cybernetics, Silver Spring, USA). Measurements of mean linear intercept and alveolar surface area obtained by micro-CT were validated by comparison to the gold standard of histology (appendix pp 5).

Terminal bronchioles were identified within the micro-CT scan by following consecutive airway branches until the first generation of respiratory bronchioles, termed transitional bronchioles,[13] were identified by the first occurrence of individual alveoli along the airway wall (appendix pp 3). Identification of transitional bronchioles subsequently enabled the parent airway to be counted as a terminal bronchiole. The numbers of terminal and transitional bronchioles per milliliter of lung (TB/ml and TrB/ml, respectively) were calculated by dividing the number of respective bronchioles by the sample volume corrected for shrinkage.

Terminal bronchioles were further classified as non-diseased, thickened or obstructed. Thickened terminal bronchioles were defined as a wall area % (airway wall area/outer area of the airway*100) greater than the 95th percentile (41·9%) of non-diseased airways in controls. Obstructed terminal bronchioles were defined as those with 100% luminal obstruction (Detailed methods provided in appendix pp 2).

Histological analysis
After micro-CT scanning, the lung samples were used for histological assessment of terminal bronchioles. The first three 5µm histological sections from the tissue sample were used to re-orientate the micro-CT volumetric data set in the same plane as the sample using image registration. The exact coordinates of the terminal bronchioles within the volumetric micro-CT scan were used to locate and section the exact regions of interest within the sample. Sections were stained using Movat's Pentachrome stain (Detailed methods provided in appendix pp 2).

Statistical analysis
All samples per patient were averaged and presented per case. Outcomes were assessed for Gaussian distribution and tested for normality using the Kolmogorov-Smirnov test, D'Agostino-Pearson omnibus normality test and Shaprio-Wilk normality test. The measures of airways/ml of lung, diseased airways/ml of lung, and alveolar surface area/ml of tissue were normally distributed, and assessed using a one-way ANOVA with a Tukey's pairwise comparison. The measures of Lm and CT density data were not normally distributed using the Shaprio-Wilk normality test and therefore a non-parametric Kruscal Wallis with Dunns post-hoc test was used. This analysis was conducted using GraphPad Software (Version 5, California, USA). Kernal density plots with multiple comparisons were generated using the Bergmann Hommel method (scmamp R package). Terminal and transitional bronchioles counts in cores with normal alveolar surface area was assessed using a linear mixed-effect model (nlme package), and Pearson's correlations with false discovery rate corrections were performed using

the statistical software R3·3·1 (RStudio, Boston, USA). Data are expressed as the mean ± standard deviation (SD), or median ± interquartile range for non-parametric data. P values <0·05 were considered significant.

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The study sponsors had no role in the design; collection, analysis and interpretation of data, writing or submission of the manuscript.

RESULTS

Patient characteristics

Table 1 summarizes the patient characteristics. There were no differences in age, height, smoking history, sex and weight between the groups. As expected, subjects with GOLD2 and GOLD4 COPD had a significantly lower diffusing capacity (DLCO/VA) compared to the controls (p<0·05). Total lung volume computed by CT was larger in GOLD4 subjects compared to all other groups (p<0·01).

Quantification of terminal and transitional bronchiole numbers in mild to moderate COPD

Compared to control smokers with a mean 4·7±1·3 terminal bronchioles per ml of lung (TB/ml) we found a decrease of 40% in GOLD1 (2·8±1·4 TB/ml, p=0·014), 43% in GOLD2 (2·7±1·1 TB/ml, p=0·036), and 68% in GOLD4 COPD patients (1·5±1·0 TB/ml, p=0·001, figure 1A). Figure 1B demonstrates that the number of respiratory transitional bronchioles per ml of lung (TrB/ml) was reduced from a mean 10·8±2·9 TrB/ml in controls by 56% in GOLD1 (4·8±2·6 TrB/ml, p=0·001), 59% in GOLD2 (4·4±2·2 TrB/ml, p=0·001) and 90% in GOLD4 COPD patients (1·1±0·9 TrB/ml, p=0·001).

Using these stereological counts we estimated the total numbers of terminal and transitional bronchioles per whole lung volume determined from the thoracic CT scan (table 2). On average smokers with normal lung function had a mean 11,091±3,059 terminal bronchioles per lung and this was reduced to 7,205±3,089 in GOLD 1, to 5,602±2,300 in GOLD2 and to 6,038±4,145 in GOLD4 patients (table 2). For transitional bronchioles this loss was even greater with an estimated number of 25,336±7,705 transitional bronchioles per lung in controls that was reduced to 12,958±5,569 in GOLD1, 8,617±3,585 in GOLD2 and 3,952±3,548 in GOLD4 COPD patients (table 2).

Quantification of emphysema in mild to moderate COPD

Quantitative analysis of the 30 preoperative CT scans was performed on the segmented right or left lung (figure 2A) and the segmented resected lobes (figure 2B), for subjects with a lobectomy sample. In both analyses, emphysema (%LAA<-910, greater than 95th percentile of controls) was only detectable in GOLD4 subjects compared to controls (p=0·0002) and GOLD1 subjects (p=0·023). Additionally, we found the CT density measurements on the 15 segmented lobes overlapped well with the density measurements of the whole right or left lung CT scans (appendix pp 6).

When micro-CT was used to assess the level of emphysema using mean linear intercept (Lm, figure 2C), we found only GOLD4 patients had a significant increase in Lm compared to controls (p<0·0001), and GOLD1 subjects (p=0·027). Importantly, when we assessed alveolar surface area per ml of lung available for gas exchange (figure 2D), we demonstrate a significant reduction in alveolar surface area per ml of lung in all COPD groups compared to controls who had a mean of 73·6±23·6cm^2/ml of lung. There was a 33% loss of alveolar surface area in GOLD1 (49·0±17·9cm^2/ml, p=0·019), a 45% loss in GOLD2 (40·3±13·6cm^2/ml, p=0·0021), and a 79% loss in GOLD4 (14·8±4·3cm^2/ml, p<0·0001) patients.

When total alveolar surface area was estimated for the whole lung (table 2), our data show that compared to smokers with healthy lung function (62·34±21m^2) the total alveolar surface area available for gas exchange was reduced to 59·13±19m^2 in GOLD1, 44·29±10·25m^2 in GOLD2 subjects, and 34·23±4·98m^2 in GOLD4 patients. We found no significant difference in the alveolar surface area between upper, lower and whole lung samples, obtained from micro-CT (appendix pp 7).

Relationship of emphysema with terminal and transitional bronchiole number in mild to moderate COPD

The kernel density plot of all 262 lung samples shown in figure 3A demonstrates when using mean linear intercept (Lm) it is only possible to distinguish emphysematous changes in GOLD4, but not GOLD1 and GOLD2 samples, compared to controls. In contrast, when the same samples were plotted using alveolar surface area per ml of lung (figure 3B), GOLD1, GOLD2, and GOLD4 samples segregate from control samples.

To determine if terminal and transitional bronchioles were lost in the absence of emphysematous destruction, we plotted the number of terminal and transitional bronchioles in the 121 out of 262 cores that had an alveolar surface area per ml of lung within the normal range (5^{th}, 41·3cm^2/ml – 95^{th} percentile, 126·2cm^2/ml) of control cases. Figure 3C, shows in lung samples that had a 'normal' alveolar surface area there was a 29% reduction in the number of terminal bronchioles in GOLD1 (3·3±2·6 TB/ml, p=0·039), and a 40% reduction in GOLD2 subjects (2·8±2·3 TB/ml, p=0·016) compared to controls (4·6±2·4 TB/ml).

There was also a significant 41% reduction in the number of transitional bronchioles in GOLD1 (3·2±4·8 TB/ml, p=0·0016), and a 53% reduction in GOLD2 (4·9±3·7 TB/ml, p<0·001) patients compared to controls (10·4±5·4TB/ml, figure 3D). Only two tissue samples from all GOLD4 lungs had an alveolar surface area within the 'normal' range therefore no statistical analysis was conducted.

Terminal bronchiole pathology

We next assessed the morphology of the 'surviving' terminal bronchioles. In control smokers' lungs 12% of the terminal bronchioles (1·0±1·4TB/ml of lung) were found to be diseased (thickened or obstructed) and that number increased to 41% in GOLD1 (1·5±1·5TB/ml, p=0·0023), 37% in GOLD2 (1·1±1·5TB/ml, p=0·08), and 77% in GOLD4 patients (1·1±0·7TB/ml lung, p=0·001, figure 4A). Moreover, figure 4B shows that the majority of diseased terminal bronchioles were thickened (blue) in GOLD1 and 2 subjects, while the majority of diseased terminal bronchioles were 100% obstructed (orange) in GOLD4 subjects (Statistical comparisons shown in appendix pp 8).

Figure 4C shows how the spatial coordinates from the volumetric micro-CT scans were used to accurately cut histological sections at regular intervals along the airway branch length as shown by the dotted lines 1, 2 and 3, which correspond to the matched micro-CT and histological cross-sectional images at locations 1, 2 and 3 shown for a representative non-diseased (figure 4D), thickened (figure 4E) and obstructed (figure 4F) terminal bronchiole observed in mild and moderate COPD patients. Figure 4F further demonstrates how the morphology of the terminal bronchioles can change significantly along its branch length, and highlights the importance of volumetric micro-CT imaging to understand the disease pathology. Movat's Pentachrome staining confirmed that 'thickened' airways quantified by micro-CT did indeed exhibit airway wall fibrosis with collagen deposition and inflammatory cell infiltration. Further, obstructed airways had thickened walls and the obstructions were not simply mucus plugs but composed of collagen infiltrated with structural and inflammatory cells.

Correlation of micro-CT measurements with lung function

Table 3 summarizes the comparisons of the micro-CT measures of terminal and transitional bronchiole number and alveolar surface area with the patients FEV_1, FEV_1/FVC, and DLCO/VA. These data show that the lung function measures used to classify the GOLD stages of COPD are significantly correlated with both the loss of terminal and transitional bronchioles and alveolar surface area in mild to moderate COPD (detailed comparison appendix pp 9).

DISCUSSION

This study provides the first direct evidence that the smallest airways within the lung; 41% of conducting terminal bronchioles and 57% of respiratory transitional bronchioles, are significantly lost in the lungs of patients with mild (GOLD1) and moderate (GOLD2) COPD, compared to age-matched smokers with normal lung function. Using a robust measurement of emphysema, alveolar surface area, which translates to the functional tissue involved in gas exchange, we also report that terminal and transitional bronchioles are lost in lung tissue where no emphysematous destruction is present indicating small airways disease is an early pathological feature of mild and moderate COPD. While several histological studies have documented airway remodeling and inflammation in the small airways of COPD patients, no study to date has been able to quantify the numbers of terminal and transitional bronchioles, and show that loss of these small airways relates to decreases in lung function in mild and moderate COPD. Further, using the combination of micro-CT and histology we demonstrate that the 'surviving' small airways that have been extensively studied in COPD[20, 23, 24] do indeed have thickened walls and when examined along their branch lengths are often completely obstructed by fibrotic tissue. This study highlights the small airways disease that occurs in patients with mild and moderate COPD, that could not be previously measured, and the importance of early intervention for disease modification.

The present results confirm and substantially extend an earlier report using micro-CT by McDonough et al.,[10] on very-severe (GOLD4) COPD patients, by providing new evidence that the destruction of terminal, for the first time transitional bronchioles, is well established in persons with mild and moderate COPD. Further, using a stereological sampling design we demonstrate that small airways disease is an early pathological feature of mild and moderate COPD and is present in regions

of the lung that have no emphysematous disease measured using alveolar surface area. These data strongly support the concept that the small airways are the major and earliest site of airflow limitation in COPD.[4-6] In 1970, Mead proposed that that the small airways may represent a "quiet zone" within the lung, where disease could accumulate over many years without being noticed.[25] In support of this hypothesis our data demonstrate that even in mild (GOLD1) COPD, classified by an $FEV_1/FVC < 0.7$ and a $FEV_1 \geq 80\%$ predicted, a patient will already have lost, on average 41% of their terminal bronchioles and 57% of their transitional bronchioles. The reason that this significant loss of small airways can occur with a limited change in lung function can be explained by the extensive parallel arrangement of the small airways within the lung. Direct measurements of small airway resistance in both post mortem human lungs[4] and living humans[6] have both shown that the small airways account for a small proportion of the total resistance to airflow in the normal human lung but become the major site of increased resistance in COPD. As the small airways are arranged in parallel a 50% reduction in their number is expected to double their resistance. Therefore, if we consider the data reported by Yanai et al.[6] on direct measurements of peripheral airway resistance in living humans they show that doubling the normal value of 0.70±0.26 cm H_2O/L/s measured in subjects with normal lung function to 1.4 cm H_2O/L/s, falls well below the 2.78 - 4.59 cm H_2O/L/s measured in severe COPD patients. Although, these findings are consistent with the much greater reduction in terminal and transitional airways reported here and previously for end-stage GOLD 4 COPD patients[10] we do not mean to imply that these are the only airways narrowed and destroyed in COPD. As indeed several previous studies have demonstrated using thoracic CT scans that airways ranging from 0.8-3 mm in diameter are also destroyed in all GOLD categories of COPD.[7, 9, 10]

There has been much debate over the relative importance of small airways disease and emphysema for lung function decline. Here we demonstrate direct evidence that small airways disease is a well-established pathological feature of patients with mild and moderate COPD. Further, these data also show that the reduction in both terminal and transitional bronchioles is well established before emphysematous destruction of the alveolar surface area can be visualised using the spatial resolution (1000μm) provided by thoracic CT scans. This strongly suggests that the reason the appearance of emphysema on thoracic CT scans appears to be a good predictor of a rapid decline in FEV_1,[18, 26, 27] is that a significant destruction of the terminal and transitional bronchioles has already occurred before the emphysematous disease can be detected. Therefore, approaches to identify susceptible smokers prior to reaching the GOLD1 classification[7, 28, 29] warrant further attention. Further, these new data substantially extend earlier reports on the pathology present in the 'surviving' small airways, by showing that the surviving airways are thickened and become more obstructed with disease progression. This finding is consistent with the concept that airways develop thickened walls and narrowed lumens with COPD progression.[20] What remains unclear is if thickening, obstruction and obliteration occur sequentially in individual airways. In several recent reviews we have postulated that terminal and transitional bronchioles may be vulnerable to deposition of fine particulate matter (especially from tobacco smoke) because they are located in the region of the lung where the transition from bulkflow to diffusion of gas occurs.[30] The answer to these questions will require much further but necessary work. These data do however suggest that small airways disease is related to the pathology of the airways themselves, and that loss of elastic recoil due to emphysematous destruction is an independent phenomenon that potentially causes further airway obstruction. It has been postulated that destruction of the axial elastic network might allow the airways to shorten and obliterate the airway lumen as visualized in an editorial by Mitzner.[31] Our current working hypothesis is that the disappearance of small airways is preceded by a localized constrictive bronchiolitis that first obliterates the airway lumen and then separates the affected airway into two parts that leaves closed end buds coming off both the parent airway and the distal daughter branch. Thus, future studies designed to answer this question using the combination of micro-CT and histology are still required to definitively understand the nature of this destructive disease process.

This study has limitations that deserve mention. We note that this study used archived lungs and lobes from surgeries conducted in the early 2000's for lung cancer resection of small peripheral, tumors with no metastasis, or lung transplantation, which excluded severe GOLD 3 patients. Therefore as the study is cross-sectional it does not allow one to observe the progress of the disease pathology over time, or to determine if the patients with mild and moderate COPD may have been born with a lower number of airways. However, only lobectomy and pneumonectomy surgeries provide the possibility to collect intact lung tissue for inflation, preoperative pulmonary function and CT data from smokers with and without mild and moderate COPD. Further, patients had an inspiratory CT scan for surgical planning but not for COPD assessment using inspiratory and expiratory CT scans. Thus, we were unable to assess the relationship of small airways disease to gas trapping in this study. We also acknowledge that for 15 of the 34 subjects, samples were obtained only from a single lobe which does not allow us to capture disease heterogeneity within the whole lung. However, when we compared the clinical CT-based density histograms of the whole lung versus the resected lung region, or alveolar surface area obtained from micro-CT measures we found no significant variation between upper and lower lung samples. Despite these limitations the numbers of terminal and transitional bronchioles and alveolar surface area reported for the control smokers with normal lung function in this study, are consistent with those previously reported in stereological studies of the normal human lung.[32, 33]

In conclusion, these findings suggest that like the kidney, where a significant proportion of nephrons are lost before renal dysfunction appears,[34] the development of airflow limitation in COPD involves progressive destruction and loss of the terminal and transitional bronchioles before a decline in lung function is observed. In addition, these new data confirm that destruction of terminal and transitional bronchioles can occur in the absence of emphysematous destruction in COPD and that the surviving airways have narrowed lumens and thickened walls. Most importantly, these data suggest that several large clinical trials investigating COPD treatments in severe COPD, may have failed because they were initiated after a substantial number of terminal and transitional bronchioles were already destroyed, and that early intervention in mild and moderate COPD patients is most likely required for disease modification.

CONTRIBUTORS

H-KK, DMV, SB, AH, NF, OLK, GZ, WME, MK, PL, IS, JAW, JDC, HOC, and TLH contributed to data collection. H-KK, DMV, SB, HOC, PDP, JCH and TLH contributed to study design, data interpretation, figures and writing.

DECLARATION OF INTERESTS

All authors declare no competing interests.

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FIGURE LEGENDS

Figure 1. Quantification of terminal and transitional bronchioles in mild to moderate COPD
Comparison of the mean number of A) terminal and B) transitional bronchioles per milliliter (ml) of lung per subject grouped by Global Initiative for Obstructive Lung Disease (GOLD) stage. Means ± SD per group are shown.* denotes p<0·05, ** p<0·01, *** p<0·001.

Figure 2. Quantification of emphysema in mild to moderate COPD
A) Comparison of percentage low-attenuation area less than -910 HU (% LAA < -910 HU) on thoracic CT per subject grouped by Global Initiative for Obstructive Lung Disease (GOLD) stage in both the whole lung and B) region of surgically resected lung lobe. C) Comparison of Mean linear intercept (Lm) measured by micro-CT per subject grouped by GOLD stage. The dotted line denotes the upper 95^{th} percentile of Lm (449·1µm) in the control group. D) Comparison of alveolar surface area / ml of tissue measured by micro-CT per subject grouped by GOLD stage. The dotted line denotes the lower 5^{th} ($41{\cdot}3cm^2/ml$) percentile of alveolar surface area / ml of tissue in the control group. Median and interquartile range per group are shown for non-parametric data (panel A,B,C), and means ± SD per group are shown for parametric data (panel D). * denotes p<0·05, ** p<0·01, *** p<0·001.

Figure 3. Relationship of emphysema with terminal and transitional bronchiole number in mild to moderate COPD
A) Kernel density plot of Mean linear intercept (Lm) [µm] using micro-CT for all 262 samples, segregated by Global Initiative for Obstructive Lung Disease (GOLD) stage. Disease severity is indicated using distinct colours; white indicates control subjects, light blue indicates GOLD1 subjects, blue indicates GOLD2 subjects and dark blue indicates GOLD4 subjects. The dotted line denotes the lower 5^{th} (267·7 µm) and upper 95^{th} (449·1 µm) percentile of Lm in the control group. * indicates p < 0.001 compared to controls. A darker colour on the shaded bar demonstrates increased Mean linear intercept indicating more emphysema. B) Kernel density plot of alveolar surface area / ml of tissue measured by micro-CT for all 262 samples, segregated by GOLD stage. The dotted line denotes the lower 5^{th} ($41{\cdot}3cm^2/ml$) and upper 95^{th} ($126{\cdot}2cm^2/ml$) percentile of alveolar surface area / ml of tissue in the control group. A darker colour on the shaded bar demonstrates decreased alveolar surface area / ml of tissue indicating more emphysema. C) Comparison of terminal and D) transitional bronchiolar number per ml of lung in all cores (n = 121) with an alveolar surface area / ml of tissue within the 5^{th} - 95^{th} percentile of controls, segregated by GOLD stage. Data were assessed using a linear-mixed effect model and the Mean ± SD per group are shown. * denotes p<0·05, ** p<0·01, *** p<0·001.

Figure 4. Terminal bronchiole pathology
A) Comparison of the percentage of diseased terminal bronchioles per total number of terminal bronchioles per subject grouped by Global Initiative for Obstructive Lung Disease (GOLD) stage. Mean ± SD per group are shown. * denotes p<0·05, ** p<0·01, *** p<0·001. B) Terminal bronchiolar number per ml of lung segregated by non-diseased (grey), thickened (blue) and complete luminal obstruction (orange). Each bar indicates one donor subject. C) Demonstrates the use of micro-CT as a scouting tool to precisely locate terminal bronchioles and enable efficient histological sectioning. The numbered dotted lines in C) indicate the corresponding histological sections taken in cross-section of a representative terminal bronchiole in panels D to F. D) Matched micro-CT and histological sections of a representative non-diseased, E) thickened and F) obstructed terminal bronchiole. Histological sections are stained with Movat's Pentachrome to highlight various components of connective tissue; elastic fibres (black), collagen and reticular fibres (yellow), fibrin and muscle structures (red).

TABLES

Table 1. Preoperative Patient Characteristics

Characteristic	Control (Smokers with normal lung function) n = 10	GOLD 1 (Mild COPD) n=10	GOLD 2 (Moderate COPD) n=8	GOLD 4 (Very Severe COPD) n=6
Lung tissue samples (n)	75	81	62	44
Sex (female:male) *	6:4	4:6	3:5	1:5
Age (years) *	62·0 ± 7·9	67·4 ± 7·3	62·9 ± 11·3	59·2 ± 2·1
Height (cm) *	167·9 ± 8·7	168·6 ± 9·9	167·4 ± 6·7	170·3 ± 6·3
Weight (kg) *	68·4 ± 14·7	77·1 ± 18·1	73·1 ± 15·6	71·5 ± 10·8
Smoking history (pack years) *	34·5 ± 10·5	45·5 ± 25·3	33·6 ± 12·7	37·5 ± 15·1
FEV_1 (% predicted) #	91·8 ± 6·4	88·3 ± 6·2	62·1 ± 9·5	22·3 ± 6·7
FVC (% predicted) ¶	96·7 ± 5·4	108·3 ± 8·5	88·5 ± 6·4	60·3 ± 19·7
FEV_1/FVC (%) †	74·9 ± 4·4	63·5 ± 4·7	60·1 ± 7·6	29·5 ± 7·8
DLCO/VA (ml/min/mmHg/L) ‡	3·85 ± 0·9	2·83 ± 0·7	2·64 ± 0·9	1·71 ± 0·8
Total lung volume (L) ¥	4·86 ± 1·4	5·37 ± 1·5	4·92 ± 0·9	7·91 ± 1·0
% LAA< -910 (HU)§	1·05 ± 3·7	1·84 ± 7·9	8·72 ± 12·4	67·7 ± 16·8
Surgical resection	x2 RUL, x2 LUL, x1 RML + RLL, x1 LLL, x2 RWL, x2 LWL	x3 RUL, x3 LUL, x1 LLL, x2 RWL, x1 LWL	x2 RUL, x1 LUL, x1 LLL, x3 LWL	x4LWL, x2RWL

Values given are the means ± standard deviation (SD). FEV_1 denotes forced expiratory volume in 1 second and FVC forced vital capacity post-bronchodilator. DLCO/VA denotes the diffusing capacity of the lung for carbon monoxide adjusted for alveolar volume. %LAA<-910HU denotes percentage low-attenuation area less than -910 HU (% LAA < -910 HU) on thoracic CT. RUL denotes right upper lobe, LUL left upper lobe, RML right middle lobe, RLL right lower lobe, LLL left lower lobe, RWL right whole lung, LWL left whole lung. The severity of disease was graded using the 2011 Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging system, based on post-bronchodilator FEV_1, where GOLD1 indicates mild ($FEV_1 \geq 80\%$ predicted), GOLD2 moderate ($50\% \leq FEV_1 < 80\%$ predicted) and GOLD4 very severe ($FEV_1 < 30\%$ predicted) airflow limitation. The control group were classified as smokers with normal lung function.

* A one-way ANOVA with a Tukey's pairwise comparison showed no significant differences in sex, age, height, weight and smoking history between all groups.

There were significant differences ($p<0{\cdot}001$) in FEV_1 between all groups except between the Control and GOLD1 groups.

¶ There were significant differences ($p<0{\cdot}01$) in FVC between all groups except between the Control and GOLD1, and Control and GOLD2 groups.

† There were significant differences ($p<0{\cdot}01$) in FEV_1/FVC between all groups except between the GOLD1 and GOLD2 groups.

‡ There were significant differences in DLCO/VA between Control and GOLD2 ($p<0{\cdot}05$), and Control and GOLD4 ($p<0{\cdot}05$) groups.

¥ GOLD4 had significantly increased total lung volumes ($p<0{\cdot}05$) compared to all groups.

§ GOLD 4 had significantly increased %LAA<-910HU compared to Controls ($p=0.0002$) and GOLD 1 ($p=0.024$).

Table 2. Estimates of small airway counts and alveolar surface area in whole lungs

	Case	Lung or Lobe location	Sex	Whole Lung Volume [L]	Terminal Bronchioles per Lung	Transitional Bronchioles per Lung	Alveolar surface area per lung [m^2]
Control	1	RML_RLL	F	2.39	13,481	32,824	64.73
	2	RUL	F	2.54	16,163	33,711	66.87
	3	LUL	M	3.14	12,848	32,121	94.20
	4	**LWL**	**M**	**2.20**	**9,204**	**26,529**	**68.71**
	5	LUL	F	1.34	7,898	15,232	37.45
	6	**RWL**	**F**	**2.87**	**10,566**	**23,114**	**58.10**
	7	**RWL**	**F**	**1.56**	**6,628**	**13,698**	**37.59**
	8	RUL	M	4.02	12,682	32,761	97.81
	9	**LWL**	**M**	**2.11**	**14,061**	**28,607**	**44.11**
	10	LLL	F	2.18	7,382	14,763	53.84
Average ± SD				2.43 ± 0.77	11,091 ± 3,225	25,336 ± 8,122	62.34 ± 21.07
GOLD 1	11	RUL	M	3.54	9,744	15,428	86.32
	12	LLL	M	2.13	12,424	20,992	51.98
	13	LUL	F	2.19	5,707	9,218	30.16
	14	**RWL**	**M**	**4.11**	**4,682**	**4,682**	**82.92**
	16	LUL	M	2.71	10,710	21,420	69.35
	18	**LWL**	**F**	**2.27**	**6,364**	**11,932**	**51.16**
	19	RUL	F	2.68	4,551	8,452	42.58
	20	RUL	M	2.45	3,462	11,541	58.59
Average ± SD				2.76 ± 0.71	7,205 ± 3,302	12,958 ± 5,954	59.13 ± 19.41
GOLD 2	21	LLL	M	2.31	9,753	14,630	49.09
	24	LUL	M	2.78	5,588	9,500	54.05
	25	LUL	F	2.19	7,109	9,297	37.29
	26	**LWL**	**F**	**1.86**	**3,260**	**9,781**	**44.43**
	27	**LWL**	**M**	**2.06**	**3,141**	**4,398**	**53.26**
	28	RUL	F	2.29	4,762	4,082	27.63
Average ± SD				2.25 ± 0.31	5,602 ± 2,520	8,615 ± 3,927	44.29 ± 10.25
GOLD 4	**29**	**LWL**	**M**	**4.30**	**4,536**	**3,402**	**41.25**
	30	**LWL**	**F**	**3.10**	**3,797**	**2,531**	**27.82**
	31	**RWL**	**M**	**4.64**	**12,897**	**11,725**	**36.13**
	32	**LWL**	**M**	**3.30**	**1,509**	**1,509**	**33.62**
	33	**LWL**	**M**	**3.95**	**3,054**	**3,054**	**29.52**
	34	**RWL**	**M**	**4.68**	**10,436**	**1,491**	**37.02**
Average ± SD				3.99 ± 0.67	6,038 ± 4,541	3,952 ± 3,888	34.23 ± 4.98

Values given are the means ± standard deviation (SD) for 30 of the 34 subjects which had a preoperative thoracic CT scan. RUL denotes right upper lobe, LUL left upper lobe, RML right middle lobe, RLL right lower lobe, LLL left lower lobe, RWL right whole lung, LWL left whole lung. Whole Lung Volume denotes the right or left lung volume obtained from the clinical CT scans in liters. Values not in bold were calculated from samples obtained within a lobe and not the whole lung and should therefore only be taken as estimates of the whole lung.

Table 3. Correlation of small airways disease and emphysema with lung function

	Alveolar Surface Area per ml of lung		Terminal Bronchioles (TB/ml)		Transitional Bronchioles (TrB/ml)	
	All subjects	Mild to Moderate COPD	All subjects	Mild to Moderate COPD	All subjects	Mild to moderate COPD
FEV_1 (%predicted)	0·72 (p<0·001)	0·502 (p=0·008)	0·523 (p=0·002)	0·326 (p=0·009)	0·64 (p<0·001)	0·423 (p=0·03)
FEV_1/FVC (%)	0·722 (p<0·001)	0·547 (p=0·004)	0·581 (p=0·001)	0·465 (p=0·019)	0·717 (p<0·001)	0·627 (p<0·001)
DLCO/VA (ml/min/mmHg/L)	0·61 (p<0·001)	0·549 (p=0·005)	0·591 (p<0·001)	0·567 (p=0·005)	0·715 (p<0·001)	0·676 (p<0·001)

Pearson's correlations were calculated to investigate the relationship between micro-CT measures (TB/ml, TrB/ml and alveolar surface area per ml of lung) and pulmonary function (FEV_1, FEV_1/FVC, DLCO/VA) with (All subjects) and without GOLD4 cases (Mild to Moderate COPD). FEV_1 denotes forced expiratory volume in 1 second % predicted and FVC forced vital capacity % predicted. DLCO/VA denotes the diffusing capacity of the lung for carbon monoxide adjusted for alveolar volume. For each correlation the r value and FDR (false discovery rate) p value in brackets are provided.

Figure 1

Figure 1.

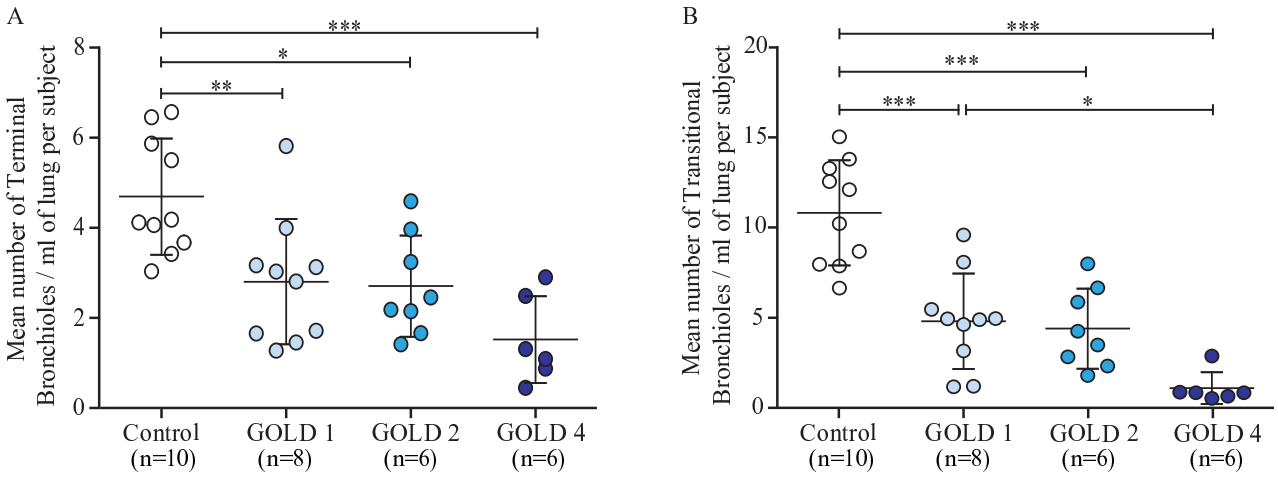


Figure 2

Figure 2.

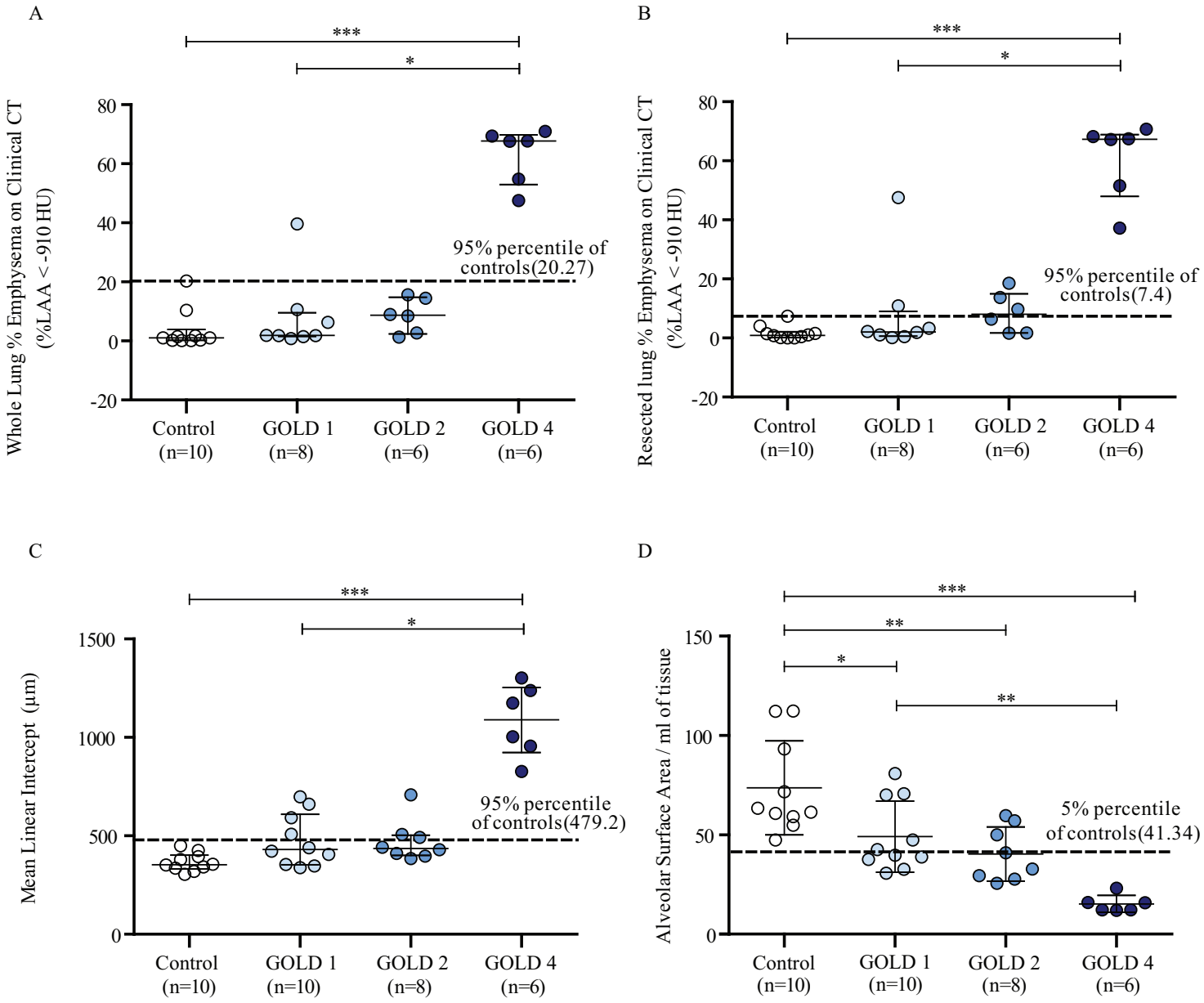
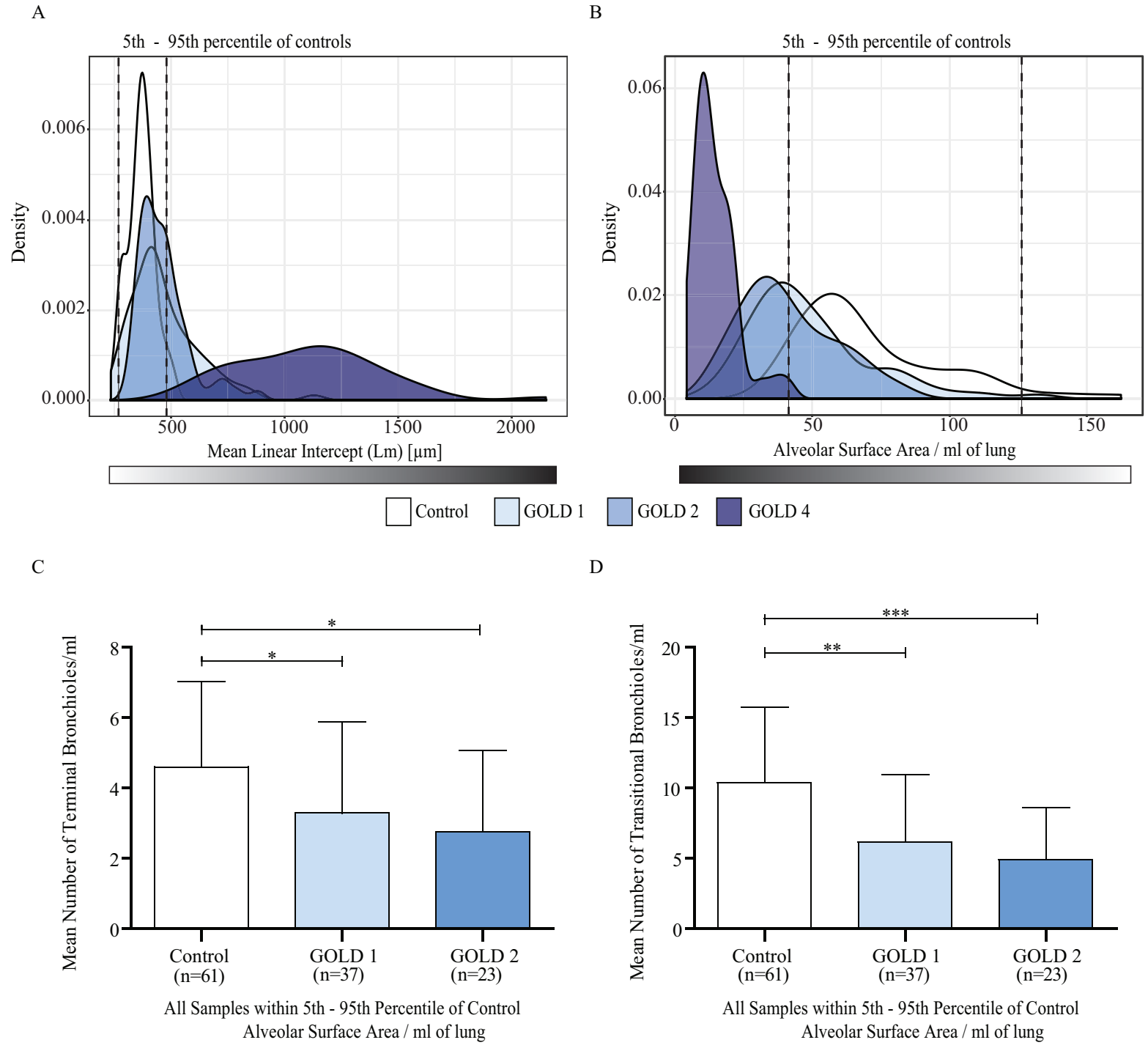


Figure 3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5th - 95th percentile of controls
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0.004
0.002
0.000
500
1000
1500
2000
Mean Linear Intercept (Lm) [µm]
B
5th - 95th percentile of controls
Density
0.06
0.04
0.02
0.00
0
50
100
150
Alveolar Surface Area / ml of lung
Control
GOLD 1
GOLD 2
GOLD 4
C
Mean Number of Terminal Bronchioles/ml
8
6
4
2
0
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Control (n=61)
GOLD 1 (n=37)
GOLD 2 (n=23)
All Samples within 5th - 95th Percentile of Control Alveolar Surface Area / ml of lung
D
Mean Number of Transitional Bronchioles/ml
20
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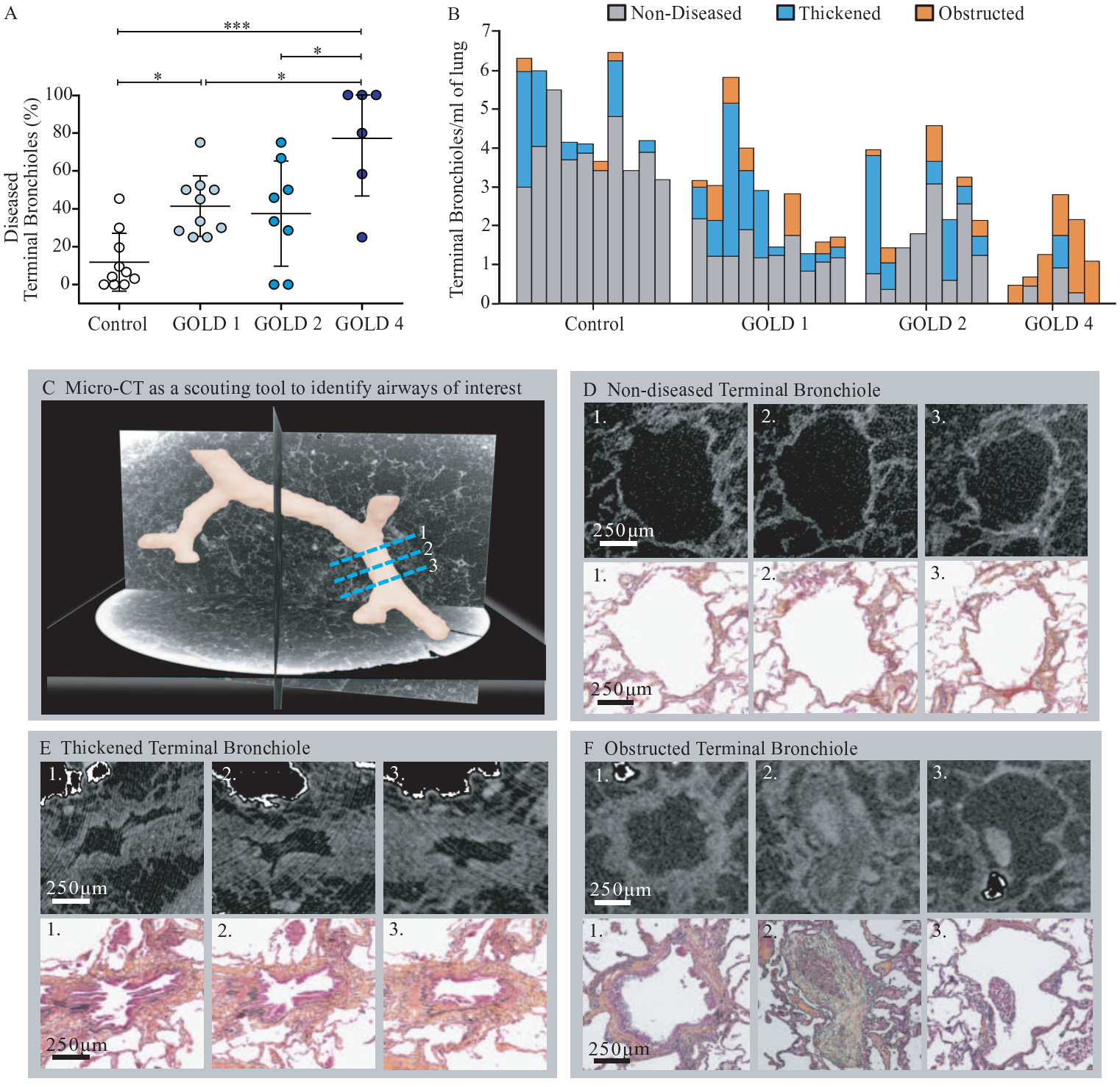
Control (n=61)
GOLD 1 (n=37)
GOLD 2 (n=23)
All Samples within 5th - 95th Percentile of Control Alveolar Surface Area / ml of lung

Figure 4

Figure 4.



Necessary Additional Data

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