Small airways disease in pre-COPD with emphysema : a cross-sectional study

Small airways disease in pre-COPD with emphysema: a cross-sectional study.

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Running head: Airway loss in pre-COPD

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ABSTRACT

Rationale: Small airway disease is an important pathophysiological feature of COPD. Recently, pre-COPD has been put forward as potential precursor stage of COPD, defined by abnormal spirometry or significant emphysema on CT in the absence of airflow obstruction.

Methods: We collected whole lungs/lung lobes from patients with emphysematous pre-COPD (n=10), COPD GOLD I (n=6), GOLD II (n=6), GOLD III/IV (n=7) and controls (n=10) which were analyzed using CT and microCT. The degree of emphysema and the number and morphology of small airways was compared between the different groups and further correlations were investigated with physiologic measures. Airway and parenchymal pathology was also validated with histopathology.

Measurements and Main Results: The number of transitional bronchioles (TrB)/mL and terminal bronchioles (TB)/mL was significantly lower in pre-COPD, GOLD I, GOLD II and GOLD III/IV compared to controls. In addition, the number of alveolar attachments of the TrB and TB was also lower in pre-COPD and all COPD groups compared to controls. We did not find any differences between the pre-COPD and COPD group in either CT or microCT measures. The % of emphysema on CT showed the strongest correlation with the number of small airways, also in patients without airflow obstruction. Histopathology showed an increase in the mean chord length and a decrease in the alveolar surface density in pre-COPD and all GOLD stages compared to control.

Conclusion: Lungs of patients with emphysematous pre-COPD already show lower small airway number and airway remodeling and in the absence of physiologic airway obstruction.

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INTRODUCTION

A recent Lancet commission pointed out the importance of chronic obstructive pulmonary disease (COPD) and the need for fundamental changes in the way we think about this disease (1). COPD is a highly prevalent disease with more than 384 million estimated cases globally. It is the third leading cause of death in 2019 according to the WHO. COPD is characterized by persistent respiratory symptoms and airflow limitation due to airway and/or alveolar abnormalities, usually caused by significant exposure to noxious particles or gases, most typically cigarette smoke (2).

COPD presents with increased alveolar airspace size (emphysema) and with small airways and terminal/transitional bronchioles that typically obstruct and disappear (3,4). Using microCT, it is now possible to analyze and quantify these morphological small airways and parenchymal changes. Studies have demonstrated a 40-50% reduction in small airway count in mild (GOLDI) and moderate (GOLDII) COPD, and a 70-90% reduction in severe COPD (GOLDIV), (3–5), as well as a reduction of alveolar attachments and narrowing of pre-terminal bronchioles in severe COPD (6). It is the current belief that this reduction in small airways leads to parenchymal alterations.

The diagnosis of COPD currently requires poorly reversible airflow limitation, defined as a post-bronchodilator forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) <0.7. However, significant lung damage may have already occurred before abnormalities in lung function are evident. Indeed, CT-detected small airway abnormalities, airway wall thickening and emphysema may be helpful in identifying patients at increased risk for disease progression to COPD (7,8). Furthermore, data from large cohorts suggested that about 35-50% of patients with Preserved Ratio Impaired Spirometry (PRISm), defined as reduced FEV_1 with normal FEV_1/FVC ratio, also progress to classically defined COPD (9,10). More recently,

the term pre-COPD has therefore been proposed to refer to these individuals in whom spirometry is unable to detect airflow obstruction but who are at increased risk of subsequently developing COPD based on their symptoms (dyspnoea, cough, phlegm), functional (i.e. low FEV_1 and/or low diffusing capacity for carbon monoxide, DlCO, without airflow obstruction) or structural abnormalities (i.e. radiologic evidence of emphysema) (8,11,12). The (airway) pathophysiology of these patients with so-called pre-COPD has not been investigated. Elucidating the morphologic changes could significantly assist in understanding why these patients progress to COPD and could provide new insights in the early pathophysiologic processes of COPD. We hypothesize that lungs of patients with pre-COPD will already show features of small airway disease. Using microCT, a cohort of explant lobes/lungs is thoroughly analysed to determine the degree and nature of (small) airway disease in pre-COPD. Some of the results of these studies have been previously reported in the form of an abstract (ECR, not published).

MATERIAL AND METHODS

Patient selection and characterization

Lungs or lung lobes from patients undergoing lung resection for primary lung cancer without evidence for metastasis (control, n=10; pre-COPD, n=10; GOLDI, n=6; GOLDII, n= 6), from severe COPD patients undergoing lung transplantation (GOLDIII, n=1, GOLDIV, n=5) or from autopsy (GOLDIII, n=1) have been prospectively collected from May 2021 till December 2022 in the Antwerp University Hospital. This study has been approved by the ethical committee of the Antwerp University Hospital and all patients provided written informed consent (approval number: EDGE001693). All lungs were from patients with European ancestry, except for two Asian patients in the pre-COPD group. Pulmonary function testing was performed between 1

week and 3 months before surgery according to the ATS/ERS guidelines. Based on smoking status, pulmonary function and chest CT, the patients were subdivided in controls (both ever and never smoker, FEV_1 and FVC >80% predicted, FEV_1/FVC>0.70 and <5% emphysema on CT), pre-COPD (smoking history >10PY, FEV_1/FVC >0.70, FEV_1 <80% predicted and/or >5% of emphysema on CT (11)), GOLDI (smoking history >10PY, FEV_1/FVC <0.70, FEV_1>80% predicted), GOLDII (smoking history >10PY, FEV_1/FVC <0.70, FEV_1 <80% and >50% predicted), and GOLDIII/IV (smoking history >10PY, FEV_1/FVC <0.70, FEV_1 <50% predicted). Lungs that showed features compatible with another chronic respiratory disease (i.e. fibrosis on CT, n=2)) or never smokers with emphysema >5% on CT (n=3) were à priori excluded.

Lung processing

Immediately following lobar/lung resection, the lungs were collected. After macroscopic investigation and resection of the tumour by the pathologist, the lungs were transported to the lab. In the lab, the bronchi were cannulated, air leaks were plugged and subsequently the lung was air-inflated using a compressed air source at a maximum pressure of 30cmH20 and fixed in liquid nitrogen fumes after gentle deflation to 10cmH20, allowing storage at -80°C, as has been described previously (figure 1A) (13,14). The inflated specimen was then subjected to ex-vivo CT scan (figure 1D), while surrounded with dry ice in a styrofoam box (GE Healthcare revolution). This CT scan was used for further scoring (see later).

Subsequently, the lung was sliced in two cm thick slices (figure 1B) after which cores with a diameter between 14 and 20 mm were systematically extracted using a core bore (figure 1C). Per lung/lung lobe, 4 cores distant from the tumor site were randomly selected. To account for regional disease differences, two of these four cores were randomly derived from slices from the upper half of the lung/lobe, while the other two cores were randomly selected from

slices derived from the lower half of the lung. These cores were scanned using microCT (FlexCT, Unitom XL, TeSCan) (15) at a resolution of 15 micron, while keeping the core frozen (figure 1E).

CT scoring

The following five features were assessed on explant CT: emphysema, bronchiectasis, bronchial wall thickness, mucus plugging and tree-in-bud changes/centrilobular nodules. Scoring was performed by an experienced chest radiologist (CM), blinded for the clinical status and further validated by a second experienced chest radiologist (JJ). The CT features were scored visually on 1 mm thick slices at 10-mm intervals, blinded for patients' information. Between 7-29 slices per lobe were evaluated depending on the size of the lung whereas from transplant lungs all lobes were assessed. More information can be found in the online supplement.

MicroCT analysis

Each lung core was used to visually quantify the number of transitional bronchioles per mL of lung and surface density as a measure of emphysema (using CTan software, Bruker; lower values represent more emphysema), which previously has been validated with the mean linear intercept (13). Transitional bronchioles were identified by following conducting airways until the airway wall was lost and alveolar buds were visually discernible. The parent airway was subsequently identified as a terminal bronchiole. Per tissue core, 3-5 airways were randomly selected (if available) to further assess airway morphometry resulting in 12 to 20 airways per lung that were analyzed in more detail. Measures were performed in the middle of the airway segment (TB, TrB) or at the end of the airway in case of the TrB. Our main analysis

focused on the measures at the end of the TrB. Cross-sectional microCT images of each of these airways perpendicular to the center line of their lumens were reconstructed at that point using Dataviewer (Bruker), and the wall thickness, minimal and maximal airway diameter, number of alveolar attachments to their wall were manually measured with FIJI (16). The ratio of the minimal and the maximal airway diameter was also calculated.

Histology

To further demonstrate the presence of emphysema or small airway disease, a portion of a core was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Subsequently, a vibratome was used to make 8 micron slices which were H&E stained. More details on the quantitative morphometry to measure chord length and alveolar surface density can be found in the online supplement (17,18).

Statistics

All results are displayed as mean±standard deviation or median (IQR) where appropriate. Airway measures are averaged over the entire lung, resulting in one value for each parameter per lung, unless otherwise indicated. Normality of the variables was assessed with the Shapiro Wilk test. Comparison of the CT and microCT measures between the groups was done using One-Way ANOVA in combination with Tukey post-hoc testing to assess significance compared to controls. Correlation analysis was performed with the Spearman correlation test. A p-value<0.05 was considered significant. Analysis was performed with Graph Pad Prism 9.0. To assess the robustness of our main findings, a linear mixed effect model with random intercept was constructed correcting for covariates including patient age, gender and smoking history using R (lme4 package, R Core Team (2021). R: A language and environment for statistical

computing. Vienna, Austria) either with the findings averaged over the entire lung or per sample basis with subject as a random effect variable.

RESULTS

Patient population

A total of 39 lungs/lung lobes were included in the study. Detailed patient characteristics are shown in table 1. Pre-COPD patients were classified as pre-COPD based on spirometry compatible with PRISm (n=3) or CT evidence of significant (>5%) emphysema (n=7). Since all PRISm patients also showed >5% emphysema, the PRISm and pre-COPD group were analyzed together. Seven pre-COPD patients also showed a low diffusion (<80%pred).

As expected, pre-COPD patients showed a FEV_1/FVC comparable to controls, while the FEV_1/FVC was decreased in GOLDI patients ($p<0.0001$) compared to both controls and pre-COPD. In addition, diffusion capacity was decreased in both pre-COPD and GOLDI patients compared to controls ($p=0.033$ and $p=0.011$) without a difference between pre-COPD and GOLDI ($p=0.49$). An overview of the CT and microCT features in the different study groups is shown in figure 2 (control: 2A; pre-COPD: 2B; GOLDI: 2C; GOLDII: 2D; GOLDIII/IV: 2E)

Radiologic assessment of ex-vivo specimen CT scan

The % of emphysema was higher in the pre-COPD ($p=0.011$), GOLDI ($p=0.017$), GOLDII ($p=0.008$) and GOLDIII/IV ($p<0.0001$) groups compared to the control group. There was no difference between the pre-COPD and GOLDI group.

The other parameters (bronchiectasis, airway wall thickening, mucus plugging and tree-in-bud) on ex vivo CT did not differ between the groups (table 2). There was a correlation between the two different radiologic observers regarding the degree of emphysema (R=0.87)

and bronchiectasis (R=0.44), while airway wall thickening (R=0.22), mucus plugging (R=0.17) and tree-in-bud (R=0.15) were not associated.

Airway and parenchymal morphology

An overview of the microCT measures is shown in table 3 and the respective effect sizes are shown in table E1.

The pre-COPD group showed a 35% reduction in the number of TB. The GOLDI, GOLDII and GOLDIII/IV group also showed significant lower number of TB/mL with a respective 32%, 53% and 95% lower number of TB compared to control (figure 3A).

Compared to the control group, there was a 45% reduction in the number of transitional bronchioles in the pre-COPD group ($p<0.0001$), a 39% decrease in the GOLDI group ($p=0.008$), a 56% reduction in GOLDII ($p<0.0001$), and 94% reduction in the GOLDIII/IV group ($p<0.001$) (figure 3B).

Regarding the surface density, a measure of emphysema, lower values were found in pre-COPD ($p=0.0002$), GOLDI ($p=0.013$), GOLDII ($p=0.0006$) and GOLDIII/IV ($p<0.0001$) compared to controls, indicative of an increase in the distance between the alveolar airspaces (i.e. emphysema) (figure 3E).

The number of alveolar attachments both at the level of terminal and transitional bronchioles was also lower in pre-COPD, GOLDI, GOLDII and GOLDIII/IV group compared to controls (figure 3C). The ratio of minimal to maximal airway luminal diameter was also different across groups with mainly lower ratios at the end of the transitional bronchioles in the pre-COPD and COPD groups compared to controls (figure 3D). Airway wall thickness was also different across the different groups both at the level of the transitional and terminal bronchioles which

specifically increased airway wall thickness in the pre-COPD group compared to controls in the middle of the terminal bronchioles and at the end of the transitional bronchioles.

A regression model adjusting for age, gender and smoking history confirmed the robustness of the observed differences in the different COPD stages (control, pre-COPD, GOLDI, GOLDII, GOLDIII/IV both using the averages over the entire lung or on the core level (Online supplement table E2).

The reproducibility of the microCT measures was formally assessed on 10 random tissue cores (controls, pre-COPD, GOLDI, GOLDII and GOLDIV, 2 each) showing excellent correlations (alveolar attachments, R=0.87, p=0.0011, wall thickness (R=0.94, p<0.0001) and minimal to maximal airway ratio (R=0.94, p<0.0001) at the end of the TrB (online supplement figure E1).

Comparison between control never smokers and ever smokers

Within our control group (n=10), there were 5 never and 5 ever smokers. There were no differences in pulmonary function parameters in ever versus never smokers. The number of TrB/mL (p=0.67), number of alveolar attachments (p=0.54), airway wall thickness (p=0.78) and ratio of minimal to maximal airway diameter (p=0.40) at the end of the transitional bronchioles were not different between ever and never-smokers.

Associations between small airway counts and physiological and radiologic measures

We also seeked to determine the associations between the number of small airways and clinical variables. The %pred values were used as these already correct for patient age, gender, and height. The number of TrB/mL were associated with FEV_1 (R=0.66, p<0.0001), FVC (R=0.36, p=0.026), FEV_1/FVC (R=0.73, p<0.0001), TLC (R=-0.62, p<0.0001), DLCO (R=0.73, p<0.0001) and RV (R=-0.66, p=0.0014) (online supplement figure E2A,B). The strongest correlation with the number of TrB/mL was however found with the % of emphysema on CT (R=-0.84,

p<0.0001) and surface density on microCT (R=0.84, p<0.0001) (Figure online supplement E2C,D). Similar associations were found for the number of TB/mL with FEV_1 (R=0.58, p<0.001), FEV_1/FVC (R=0.72, p<0.0001, TLC (R=-0.69, p<0.0001, DLCO (R=0.65, p<0.001), RV (R=-0.62, p=0.001) and emphysema on CT (R=-0.83, p<0.0001).

Considering only the patients without airflow limitation (controls and pre-COPD, n=20), the number of TrB/mL did not correlate with FEV_1 (p=0.99), FVC (p=0.72), TLC (p=0.22) and RV (p=0.10). There was however an association between the number of TrB/mL and the DLCO (R=0.46, p=0.041) and emphysema on CT (R=-0.77, p<0.001). The number of TB/mL correlated with TLC (R=-0.51, p=0.020), DLCO (R=0.45, p=0.045) and degree of CT emphysema (R=-0.72, p=0.0005). Correlation analysis including only pre-COPD, GOLDI and GOLDII (n=22 in total) demonstrated no association between the number of alveolar attachments and FEV_1 % pred (p=0.94) and FEV_1/FVC (p=0.25).

Histopathologic validation of small airway pathology

To further demonstrate the histopathological presence of emphysema, figure 4 shows representative H&E images of the different study groups illustrating control lung tissue (figure 4A) mild microscopic emphysema in pre-COPD and GOLDI (figure 4B and 4C), which is getting more severe in GOLDII and GOLDIII/IV (figure 4D and 4E). Assessment of the chord lengths showed that the airspace was increased in pre-COPD (p=0.0043), GOLDI (p<0.0001), GOLDII (p<0.0001) and GOLDIII/IV (p<0.0001) compared to the controls. Alveolar surface density was also decreased in pre-COPD (p=0.032), GOLDI (p=0.013), GOLDII (p=0.0037) and GOLDIII/IV (p<0.0001) compared to controls (online supplement figure E3A, B). To further validate our microCT findings of small airway disease in pre-COPD, serial sectioning was performed on an area showing airway disease typical for COPD on microCT, where the presence of airway wall

289 thickening, airway narrowing, complete mucosal obliteration was observed on
290 histopathological examination (figure 5).
291
292 **DISCUSSION**
293 This is the first study showing that patients with pre-COPD with emphysema show significant
294 small airway loss and remodeling. The number of TB/mL or TrB/mL of lung in pre-COPD is in
295 fact similar to what is found in patients with established GOLDI. In addition, we found strong
296 correlations between the number of TB/mL and TrB/mL and physiologic measures such as
297 pulmonary function and radiologic scorings. Significant emphysema on CT (>5%) constitutes
298 an important parameter as the lungs with emphysema in the absence of physiologic
299 obstruction already show a lower number of small airways.
300 Currently, it is not clear why airflow obstruction is not yet observed in patients with pre-COPD
301 since the extent of emphysema on CT, microCT and histopathology as well as the number of
302 small airways is comparable between pre-COPD and GOLDI. Next to the number of TB and
303 TrB/mL, the number of alveolar attachments also show significant decreases in the pre-COPD
304 group compared to control group. In fact, a recent pathology-based study indicated that loss
305 of alveolar attachments of the small airways is mostly related to airflow obstruction in COPD
306 (19), which was recently also confirmed by Booth et al (20). Interestingly, the number of
307 alveolar attachments also showed a strong correlation with the FEV_1/FVC in our study. It is,
308 however, of interest that the number of alveolar attachments was already decreased in pre-
309 COPD as well, where the FEV_1/FVC was not (yet?) decreased.
310 It is common knowledge that there is a significant proportion of (heavy) smokers without
311 airway obstruction although emphysema is already present on CT. These patients show
312 greater rates of lung function decline in the future and therefore, they are more likely to

develop airway obstruction in the (near) future (21). Similarly, population-based studies have demonstrated that patients with PRISm have a higher mortality risk and higher risk to develop airway obstruction (22–24). Indeed, between 10 to 25% of patients with PRISm will continue to develop established COPD, making this a very relevant and interesting population to study as a pre-cursor stage of COPD (25). These observations are also paired with physiologic observations in symptomatic smokers with respiratory symptoms without COPD, who show physiological changes (maximum mid expiratory flow between 25 and 75% of vital capacity) comparable with pathological changes in the lung prior to the diagnosis of COPD (26). Impulse oscillometry has similarly demonstrated that small airway disease was present in a significant proportion of heavy smokers without airflow obstruction (27). Specifically for patients with PRISm, Zhao et al. recently showed that patients with PRISm showed spirometric small airway disease assessed with maximal mid-expiratory flow, forced expiratory flow 50%, and forced expiratory flow and additionally also increases in CT identified airtrapping (28). Given all this indirect evidence of ongoing pathophysiological processes in the lungs of patients with pre-COPD, this is the first direct evidence of small airway involvement in a group of patients who are at risk of developing COPD. Our observations can also lead to question earlier studies that included healthy smokers as controls as these smokers were likely only considered healthy based on the absence of airflow obstruction. We are convinced that our observations of ongoing airway remodeling in pre-COPD will also lead to a better investigation of the immunological and molecular changes in those lungs with pre-COPD and especially the presence of inflammatory changes in those lungs. This was however, beyond the scope of the current work. It is noteworthy that 2/10 patients with pre-COPD showed a normal number of small airways, which we speculate are those patients with a low risk of future progression to COPD. It also noteworthy that within our cohort, the majority of patients with significant

smoking history without COPD showed emphysema on CT and were hence classified as pre-COPD instead of a healthy smoker.

We acknowledge certain limitations in our study. Most importantly, we were not able to sample the resection specimens according to the standard of stereology because of logistical constrains related to the further processing of the lung to obtain fast pathological staging of the concurrent tumor. Secondly, we only used 4 samples per lung which could be considered a low number. We however leveraged this strategy previously and demonstrated limited differences when including more samples per lung (13). In addition, we formally tested whether there are differences in the microCT measures of 4 samples from a GOLDI lung compared to 9 samples of the same lung, but could not find any differences in the number of TrB/mL, surface density, wall thickness, minimal to maximal airway diameter, and number of alveolar attachments at the end of the TrB (figure online supplement E4). In addition, we were not able to quantify the degree of small airway disease and emphysema on the in vivo CT using standardized software packages because the pre-operative imaging was not standardized across the different study groups (transplantation, tumor resection) resulting in different slice thickness and different imaging modalities (CT vs PET/CT) across the patients. However, we have used rigorous scoring of a standardized ex-vivo CT by an experienced chest radiologist as an alternative. In addition, we do not know the pulmonary function trajectory nor the respiratory symptoms of the patients, so we cannot be certain what proportion of patients will formally develop COPD in the future. Also, it is possible that pre-COPD patients start with a lower number of small airways and do not lose small airways. It remains to be established if these findings can be generalized to all patients with pre-COPD. We also combined patients with GOLDIII and IV into a single group as the number of subjects with GOLDIII was low (n=2) because these patients often do not qualify for lobectomy because of their poor respiratory

function, while their COPD is mostly not severe enough to be eligible for lung transplantation. Our analysis was by design only limited to the analysis of a single lung lobe, while COPD is known to be a heterogeneous disease. The scanning resolution is also on the lower end to reliably identify the appearance of alveolar buds. Lastly, the number of included lungs is also relatively low, especially given the fact that we included a lot of groups. Nevertheless, our analysis resulted in statistical meaningful comparisons.

In conclusion, we have shown that patients with pre-COPD, who, based on population-based studies are at risk of developing COPD, already have significant small airways disease and emphysema. Identifying pre-COPD and focusing on mechanistic investigation in pre- COPD could revolutionize our understanding and treatment for COPD (29).

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REFERENCES

1. Stolz D, Mkorombindo T, Schumann DM, Agusti A, Ash SY, Bafadhel M, et al. Towards the elimination of chronic obstructive pulmonary disease: a Lancet Commission. The Lancet. 2022 Sep;400(10356):921–72.
2. Venkatesan P. GOLD report: 2022 update. The Lancet Respiratory Medicine. 2022 Feb;10(2):e20.
3. Koo HK, Vasilescu DM, Booth S, Hsieh A, Katsamenis OL, Fishbane N, et al. Small airways disease in mild and moderate chronic obstructive pulmonary disease: a cross-sectional study. Lancet Respir Med. 2018 Aug;6(8):591–602.
4. McDonough JE, Yuan R, Suzuki M, Seyednejad N, Elliott WM, Sanchez PG, et al. Small-Airway Obstruction and Emphysema in Chronic Obstructive Pulmonary Disease. N Engl J Med. 2011 Oct 27;365(17):1567–75.
5. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, et al. The Nature of Small-Airway Obstruction in Chronic Obstructive Pulmonary Disease. N Engl J Med. 2004 Jun 24;350(26):2645–53.
6. Tanabe N, Vasilescu DM, Kirby M, Coxson HO, Verleden SE, Vanaudenaerde BM, et al. Analysis of airway pathology in COPD using a combination of computed tomography, micro-computed tomography and histology. Eur Respir J. 2018 Feb;51(2):1701245.
7. Young AL, Bragman FJS, Rangelov B, Han MK, Galbán CJ, Lynch DA, et al. Disease Progression Modeling in Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med. 2020 Feb 1;201(3):294–302.
8. Han MK, Agusti A, Celli BR, Criner GJ, Halpin DMG, Roche N, et al. From GOLD 0 to Pre-COPD. Am J Respir Crit Care Med. 2021 Feb 15;203(4):414–23.
9. Wijnant SRA, de Roos E, Kavousi M, Stricker BH, Terzikhan N, Lahousse L, et al. Trajectory and mortality of preserved ratio impaired spirometry: the Rotterdam Study. The European respiratory journal. 2020 Jan 1;55(1).
10. Young KA, Strand MJ, Ragland MF, Kinney GL, Austin EE, Regan EA, et al. Pulmonary Subtypes Exhibit Differential Global Initiative for Chronic Obstructive Lung Disease Spirometry Stage Progression: The COPDGene® Study. Chronic obstructive pulmonary diseases (Miami, Fla). 2019;6(5):414–29.
11. Celli B, Fabbri L, Criner G, Martinez FJ, Mannino D, Vogelmeier C, et al. Definition and Nomenclature of Chronic Obstructive Pulmonary Disease: Time for its Revision. Am J Respir Crit Care Med. 2022 Aug 1;
12. Agustí A, Celli BR, Criner GJ, Halpin D, Anzueto A, Barnes P, et al. Global Initiative for Chronic Obstructive Lung Disease 2023 Report: GOLD Executive Summary. Am J Respir Crit Care Med. 2023 Apr 1;207(7):819–37.
13. Verleden SE, Kirby M, Everaerts S, Vanstapel A, McDonough JE, Verbeken EK, et al. Small airway loss in the physiologically ageing lung: a cross-sectional study in unused donor lungs. Lancet Respir Med. 2020 Oct 5;

14. Verleden SE, Vasilescu DM, Willems S, Ruttens D, Vos R, Vandermeulen E, et al. The site and nature of airway obstruction after lung transplantation. Am J Respir Crit Care Med. 2014 Feb 1;189(3):292–300.
15. Samber BD, Renders J, Elberfeld T, Maris Y, Sanctorum J, Six N, et al. FleXCT: a flexible X-ray CT scanner with 10 degrees of freedom. Opt Express. 2021 Feb 1;29(3):3438.
16. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012 Jun 28;9(7):676–82.
17. Conlon TM, John-Schuster G, Heide D, Pfister D, Lehmann M, Hu Y, et al. Inhibition of LTβR signalling activates WNT-induced regeneration in lung. Nature. 2020 Dec 3;588(7836):151–6.
18. Günes Günsel G, Conlon TM, Jeridi A, Kim R, Ertüz Z, Lang NJ, et al. The arginine methyltransferase PRMT7 promotes extravasation of monocytes resulting in tissue injury in COPD. Nat Commun. 2022 Mar 14;13(1):1303.
19. Polosukhin VV, Gutor SS, Du RH, Richmond BW, Massion PP, Wu P, et al. Small airway determinants of airflow limitation in chronic obstructive pulmonary disease. Thorax. 2021 Nov;76(11):1079–88.
20. Booth S, Hsieh A, Mostaco-Guidolin L, Koo HK, Wu K, Aminazadeh F, et al. A Single-Cell Atlas of Small Airway Disease in Chronic Obstructive Pulmonary Disease: A Cross-Sectional Study. Am J Respir Crit Care Med. 2023 Aug 15;208(4):472–86.
21. Mohamed Hoesein FAA, de Hoop B, Zanen P, Gietema H, Kruitwagen CLJJ, van Ginneken B, et al. CT-quantified emphysema in male heavy smokers: association with lung function decline. Thorax. 2011 Sep 1;66(9):782–7.
22. Washio Y, Sakata S, Fukuyama S, Honda T, Kan-O K, Shibata M, et al. Risks of Mortality and Airflow Limitation in Japanese with Preserved Ratio Impaired Spirometry. Am J Respir Crit Care Med. 2022 May 12;
23. Wan ES, Balte P, Schwartz JE, Bhatt SP, Cassano PA, Couper D, et al. Association Between Preserved Ratio Impaired Spirometry and Clinical Outcomes in US Adults. JAMA. 2021 Dec 14;326(22):2287–98.
24. Higbee DH, Granell R, Davey Smith G, Dodd JW. Prevalence, risk factors, and clinical implications of preserved ratio impaired spirometry: a UK Biobank cohort analysis. Lancet Respir Med. 2022 Feb;10(2):149–57.
25. Wan ES, Fortis S, Regan EA, Hokanson J, Han MK, Casaburi R, et al. Longitudinal Phenotypes and Mortality in Preserved Ratio Impaired Spirometry in the COPDGene Study. Am J Respir Crit Care Med. 2018 Dec 1;198(11):1397–405.
26. Bazzan E, Semenzato U, Turato G, Biondini D, Cubero P, Marin-Oto M, et al. Symptomatic smokers without COPD have physiological changes heralding the development of COPD. ERJ Open Res. 2022 Apr;8(2):00202–2022.
27. Pisi R, Aiello M, Frizzelli A, Calzetta L, Marchi L, Bertorelli G, et al. Detection of Small Airway Dysfunction in Asymptomatic Smokers with Preserved Spirometry: The Value of the Impulse Oscillometry System. COPD. 2021 Sep;Volume 16:2585–90.
28. Zhao N, Wu F, Peng J, Zheng Y, Tian H, Yang H, et al. Preserved ratio impaired spirometry is associated with small airway dysfunction and reduced total lung capacity. Respir Res. 2022 Oct 31;23(1):298.
29. Martinez FJ, Agusti A, Celli BR, Han MK, Allinson JP, Bhatt SP, et al. Treatment Trials in Young Patients with Chronic Obstructive Pulmonary Disease and Pre–Chronic Obstructive Pulmonary Disease Patients: Time to Move Forward. Am J Respir Crit Care Med. 2022 Feb 1;205(3):275–87.

FIGURE LEGENDS

Figure 1: Study design illustrated with a lung from a patient with pre-COPD. (A) Explant lungs/ lung lobes were collected during surgery, cannulated, air-inflated and frozen solid in liquid nitrogen vapors. (B) Following CT and microCT scanning, the specimens were sectioned in 2 cm lung slices and (C) cores were randomly taken from these slices, where the location of every sample is carefully tracked. (D) Representative ex-vivo CT image of a pre-COPD lung (D) which was used to assess the extent of emphysema. (E) Four random cores per lung were selected (2 from the upper lung areas and 2 from the lower lung areas) for microCT analysis to characterize transitional bronchiole number/mL of lung and airway morphology.

Figure 2: Overview of the CT and microCT presentation of the different study groups (control: A; pre-COPD: B; GOLDI: C; GOLDII: D; GOLDIII/IV: E) demonstrating visible emphysema from the pre-COPD group onwards which advances with more advanced GOLD grade. The airways (Awy) are shown with their accompanying blood vessel (BV) showing a more irregular lumen and fewer alveolar attachments with more severe COPD.

Figure 3: overview of main results. (A) the number of TB/mL was decreased in pre-COPD, GOLDI, GOLDII and GOLDIII/IV compared to controls. (B) the number of TrB/mL was decreased in pre-COPD, GOLDI, GOLDII and GOLDIII/IV compared to controls. (C) Number of alveolar attachments was lower in pre-COPD, GOLDI and GOLDIII/IV compared to controls. (D) Minimal to maximal airway diameter ratio was decreased in GOLD I and GOLD II compared to controls (E) Surface density (SD) was decreased in pre-COPD, GOLDII and GOLDIII/IV compared to controls.* $p<0.05$; **$p<0.01$; ***$p<0.001$. Never smokers are shown in red.

Figure 4: Histopathological correlates of the different study groups. Overview of representative H&E staining of the cores that were scanned with microCT showing

inconspicuous parenchyma in controls (A) mild emphysema in pre-COPD (B) and GOLDI (C) which gets more severe in GOLD II (D) and III/IV (E).

Figure 5: Small airway pathology in pre-COPD. The airway (Awy) showed variable airway pathology across a short segment, while the blood vessel (BV) is shown as a reference. Firstly, diffuse thickening of the airway wall was observed (A-B), prior to narrowing (C-H) and eventual complete obliteration of the small airway lumen with mucus (*) (I-J). Eventually the airway re-opened and gave rise more distally to a normal appearing transitional bronchiole and respiratory bronchioles (K-L).

	Control	Pre-COPD	GOLD I	GOLD II	GOLD III/IV
Number of pts	10	10	6	6	7
Age, Y	65±10	66±7	64±7	67±6	63±4
BMI (kg/m^2)	26.1±4.1	25.0±3.0	22.4±3.3	23.5±2.5	22.1±3.0
Gender (M//F)	4//6	5//5	3//3	5//1	0//7
Ever smoker	5	10	6	6	7
>5% CT emphysema	0	10	5	6	7
Smoking, PY	37±29	38±17	33±12	40±8	35±10
Tissue					
LUL	2	5	2	1	0
RUL	2	2	2	4	0
LLL	1	0	1	1	0
RLL	5	3	1	0	0
Left lung	0	0	0	0	3
Right lung	0	0	0	0	4
FEV_1 (L)	2.72±0.84	2.60±0.94	2.60±0.60	2.08±0.26	0.56±0.17
FEV_1 (%pred)	98±13	93±20	92±11	70±9	24±8
FVC (L)	3.41±1.24	3.12±1.11	4.10±1.14	3.70±0.64	1.89±0.52
FVC (%pred)	98±14	90±19	113±16	96±17	65±19
FEV_1/FVC	0.80±0.06	0.79±0.06	0.64±0.03	0.57±0.08	0.29±0.02
DLCO (%pred)	100±14	81±16	74±21	75±15	36±8
TLC (%pred)	102±10	104±20	119±13	110±10	144±14
RV (%pred)	112±22	128±37	134±27	137±38	237±22

Table 1: **Patient characteristics**. Abbreviations: PY=packyear; LUL=left upper lobe; RUL= right upper lobe; LLL= left lower lung; RLL= right lower lung; FEV_1=forced expiratory volume in 1 second; FVC= forced vital capacity; DLCO= diffusing capacity for carbon monoxide; TLC= total lung capacity; RV= residual volume. Values present mean±standard deviation.

	Control	Pre-COPD	GOLD I	GOLD II	GOLD III/IV	Correlation 2nd observer
% of emphysema	1.3±1.9	22.0±16.9	22.9±16.0	31.3±12.7	85.2±11.2	R=0.87 (0.75-0.93)
% BRECT	4.3±4.1	3.5±3.0	4.2±3.6	2.3±1.5	1.7±1.4	R=0.44 (0.13-0.67)
% airway wall thickening	4.7±2.8	7.8±6.1	9.7±6.4	5.7±1.9	10.5±3.3	R=0.22 (-0.12-0.51)
% mucus plugging	0.0±0.0	1.7±4.4	0.0±0.0	0.5±1.3	1.4±1.3	R=0.17 (-0.17-0.47)
% of tree in bud	2.5±7.9	6.1±9.4	4.0±7.6	0.7±1.6	1.4±2.5	R=0.15 (-0.19-0.46)

Table 2: Summary of the ex-vivo CT scoring. BRECT=Bronchiectasis. The scoring system is described in more detail in the material and method section and in the online supplement. The % indicates the % of the total lung/lobe. Correlation analysis was performed with Spearman-rank test and the result is shown as R-value (95%CI)

	Control	Pre-COPD	GOLD I	GOLD II	GOLD III/IV	ANOVA p
MicroCT measures						
Number of TrB/mL, n	11.45±1.90	6.32±3.03***	6.97±1.75**	5.00±1.77***	0.70±0.54***	<0.0001
Morphometry TrB middle						
Alveolar attachments, n	9.2±0.6	7.5±1.***	7.4±1.0**	7.1±1.0***	4.8±0.8***	<0.0001
Airway wall thickness (μm)	52.4±6.2	81.0±15.4	65.5±13.2	85.4±14.1	108±68**	0.0101
Min/ max airway diameter	0.86±0.03	0.79±0.03	0.83±0.04	0.77±0.04	0.80±0.17	0.0009
Morphometry TrB end						
Alveolar attachments, n	8.9±0.5	7.5±1.1**	7.0±1.0***	6.9±1.0***	5.1±0.4***	<0.0001
Airway wall thickness (μm)	53.9±10.6	97.1±39.1*	68.1±9.7	97.3±19.4*	82.5±48.6	0.017
Min/ max airway diameter	0.84±0.02	0.79±0.03*	0.77±0.05*	0.74±0.04***	0.77±0.07*	0.0007
Number of TB/mL, n	5.53±0.94	3.63±1.47***	3.79±0.70**	2.61±1.05***	0.27±0.17***	<0.0001
Morphometry TB middle						
Alveolar attachments, n	10.0±0.7	8.2±1.3*	7.8±1.2**	7.9±1.1*	4.8±2.2***	p<0.0001
Airway wall thickness (μm)	61.5±7.8	97.5±40.0*	75.3±14.9	98.3±21.1*	68.2±31.1	0.022
Min/ max airway diameter	0.86±0.03	0.81±0.04	0.84±0.03	0.76±0.03*	0.56±0.20***	<0.0001
Surface density (1/μm)	0.0157±0.0016	0.0117±0.0035***	0.0122±0.0036*	0.0109±0.0024***	0.0046±0.0008***	<0.0001
Histologic measures						
Chord length (μm)	96.3±7.9	132.6±12.1**	164.2±11.9***	170.2±25.0***	314.3±44.9***	<0.0001
Alveolar surface density (1/μm)	0.0112±0.0097	0.080±0.0080*	0.0747±0.0089*	0.0715±0.0097**	0.0049.0±0.0117***	<0.0001

552 **Table 3: Summary of microCT and histologic measures** shown as mean±SD. *p<0.05; **p<0.01;

553 ***p<0.001

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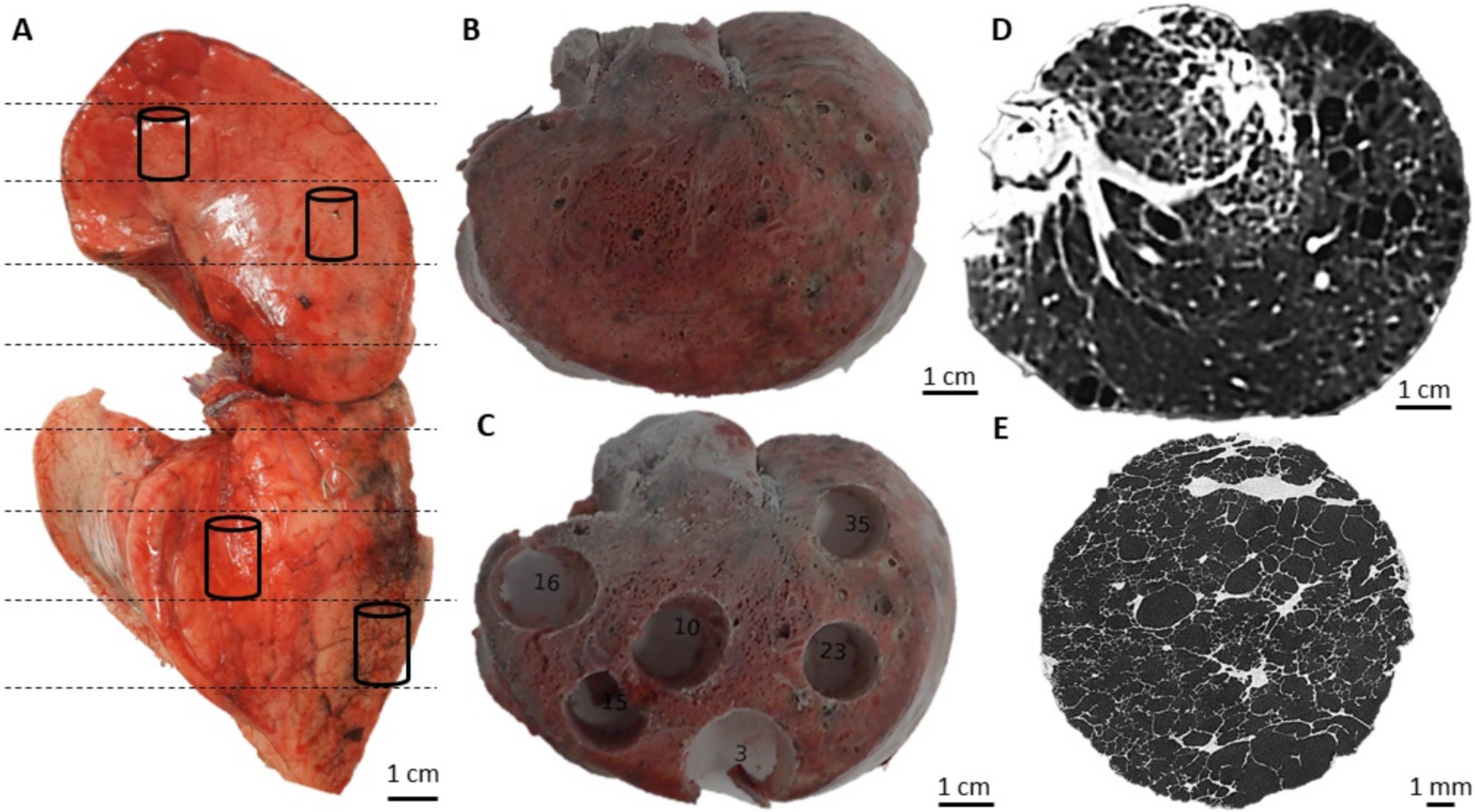
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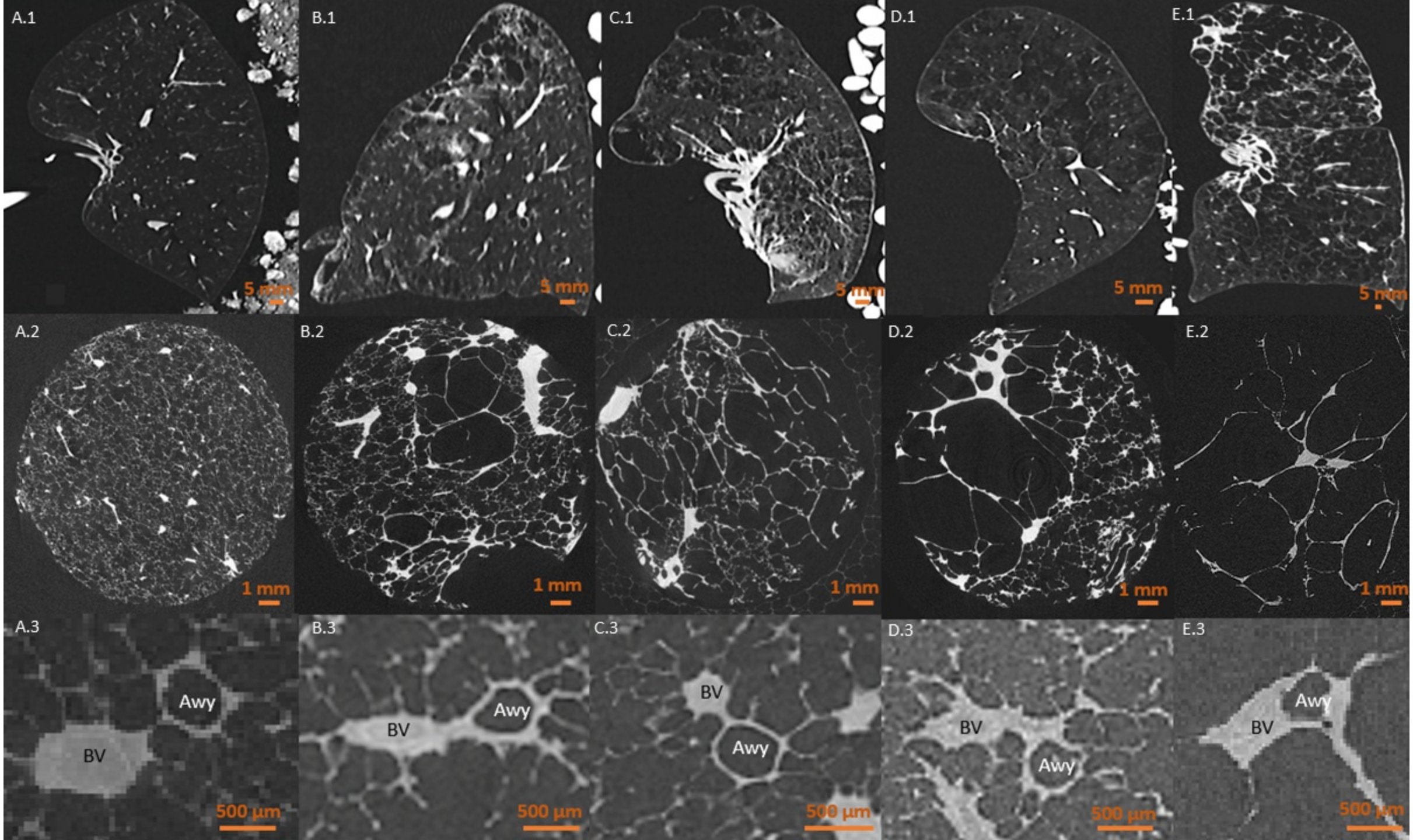
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Awy
BV
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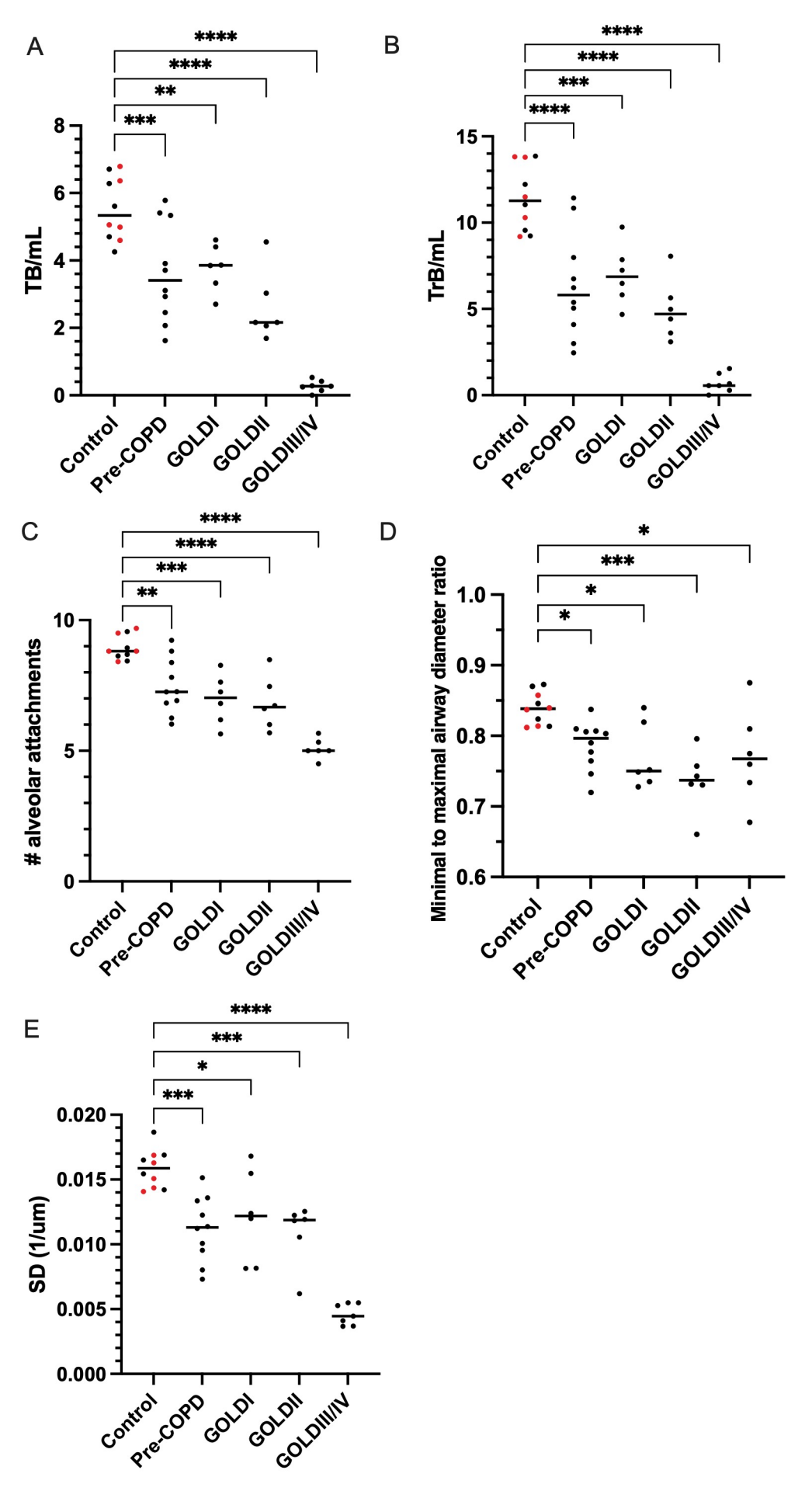
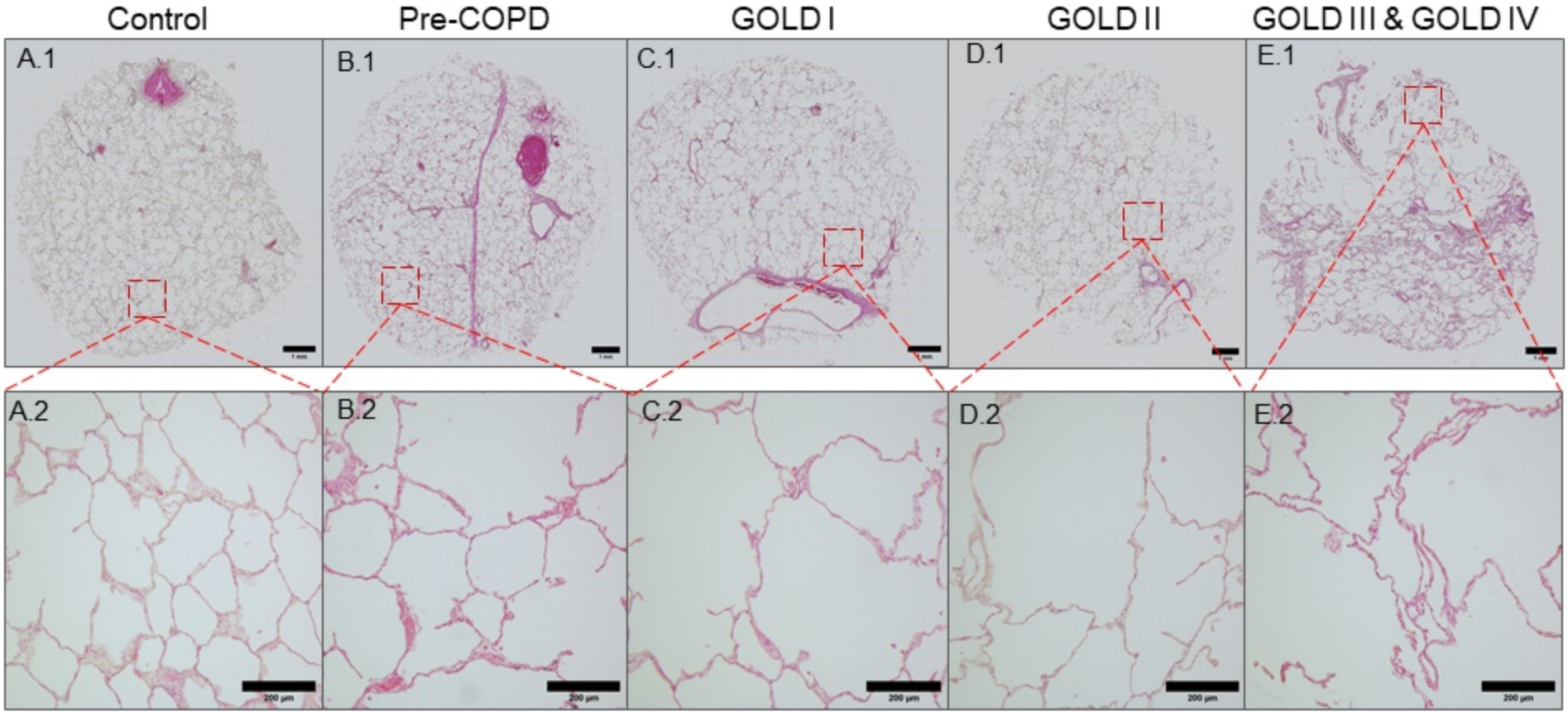

A
TB/mL
Control
Pre-COPD
GOLDI
GOLDII
GOLDIII/IV
B
TrB/mL
C
alveolar attachments
D
Minimal to maximal airway diameter ratio
E
SD (1/um)


Control
Pre-COPD
GOLD I
GOLD II
GOLD III & GOLD IV
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B.1
C.1
D.1
E.1
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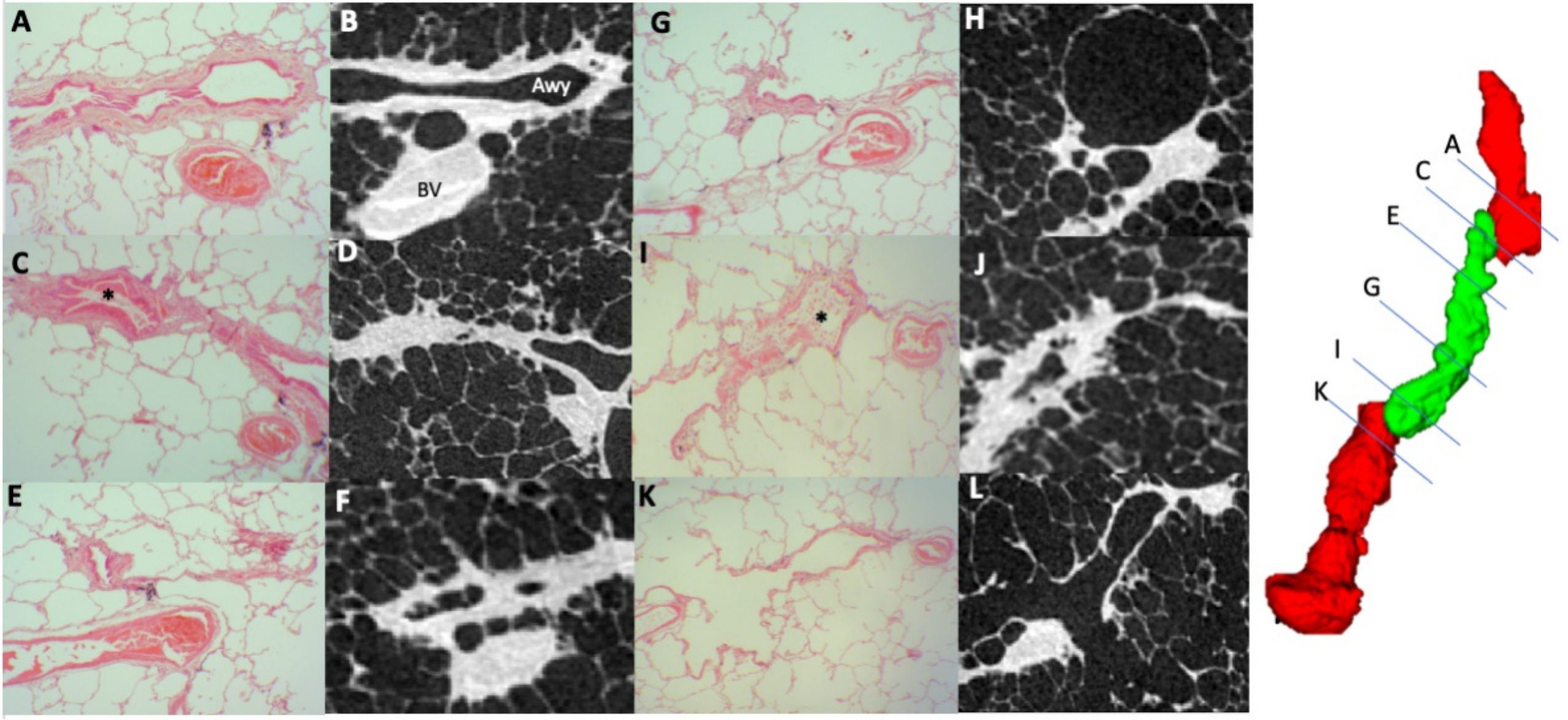
D.2
E.2
200 µm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