Appendix 1

Ex vivo characterization of sequential amputation lesions

Study setup

Using an *ex vivo* setup with ovine lungs, the repeatability of sequential lung edge amputations or lung incisions for attaining bronchioles of ≥1.5 mm but lower than 3.0 mm was investigated (same inclusion criteria as the model used by Ranger *et al.* in 1997). Next, the defect which was found most optimal was investigated in an *in vivo* acute aerostasis experiment and the intrinsic sealing capabilities were tested, by measuring minimal leaking pressures (MLP) at obduction and air leakage using a digital drain (see main manuscript).

Outcome measures

Bronchial diameters (lumen) were measured using a ruler with markings every 0.5 mm (Aesculap AA804R), and approximated in 0.5 mm increments. Bronchioles <0.5 mm but approximately larger than 0.25 mm were noted as 0.5 mm. If the bronchial lumen was approximately smaller than 0.25 mm or if no bronchiole lumen could be identified macroscopically, this was noted as 0 mm. MLP was determined using a Servo-I mechanical ventilator (Maquet critical care). Under pressure control settings, positive end-expiratory pressure (PEEP) was put on 10 cmH_2O while air leakage was assessed using water immersion or dripping water over the lesion. PEEP was gradually dialed down in steps of 1 cmH_2O until air leakage disappeared and the last plateau ventilatory pressure (Pplat) at which air leakage was still present was noted as MLP. In the case of MLP >10 cmH_2O, the pressure was gradually increased until leakage was observed. The dimensions (length and width) of sequential amputation lesions were measured in whole millimeters (Figure S1).

Sequential amputations versus incisional defects

First, two methods for attaining bronchiole lesions were compared *ex vivo*: sequential amputations versus lung incisions. For this, lungs of two sheep (54 and 84 kg) sacrificed for a different experiment were harvested immediately after death. Sequential lung amputations perpendicular to bronchial branching were compared with lung incisions regarding repeatability and leaking capability. Right upper (RUL), right middle (RML), right lower (RLL), left upper (LUL) and left lower lobes (LLL) were selectively intubated and fully recruited until Pmax =40–50 cmH_2O. All lesions were marked and created on a static lung with PEEP =10 cmH_2O. First, sequential amputations (*Figure S1*) were performed starting at 1 cm from the edge and measuring maximum bronchiole diameter and MLP during each increment until bronchioles of ≥1.5 mm were encountered. Then, a large clamp was placed across the defective area and the procedures were repeated on the same lobe for incisional defects (25 mm long, 5 mm deep and increasing depth in steps of 5 mm).

On the two lung specimens, a total of N=31 variations of the sequential amputation defect (N=10 1 cm from edge, N=10 2 cm from edge, N=7 3 cm from edge and N=4 4 cm from edge) and N=34 variations of the incision defect (N=10 at 5 mm depth, N=9 at 10 mm depth, N=10 at 15 mm depth, N=4 at 20 mm depth and N=1 at 30 mm depth) were created, N=2 for each RUL, RML, RLL, LUL and LLL. For the sequential amputations, a significant strong positive correlation was found between distance from the edge (1–4 cm) and maximum bronchiole diameter in the defect (Pearson correlation coefficient =0.635, P<0.001). This was also the case for incisional defects between incisional depth (5–30 mm) and maximum bronchiole diameter (Pearson correlation coefficient =0.678, P<0.001). However, the incisional defect produced outliers in maximum bronchiole diameter of 3–5 mm, while the sequential amputations showed a more predictable pattern of bronchiole diameter increase upon further amputations. In both cases, the maximum bronchiolar diameter showed a significant strong negative correlation with the MLP. For sequential amputations, the Pearson correlation coefficient was −0.739 (P<0.001) and for incisional defects −0.678 (P<0.001). These relations are shown visually in Figure S2. Based on this experimental data, it was decided that sequential amputation lesions will result in a more repeatable model regarding bronchiole diameters, and this defect was used in the following experiments. The RUL was no longer considered for further experiments, due to limited access and to increase comparability between right and left lung lesions *in vivo* (RML/LUL and RLL/LLL lesions are more comparable regarding their geometry).

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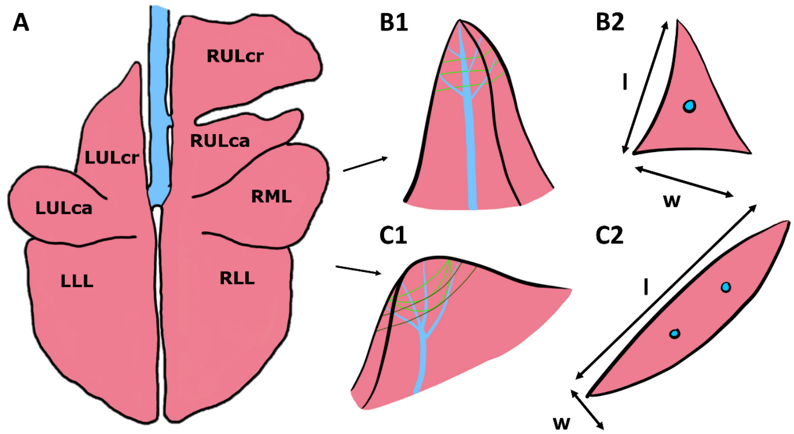

Figure S1 Schematic overview of lesion geometry. (A) Sequential amputation lesions are best performed on the RML/LULca/LLL/RLL in the *in vivo* situation, and are roughly made perpendicular to bronchial branching. (B1) Lesions on the RML/LULca are performed by sequentially increasing the cut line with 1 cm from the previous cut line (green lines), resulting in a triangular defect (B2). (C1) Lesions on the RLL/LLL are performed by increasing the cut depth, but limiting the cut width to allow for future patch placement in the model (light green lines, corresponds to the maximum defect width of 5 cm). This results in a roughly oval shaped defect (C2). In the initial *ex vivo* characterization experiment, the dark green cut lines were followed, leading to increased defect lengths.

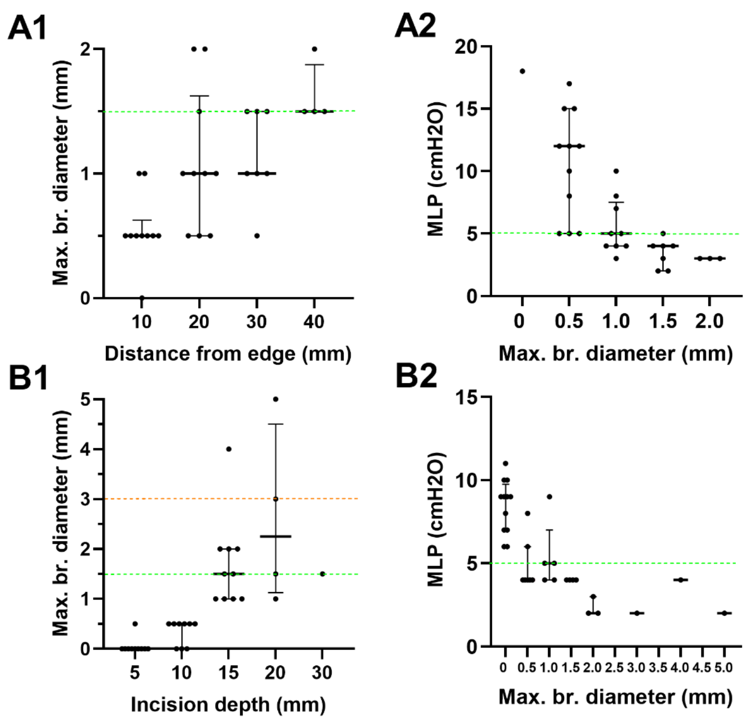

Figure S2 Sequential amputations versus incisional defects characterization. (A1) Sequential amputations defect shows a positive correlation between cut distance from edge, which follows a roughly linear and predictable pattern. From 2 cm distance from edge on, the 1.5 mm threshold for a suited model is passed (green line). (A2,B2) The bronchiole diameter in the defect is negatively correlated with the MLP. From 1.5 mm on, the MLP is consistently 5 cmH_2O or lower (green line). (B1) Incisional defect show a positive correlation with the incision depth, cut following a less predictable pattern. As can be seen, from 15 mm depth on, the 1.5 mm threshold for bronchial diameter is passed (green line), but the 3.0 mm upper limit (orange line) may also be exceeded.

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Anesthesia protocol in vivo studies

Housing remarks

Animals were transported to the research facility from a farm one day before the experiment together with two buddy sheep. Housing was in accordance with institutional protocols, and animals were fed ad-libitum up until the surgery.

Anesthesia

In these model development studies, anesthetic protocols were being optimized for the acute aerostasis model, which lead to some heterogeneity in used methods. P1 and P2 were pre-medicated using midazolam and carprofen, followed by induction of anesthesia using propofol in the jugular vein. P4, P5, P6, E2 and E3–E6 were pre-medicated using midazolam, ketamine and carprofen and anesthesia was induced using remifentanil and propofol through an intra-venous canula. They were then intubated and put on isoflurane during shaving and placement of a urinary catheter. Mechanical ventilator settings were adjusted based on the ventilation and oxygenation requirements. Propofol and remifentanil titrated for adequate blood pressure regulation (target mean arterial blood pressure during surgery: 50–100 mmHg) were used for anesthetic maintenance during surgery in P1 and P4, P5, P6, E2 and E3–E6, and propofol and sufentanil in P2. In P2, propofol was swapped out for pentobarbital halfway through the surgery to achieve a more stable blood pressure. Additionally, an arterial blood pressure line and ventilation line in the rumen (through a small epigastric incision in P1 and P2 and trans-esophageal in the remaining) were placed. The first sheep (P1) received a percutaneous tracheotomy canula to facilitate insertion of an endo-bronchial blocker (EZ-blocker) for single-lung procedures. Intermitted hyperoxygenation followed by apnea to facilitate defect creation on the lung was utilized for P2 and P4, P5, P6, E2 and E3–E6 as a less complicated alternative. An intercostal block was placed in all animals at three levels with a combination of lidocaine and bupivacaine. After surgery, the sheep were placed in an abdominal position, buprenorphine was administered and ketamine and midazolam were infused continuously to allow the sheep to breath spontaneously while still under anesthesia, and propofol was administered if required to maintain a deep anesthetic plane. During this spontaneous observation period, the animal was continuously monitored by an experienced biotechnician. Anesthetic monitoring throughout the procedure included end-tidal CO_2, oxygen saturation, pulse, blood pressure, arterial blood gasses and reflexes. At the end of the experiment, the sheep was euthanized by an overdose of pentobarbital.

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Histology of bronchiolar lesions

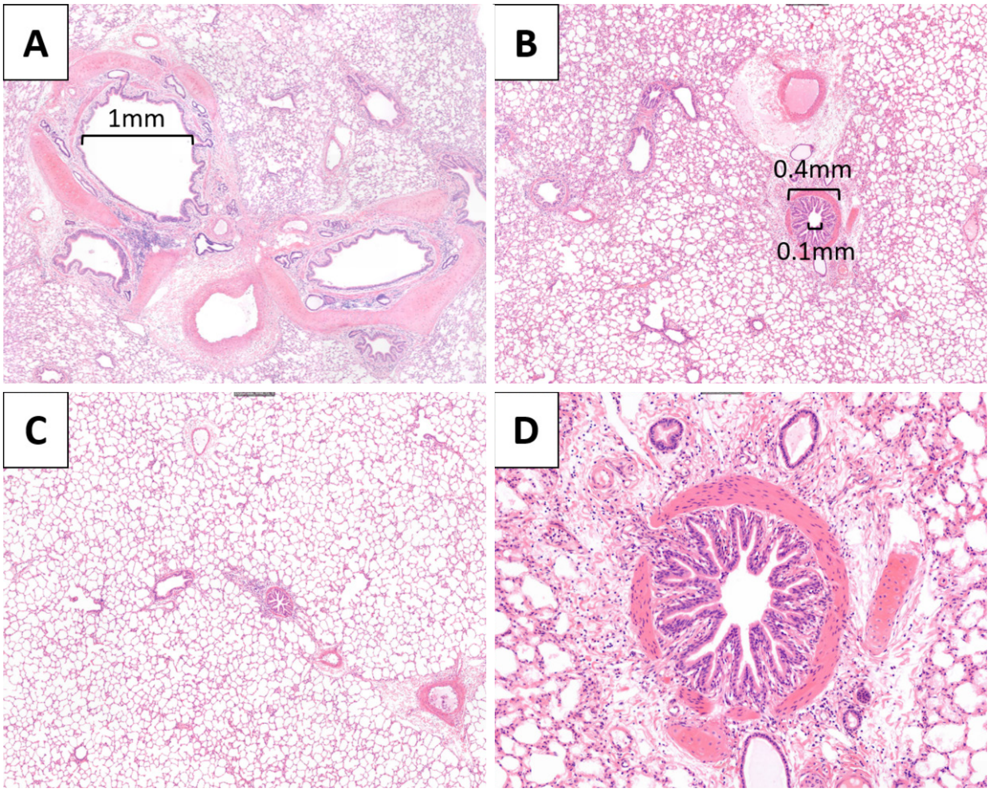

Figure S3 Histology of lesions including bronchioli. (A) Large bronchi are noted in this section, corresponding to macroscopic bronchi of ⌀1.5 mm. Note the hyaline cartilage around the lumen. Compared to normal parenchyma (C, control section), the alveoli in this section appear less aerated due to intra-alveolar bleeding and alveolar collapse. (B) Small bronchioles are seen, which appear contracted, and only a very small part of hyaline cartilage is seen (detail in D). However, the bronchioles in the normal parenchyma also have this similar contracted appearance (C). H&E staining: A, 1.6×; B, 2×; C, 2×; D, 5×.

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Literature table of previous animal studies with negative control groups

Table S1 Intrinsic sealing mechanisms from untreated pulmonary parenchymal lesions described in previously literature (based on comprehensive literature survey)

Author, year	Species, N	Lesion	Intrinsic sealing findings	Histology (intrinsic sealing)
Joannides, 1949 (41)	Dogs[a], ND	Crushing, punctures, tears, incisions, wedges, resection of lung tissue	Secretion plugging bronchioles. Alveolar compression due to air leak collapsing the lung after injury or after gentle compression[b]	Extended intra-alveolar bleeding. Compressed alveoli
Findlay, 1950 (42)	Cats, ND	Segmental lobectomy, residual parenchymal defect 2×1.5×1.5 cm. Middle lobe tip amputated and rotated into wound	Minimal air leak or pneumothorax. One animal died from BPF	Partial aeration of alveoli. Widening of the septa. Inflammatory response. Intra-alveolar blood
Kausel, 1955 (19)	Dogs, N=8	Segmental lobectomy, residual parenchymal defect 9–15 cm^2	No pneumothorax or air leaks. One animal died from BPF	Compressed alveoli. Intra-alveolar bleeding. Massive capillary enlargement. Alveolar cell enlargement
Wilder, 1963 (34)	Dogs, N=9	5×2 cm amputation of upper lobe	No drains left in place, 6/9 animals survived (compared to 3/9 in glue-treated group). In survivors, lesions were adherent to chest wall	ND
Poticha, 1965 (18)	Dogs, N=6	Removal of entire parietal surface of lobe at 3 mm depth	No drains left in place, no air leak complications. One death due to other pulmonary complications. Exudate of blood and fibrin sealed all air leaks, at 1 week leading to fibrin adhesions to chest wall and atelectasis adjacent the injury	ND
Nuchprayoon, 1968 (35)	Dogs, N=8	Removal of lung tissue at costophrenic margin at 1.5–2 cm depth, inducing 12×1.5 cm raw surface	Bronchi >1.0 mm suture ligated and three hours thoracic drainage postoperatively. 6/8 animals died due to tension pneumothorax. 2/8 developed chronic lower lobe atelectasis due to air leak	Alveolar congestion
McCarthy, 1988 (24)	Dogs, N=8	9×9 cm defect at 0.5 cm depth on left lower lobe	Air leak sealed on thoracic drainage in 4/8 control animals (also in 4/8 sealed animals)	ND
Feito, 2000 (37)	Rabbits, N=10	Several superficial incisions (average 7) at 1.5 mm depth	Uneventful postoperative course in all animals. At day 0–1, mean 19.4% pneumothorax, reduced to 3.3% at day 6–7. Adhesions present at obduction	ND
Luh, 2004 (40)	Pigs, N=5	Bilateral 5×5 cm <0.5 cm depth lesions on left and right upper lobes	Critical leak pressure <5 mmHg at 0.5 and 72 h. Air leakage on thoracic drain until 72 h	Poor pleural coverage
Getman, 2006 (38)	Rats, N=10	5 mm parietal defects	Uneventful postoperative course. Adhesions of defect area to chest wall	ND
Kanzaki, 2007 (36)	Rats, N=4	5 mm long incision at 3 mm depth	4/4 animals died due pneumothorax within 1 h	ND
Buyukkale, 2017 (39)	Rats, N=8	Linear incision left upper zone 0.2×0.1 cm (length × height)	No postoperative complications. Air leak pressure mean 43.5 mmHg at day 7	ND

[a], species used not well described in paper; [b], since sutures were used, healing cannot be attributed to intrinsic sealing. However, histology findings were representative of intrinsic sealing mechanisms in this study. ND, not described; BPF, bronchopleural fistula.

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