

2 **The Total Number of Acini Remains Constant throughout**
 Postnatal Rat Lung Development

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Running Head. Constant Number of Acini during Rat Lung Development

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24 **Abstract**

The pulmonary airways are subdivided into conducting and gas-exchanging airways. The small tree of gas-exchanging airways which is fed by the most distal conducting airway represents an acinus. Very little is known about the development of the number of acini. The goal of this study was to estimate their number throughout rat postnatal development. Right middle rat lung lobes were obtained at postnatal day 4-60, stained with heavy metals, paraffin embedded, and scanned by synchrotron radiation based X-ray tomographic microscopy or imaged using micro computed tomography after critical point drying. The acini were counted by detection of the transitional bronchioles (bronchioalveolar duct junction; BADJ) using morphological criteria (thickness of the walls of airways and appearance of alveoli) during examination of the resulting 3D image stacks. Between postnatal days 4-60, the number of acini per lung remained constant (5840 ± 547 acini), but their volume increased significantly. We conclude that the acini are formed before the end of the saccular stage (before postnatal day 4) and that the developmental increase of the lung volume is achieved by an increase of the acinar volume and not by an increase of their number. Furthermore, our results propose that the bronchioalveolar stem cells, which are residing in the BADJ, are as constant in their location at the BADJ itself.

44 **Keywords.** Pulmonary acinus,
Lung development,
46 bronchioalveolar duct junction, BADJ
bronchioalveolar stem cells, BASCs

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Introduction

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In 1974, P. H. Burri explained (8): “Another type of alveolar formation consists in the
52 transformation of originally purely conducting into respiratory airways” and in 1984
(7): “The branching pattern and structure of conducting airways are mature at birth,
54 so that except for the terminal bronchiole (where transformation into respiratory
passage may occur) the adult bronchial tree is the replica of the newborn one”. His
56 statements were based on observations made by Boyden and Tompsett on bronchial
trees of human infants and dog puppies (5, 6). To our best knowledge — due to the
58 complexity of lung architecture and to technical limitations — nobody has
quantitatively followed the development of the pulmonary acini until now.

60

The term acinus (plural: *acini*) is generally used to describe the few generations of
62 the bronchial tree which are located distally of a terminal bronchiole. The latter is
defined as the most distal purely conducting generation of airways. One acinus
64 represents the functional unit of the lung. Because rat belong to the animals which do
not possess respiratory bronchioles (41) a transitional bronchiole opens directly into
66 the most proximal alveolar duct(s) of the acinus. The transitional bronchioles contain
the bronchioalveolar duct junction where the bronchioalveolar stem cells are located.
68 In human lungs, which possess respiratory bronchioles (41) the entrance of the acini
is defined at the junction of the terminal and respiratory bronchioles. The
70 bronchioalveolar junction is defined as the entrance of a ventilator unit (38). Again at
this junction the bronchioalveolar stem cells are located. Therefore, the best human
72 correlate of the rat acinus is the human ventilator unit.

74 Due to their complex three-dimensional structure, acini cannot be detected by two-
dimensional investigations. Therefore, several approaches were proposed to
76 overcome this limitation. Casting methods were used by Yeh et al. (46) and by
Rodriguez et al. (24). Serial-sectioning was used by Mercer and Crapo (21), while
78 Wulfsohn et al. (45) developed an estimation technique based on a disector of five
consecutive sections. However, as explained in Barré et al. (3) these methods
80 require tedious work, and thus are not suited for studies with larger number of
samples. Several publications proposed approaches based on X-ray tomography
82 (10, 17, 18, 20, 34-36, 42). Even if the latter approach would be suitable for the
analysis of larger number of sample, to our best knowledge, it has not been done
84 until now, especially not for the analysis of the development of the acini.

86 Lung developmental is divided in the embryonic, fetal, and postnatal periods (7, 30,
33, 44). In the embryonic period, the lung anlage appears as two independent
88 outpouchings of the foregut forming the two lung buds. The formation of the airways
starts shortly afterwards by branching morphogenesis which is defined as a repetitive
90 branching and outgrowth of the future airways. Typically, in human lungs, the
branching of the airways follows a dichotomous pattern. This means that the airways
92 symmetrically branch into two smaller airways of the same diameter. With the
appearance of the pleura the embryonic period blends over into the fetal period.
94 Branching morphogenesis continues and at the end of the pseudoglandular stage
approximately 20 airway generations are formed in humans (16). The airway
96 formation continues during the next step, the canalicular stage. At the end of this
stage the transition between conducting and gas-exchanging airways is detectable,
98 resulting in the “birth of the acinus” (4) even if the proximal part of the acinar airways
is already formed during the pseudoglandular stage. Branching morphogenesis

100 comes to its end at latest during the saccular stage, by the arising of saccules and of
the type 1 and 2 alveolar epithelial cells at the distal ends of the bronchial tree. The
102 next and final stage, alveolarization, is composed of two phases (classic and
continued) (32, 39) which ends during young adulthood (12, 14, 31). This description
104 of the stages of lung development is in general valid for all mammals
independently of their airways branching pattern (28). The main differences are found
106 in the duration of the stages and at the time point of birth.

108 While human lungs possess a dichotomous branching pattern, lungs of rats and
other rodents possess a monopodial one (23, 46). At most of the branching points
110 the airway divides asymmetrically into one larger and one smaller airway. In rats, the
trachea divides into two main bronchi from which main lobar bronchi, one per lobe,
112 emerge to ventilate the five lung lobes. A system of secondary and tertiary lobar
bronchi arises from the next larger category to ventilate the peripheral parts of the
114 lobes. Acini are directly connected by transitional bronchioles to purely conducting
airways of various generations, even including secondary lobar bronchi (3, 24).

116

The goal of this study was to estimate the number of acini for rats at different ages
118 (postnatal days 4, 10, 21, 36, and 60). Taking P.H. Burri's statements (7, 8) as
starting point, we studied the development of the pulmonary acini in rats. We used a
120 time-efficient estimation protocol developed in our laboratory (3) in order to
investigate large amounts of samples. Surprisingly, the number of acini showed no
122 statistical differences between the days studied indicating that the number of acini
remains constant during lung development. Furthermore we conclude that the
124 positions of the entrance of the acini, as well as the sites where the bronchioalveolar

stem cells are residing, are defined in the bronchial tree before the end of the
126 saccular stage.

128

130 **Materials and Methods**

132 **Animals.** Lungs of twenty-seven male rats (postnatal days 4, 10, 21, 36, and 60;
Wistar Bern) were obtained after fixation with 4% paraformaldehyde in phosphate
134 buffered saline via tracheal instillation at a constant pressure of 20 cm water column.
The lung was removed from the chest cavity and the pressure was maintained during
136 fixation for a minimum of two hours at 4°C in order to prevent a recoiling of the lung
(19, 22, 40). After fixation, all lobes were separated and their volume was measured
138 by water displacement (26). A second volume measurement was performed using
the Cavalieri principle (9) on the tomographic dataset to determine the shrinkage
140 factor of the samples.

142 All animal studies were approved by and conducted in accordance with the
Veterinary Service of the Canton of Bern, Switzerland and the Swiss Federal Agency
144 for Environment, Forest and Landscape.

146 **X-ray Tomography.** Two tomographic methods were used during this study:
synchrotron radiation based x-ray tomographic microscopy (SRXTM) and micro
148 computed tomography (μ CT). The right middle lung lobes were prepared, as already
described (3, 25), either by critical point drying for μ CT (15) or by heavy metal

150 staining with osmium tetroxide and uranyl acetate (33), and paraffin embedding for
SRXTM.

152

Synchrotron radiation based x-ray tomographic microscopy. 20 samples were
154 scanned at the TOMCAT beamline (37) of the Swiss Light Source synchrotron facility
at the Paul Scherrer Institute (Villigen, Switzerland). X-rays at energy of 20 keV were
156 converted to visible light by a scintillator (20 μm thick LuAG:Ce or 18 μm thick
YAG:Ce, Crytur Ltd., Turnov, Czech Republic) after passing the samples. An optic
158 microscope magnified the visible light in order to obtain effective voxel size lengths of
1.75 to 3.5 μm . In order to visualize the entire lung lobe, the field of view of the
160 microscope was increased perpendicular to the rotational axis using 'wide-field
SRXTM' (three field of views) (11) or 360° scans (two field of views). In addition, five
162 to seven wide-field scans were stacked parallel to the rotational axis, resulting in 3D-
stacks of 8 bit grayscale images of up to 7500x7500 pixels per slice.

164

Micro computed tomography. Seven samples were imaged by a μCT device
166 (SkyScan 1172, Bruker, Billerica, MA, USA) at 33 kV and 204 μA without filtering.
Two to three oversize scans in the vertical direction were needed to visualize the
168 entire sample at 2.5-3.5 μm voxel side length. The GPU reconstruction software
(NReconServer64bit, Bruker, Billerica, MA, USA) was used on a GeForce GTX 680
170 graphic card (Nvidia Corp., Santa Clara, Ca, USA) to create 8 bit grayscale image
stacks of approximately 4000x4000x2500 voxels per scan. Additional samples (right
172 upper, right lower, cardiac lobe and left lung) at days 4 and 60 were also scanned in
the μCT .

174

Detection and Counting of Acini. The manual acini counting followed the protocol described by Barré et al. (3). The acini entrances (i.e. transition from conducting to gas-exchanging airways) were detected based on morphological criteria (thickness of airway walls and appearance of alveoli) by scrolling through tomographic data sets representing right middle lung lobes (n = 27). In addition, three entire lungs were counted at days 4 and 60 to validate that the right middle lobe is a valid estimator of the entire lung. The software Fiji (27) was used to crop and display sub-stacks of 250 to 500 images, in order to reduce the computing power requirement, and to manually label the detected acini entrances. The labels were manually counted using a laboratory counter (Clay Adams, New York, USA). After one sub-stack was counted, labels of overlapping acini were reported to the next sub-stack to exclude double counting. All data were analyzed on a Dell Precision T5500 work station (Intel Xeon X5650 (six Core, 2.67 GHz), 24 GB RAM, Windows 7 Professional 64).

188

Three-dimensional Visualization. The conducting airways of five right middle lung lobes (one per developmental time point) were visualized three-dimensionally adapting a protocol described by Barré et al. and Haberthür et al. (3, 10). Briefly, several images stacks of 250-500 eight bit greyscale images were analyzed using MeVisLab (version 2.1, 2010-07-26 Release, MeVis Medical Solutions and Fraunhofer MEVIS-Institute for Medical Image Computing, Bremen, Germany). The sub-stacks were loaded as TIFF files and segmentation stoppers were manually set at the acinar entrances to separate them from the conducting airways. The conducting airways were segmented using a gray-level threshold-based region growing algorithm (47) after down-sampling with a factor of 2. The segmentation seed points were manually set within the conducting airways. All segmentations were reconstructed as one 3D model using a custom-made MeVisLab pipeline. This basic

pipeline stacked all segmentations and displayed spheres at the position of the
202 segmentation stoppers.

204 **Statistics.** The statistical analysis was done using Microsoft Excel (version
14.0.7106.5003, 32-bit) and Prism (version 5.04, GraphPad Software Inc.). The
206 values are expressed as means (\pm standard deviation). The linear regression of the
observed values and the R^2 (coefficient of determination) were calculated. This
208 coefficient indicates how well the observed values fit the ideal values of a linear
regression. In this case, R^2 is the square of the Pearson correlation coefficient (r). No
210 correlation between parameters was assumed when $R^2 \leq 0.5$ ($|r| \leq 0.7$), weak
correlation when $0.5 < R^2 \leq 0.7$ ($0.7 < |r| \leq 0.84$), and strong correlation when $0.7 <$
212 R^2 ($0.84 < |r|$). In addition, multiple regression and ANOVA F-tests were used (both
using the data analysis toolbox on Microsoft Excel)(1). A multiple regression test was
214 used to determine if observations groups were correlating. To do so, one group was
expressed as the dependent variable and the others as independent variables. The
216 predicted values (i.e. regression results) were compared to the values of the group
set as dependent variable using the coefficient of determination. The standard score
218 (difference of observation and group mean divided by group standard deviation) was
used to normalize the data prior to regression analysis. The significance of difference
220 between the means of observation groups was achieved using a T-test or using one
way ANOVA F-test. A significance level of $\alpha = 95\%$ was used for both tests, detecting
222 significant difference if $p \leq 0.05$ or non-significant difference if $p > 0.05$. In addition to
ANOVA, a Bonferroni's multiple comparison tests was performed to define which
224 group was significantly different from the other groups (1). T-test and ANOVA require
normal distributed data. Therefore QQ-plots were used to test for normal-distributed
226 values.

228 **Calculations.** The total number of acini present in a rat lung was calculated by
dividing the counted number of acini in the right middle lobe by the parenchymal
230 volume of the corresponding lobe and by multiplying this result with the total
parenchymal volume. The mean acinar volume (tissue plus airspace of an acinus)
232 was calculated by dividing the lobe volume by the counted number of acini and
multiplied by the volume density of parenchyma (Tab. 1). The parenchymal volume
234 density was estimated according to the ATS guidelines (13).

236

238 **Results**

240 **Detection of the acinar entrance throughout lung development.** In tomographic
datasets morphological criteria (thickness of the airways walls and appearance of
242 alveoli) were used to detect the entrances of the acini (Fig. 1) as described in Barre
et al. (2). Because the acini are not fully developed and the alveoli not yet formed at
244 day 4, we used the appearance of the gas-exchanging capillaries in the walls of the
airspaces as additional, newly introduced criterion for the detection of the acinus
246 entrance. Due to the iron contained within the erythrocytes, the contrast inside the
capillaries was large enough to be safely detected. This criterion was also used at
248 day 10, while it was not necessary to be used for lungs at older age. Although acinar
airways are still immature, the alveolar duct already presents its typical shape (airway
250 walls covered with uprising new alveolar septa) at the beginning of the stage of
alveolarization. The latter contrasted with the tubular wall of the conducting airways.

252 Therefore, we used it as the second additional criterion for the detection of the
transition between conducting and gas-exchanging airways.

254

Growth of lobe volume. We compared the growth of the five lung lobes throughout
256 postnatal lung development (Tab. 2). In particular, we asked if its fraction of the total
lung volume changes during development. No significant changes were observed for
258 the right upper, right middle, and right lower lobe (Tab. 2). However, the fraction of
the left lung decreased between postnatal days 4-10 and subsequently stays
260 constant. The volume fraction of the cardiac lobe increases inversely proportional to
the left lung between postnatal days 4-10. Afterward only a small additional increase
262 was observed until day 60.

264 **Validation of the sampling.** According to Barré et al. (3), the right middle lung lobe
is a valid estimator of the number of acini for the entire lung for adult rats. The
266 estimation was based on the parenchymal volume of the entire lung and of the right
middle lobe. In order to test if the right middle lobe is also a valid estimator for
268 immature lungs, all acini of three entire lungs at postnatal day 4 were counted
manually. This counting showed that the mean acinar volume of the right middle lobe
270 (0.066 μl) was not statistically different from the mean acinar volume of right upper
(0.065 μl) and right lower lobes, as well as of the entire lung (0.073 μl) and of the
272 sum of the left lung and the cardiac lobe (0.081 μl / Tab. 3). We chose this sum,
because during postnatal development the volume of the cardiac lobe growth
274 unproportional to the three other right lung lobe (see above). However, the sum of
the volume of the left lung and the cardiac lobe growth proportional to the three other
276 lobes (Tab. 2). Based on the mean acinar volume the mean number of acini ($6469 \pm$
720 acini) was estimated for these three samples following the method presented

278 previously (3). No statistical difference was observed between the counted ($5865 \pm$
465 acini) and the estimated (6469 ± 720 acini) number of acini at postnatal day 4.
280 The counted number of acini per lobe (N=3 for right upper, right middle, right lower,
cardiac, and left lung) between days 4 and 60 (3) showed no statistical differences. In
282 addition, the mean of the observed difference between estimated and counted total
number of acini was approximately 10%. Collectively, these findings support the view
284 that the right middle lung lobe is a valid estimator for the total number of acini at any
stage of postnatal lung development.

286

Number of Acini. The number of acini in the right middle lung lobes were counted at
288 postnatal days 4, 10, 21, and 36 as previously described and compared to data of the
adult lung (day 60) (3). No significant differences were observed between the five
290 age groups (Tab. 4). Therefore, in opposite to body weight and lung volume, the
number of acini is constant throughout postnatal lung development. We detected no
292 direct correlation between these three lung parameters. However, the variation to the
mean can be calculated for all individuals over the five time points. To do so, in a
294 multiple regression analysis the standard scores (difference of mean and individual
value divided by the standard deviation) of body weight and lung volume of all 27
296 animals were set as the independent variables, and the number of acini of the right
middle lobe was set as the dependent variable. The analysis provided a value (Fig. 2,
298 predicted curve) for every sample. Assuming a linear relationship between all three
tested parameters, the predicted values should match with the observed number of
300 acini (Fig. 2). This was not the case ($R^2 = 0.08$), and thus the number of acini, body
weight, and lung volume showed **no** linear correlations.

302

Mean acinar volumes. The mean acinar volume (Tab. 5), defined as the
304 parenchymal lobe volume divided by the counted number of acini, was calculated for
the right middle lobe at day 4, 10, 21, 36, and 60. Based on the specific acinar
306 volume (Tab. 5) a bi-phasic growth of the acinar volume was observed (Fig. 3).
Hence, the acinar volume increases proportionally to body weight until day 21, while
308 after day 21 body weight grows faster than acinar volume. This kind of bi-phasic
growth was already reported for the lung volume, the anlage of new alveolar septa,
310 and for the number of alveoli by Burri (8), Schittny (32), and Tschanz (40),
respectively.

312

Shrinkage of samples. Large shrinkage factors have been reported for paraffin
314 embedding ($28.70 \pm 0.62 \%$) and for critical point drying ($62.0 \pm 1.5 \%$) both
measured at day 60 (3). In the present study, we analyzed the shrinkage factor of the
316 right middle lobe for all five remaining time points. The volume of paraffin embedded
samples showed a reduction of 24.6% (± 4.4) and the ones of the critical point dried
318 samples decreased by 62% (± 1.5).

Reconstruction of conducting airways. In order to visualize our results, we
320 reconstructed the conducting airways of one right middle lobe per time point (Fig. 4).
The visualization highlighted the high similarity of the conducting airways pathway at
322 any time point and between the individuals. Most of the conducting airways ended
324 with one acinar entrance being located on a transitional bronchiole. However, we
also detected acinar entrances very close to each other. In this case, a branching
326 point was located inside the transitional bronchiole (Fig. 4) as already reported in
Barré (3).

328

330 **Discussion**

332 Previous studies by Burri et al. (7, 8) and Boyden & Tompsett (5, 6) proposed that
originally purely conducting bronchioles may be transformed into respiratory airways
334 during early alveolarization. To our best knowledge the presented study represents
the first quantitative investigation of the development of the number of acini
336 throughout lung development in rats. We were able to show that the number of acini
stays constant from day 4 to young adulthood (Tab. 4). From the structural point of
338 view we did not observe any shift of the acinus entrance or the bronchoalveolar duct
junction within the bronchial tree. Initially, we expected a proximal shift of the acinus
340 entrances and a dramatic, approximately factor of 2 decrease of the number of acini.
Our investigations throughout postnatal lung development did not show any statistical
342 difference of the number of acini. At an early stage of alveolarization (day 4
postnatal) 6326 ± 497 acini were estimated for the entire lung. A similar number
344 (5612 ± 547) was observed in young adult rats at postnatal day 60. We conclude that
the acini are formed before the end of the saccular stage (before postnatal day 4)
346 and that the developmental increase of the lung volume is achieved by an increase of
the acinar volume and not by an increase of the number of acini. According to
348 Kitaoka et al. (16) the formation of the airways is completed up to the acinar airways
at the end of the pseudoglandular stage. During the canalicular stage, epithelial
350 differentiation takes place and the bronchioalveolar duct junction, the border between
the conducting airways and the gas-exchange region, is formed (29). Because no
352 shift of the acinus entrance occurs and no additional conducting airways are created,
we conclude that the number of acini will not change during alveolarization.

354 The bronchioalveolar duct junction contains the so called bronchioalveolar stem cells
which are very important for homeostasis and repair. Because the location of the
356 acinar entrance or better the bronchioalveolar duct junction does not move during
alveolarization, the site where these stem cells are residing, stays also constant.

358

Our conclusions, based on rat lungs, are at a first view in contradiction with the
360 observations of Boyden and Tompsett (5, 6). They observed a reduction of the non-
respiratory generation between newborn and adult in humans and dogs. The
362 difference may be explained by the different architecture of rat, dog, and human
airways. Rats possess one generation of transitional bronchioles instead of few
364 generations of respiratory bronchioles (24) which are found in dogs and humans.
Thus, for rats, the acinus entrance is located at the bronchioalveolar duct junction
366 where the epithelium of the bronchioles (club cells and ciliated cells) blends over into
the alveolar epithelium. The correlating structural in humans is the ventilatory unit
368 and not the acinus (see introduction). Therefore, Boyden and Tompsett (5, 6)
described the alveolarization of the respiratory bronchioles and not a movement of
370 the bronchioalveolar duct junction. If the correlating structure of humans and rat are
correctly compared, no contradiction between the presented data and Boyden and
372 Tompsett observations (5, 6) are present.

374 Our detection method did not only focus on the appearance of alveoli for the
detection of the acini entrances, but also on the airway wall thickness, on the
376 appearance of alveolar capillaries, and on the shape of the airways. Because of that,
we were able to detect the transition between the conducting and the gas-
378 exchanging parts of the transitional bronchiole even before alveoli were present. As a
consequence, the number of acini could be counted prior to the beginning of the

380 alveolarization. We conclude that, for rat lungs, the final number of acini is reached at
the latest by the end of the saccular stage.

382

The localization of the acini entrances represents an important additional observation
384 (Fig. 4, colored spheres). They are inhomogenously distributed over the entire lobe,
in close proximity to the larger airways. As a result a cortical region exists near the
386 pleura which is free of any acinus entrances. Due to the monopodial branching
pattern (46) the most proximal acinus is located at intra-lobar generation four,
388 whereas distal acini are located at much higher generations (Fig. 4). Lee et al. (17,
18) used X-ray tomography of silicon casts of the airways to analyze and count the
390 generations and segments of the conducting airways. We propose that an estimation
of the number of acini, based on their method may be biased due to the
392 inhomogeneous distribution of the entrances of the acini in species possessing a
monopodial branching pattern. To eliminate any dependency of the results on the
394 kind of branching pattern, we counted all acinar entrances in one lobe.

396 We compared the bronchial tree down to the acinar entrances between days 4 and
60 (Fig. 4). The shown bronchial trees were obtained of five different individuals.
398 Therefore, we are comparing different developmental stages and individual animals
at the same moment. We observed a high similarity and no change in complexity
400 between the five analyzed bronchial trees. We conclude that once the bronchial tree
is formed it stays very constant during lung development and that a proportional
402 growth takes place during postnatal rat lung development. In addition, the individual
alterations appear to be small and at a similar level as variations observed in the
404 branching of larger blood vessels. The number and the localization of the secondary
and tertiary lobar airways (43) demonstrated this similarity.

406

Previously, we demonstrated that the right middle lung lobe is a valid estimator for
408 the number of acini for the entire lung (3). However, our method assumes a direct
correspondence between the number of acini per lobe and the fraction of total lung
410 volume of this particular lobe. A variation of the fraction of total lung volume was
observed for the left lung and the cardiac lobe between days 4 and 10 (Tab. 2). To
412 investigate if these variations influenced the estimation method, the number of acini
of three entire lungs were manually counted at day 4 (5865 ± 465 acini) and
414 compared with the results obtained at day 60 (5943 ± 521 acini) (3). No statistical
differences were observed between the counted numbers of acini at these two time
416 points. This demonstrated that at day 4 the acinar development of cardiac lobe is at a
similar state as the other four lobes. The observed variations of the fraction of total
418 lung volume had no influence on the estimation of the total number of acini based on
the right middle lobe. However, due to these variations the number of acini cannot be
420 estimated for the single lobes at postnatal day 4, as proposed in Barré et al. (3). To
overcome this problem cardiac lobe and left lung have to be considered as one entity
422 (Tab. 3) for the calculations. When the cardiac lobe and the left lung are combined as
one entity, the mean acinar volume of the right middle lobe does not statistically differ
424 with the mean acinar volume of any other lobe ($p = 0.198$, Tab. 3). Thus, we
conclude that the right middle lobe is also a valid estimator for the entire lung
426 development.

428 In summary, we conclude that the total number of acini is constant throughout lung
development but can differ between individuals. No relationships were detected with
430 other parameters like total lung volume, surface area, etc. Combining our method
with others (10, 17) it will be possible to further characterize conducting airways and

432 gas-exchanging regions. This will hopefully lead to a better understanding of clinical
relevant topics, for instance air-flow within the lung, pulmonary particle depositions,
434 or lung regeneration.

436

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438

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446 **Author Contributions.** S.F. Barré, obtained and scanned samples, developed the
procedure and performed the counting of the acini, analyzed data and drafted the
448 manuscript. D. Haberthür obtained and scanned samples, contributed to the
development of the procedure of the counting of the acini and to the writing of the
450 manuscript. T.P. Cremona contributed to writing. M. Stampanoni designed and built
the beamline. J.C. Schittny conceived and designed the study, obtained and scanned
452 samples, analyzed data and contributed to writing.

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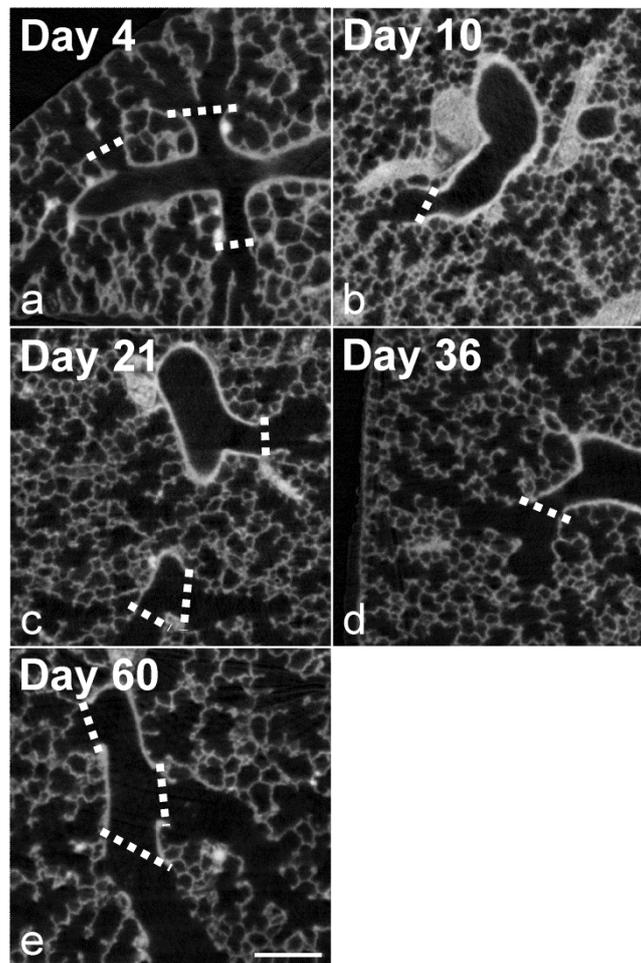
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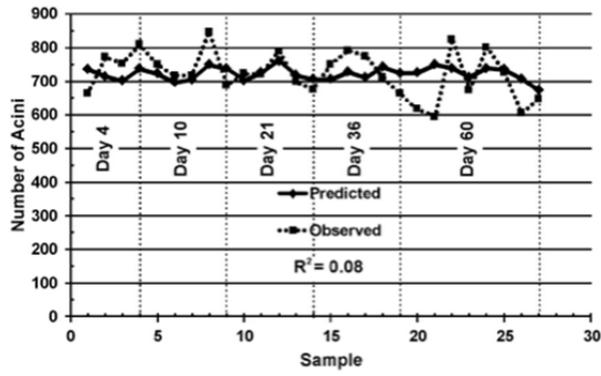
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Figures



586

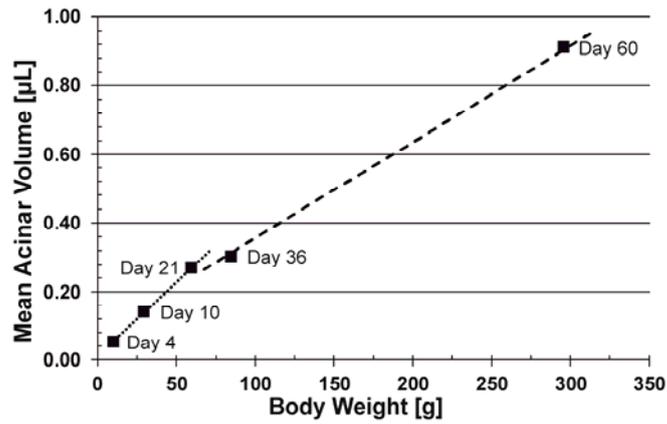
Figure 1. Acini, as they appear throughout lung development. At day 4 (**a**) only
588 sacculi and no alveoli are present. The walls of the conducting airways are smooth
and not much, but significantly thicker than the inter airspace wall of the parenchyma.
590 At day 10 (**b**) alveolarization evidently started; resulting in smaller parenchymal
airspace and a larger difference between the thickness of the walls of the conducting
592 and gas-exchanging airways. At the later days (21 – 60, **c-e**) the difference of the
wall thicknesses are becoming even more pronounced. Dotted lines mark acinar
594 entrances. Scale bar 250 μm .



596

Figure 2. Relationship between number of acini, lung volume, and body weight.

598 No linear correlation was observed between number of acini, body weight, and lung volume. Thus, the number of acini cannot be estimated by these parameters.



600

Figure 3. Bi-phasic growth of the mean acinar airspace volume. The mean

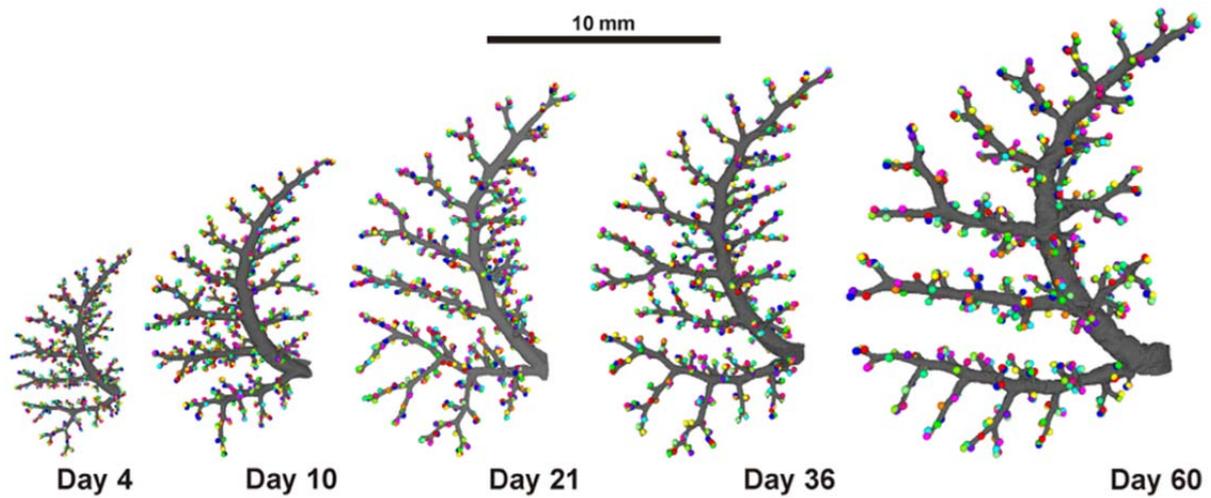
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acinar volume (tissue and airspace) grows in two distinct phases. Days 4-21:

proportional growth of acinar volume and body weight ($R^2 = 0.954$, dotted line). Days

604

36-60: no proportional growth ($R^2 = 0.887$, dashed line).



606 **Figure 4. Trees of conducting airways throughout postnatal lung development.**
 The walls of the conducting airways are shown in grey and the spheres represent the
 608 acini entrances. These three-dimensional visualizations show the large similarity of
 the conducting airways structure at days 4, 10, 21, 36, and 60 and between different
 610 individuals.

Tables

| Volume density of parenchyma | | | | | |
|------------------------------|-------|-------|-------|-------|-------|
| Postnatal day | RUL | RML | RLL | LC | LL |
| 4 | 0.865 | 0.818 | 0.856 | 0.827 | 0.865 |
| 10 | | 0.846 | | | |
| 21 | | 0.869 | | | |
| 36 | | 0.870 | | | |
| 60 | 0.888 | 0.839 | 0.843 | 0.865 | 0.862 |

612

Table 1. Volume density of the parenchyma. No significant differences were

614 observed. RUL: right upper lobe; RML: right middle lobe; RLL: right lower lobe; LC:
cardiac lobe; LL: left lung.

| Postnatal Day | Fraction of Total Lung Volume [%] | | | | | | | | | |
|-----------------------------|-----------------------------------|------|-------|------|-------|------|--------|------|--------|------|
| | RUL | | RML | | RLL | | LC | | LL | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| 4 | 11.15 | 0.81 | 12.37 | 0.88 | 29.68 | 2.50 | 5.86* | 0.80 | 40.94* | 2.35 |
| 10 | 11.75 | 1.27 | 13.08 | 1.49 | 30.72 | 2.89 | 11.87 | 1.59 | 32.59 | 5.80 |
| 21 | 11.04 | 0.75 | 12.68 | 0.51 | 29.69 | 0.72 | 10.69 | 0.28 | 35.90 | 0.99 |
| 36 | 10.74 | 0.70 | 12.37 | 0.60 | 30.40 | 0.54 | 10.87 | 0.66 | 35.62 | 0.92 |
| 60 | 10.89 | 0.83 | 12.49 | 1.14 | 28.49 | 1.17 | 12.69* | 0.70 | 35.44 | 1.04 |
| Mean of all days | 11.09 | 0.89 | 12.59 | 0.97 | 29.66 | 1.80 | - | - | - | - |

616

Table 2. Fraction of the lung lobes of the total lung volume throughout lung

618 **development.** Except of the cardiac lobe and the left lung the lobe volume increases
in parallel to the growth of the total lung volume. At the cost of the left lung, the
620 cardiac lobe increases disproportionately. RUL: right upper lobe; RML: right middle
lobe; RLL: right lower lobe; LC: cardiac lobe; LL: left lung; SD: standard deviation; *:
622 differs significantly (ANOVA) from the other time points for the same lobe.

| | Mean acinar volume | | | Manual counting | |
|--------------------|--------------------|-------|---------|-------------------|-----|
| | Mean [μ L] | STD | t-test | Mean [# Acini] | STD |
| RUL | 0.065 | 0.014 | 0.556 | 766 | 70 |
| RML | 0.066 | 0.008 | | 777 | 29 |
| RLL | 0.069 | 0.014 | 0.917 | 1841 | 175 |
| LC | 0.030 | 0.003 | 0.027 * | 769 | 24 |
| LL | 0.105 | 0.017 | 0.001 * | 1711 | 214 |
| LC + LL | 0.081 | 0.011 | 0.198 | 2481 | 210 |
| Entire Lung | 0.073 | 0.011 | 0.674 | 5865 | 465 |

624

Table 3. Comparison of the mean acinar volume and the number of acini per

626 **lobe at postnatal day 4.** To validate the right middle lobe as an estimator for the entire lung, three entire lungs were manually counted. We observed that the right
628 upper, middle, and low lobe, but not the cardiac lobe and the left lung represent a valid sample for the entire lung. RUL: right upper lobe; RML: right middle lobe; RLL: right lower lobe; LC: cardiac lobe, LL: left lung, SD: standard deviation, *: significantly
630 differs (ANOVA) from the other time points for the same lobe.

| Postnatal Day | Number of Samples | Body Weight | | Lung Volume | | Number of Acini (RML) | |
|-------------------------|-------------------|-------------|-------|-------------|-------|-----------------------|----|
| | | [g] | | [ml] | | [# Acini] | |
| | | Mean | SD | Mean | SD | Mean | SD |
| 4 | 4 | 10.15 | 0.54 | 0.506 | 0.030 | 749 ⁺ | 62 |
| 10 | 5 | 29.42 | 3.22 | 1.32 | 0.102 | 743 ⁺ | 61 |
| 21 | 5 | 59.65 | 2.05 | 2.32 | 0.136 | 721 ⁺ | 42 |
| 36 | 5 | 84.75 | 9.65 | 2.80 | 0.271 | 738 ⁺ | 52 |
| 60 | 8 | 295.82 | 16.77 | 7.51 | 0.544 | 686 ⁺ | 89 |
| Mean of all days | 27 | | | | | 722 | 67 |

632

Table 4. Comparison of the body weight, lung volume and number of acini.

634 RML: right middle lobe, SD: standard deviation, ⁺: no significant difference.

| Postnatal Day | Mean Acinar Volume | | Specific Mean Acinar Volume | |
|---------------|--------------------|-------|-----------------------------|-------|
| | [μl] | | [μl / 100 g] | |
| | Mean | SD | Mean | SD |
| 4 | 0.069* | 0.007 | 0.678 | 0.075 |
| 10 | 0.196* | 0.017 | 0.677 | 0.115 |
| 21 | 0.355 [†] | 0.027 | 0.596 | 0.055 |
| 36 | 0.410 [†] | 0.050 | 0.484* | 0.019 |
| 60 | 1.157* | 0.138 | 0.393* | 0.064 |

636 **Table 5 Mean acinar volume.** Parenchymal lobe volume divided by the counted
number of acini, Specific mean acinar volume: mean acinar volume per 100 g body
638 weight, *: differs significantly (ANOVA) from the other time points, [†]: the mean acinar
volume of days 21 and 36 are not significantly different from each other, however
640 they are both significantly different from the three other time points.