Estimation of the number of alveolar capillaries by the Euler number (Euler-Poincaré characteristic)

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The lung parenchyma provides a maximal surface area of blood-containing capillaries that are in close contact with a large surface area of the air-containing alveoli. Volume and surface area of capillaries are the classic stereological parameters to characterize the alveolar capillary network (ACN) and have provided essential structure-function information of the lung. When loss (rarefaction) or gain (angiogenesis) of capillaries occurs, these parameters may not be sufficient to provide mechanistic insight. Therefore, it would be desirable to estimate the number of capillaries, as it contains more distinct and mechanistically oriented information. Here, we present a new stereological method to estimate the number of capillary loops in the ACN. One advantage of this method is that it is independent of the shape, size, or distribution of the capillaries. We used consecutive, 1 μm-thick sections from epoxy resin-embedded material as a physical disector. The Euler-Poincaré characteristic of capillary networks can be estimated by counting the easily recognizable topological constellations of “islands,” “bridges,” and “holes.” The total number of capillary loops in the ACN can then be calculated from the Euler-Poincaré characteristic. With the use of the established estimator of alveolar number, it is possible to obtain the mean number of capillary loops per alveolus. In conclusion, estimation of alveolar capillaries by design-based stereology is an efficient and unbiased method to characterize the ACN and may be particularly useful for studies on emphysema, pulmonary hypertension, or lung development.

capillary number; stereology; Euler number

QUANTITATIVE INFORMATION ABOUT the alveolar capillary network (ACN) using stereology has a long and successful tradition (43, 44). Volume of the capillary lumen and surface area of the luminal endothelium are functionally important parameters that are closely related to the blood volume inside of the capillaries and the diffusion area for respiratory gases, respectively (7, 18). In combination with information on the alveolar epithelium, these parameters have greatly enhanced our understanding of pulmonary physiology (6).

However, under conditions of rarefaction or angiogenesis of capillaries within the ACN, volume and surface area may not always be the most suitable parameters. They do provide an answer to the questions of how large the loss of functionally active diffusion area or blood volume is, but they fall short in answering the question of how this loss occurs (12, 46). The latter could be better assessed by estimation of the length and number of alveolar capillaries. However, as was shown a few years ago, the three-dimensional (3D) characteristics of the ACN impede the estimation of capillary length by design-unbiased stereology (25); due to the sheet flow concept (4), which guarantees a large contact area between endothelial and epithelial surface, the capillary segments of the ACN nearly have a diameter that equals their length (45). Thus the generally accepted rule of thumb for stereological length estimations of an approximate 1:10 ratio between diameter and length is not fulfilled (25). In other words, for a network composed as a sheet with interposed pillars of connective tissue, length is not an appropriate characterization of the composing elements of the network. The number of capillaries or capillary segments, however, would help to characterize the ACN and provide useful information to understand how an increase or decrease of alveolar surface area is achieved, namely, by alteration of the characteristics of the existing network or by modulation of the amount of components used to build up the network. Particularly, research questions related to chronic obstructive pulmonary disease and emphysema development (42) or pulmonary hypertension (41) could greatly benefit from a new, quantitative approach to study the microvessels of the lung (22, 23, 30).

As was shown for other capillary (e.g., in the kidney or the heart) or biological networks (e.g., trabecular bone, mitochondria), the Euler number or Euler-Poincaré characteristic, as a measure of the connectivity of the connected parts of the network, gives information on the number of objects needed to build up the network (3, 9, 16, 21, 26, 27, 40). Therefore, the present study established the concept of the Euler-Poincaré characteristic for estimation of the number of capillary loops of the ACN. For this purpose, design-based estimation of the number of capillary loops was performed on samples from the gas-exchange region of postnatal rat lungs, aged 6 or 42 days. Additionally, we validated the stereological estimation with data from a 3D reconstructed data set of the alveolar region.

MATERIALS AND METHODS

Animals and tissue preparation. The samples used for the present study were taken from rats that were part of previous studies (35, 39). The experiments were approved by the local government authorities...
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and comply with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85, reprint 2002) and with the current laws.

Two groups of rats, aged 6 (n = 6) or 42 (n = 5) days after birth, were used for the present study. The rats were euthanized under anesthesia, as described in the original study (35). Lung fixation was performed by vascular perfusion of a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.2 M HEPES buffer at a perfusion pressure of 20 cm H2O and a positive end-expiratory pressure of 3 cm H2O.

After excision, volumes of left and right lung [V(lung)] were determined separately by fluid displacement, according to Archimedes’ principle (33, 36), before embedding in 2% aqueous agar-agar (sampling method; see below). Following a standard procedure (24), samples were subsequently postfixed in osmium tetroxide (1%), incubated in half-saturated uranyl acetate overnight, and dehydrated in an ascending acetone series (70, 90, and 100%). Finally, tissue blocks were embedded in epoxy resin.

Randomness of location and orientation. Representative specimens will be guaranteed if an appropriate sampling scheme, such as systematic uniform random sampling (SURS), is carried out. SURS starts with the selection of the slices and ends with the selection of the field of view. Each part of the organ gets an equal chance of being selected, which makes it uniform in randomness of location. Every level of sampling begins at a randomly chosen point, but to be systematic, each step within the sampling level follows a preassigned pattern and thereby, determines the location of all following items (10, 20). Therefore, lungs were cut in slices from apex to base with a thickness of 3 mm (sampling thickness) at a random position between 0 and 3 mm. The slices were numbered, and every second slice was collected, starting with a randomly determined number between one and two. An area grid was then mounted on the chosen slices, and at each grid area hitting lung tissue, a tissue block with a size of ~1–2 mm3 was taken.

The estimation of surface area and length using thin sections requires the use of isotropic uniform random (IUR) sections. Depending on the size of the tissues, IUR sections can either be generated with the orientator (19) or the isector (28). However, as global isotropy is widely accepted in the parenchyma of mammalian lungs (37) and stained with toluidine blue for 1 min at 60°C.

The investigation was accomplished using a light microscope DM6000B (Leica, Wetzlar, Germany) equipped with a digital camera DP72 (Olympus, Hamburg, Germany), a slide scanner Axio Scan.Z1 (Zeiss, Göttingen, Germany), and a computer with newCAST stereology software (Visiopharm, Horsholm, Denmark).

One important feature of the disector principle is that no counting events are fully contained in the disector volume or lost between the two sections. We performed a pilot study on consecutive, 100 nm-thick ultrathin sections by transmission electron microscopy to analyze if there are any topological events within 1 μm in z direction. This was not the case, which is why we considered the disector height of 1 μm as reasonable (Fig. 1).

Estimation of classical stereological parameters. The volume fraction of parenchymal tissue (defined as the gas-exchange region) was estimated at an objective lens magnification of 10×, with a test grid consisting of 4×4 points. The number of points hitting parenchyma [P(par)] and nonparenchymal structures [P(np)] was counted and used to calculate the total volume of parenchyma of the lung [V(par,lung)]

\[
V_{\text{par, lung}} = \frac{P_{\text{par}}}{P_{\text{par}} + P_{\text{np}}} \times V_{\text{lung}}
\]

The surface area of the capillary endothelium was estimated at a magnification of 40× by the intersection counting method (13). A test system consisting of a single test line with a length \( l \) of 93.4 μm was mounted on each field of view. The number of intersections \( I \) of the test line with capillary luminal endothelium was counted, as well as the number of line points \( N_{\text{lp}} \); the left and right end of each line, with a length per point \( l_{\text{lp}} \) of \( l/2 = 46.7 \) μm hitting parenchyma. From these counts, the surface density of capillary endothelium \( S_{\text{v, (cap/par)}} \) was calculated

\[
S_{\text{v, (cap/par)}} = \frac{2 \times I}{l_{\text{lp}} \times N_{\text{lp}}}
\]

The total surface area can be calculated by \( S_{\text{cap,par}} = S_{\text{v, (cap/par)}} \times V_{\text{par, lung}} \).

Fig. 1. Serial sections of lung parenchyma. This figure shows transmission electron micrographs of a series of ultrathin sections (100 nm thickness). It demonstrates that no counting events are lost when the disector height is 1 μm.
Furthermore, at a magnification of 40×, the volumes of various components of the gas-exchange region (alveolar duct airspace, alveolar airspace, septal capillaries, and septum, in general) were estimated using the point counting method with a point grid consisting of 32 test points, as described above. The number of alveoli was estimated using the physical disector and the Euler-Poincaré characteristic, as described previously (14, 31). At birth, the rat lung is still in the saccular phase of development (1), but at 6 days, alveoli are present and can be counted. The volume of alveolar airspace was divided by the number of alveoli to obtain an estimate of the mean volume of alveoli.

Estimation of the number of capillaries. The 3D probe of the disector allows the sampling of capillaries proportional to their number without making assumptions on size or shape. The Euler-Poincaré characteristic/Euler number is a rather universal tool to determine the zero-dimensional parameter number of the components of a complex network, taking into consideration the connectivity of the network. Practical examples are illustrated by glomerular capillaries (27, 29) and bone trabeculae (9). As described earlier (26), one can find three different topological phenomena when viewing capillary profiles in a physical disector. Generally, objects can occur as new, isolated parts [island (I)] and as enclosed cavities within an existing profile [hole (H)], and in the majority of cases in this study, they can form extra connections between two isolated structures [bridge (B)].

At an objective lens magnification of 40×, two closely spaced, parallel sections with a physical disector height of 1 μm were aligned, where one section was preassigned for counting corners of the counting frame \((A = 3.920 \mu \text{m}^2)\) hitting parenchyma to estimate the reference volume. The unbiased counting frame consists of an exclusion line and an inclusion line. Structures are only counted if they lie completely or partly within the frame and do not touch the exclusion line or its extensions (8). Figure 2 illustrates the possible appearance of capillaries within the disector. In the first scenario, two capillary profiles can be found in the alveolar septum in the sampling section. They can either continue to occur as separate profiles (no counting event), or they can become connected and appear as one capillary profile with a single lumen \((B = 1 \text{ counting event})\) in the look-up section. Furthermore, capillaries can be present in the parenchyma in one section and be absent in the other \((I = 1 \text{ counting event})\). Theoretically, the counting event “hole” is also possible but was not noticed throughout the counting procedure of this study.

From the raw counts, the Euler number of capillaries \([\chi(\text{cap})]\) was determined by

\[
\chi(\text{cap}) = \sum (\text{islands}) - \sum (\text{bridges}) + \sum (\text{holes})
\]

This stereological formula originates from the general \(\chi(\text{cap}) = c - h + v\) (where \(c\) is the number of connected components; \(h\) is the number of handles/loops; and \(v\) is the number of voids). For the ACN, it is known that the whole ACN is one single connected component and cannot contain any voids if regarded as (blood) filled. Therefore \(\chi = 1 - h\), thus deducing the number of capillary loops as \(h = 1 - \chi\).

Because of the absence of the counting event hole, the equation was simplified by setting \(\sum (\text{holes}) = 0\)

\[
\chi(\text{cap}) = \sum (\text{islands}) - \sum (\text{bridges})
\]

The numerical density \([N_v(\text{cap/lung})]\) was then calculated using \(\chi(\text{cap})\), as well as the total volume of the disector \([v(\text{dis})] = 2 \times (P(\text{ref})/4) \times A(\text{frame})\). The counting of the disector in both directions, i.e., first using one section as the look-up section, the other one as the sampling section, and then vice versa, achieves greater efficiency (16), and thus it is reflected by the factor of two in the denominator. Moreover, all corners of the counting frame were included, so the sum of reference points \([P(\text{ref})]\) had to be divided by four.

Fig. 2. Illustration of the observed counting events. One field of view in a disector; i.e., the left and right images are taken from the 2 consecutive semithin sections. A: the topological event of a bridge is visible. The arrows highlight the capillaries that are seen as individual capillary profiles in the left section and are connected in the right section. B: the topological event of an island can be seen. The arrow points at a capillary profile that is present in the right section but not in the left section.
The total number of capillaries \([N_{\text{cap/lung}}]\) is gained by multiplying the numerical density by \(V_{\text{lung}}\).

**Digital analysis.** To verify the stereological \(\chi\) estimation of the ACN, a 3D dataset was created from a consecutive series of \(\sim 300\) semithin sections of an alveolar cluster and its ACN. The scanned sections were stacked and aligned with Fiji (34). The 3D dataset was processed further with Insight Segmentation and Registration Toolkit (ITK) filters (15). Further processing methods, such as watershed segmentation, were used to create a segmentation of the blood vessels with a voxel size of \(\sim 0.33\) \(\mu\)m. This segmentation was manually checked and corrected where necessary with ITK-SNAP (47) and finally filtered, such that the segmentation only consisted of voxels that have at least 50\% (13 of 26) of their neighboring voxels belonging to the segmentation. This prevents most topological details below those of the ACN and insusceptible to visual inspection, i.e., during stereological counting. A stronger filter condition would be the enforcement of a minimum inscribed sphere radius inside of the vessels and/or a minimum pillar “width.”

The \(\chi\) of this segmentation was then calculated with octave (2) using the implementation of Legland et al. (17). The use of a 3D binary thinning algorithm that preserves topology, such as implemented for ITK by Homann (11), allows the reduction of the segmentation to its centerlines, which represent a simplified version of the ACN with the same \(\chi\). The removal of trailing branches that originate from the connection of the ACN to the adjacent alveoli (and from perturbations) does not change the \(\chi\) either. The transformation of the voxel representation of this centerline graph of the ACN into a vector representation made up of only vertices (\(V\)) and connecting lines (\(L\)) allows rechecking of the \(\chi\) with the general formula for graphs: \(\chi = #V - #L\). Additionally, this representation allows a unique definition of capillary segments, which are the connections between branching points. A loop is defined as the shortest cycle around a pillar and as can be seen in Fig. 3, can have a varying amount of branching points.

**Statistical analysis.** Differences between 6- and 42-day-old rats were analyzed by a nonparametric Mann-Whitney \(U\)-test. When \(P < 0.05\), a significant difference between the differently aged animals was
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Table 1. Summary of stereological data

<table>
<thead>
<tr>
<th></th>
<th>6 Days</th>
<th>42 Days</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>V(par,lung), ml</td>
<td>0.71 (0.18)</td>
<td>6.8 (4.8)</td>
<td>0.0043</td>
</tr>
<tr>
<td>V(ductair,lung), ml</td>
<td>0.136 (0.047)</td>
<td>1.91 (1.7)</td>
<td>0.0043</td>
</tr>
<tr>
<td>V(alvair,lung), ml</td>
<td>0.339 (0.096)</td>
<td>3.91 (2.45)</td>
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</tr>
<tr>
<td>V(sept,lung), ml</td>
<td>0.148 (0.065)</td>
<td>0.715 (0.224)</td>
<td>0.0043</td>
</tr>
<tr>
<td>V(cap,lung), ml</td>
<td>0.094 (0.042)</td>
<td>0.585 (0.534)</td>
<td>0.0173</td>
</tr>
<tr>
<td>S(cap,lung), cm²</td>
<td>303 (98.4)</td>
<td>2091 (1656)</td>
<td>0.0043</td>
</tr>
<tr>
<td>N(alv,lung), × 10⁶</td>
<td>2.77 (1.35)</td>
<td>35.8 (43.4)</td>
<td>0.0173</td>
</tr>
<tr>
<td>V(alvair,lung)/N(alv,lung), µm³ × 10³</td>
<td>143 (68)</td>
<td>254 (120)</td>
<td>0.7922</td>
</tr>
<tr>
<td>Nv(cap,lung), µm³/10⁹</td>
<td>1.43 (0.44)</td>
<td>1.89 (0.52)</td>
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</tr>
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<td>N(cap,lung), × 10⁸</td>
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Data are given as mean (SD). V(par,lung), volume of lung parenchyma; V(ductair,lung), volume of alveolar duct air; V(alvair,lung), volume of alveoli; V(sept,lung), volume of septal tissue in the parenchyma; V(cap,lung), volume of septal capillaries; S(cap,lung), surface area of septal capillaries; N(alv,lung), number of alveoli; Nv(cap,lung), numerical density of septal capillary loops; N(cap,lung), number of septal capillary loops.

Table 1 summarizes the means of the stereological parameters obtained in this study. Figures 4 and 5 demonstrate the data of the individual animals for various stereological parameters. As expected, all global parameters (volume, surface area, and number) increased from the early postnatal to the young adult rats. However, differences were noted in the degree of changes. Whereas volume and surface area increased by a factor of 6.2 and 6.9, respectively, the number of capillary loops was 9.1 times higher in the young adult than in the 6-day-old rats. The number of alveoli increased by a factor of 12.9, thus leading to a similar number of capillary loops per alveolus in both age groups. The mean volume of alveoli calculated from volume and number was not significantly higher in the 42-day-old than in the 6-day-old rats.

All parameters were estimated from digital slides generated by a slide scanner. This procedure increased the efficiency compared with the analysis directly at the microscope. For an experienced observer, it is possible to generate the data of all parameters of this study within ~8 h per animal. For the disector analysis of the capillaries, 100–200 events were counted from 4 independent tissue blocks, which took ~2.5 h per animal. As shown in Table 2, this procedure was sufficient to guarantee that the variation between the study subjects was not dominated by the stereological method. It should be noted that the high variation in the adult age group is caused by a high degree of variation in the lung volume. For comparison, the numerical density of capillaries shows a much lesser variation.

With respect to the digital analysis that was used to validate the stereological approach, the χ calculated from the segmentation, as mentioned above, gave: χ = 232; i.e., the ACN of this alveolar cluster consists of ~233 capillary loops, i.e., has 233 pillars. The stereological estimation of the χ yielded: χ = 219. Figure 3 shows three cross-sections through the dataset overlayed with its segmentation, a 3D rendering of the segmentation of the ACN.

The centerline network of the ACN shown in Fig. 3 has 440 branching points and 672 capillary segments; according to the general formula for graphs (see above), χ = 440 − 672 = 232. The number of branches per branching point (degree of the branching point) strongly depends on the resolution below which branching points are regarded as merged, i.e., not separable. For example, at the given voxel size, there is one branching point of degree 4 visible in Fig. 3E. Under the assumption that the position of the branching points is even less well resolved, other pairs of branching points will merge.

Of note, the digital analysis of the ACN from one region of one lung required several months of work. This approach can therefore not be considered as an alternative to the stereological estimation.

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Fig. 4. Stereological estimates of capillary volume (A), surface area (B), and number (C). Each data point represents 1 animal; each horizontal bar is group mean. *Statistically significant difference.
false loops, as can other segmentation errors. It needs to be emphasized that a distinction must be made among the terms capillary segment, capillary loop, and capillary, in general. A capillary as an anatomical entity is defined by its wall composition, which is made of endothelium, basal lamina, and a few pericytes (5); thus it does not have a defined beginning or an end. Imagine a capillary coming from an arteriole and then branching into a number of vessels that flow together before opening out into the venous system. Are the branches of the capillary then separate capillaries or capillaries of different order? One way is to define each part of a capillary bed that is confined by two branching points as a capillary segment (see Fig. 3). The number of capillary segments in the human ACN was estimated to be $\sim 2.77 \times 10^7$, based on a hexagonal model (45). Whereas this number appears to be accurate, it relies on the correctness of the model assumption, which may be true for a healthy human lung but may be different in other species or altered by diseases in a nonpredictable fashion. The design-unbiased approach presented in this study does not rely on a model assumption, which makes it suitable for healthy as well as pathologically altered lungs. Nevertheless, its “readout” parameter, the number of capillary loops, cannot be understood as intuitively as the number of segments. As outlined in Fig. 3, the number of loops and segments is not the same in a network and depends on the number of branches that leave a branching point. Therefore, there is no fixed relationship between the number of capillary loops and segments, and the number of segments cannot be deduced from the number of loops without a generalized model assumption. However, as explained by the definition of a capillary loop (Fig. 3), the number of loops equals the number of connective tissue pillars. Furthermore, the present investigation of postnatal rats has highlighted that in particular, when used together with other parameters, such as alveolar number, the number of capillary loops provides additional and important information that conveys mechanistic insight into the processes that lead to an enlargement (or rarefaction) of a capillary bed.

**DISCUSSION**

The present study demonstrates a new design-unbiased and efficient approach to characterize the ACN quantitatively by the use of the Euler number, which provides a measure of the connectivity and number of the structures that form a network (9). As a result, this estimator delivers the number of capillary loops in the ACN, a parameter that has been used successfully for other microcircuits, such as myocardial capillaries (21, 40) or kidney glomeruli (26, 27). The ACN, however, differs strongly from other capillary beds, such as the myocardial or (to a lesser extent) the glomerular capillary bed in that the ACN rather consists of a sheet of blood that is interrupted by pillars of connective tissue. This network can be described by a model of hexagonally arranged capillary segments, whose length is nearly the same as their diameter (45). Although the estimation of the Euler-Poincaré characteristic is mathematically based on solid grounds, we validated its applicability to the ACN by segmenting and reconstructing the ACN of two adjacent alveoli and determined the Euler number both digitally (reference number) and by the disector method. Both ways of estimation yielded a similar result for the Euler number, thus validating the counting procedure. However, even though the calculation of $\chi$ from a 3D segmentation (or the vector representation of its centerline graph) is accurate, uncertainties enter from the (in-)correctness of the segmentation of the blood vessels at the scale of the voxel size. Besides that, remaining erythrocytes that fully block a capillary segment are difficult to identify for the software and sometimes even for humans. This may lead to missing connections within the ACN segment and therefore, an underestimation of the number of capillary loops. On the other hand, erythrocytes that do not fully block a vessel can create

**Table 2. Estimates of coefficients of variation for the number of capillaries**

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<td>0.255</td>
<td>0.831</td>
</tr>
<tr>
<td>CE(met)</td>
<td>0.142</td>
<td>0.180</td>
</tr>
<tr>
<td>CV(bio)</td>
<td>0.212</td>
<td>0.811</td>
</tr>
</tbody>
</table>

CV(obs), coefficient of observed variation; CE(met), coefficient of error of the method; CV(bio), coefficient of biological variation.
may be necessary to switch to a different embedding medium that allows both the preparation of thin sections (maximum 1 μm) and the application of the staining method. Second, the estimation of the Euler number requires the use of the disector technique (37). In general, it is recommended that the disector height is ~30–40% of the projected height of the object to be counted. This recommendation fulfills two criteria: that no counting event is completely enclosed between the two sections and that it is efficient (i.e., that it creates a reasonable number of counting events in relation to the amount of working time). For isolated particles, such as nuclei, it is possible to perform a few measurements on the diameter of the particle and then determine the optimal disector height. In the present case, we rather focused on the aspect that no counting event escapes the observer by being entirely lost between the sections. Therefore, we generated serial ultrathin sections and visualized them in the electron microscope (Fig. 1). As such, we think that a disector height of 1 μm is safe for not losing a significant amount of topological events in between. However, when the diameter of the capillaries becomes smaller, either physiologically or as an artefact of the fixation procedure, this may not be so. In such cases, it is better to use a smaller disector height, such as 0.5 μm.

The estimation of the number of capillary loops in the ACN is more time consuming than the traditional parameters, volume and surface area, which will remain the physiologically most relevant ones as they define the blood volume and—together with alveolar epithelium—the diffusion area for gases in the alveolar region, respectively. As outlined a few years ago, the length of the capillaries is not an unbiased estimator of the ACN, because the 3D composition of the ACN does not fulfill the requirements of the stereological counting rule. In other words, capillary length is not an inherent characteristic of the ACN (20). However, the volume and the surface area of the capillaries are greatly influenced by the perfusion or inflation pressures so that changes in the vascular and airway mechan-isms, for instance, between different experimental groups, may affect the estimates substantially. The number of alveolar capillary loops is not affected by these variables, as long as the counting events are clearly identifiable (see above). Volume and surface area of the capillaries may also be influenced by changes in the diameter of the vessels (due to experimental setting or developmental state) but also by the resolution during analysis (32). In such cases, these parameters do not provide a clear picture of the ongoing structural process. Therefore, our new approach offers useful and mechanistic information on structural processes happening in the ACN that cannot be achieved by other morphometric approaches. Importantly, the number of capillary loops is not thought or intended to be a more sensitive parameter than volume and surface area under pathological conditions. The data presented in this study show that the new parameter contains an equally high degree of variation as volume and surface area. One example is the question of whether hypoxic conditions cause angiogenesis in the lung (as in the systemic circulation). The existing controversy about this issue could be solved by the Euler-Poincaré characteristic, as number is the only parameter that is able to prove angiogenesis. On the other hand, there is evidence that during the development of emphysema, a loss of capillaries in the septa precedes the loss of elastic properties and of alveolar septa. This so-called “vascular hypothesis” could well be addressed by investigating whether the number of capillary loops per alveolus truly is decreased during emphysema development. Therefore, we believe that this method will be a useful tool for studies on lung development, emphysema, pulmonary hypertension, and many other areas of pulmonary research.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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