Rat lungs show a biphasic formation of new alveoli during postnatal development

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Submitted 16 December 2013; accepted in final form 17 April 2014

The structural properties of the lung are well suited for its function as gas exchanger. For optimal diffusion, a maximized surface area of the interface and a minimized barrier thickness between the two phases—air and blood—are needed and achieved by an ingenious and delicate architecture of parenchymal lung tissue. The key functional elements of the lung are the alveoli and their delimiting interalveolar septa. The number and geometry of alveoli essentially influence the amount of available gas-exchange surface capillaries, thus affecting the diffusion capacity of the lung.

Stages of lung development. The developing lung of mammals undergoes well-defined stages that chronologically describe the dramatic changes within lung tissue from its embryonic appearance until the organ reaches its maturity (Fig. 1). We distinguish pre- and postnatal stages, where every stage is named according to characteristic morphological features. The consecutive developmental stages during fetal lung development are the pseudoglandular, canalicular, and saccular stage. During the next stage, the stage of alveolarization, the gas-exchange surface increases dramatically due to the subdivision of the existing airspaces (sacculi) by the formation of new inter-airspace walls resulting in the appearance of the alveoli.

Depending on species, alveolarization can be pre- or postnatal: in precocial animals like guinea pigs (36) and sheep (1), alveolar formation starts well before birth, and at term their lungs appear almost mature. In altricial species, alveolar formation is rather a postnatal event, like in rats (4, 6), mice (2), and humans (46, 47). Finally, during the stage of microvascular maturation the double-layered capillary network of the inter-airspace walls is transformed to a single-layered capillary network by a combination of capillary fusion and preferential growth [rats, postnatal days 14–21; humans, term to 2–3 yr postnatal (4, 30, 32, 46)].

Start of alveolarization. At birth, the gas-exchange units in the rat lung consist exclusively of sacculi, which are delimited by rather thick but smooth inter-“saccular” walls, the “primary septa.” A few days after birth, numerous small ridges arise from the saccular wall, the so-called “secondary septa,” which grow in a centripetal manner into the sacculi in order to subdivide them into alveoli (4). At this stage, both primary and secondary septa show a double capillary layer and are termed “primitive” or “immature” septa, as opposed to the mature ones in adult lungs. The human lung shows already at birth such secondary septa, but the main phase of septation happens clearly postnatally (46). Shortly after birth (day 4) the morphology of ongoing septation does not allow to distinguish between sacculi and alveoli.

End of alveolarization. Burri et al. (4, 6) postulated that the formation of secondary septa requires a double-layered capillary network in the preexisting septum from which the new septum is lifted off. Each side has its “private” capillary bed. This structural condition allows an upfolding of the capillary network of one septal side, still ensuring blood supply of the other side. Furthermore, after the maturation of the alveolar microvasculature from a double-layered to a single-layered capillary network, the formation of new alveoli was not detected anymore in developmental studies performed by morphological observation of lung sections. Consequently, it was assumed that after microvascular maturation has completed the formation of new alveolar septa/new alveoli is discontinued. Until recently, it was not possible to verify or falsify this latter hypothesis, because the required imaging tools [synchrotron radiation-based X-ray tomographic microscopy (33)] and the required quantitative methods [the estimation of the number of alveoli (20, 26) or the estimation of the length of the free septal
edge (33)) were not available. As soon as these tools had been developed it turned out that new alveoli are formed until young adulthood, as shown by now for Rhesus monkeys, rats, mice, and humans (10, 19, 23, 25, 33). Using high-resolution synchrotron radiation-based X-ray tomographic microscopy we (33) showed recently that for alveolarization the postulated requirement of a double-layered capillary network is still valid and that, if necessary, the required capillaries may be newly formed locally by angiogenesis. It is well accepted that the formation of new alveolar septa continues at least until young adulthood.

**Aim of study.** In a previous study we (33) quantitatively investigated postnatal lung development of the rats, focusing on the formation of alveolar septa. Alveolarization was assessed by tracking septation in terms of lengthening of the free septal edges, which represents the formation of new alveolar walls.

The aim of this study was to directly elucidate the time course of alveolarization by an unbiased estimation of the alveolar number during postnatal rat lung development and to compare it to our previously obtained rat data. In contrast to the developmental increase of the length of the free septal edge (33), we observed a much more pronounced biphasic formation of new alveoli.

**MATERIALS AND METHODS**

**Animals and lung tissue preparation.** Male Sprague-Dawley rats were lactated until weaning at about day 21 and afterwards fed with standard rat pellets and water ad libitum. The animal experiments were approved by the Swiss Federal Act on Animal Protection and the Swiss Animal Protection Ordinance.

At each of postnatal days 4, 10, 21, 36, and 60, five animals were taken for lung fixation. After deep anesthesia a pneumothorax was performed and lungs immediately fixed in situ by intratracheal instillation of 2.5% glutaraldehyde in potassium phosphate-buffered solution at a constant pressure of 20 cmH₂O column (42). The pressure in the lungs was maintained for at least 24 h. Thereafter the volumes of the separated lung lobes were assessed by water displacement (31) and the total lung volume calculated. Shrinkage due to embedding was calculated by relating the volume before embedding (water displacement) to the volume after embedding. The latter was based on the Cavalieri principle (13) on paraffin sections. Shrinkage was on average 63.2% (SD 4.7%) without significant deviation between the days. This resulted in a mean volume after shrinkage of 36.8% (shrinkage factor) of the original volume.

The animals used were part of our studies about the pulmonary effects of a neonatal high-dose short-term glucocorticoid treatment of rats (11, 22, 29, 30, 34, 42). In addition, the controls of this experiment were used in other studies analyzing lung development (14–16, 27, 33, 43).

**Section sampling.** The right middle lobe of each animal was embedded in paraflin. Earlier studies have indicated that in rats one entire lobe represents a valid sample for the whole lung (45). With respect to alveolar structures lung parenchyma can be considered as isotropic (18); therefore isotropic uniform random sections were cut perpendicular to the longitudinal axis of the lobe without special attention to orientation. Sections were cut at a thickness of 4 μm, stained with fuchsin, and numbered. Approximately 10 section pairs equidistantly spaced over the whole lobe were used for stereology. Image pairs were recorded and analyzed using the newCAST software (Visiopharm, Hørsholm, Denmark) and a 20× lens on an Olympus AH-2 light microscope (Olympus Schweiz AG, Volketswil, Switzerland).

**Alveolar number.** Alveolar number was assessed in the lung parenchyma by counting the rings shaping the alveolar openings (alveolar entrance rings) (20, 26). These rings are part of a three-dimensional network. According to its Euler characteristics, every additional connection within the network represents an additional ring and thus one alveolus. The unbiased counting of connections (“bridges”) in space from sections requires a three-dimensional sample. This was achieved by using the disector principle based on pairs of sections (37). “New” appearing septal bridges from an image on one section to the corresponding image on the consecutive section were counted, and this resulted directly in the number of alveoli per probe volume. In order to get the absolute number of alveoli the alveolar density was multiplied by the reference volume, that is, the parenchymal density (entire lung minus pleural tissue and vessels/bronchi larger than 25 μm) times the lung volume (shrinkage corrected), according to the following formula:

\[ N_{\text{alv}} = \left( \sum \text{bridges} / V_{\text{disector}} \right) \times V_{\text{parenchyma}} \times V_{\text{lung}} \times (\text{shrinkage factor}) \]

where \( N_{\text{alv}} \) is the number of alveoli, \( V_{\text{disector}} \) is the total volume of all dissectors, \( V_{\text{parenchyma}} \) is the volume density of lung parenchyma, and \( V_{\text{lung}} \) is total lung volume.

This approach is design-based and free of any assumptions on shape, size, or distribution of alveoli. The distance of section pairs set at 4 μm was realized by taking consecutive sections of a thickness of 4 μm (26). The calculated coefficient of error depending on the number of estimated alveoli per animal was ~7%. Compared with the observed overall coefficients of variations from 10 to 20%, the stereological approach appears to be precise enough.
Valveoli is the volume density of alveolar air space, $V_v$; parenchyma is dividing the absolute alveolar volume by the total number of alveoli: $V_v$ is not density estimates not affected by shrinkage. Water displacement was multiplied by dimensionless volume shrinkage correction was needed as the lung volume before embedding. Density of lung parenchyma, and $V_{lung}$ is total lung volume. Here no volume density of the subcomponent $(Bitplane AG, Zürich, Switzerland).$ Visualization and surface rendering we applied the software Imaris $(Paul-Scherrer-Institute, Villigen, Switzerland)$ (29). For 3D visualization station of the Materials Science Beamline at the Swiss Light Synchrotron radiation-based X-ray tomography. Lung samples, obtained as described above, were embedded in paraffin and scanned by a monochromatic X-ray beam $(12.398 \text{ keV})$ at the microtomography station of the Materials Science Beamline at the Swiss Light Source $(Paul-Scherrer-Institute, Villigen, Switzerland)$ (29). For 3D visualization and surface rendering we applied the software Imaris $(Bitplane AG, Zürich, Switzerland).$

RESULTS

Volume of parenchymal compartments. The parenchymal volume was categorized into the following volumetric parts: 1) alveolar volume: air space volume surrounded largely by interalveolar walls, 2) ductal volume lying in between alveoli, and 3) septal volume of lung parenchyma (see Table 1 and Fig. 2).

Figure 2 shows the progression of the various volume fractions in the observed period. All components enlarged significantly until the end of the observation period. The net enlargement of ductal $(871\%)$ and septal volume $(802\%)$ tended to parallel total lung volume $(891\%)$ from days 4 to 60 while the volume increase of alveolar space by $1,061\%$ in the same period was disproportionately higher. The pattern of volume increase was slightly different in the three fractions. Septal volume density (data not shown) had its peak at day 10 $(28.8\%)$ with a significant drop to $20.3\%$ toward day 21 and staying on the lower level until the end of the experiment. Thus septal volume had the lowest increase rate from days 10 to 21 $(+43\%)$, while air space volumes (ductal and alveolar) showed maximal gains $(+125\%)$.

Alveolar number. At the beginning of the observation period at day 4, some $0.82 \times 10^6$ alveoli were counted. However, at this early maturational state, it was not evident to distinguish between original sacculi and “true” newly formed alveoli with our quantification method (see Introduction). At day 21, $14.3 \times 10^6$ alveoli were present and at day 60 there were $19.3 \times 10^6$. The progression curve of alveolar number during the observation (Fig. 3) showed a characteristic biphasic shape with a steep increase from day 4 to day 21 $(0.82 \times 10^6$ to $14.3 \times 10^6$, $+1,640\%)$. From days 21 to 60 the number increased further and significantly, but less steeply $(+35\%)$ to $19.3 \times 10^6$.

Mean volume of alveoli. The size of alveoli varied considerably during the observation period (Figs. 4 and 5). However, as mentioned previously, at day 4 the estimation procedure for

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<tr>
<th>Day 4</th>
<th>Mean ± SD</th>
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<tr>
<td></td>
<td>Body Wt, g</td>
<td>Lung Volume, cm$^3$</td>
<td>Alveolar Airspace Volume, cm$^3$</td>
<td>Ductal Airspace Volume, cm$^3$</td>
<td>No. of Alveoli, × 10$^6$</td>
<td>Mean Volume of Alveoli, µm$^3$</td>
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<td>13.1 ± 0.8</td>
<td>1.03 ± 0.07</td>
<td>0.486 ± 0.073</td>
<td>0.133 ± 0.020</td>
<td>0.823 ± 0.07</td>
<td>592,643 ± 89,339</td>
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<td>Day 10</td>
<td>27.0* ± 3.8</td>
<td>1.81* ± 0.23</td>
<td>0.880* ± 0.117</td>
<td>0.230* ± 0.044</td>
<td>3.538* ± 1.26</td>
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<td>Day 21</td>
<td>79.2* ± 4.5</td>
<td>3.61* ± 0.34</td>
<td>1.910* ± 0.154</td>
<td>0.594* ± 0.108</td>
<td>14.303* ± 3.08</td>
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<td>Day 36</td>
<td>209.0* ± 8.9</td>
<td>7.28* ± 0.49</td>
<td>3.956* ± 0.360</td>
<td>0.973* ± 0.229</td>
<td>19.279† ± 3.08</td>
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<td>Day 60</td>
<td>418.0* ± 24.9</td>
<td>10.21* ± 0.59</td>
<td>5.645* ± 0.323</td>
<td>1.292* ± 0.225</td>
<td>19.279† ± 3.08</td>
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Values are means and SD from $n = 5$ animals. Significance: *$P \leq 0.05$ to the preceding day. †$P \leq 0.05$ day 60 vs. day 21.

Fig. 2. Lung volume fractions in the progression of time. Increase of absolute volumes of parenchymal regions. Increases of alveolar ($V_{alv}$), ductal ($V_{duct}$) (including alveolar sac) airspace volumes, and septal volume ($V_{sept}$) from all consecutive day-to-day comparisons were all significant; only $V_{sept}$ from day 10 to day 21 remained just below the significance level $(n = 5)$. Error bars indicate ± 1 SD.
the parenchymal airspaces did not allow to distinguish between sacculi and alveoli. This also explains why the observed "airspace" volume was 2.2 times larger (0.59 \times 10^6 m^3) than the alveoli at day 10 (0.27 \times 10^6 m^3). The smallest mean alveolar volume was found at day 21, being only 0.14 \times 10^6 m^3. At day 60 the mean volume reached 0.30 \times 10^6 m^3.

**DISCUSSION**

The characteristic structural subunits of the mammalian lung are the alveoli. The number of alveoli, as well as the rate and time course of alveolar formation, are important parameters that define the maturational and developmental state of the lung and its function after growth. The schedule of lung maturation has been well described in rats (4, 6), mice (2), and humans (39, 46, 47) by means of quantitative and morphological observations. In these studies, however, unbiased alveolar numbers have never been obtained. Usually the outgrowth of new septa was taken as the sign for the onset of alveolar formation, while the end of alveolarization was inferred from the degree of septal maturation and from the missing of new septal buds. As an alternative to the estimation of the number of alveoli (quantification of alveolarization) the formation of new alveolar septa (quantification of septation) was followed throughout postnatal lung development in rats and mice by means of quantification of the length of the free septal edge (23, 33). The length of the free septal edge corresponded to the total length of the alveolar entrance rings. Both studies showed that new septa are formed until young adulthood, but the precise correlation to the formation of the alveoli remained open. The present study defines for the first time the timetable of alveolar formation by a direct and unbiased assessment of the number of alveoli in the rat model. Starting at postnatal day 4 with only \( \frac{0.82 \times 10^6}{10^6} \) alveoli (or more correctly sacculi) the number increased to \( \frac{3.5 \times 10^6}{10^6} \) at day 10 and almost \( \frac{14.3 \times 10^6}{10^6} \) at day 21. This means that within 17 days \( \frac{13.5 \times 10^6}{10^6} \) alveoli were newly formed what represents a rate of 800,000/day (or 33,000/h). In this period lung volume increased less...
than four times, which hints at the enormous transformation of the inner lung structure. Alveolarization corresponds to a phenomenal increase in gas-exchange surface area (6). In the 39 days from day 21 to day 60 only 5 × 10^9 alveoli were added, clearly underlining the continuation of alveolarization. The mean rate of alveoli formed in this second or late phase, assuming a theoretical linear increase, dropped to 128,000 per day at what corresponds to roughly one-sixth of the rate in the early phase. However, these rates are just theoretical values not taking into account any slow-down of formation. The recognition of a biphasic behavior of alveolarization with the term of “bulk alveolarization” for the first phase and “continued alveolarization” for the second phase is of high developmental and clinical importance.

Just at the beginning of alveolarization the size of airspaces, still being predominantly sacculi, is at its highest level. During the first phase of alveolar formation (days 4–10), when there is a transition from sacculi to alveoli, the mean individual volume of alveoli dropped heavily. Septation is so intense that the volumetric expansion of the alveolar air space does not follow, and therefore the alveoli are becoming much smaller. An inverse situation appears in the phase of “continued” alveolarization when the formation of new alveoli is less important than the growth of the alveoli, leading to an increase of the mean airspace volume.

Between days 21 and 36, the minute increase of alveolar number and the following increase to day 60 can be interpreted as a transitional period where early “bulk” alveolarization has faded out and continued alveolarization is not as efficient to be detected. As lung volume still increases considerably between days 21 and 36 the mean size of alveoli increases, too.

While the formation of new alveoli during the early or “bulk” phase is morphologically well described, the question arises which potential mechanisms allow new alveoli to be formed in the phase of continued alveolarization, where the microvascular pattern is that of a mature lung. Three mechanisms were proposed so far (5, 33). First, the formation of new alveolar septa at sites where the alveolar microvasculature never fully matured, and therefore the alveoli are becoming much smaller. An inverse situation appears in the phase of “continued” alveolarization when the formation of new alveoli is less important than the growth of the alveoli, leading to an increase of the mean airspace volume.

Second, at sites of mature single-layered capillary networks a focal and temporary reduplication of the capillary bed can establish conditions for septal upfolding. This reduplication was explicitly found in vascular casts at locations where new interalveolar septa started to emerge (33). The second mode can operate everywhere in the lung parenchyma. In a rat model alveolarization was experimentally depressed by various glucocorticoid regimes. After the treatment immature septa with two capillary layers reappeared and a marked recovery of alveolarization was found (30, 41). This might be an enhanced form of the second mode of late alveolarization.

Third, at the parenchymal border where alveoli neighbor nonparenchymal structures, i.e., next to bronchi, vessels, and pleural tissue, alveolar walls have their own, private capillary bed, and it was postulated that an upfolding of new septa may take place throughout life at these sites (5). The latter represents the only kind of alveolarization that does not require a double-layered capillary network, because this capillary network exchanges the gas only at one side and not at two opposing sides like the capillary networks of mature alveolar septa. It is postulated that all of the three modes of late alveolarization take place throughout postnatal lung development after maturation of most alveolar septa.

All listed mechanisms fulfill the requirement of a “private” capillary bed per septal side where one side intends to fold up and form a new interalveolar septum. Our finding of a continued alveolarization after the completion of microvascular maturation could provide an explanation for the lung’s potential to functionally and structurally adapt to environmental changes, like hypoxia (8, 9, 28), and to recover following resection of lung tissue. Functional recovery of the lung was shown to take place after pneumonectomy and other clinical/experimental situations for mice, rats, dogs, and humans (3, 7, 10, 12, 17, 24, 38, 44). This means that the lung is not only capable of partially restoring the diffusion surface area by expanding its airspaces but also by forming new ones. Even in lungs showing a stable compartmental volume and/or alveolar structure, a constant remodeling of the parenchyma without changing the net number of alveoli cannot be excluded.

The two different phases of alveolarization are not only distinguished in morphological investigation, but also observed during the studies of protein expressions, e.g., while tenasin-C is strongly expressed close to the free septal edge during bulk alveolarization, the tenasin-C expression is reduced to a weak and diffuse expression during continued alveolarization. An early short-term treatment with dexamethasone (postnatal days 1–4) did not only postpone and prolong bulk alveolarization, but also the strong expression of tenasin-C was shifted to later days (29). The expression of elastin, which is at least essential for the bulk alveolarization (21), does not change towards or during continued alveolarization (29). The latter may be taken as evidence that elastin is essential for any kind of pulmonary alveolarization.

Semmler-Behnke et al. (35) showed that the deposition of nanoparticles in infant rat lungs shows a peak at postnatal day 21, at the end of bulk alveolarization (35). Comparing their deposition pattern with our data describing the size of the alveoli a striking negative correlation between these two datasets is observed. Between days 4 and 21, we estimated a decrease of the alveolar volume by a factor of 4, which correlates to the observed five times increase of the deposition of 20-nm particles. While between days 21 and 36 (or 60), the size of the alveoli roughly doubled, roughly half of the deposition was reported comparing days 21 and 36 (or 60). The negative correlation with our results quantitatively validates the computational simulations of nanoparticle retention of Semmler-Behnke et al. (35), where they predicted that the deposition of nanoparticles depends on the size of the alveoli and that small alveoli cause a higher retention.

In our study the mean number of alveoli present in rats at day 21 was 14.3 ± 3.1 × 10^6. This is more than half of the 34 × 10^6 estimated using a model-based approximation by Burri et al. (6). At day 60, at the end of adolescence, we observed 19.2 ± 3.1 × 10^6 alveoli which corresponds to 20 × 10^6 alveoli published by Hyde et al. (20) in adult rats using the disector counting principle, too. Using a disector-based approach on virtual sections from X-ray tomographic scans of lungs obtained from the same rats (42) but studying only the medial lower edge of the right lower lung lobe, Haberthür et al. (14) estimated 46.3 million alveoli in 60-day-old rats (14). This
twofold difference may be explained as follows. First, for technical reasons, Haberthür et al. did not use a sampling that represents an entire lung lobe. Regional differences inside one lung lobe, be it biological variability or an uneven filling of the lung, may contribute to the different numbers of alveoli. Second, Haberthür et al. used a shrinkage factor which resulted in half of the shrinkage as the one we observed. However, in both studies the shrinking factor was determined without obvious error using the same stereological methods. The only difference was that the lungs Haberthür et al. use were stored in fixative of 15 years, and our material was embedded directly after the tissue was obtained and stored for the same time as paraffin blocks.

With respect to mean alveolar volume the differences between our smaller volume at day 60 (0.30 ± 0.04 × 10^6 μm) and the values of Hyde et al. (0.5 ± 0.18 × 10^6 μm^3) can be explained with the higher body weight and lung volume in the probably older rats (no age information) in the work of Hyde et al. (20). A similar increase was observed comparing the total alveolar volume (our day 60 rats, 5.6 ± 0.3 cm^3; those of Hyde et al., 8.5 ± 1.98 cm^3). The disproportionate volume increase with only slight number increase of the number of alveoli explains the higher mean volume of alveoli in the work of Hyde et al.

Figure 6 compares key parameters of alveolarization. Alveolarization shows a strong biphasic behavior with a fast formation of alveoli before day 21 (~73% of the absolute number increase) and a slow one afterwards (~27%). This phenomenon is much stronger for alveolarization than for the increase in lung parenchyma and the total length of the free septal edge. Comparing the estimation of the number of alveoli to the estimation of the length of the free septal edge or the anlage of septa, respectively, we could expect a very good correlation as long as the number weighted mean alveolar volume does not change, e.g., in humans (19). With changes in the mean alveolar volume, the correlation between these two parameters decreases [for an illustration of this phenomenon see Fig. 5 and Schittny at al. (33)].

A model calculation may illustrate the close, but not 1-to-1, relation of alveolar number and the length of the free septal edge. Taking the alveolar entrance rings (= free septal edge) as circles, the total length of them corresponds to:

\[
\text{Length(septal edge)} = N_{\text{alv}} \times 2\pi r/2
\]

where \(N_{\text{alv}}\) is the number of alveoli and \(r\) is the radius of the entrance ring. The division by 2 takes into account that each septal crest is part of two entrance rings of adjacent alveoli. This formula shows that the length of the free septal edge is dependent on alveolar number and alveolar size.

Therefore, both parameters represent two independent entities which are based on two different points of view: alveolarization vs. septation. Consequently, they should not be taken as substitutes to each other, and both should be determined in order to fully characterize structural changes during lung development, injuries, and/or regeneration.

In summary we would like to conclude from our unbiased quantitative estimation of the alveolar number that the early phase of alveolarization corresponds to the known mass (“bulk”) production rate of alveoli, which is in good agreement with the morphological findings (4, 6). Because most of the alveolar microvasculature is still immature in this phase (days 4–21), the alveoli will be predominately formed according to the mechanism of classical alveolarization. After day 21 alveolarization continues at a slower pace and follows the three possible mechanisms of late alveolarization, discussed above. Therefore, we would like to call this latter phase continued alveolarization.

ACKNOWLEDGMENTS
We thank M. Hofstetter, B. de Breun, B. Krieger, and B. Haenni for expert technical assistance. Microscopy was performed on equipment supported by the Microscopy Imaging Center (MIC) of the University of Bern.

GRANTS
This work was supported by Swiss National Science Foundation Grants 3100-045831, 3100A0-109874, and 310030-125397.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
REFERENCES


