Kinetic analysis of pulmonary neutrophil retention in vivo using the multiple-indicator-dilution technique

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Schwab, Andreas J., Agnès Salamand, Yahye Merhi, André Simard, and Jocelyn Dupuis. Kinetic analysis of pulmonary neutrophil retention in vivo using the multiple-indicator-dilution technique. J Appl Physiol 95: 279–291, 2003. First published March 14, 2003; 10.1152/japplphysiol.00783.2001. 2001—Multiple-indicator-dilution experiments were performed in the lungs of 13 anesthetized dogs by simultaneous bolus injection of 111In-labeled neutrophils, 51Cr-labeled red blood cells, and Evans blue-labeled albumin. Concomitant counts of unlabeled neutrophils were similar in pulmonary artery and aortic blood samples, demonstrating a dynamic balance across the lungs in the physiological state. Outflow profiles of labeled neutrophils were analyzed on the basis of a recirculatory pharmacokinetic model of labeled albumin. The outflow profiles of the recovered neutrophils were composed of a throughput component of circulating neutrophils and a component of reversibly marginated neutrophils. They were interpreted by a model incorporating neutrophil margination (transfer coefficient \( = 0.195 \pm 0.081 \text{ s}^{-1} \)), rapid demargination (0.054 \( \pm 0.027 \text{ s}^{-1} \)), and transfer to a slow marginated pool (0.023 \( \pm 0.018 \text{ s}^{-1} \)). It will be interesting to apply the analysis in future studies aimed at determining whether it could be a useful research tool to investigate the interactions between the pulmonary endothelium and neutrophils in physiological and diseased states.

pulmonary circulation; mathematical modeling; microcirculatory exchanges; neutrophil margination; recirculatory model

THE PULMONARY CIRCULATION is an important site for retention of neutrophils. The concentration of neutrophils within pulmonary capillaries exceeds that in the large vessels of the systemic circulation by a factor of 35–100 (6, 7, 16). This accumulation of neutrophils within the lung is usually called margination. This expression, originally coined to describe association of neutrophils with the endothelium of venules, is commonly used also in the case of the lung, although the mechanisms of neutrophil enrichment are quite different (see below). Neutrophils have been shown by intravital microscopy to become immobilized within capillaries for 1–1,200 s before being released (29, 33, 49). After intravenous injection of radiolabeled neutrophils, 88% (pig), 78% (rabbit), or 72% (dog) are retained during the initial pulmonary transit. After 10 min, the proportion of neutrophils retained in the lungs falls to 60% (pig), 23% (rabbit), or 30% (dogs and humans) (6, 22, 35, 37, 40). To interpret the latter values, we must consider the fact that neutrophils released by the lung are constantly replaced by those delivered from the recirculating blood pool. As a result, the total pulmonary marginated pool in dogs and rabbits is about three times larger than the total body circulating pool (6, 22).

In the lung, the bulk of marginated neutrophils is found within capillary segments (15, 27, 32–34, 44). This contrasts with the systemic circulation, where the marginated pool resides essentially within the postcapillary venules (26). Only a very small portion of neutrophils within the healthy lung is found outside the vascular space within the interstitium or the alveoli (13). Pulmonary margination of neutrophils is believed to be caused mostly by mechanical constraint within the capillary segments (15, 44). Neutrophils within the peripheral circulation show an approximately spherical shape with a diameter (7 \( \mu \text{m} \)) that exceeds the average pulmonary capillary diameter (5 \( \mu \text{m} \)) (7, 8). They are far less deformable than erythrocytes (RBCs) because of a much more rigid cytoskeleton, which has to be reorganized for cell deformation to occur (42). In keeping with this, subsequent mobilization (demargination) requires a shape change from spherical to oblong, a process that depends on neutrophil deformability (15, 42). The contribution of selectins and integrins to margination in the healthy lung is unimportant. The pulmonary capillary endothelium lacks selectins. L-selectin, expressed on the surface of leukocytes, does not seem to play a major role in the immobilization of neutrophils in the lung, although a moderate decrease of intracapillary leukocyte velocity after infusion of fucoidan (an inhibitor of selectin activity) has been observed in the pulmonary capillaries of rabbits (28). Yoder et al. (50) observed the behavior of neutrophils obtained from a dog deficient in the adhesion-promoting glycoproteins CD11 and CD18 after injection into a healthy dog. They found no difference in the transit times or the number of stops between these cells and cells from a healthy animal, except when the cells were

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activated. Similarly, in L-selectin-deficient mice, no defect in pulmonary neutrophil margination was observed (9).

During inflammation, increased pulmonary neutrophil retention has been attributed to decreased neutrophil deformability (4), and cell adhesion molecules of the selectin and integrin families are involved in migration of neutrophils into the interstitial and alveolar space (1, 39).

The multiple-indicator-dilution technique has previously been applied to quantify the apparent single-pass fractional retention of neutrophils by the lungs in vivo (5, 6, 37, 40). However, evaluation of these studies yielded information on first-pass immobilization of neutrophils but not on their subsequent return to the vasculature because of the interference of recirculation. In the present work, the impact of recirculation on the measured outflow profiles of neutrophils was estimated by comparison with the outflow profiles of albumin, so that a more detailed mathematical analysis could be undertaken. A simple parametric model with linear kinetics was used to obtain quantitative parameters describing the processes of margination and early demargination of neutrophils. The objective of this approach is to characterize neutrophil kinetics in the intact organ within the few seconds of a single pulmonary transit time.

MATERIALS AND METHODS

Animal Preparation

The study protocol was approved by the Animal Research Ethics Committee of the Montreal Heart Institute in accordance with the Canadian Council of Animal Care guidelines. The study group was composed of 13 healthy mongrel dogs with a mean weight of 27 ± 4 kg. The animals were anesthetized with pentobarbital sodium (30 mg/kg iv), with additional dosing to maintain anesthesia if needed, intubated, and mechanically ventilated with room air. Under sterile conditions, a right paramedial cervical incision was performed, and the right jugular vein and the carotid artery were isolated and ligated distally. A Swan-Ganz catheter was then inserted into the jugular vein and advanced into the main pulmonary artery. A modified pigtail catheter (with tail cutoff and carving of additional holes) was inserted into the carotid artery and positioned 2 cm above the aortic valve under fluoroscopic guidance. Pulmonary arterial pressure and aortic pressure were measured with a polygraph (Gould Instruments, Valley View, OH) and recorded with a zero reference at midchest at the level of the right atrium. Heart rate was also continuously recorded by using electrocardiographic limb leads. Heparin (100 U/kg) was administered to prevent clotting of the catheters and the samples to be collected.

Isolation and Labeling of Neutrophils

All procedures of isolation and labeling of neutrophils were performed under sterile conditions, as previously described (38). Venous blood (120 ml) was drawn from each animal in syringes containing 5:1 acid citrate-dextrose. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 500 g for 15 min. Neutrophils were isolated from the pellet obtained after removal of PRP. The method involves sedimentation of RBCs with 4% dextran for 45 min. The leukocyte-rich suspension was washed with citrated Hanks'HEPES buffer (pH 7.4), layered over an equal volume of Ficol-Hypeaque gradient separation medium, and centrifuged at 400 g for 30 min. After hypotonic lysis of the contaminating RBCs from the recovered neutrophils, the isolated neutrophils were resuspended in 5 ml of citrated Hanks'HEPES buffer and incubated with 250 μCi 51Cr-labeled RBCs for 30 min. The suspension was centrifuged to remove unbound 111In, and the radiolabeled neutrophils were then suspended in 2.5 ml of PRP. This procedure yielded a neutrophil preparation that is >95% pure and viable, as assessed by an electronic Coulter counter and the trypan blue exclusion test. Mean neutrophil recovery was 59.9 ± 15.5 × 109/ml with a labeling efficiency of 62 ± 10%.

In preliminary experiments, it was verified that no detectable 111In was released to the supernatant.

Multiple-Indicator-Dilution Experiments

An injection mixture was prepared by combining 2 ml of 111In-labeled neutrophils with 4 ml of 51Cr-labeled RBCs and 3 ml of isotonic Evans blue (5 g/ml) as a label for albumin. Isotonic saline and/or unlabeled RBCs were added to the mixture to obtain a final hematocrit matching that of the dog, with a final volume of ~10 ml. Bovine serum albumin was added to the mixture to a final concentration of 4 g/100 ml of noncellular fluid. Three milliliters of the mixture were allocated for each of the two injections, and the remainder was used to prepare injection-dose standards that were diluted with blood (1:10). Samples obtained by addition of small amounts of each of the original suspensions of 111In-labeled neutrophils and 51Cr-labeled RBCs to the animal's blood were used for the extent of crossover of 111In counts into the 51Cr channel (there was no crossover of 51Cr counts into the 111In channel). Counts were ≥10 times above background values obtained immediately after the injection before the first appearance of tracer.

Before each experiment, blood samples were obtained from the pulmonary artery and aorta to determine complete blood cell counts and blood gases. A Swan-Ganz catheter was positioned in the right ventricular outflow just below the pulmonary valve, and the injection mixture was introduced into the dead volume of the catheter. The bolus was then flushed with 10 ml of the animal's blood. Timed samples were simultaneously collected from the aortic catheter with a roller pump and a linear fraction collector (60 tubes in 40 s) with 1.3 ml of blood per tube, starting at the time of injection. In 11 animals, two similar experiments (experiments I and II) were performed sequentially in the same animal with a 15-min interval between experiments to test experimental reproducibility.

Analysis of Indicator-Dilution Samples

Each collected sample was processed to determine the fractional recovery of each tracer. Dose and crossover standards were treated in a manner identical to the other samples. A 100-μl aliquot was taken from each sample and placed into a gamma counter (Cobra 5002, Canberra-Packard, Meriden, CT) to determine 111In activity. To minimize isotope crossover, the tubes were counted again 48 h later to obtain 51Cr activity. The remaining blood sample was centrifuged for 10 min at 870 g, and 0.2 ml of the resulting plasma was added to 1 ml of physiological saline in a standard disposable spectrophotometer cuvette (1-cm path length; Fisher Scientific, Montreal, QC, Canada) for determination of Evans blue
absorbance at 620–740 nm in a spectrophotometer (model HP 8452A, Hewlett-Packard, Palo Alto, CA).

The fractional recovery of each tracer per milliliter of blood (concentration normalized to dose amount) was calculated and plotted as a function of time to construct the indicator-dilution curves. The mean transit times for tracer RBCs and albumin, the cardiac output, and the central blood volume were then computed as previously described (10).

Theory

Model of pulmonary neutrophil retention. It has been shown in previous studies that neutrophils remain motionless for variable times, from fractions of a second to several minutes (34). Analysis of these data reveals a deviation from simple Poisson statistics, such that the probability that a neutrophil that has been immobilized in the lung capillaries returns to the circulation is high for the first few seconds and gradually decreases with longer immobilization times. To account for this peculiarity, we used a simplified model that can be applied more easily by using conventional mathematical methods. For this, we assign the immobilized neutrophils to two separate pools. Neutrophils within the rapid marginalized pool show a high time-independent probability to return to the vasculature, whereas neutrophils within the slow marginalized pool are immobilized quasi-permanently; i.e., their probability of being demarginated in the time scale of the experiment is considered to be negligible. Neutrophils migrating into the interstitial and the alveolar space would be included in the slow marginalized pool, although, in the healthy lung, migration of neutrophils is insignificant.

Modeling of the retention of neutrophils within the pulmonary capillary bed was accomplished by a variation of the barrier-limited model of Goresky and Nadeau (18), which is based on the Sangren-Sheppard equation (43). The model was similar to that previously used to evaluate the pulmonary disposition of serotonin (11, 18). The pulmonary capillaries were regarded as the site where the three indicators separate from each other. The other components, namely, the pulmonary large vessels (the pulmonary artery downstream from the injection site, the pulmonary vein, and their branches, possibly including arterioles and venules) and the left heart, were combined to form a simple time delay (τ0). In perfused rabbit lungs, large-vessel transit times have previously been found to be uniform (2). The mean transit time through the left heart can be approximated as the inverse of the product of cardiac frequency and ejection fraction. For a healthy dog, this yields a monoexponential washout with mean transit time of \( \sim 1/(1.25 \, s^{-1} \times 0.5) = 1.6 \, s \). These values are small compared with the pulmonary transit times for albumin, such that the error introduced by assuming a simple delay in the nonseparating part of the central circulation was considered to be minor.

Aortic concentration profiles of labeled neutrophils are shaped, apart from pulmonary retention, by the characteristics of blood flow within the central and peripheral circulation. These characteristics were assessed separately by using a simultaneously injected reference tracer: albumin labeled with Evans blue. The pulmonary impulse response for neutrophils, \( h_c^{\text{N}}(t) \) (the normalized vascular concentration of neutrophils after a single injection excluding recirculation), can be derived from the pulmonary impulse response for albumin, \( h_c^{\text{Alb}}(t) \), and is expected to be (11, 18)

\[
h_c^{\text{N}}(t) = h_c^{\text{Alb}}[v_{\text{rel}}(t - \tau_0) + t_0]e^{-(k_c - e^{-\frac{t}{t_0}}) - e^{-\frac{t}{t_0}}} \\
\times \sum_{n=1}^{\infty} \frac{(k_{m-c})^n(t - \tau_0 - \tau)^{n-1}}{n!(n-1)!} d\tau \tag{1}
\]

The parameters to be optimized are as follows: common large-vessel transit time (\( \tau_0 \), in s), ratio of the velocity of neutrophils to the velocity of RBCs (\( v_{\text{rel}}/v_0 \) or \( v_{\text{rel}} \), dimensionless), coefficient for transfer of neutrophils from the circulating to the marginalized pool of neutrophils (\( k_{m-c} \), in s\(^{-1} \)), coefficient for transfer of neutrophils from the rapid marginalized to the circulating pool (\( k_{m-c} \), in s\(^{-1} \)), and coefficient for transfer of neutrophils from the rapid to the slow marginalized pool (\( k_{m-s} \), in s\(^{-1} \)). Correction for the dispersion of the tracers in the collection catheter was performed as described previously (17, 19).

Recirculatory pharmacokinetic modeling. Later in time, the tracers reach the lungs a second time after passing through the peripheral circulation. Two different modeling approaches were conceived. According to one approach, the recirculating neutrophils were excluded by omitting the experimental values obtained after onset of recirculation: we will call this the truncated analysis. A second, more detailed and, possibly, more informative approach was to include the entire neutrophil curve in the analysis. Discrimination between returning and recirculating neutrophils deserves special consideration if one wishes to obtain maximal information from the later portion of the outflow curves. To accomplish this, recirculatory pharmacokinetic analysis (24, 25) was applied to tracer neutrophils. We will call this the recirculation analysis.

The circulation of the whole animal was regarded to be composed of two parts: the central circulation and the systemic (or peripheral) circulation. According to the experimental protocol used, the central circulation was regarded to be the part situated between the point of injection within the pulmonary artery and the point of sample collection in the aorta and was thus deemed to include the lung and the left heart, excluding the right heart. Conversely, the right heart was regarded to be part of the systemic circulation together with all other organs and tissues. The minimal contribution of the coronary circulation was neglected.

Recirculatory pharmacokinetic model for albumin. The concentration curve for labeled albumin collected from the aorta was considered to be composed of two components: a first-pass component consisting of tracer entering the lungs directly from the injection that had not undergone recirculation and a recirculating component originating from label that had traversed the right heart after peripheral recirculation. The first-pass component, \( c_{\text{Alb}}^{\text{IP}}(t) \), was taken to be represented by the observed concentration curve up to the time of beginning recirculation (determined as the inflection point within the downslope of the semilogarithmic graph), augmented by a tail obtained by monoexponential (semilogarithmic) extrapolation as suggested by Hamilton et al. (20). The recirculating component was considered to be the difference between the experimental curve and the extrapolated first-pass component: \( c_{\text{Alb}}^{\text{REC}}(t) = c_{\text{Alb}}^{\text{IP}}(t) - c_{\text{Alb}}^{\text{REC}}(t) \). Cardiac output (\( F \)) was obtained as the reciprocal of the area under the first-pass component, normalized to the injected amount (31).
\[ F = \frac{q}{\int_0^\infty c_{\text{Alb}}^\text{in}(t) \, dt} \]  

and the impulse response of the central circulation was taken as the concentration of the first-pass component, multiplied by \( F \) and divided by the amount of tracer injected \( (q) \)

\[ h_{\text{Alb}}^\text{in}(t) = (Fq)c_{\text{Alb}}^\text{in}(t) \]

The theoretical curve for the normalized concentration of albumin at the collection point (the aorta), \( c_{\text{Alb}}^\text{in}(t) \), was thus calculated by convolution of the impulse response of the concentration profiles of the combined injected (represented by an impulse function, \( \delta(t) \)) and recirculating tracer with the impulse response of the central circulation, \( h_{\text{Alb}}^\text{in}(t) \), as follows

\[ c_{\text{Alb}}^\text{in}(t) = [(q/F)\delta(t) + c_{\text{Alb}}^\text{in}(t) \cdot h_{\text{Alb}}^\text{in}(t)] \cdot h_{\text{Alb}}^\text{in}(t) \]

which expands to

\[ c_{\text{Alb}}^\text{in}(t) = c_{\text{Alb}}^\text{in}(t) + (F/q)c_{\text{Alb}}^\text{in}(t) \cdot c_{\text{Alb}}^\text{in}(t) \cdot h_{\text{Alb}}^\text{in}(t) \]

As a convenient parametric model for approximating the impulse response of the systemic circulation, \( h_{\text{Alb}}^\text{in}(t) \), we used a compartmental system as previously proposed by Krejcie et al. (24, 25). This system consists of two pathways in parallel: a slow pathway with two compartments in series and a fast pathway with six compartments in series (Fig. 1). It should be noted that these compartments have no physical meaning, and other parameterized functions with similar shape could have been used. However, a single Erlang function did not yield satisfactory fitting results, indicating that a substantial portion of the tracer had prolonged transit times, presumably caused by poorly perfused tissues. All the compartments in a pathway are assumed to have the same volume and, therefore, also the same turnover rate. Accordingly, \( h_{\text{Alb}}^\text{in}(t) \) is obtained as a weighted sum of two Erlang functions

\[ h_{\text{Alb}}^\text{in}(t) = P_F \frac{k_F^n e^{-kt}}{(nF - 1)!} + (1 - P_F) \frac{k_S^n e^{-kt}}{(nS - 1)!} \]

where \( P_F \) is the proportion of cardiac output flowing through the fast pathway, \( k_F = nFE/F_V, k_S = nSE/F_S \), \( F \) is cardiac output, \( F_V \) and \( F_S \) are the volumes of the fast and slow pathways, respectively, and \( n_F = 6 \) and \( n_S = 2 \) are the number of compartments comprising each of the pathways, respectively. The parameters to be fitted with this model are \( P_F, V_F, V_S, \) and \( P_F \).

The unknown function \( c_{\text{Alb}}^\text{in}(t) \) occurs implicitly in Eq. 5, and solving for \( c_{\text{Alb}}^\text{in}(t) \) would require deconvolution. The following procedure allows estimation of the unknown parameters, \( P_F, V_F, V_S, \) and \( P_F \), without requiring explicit deconvolution. Values for the expression represented by the right-hand side of Eq. 5 were calculated by using spline approximations for \( c_{\text{Alb}}^\text{in}(t) \) and \( c_{\text{Alb}}^\text{in}(t) \) and with \( h_{\text{Alb}}^\text{in}(t) \) approximated according to Eq. 6. Convolution integrals were calculated by using a fast Fourier algorithm from International Mathematical and Statistical Libraries (IMSL, Visual Numerics, Houston, TX) after discretization of the curves using time intervals of 1 s. The resulting approximations of \( c_{\text{Alb}}^\text{in}(t) \) were fitted to the experimental values of \( c_{\text{Alb}}^\text{in}(t) \) by using a least-squares fitting procedure from IMSL by varying the values of the parameters \( P_F, V_F, \) and \( V_S \) in Eq. 5.

**Recirculatory pharmacokinetic model for neutrophils.** To obtain some information on pulmonary demargination, a recirculation model was applied to the neutrophils as well. Neutrophils can be sequestered by extravasation in the lungs as well as within organs that are supplied by the peripheral circulation. Quasi-irreversible systemic retention of neutrophils was modeled by including irreversible disappearance of tracer from each systemic compartment with a common quasi-irreversible retention coefficient \( k_{\text{so}} \). This leads to the following sum of modified Erlang functions

\[ h_{\text{N}}^\text{in}(t) = P_F \frac{k_F^n e^{-kt}}{(nF - 1)!} + (1 - P_F) \frac{k_S^n e^{-kt}}{(nS - 1)!} \]

The arterial neutrophil concentration \( [c_{\text{N}}^\text{in}(t)] \) is then modeled by convolution in a way analogous to Eq. 3

\[ c_{\text{N}}^\text{in}(t) = [(q/F)\delta(t) + c_{\text{N}}^\text{in}(t) \cdot h_{\text{N}}^\text{in}(t)] \cdot h_{\text{N}}^\text{in}(t) \]

where \( h_{\text{N}}^\text{in}(t) \) is calculated by using Eq. 3. Again, \( c_{\text{N}}^\text{in}(t) \) occurring implicitly in Eq. 8, a spline approximation of the experimental curve, is used to evaluate this function on the right-hand side of Eq. 8. The neutrophils were considered to show a transit time distribution through the peripheral circulation identical to that of albumin. Accordingly, the values for \( P_F, V_F, \) and \( V_S \) obtained from the evaluation of the albumin curves were used as fixed parameters for the evaluation of the neutrophil curves, and the parameters \( n_F, k_F, n_S, k_S, k_{\text{m-e}}, \) and \( k_{\text{m-s}} \) were optimized. This procedure is a modification of that used by Krejcie et al. (24, 25), who fitted recirculation parameters separately for the vascular reference (indocyanine green) and for the drugs studied.

**Statistical Analysis**

Differences between variables were analyzed by using two-tailed unpaired or paired Student's t-tests where appropriate. Values are means ± SD. Differences were considered significant if \( P < 0.05 \). The model-derived parameters were
optimized by using a least-squares algorithm (Levenberg-Marquart, from IMSL). In performing the optimization, individual fractional recovery points were weighted according to the square root of their values, which is proportional to radioactive count errors. Coefficients of variation as a measure of the precision of the parameter identification were obtained from the information matrix provided by the fitting procedure (30).

RESULTS

Hemodynamics and Gas Exchange

The anesthetized animals had slightly elevated mean pulmonary arterial pressure (25 ± 4 mmHg). Heart rate and mean systemic pressure were also elevated at 193 ± 22 beats/min and 167 ± 8 mmHg, respectively, presumably as a consequence of the surgical procedure, which included ligation of the carotid artery. Previously, we had measured in similarly anesthetized animals without carotid ligation (n = 14) a mean pulmonary arterial pressure of 17 ± 2.5 mmHg, a heart rate of 73 ± 13 beats/min, and a mean arterial pressure of 119 ± 14 mmHg (12). Cardiac output varied over a wide range (2.51 ± 1.32 l/min, range 0.56–5.54 l/min). This experimental variation, probably due to varying surgical stress (increasing cardiac output) and varying pharmacodynamics of the anesthetic (decreasing cardiac output), was used to assess the influence of cardiac output on neutrophil kinetics. Acid-base balance was normal, with pH 7.38 ± 0.07 and 23 ± 2 mM HCO3. The PO2 (103 ± 10 Torr), PCO2 (35 ± 7 Torr), and O2 saturation (0.98 ± 0.01) were also within the normal range, indicating that near-physiological gas exchange was achieved. The alveoloarterial PO2 difference was also normal at 4 ± 8 Torr.

Blood Neutrophil Counts

Blood neutrophil counts were slightly higher in the aorta than in the pulmonary artery only for experiment II (Table 1). In contrast, there was no difference in neutrophil counts across the pulmonary circulation between experiments I and II.

Tracer Outflow Profiles and Derived Parameters

Typical multiple-indicator-dilution experiments from three different animals are displayed in Fig. 2. To demonstrate the effect of varying cardiac output on the outflow profiles, experiments with spontaneous low, intermediate, and high cardiac output were selected. The RBCs appear and peak first, followed closely by tracer albumin, which is slightly delayed and reaches a comparatively lower peak. This well-known behavior is attributed to the presence of a slip layer of plasma between the capillary wall and the RBCs (11, 41). The recoveries of these two vascular tracers after a single pulmonary transit (evaluated as areas under the first-pass curves with monoexponential extrapolation) were identical and were assumed to be complete. Data derived from the analysis of these two vascular references are assembled in Table 2. In duplicate experiments performed 15 min later in the same animal, cardiac output and central blood volume were slightly reduced, with concomitantly longer transit times.

Recirculation Analysis of Albumin Kinetics

Because recirculation could not be observed within the time of sample collection, recirculation analysis could not be applied to three results with low cardiac output: one in experiment I and two in experiment II. In the others, recirculation analysis provided a good fit to the data (Table 3). Typical calculated curves for systemic circulation are presented in Fig. 3. The model enabled us to separate the tail end of the albumin curves into first-pass and recirculating components (Fig. 4).

Model Analysis of Pulmonary Neutrophil Margination

Influence of quasi-irreversible systemic retention on the fitted parameters. Because albumin undergoes negligible systemic sequestration, complete recirculation of this tracer is presumed. Neutrophils, however, exhibit sequestration in the systemic circulation, and an unknown proportion of the initial pulmonary outflow is expected to recirculate. With this in mind, neutrophil concentrations in the aorta were modeled with the assumption of quasi-irreversible retention of neutrophils in the lungs only, in the systemic circulation only, or in both.

In regard to the identifiability of quasi-irreversible systemic retention, three cases were distinguished, depending on cardiac output (experiments I and II were pooled). At low cardiac outputs (3 experiments with cardiac output values between 0.36 and 0.59 ml·s⁻¹·kg⁻¹), the tail part of the aortic concentration curves was not observable; therefore, the extent of recirculation was unknown. In these experiments, re-

Table 1. Aortic and pulmonary leukocyte counts

<table>
<thead>
<tr>
<th>n</th>
<th>WBCs, 10⁶ ml⁻¹</th>
<th>Neutrophils, 10⁶ ml⁻¹</th>
<th>Lymphocytes, 10⁶ ml⁻¹</th>
<th>Monocytes, 10⁶ ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>8.9 ± 3.0</td>
<td>6.6 ± 2.5</td>
<td>1.4 ± 0.9</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Aorta</td>
<td>9.1 ± 2.9*</td>
<td>6.6 ± 2.4</td>
<td>1.4 ± 0.6</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>8.8 ± 4.0</td>
<td>6.9 ± 3.8</td>
<td>1.1 ± 0.5</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Aorta</td>
<td>9.0 ± 3.9</td>
<td>7.3 ± 3.8</td>
<td>0.9 ± 0.8†</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SD. WBCs, white blood cells. *P < 0.05 compared with pulmonary artery. †P < 0.05 compared with experiment II.
Table 2. Parameters obtained from analysis of experimental profiles

<table>
<thead>
<tr>
<th>Experiment</th>
<th>n</th>
<th>Body Mass, kg</th>
<th>Hct</th>
<th>Cardiac Output, ml·s⁻¹·kg⁻¹</th>
<th>Central Blood Volume, ml/kg</th>
<th>Mean Transit Time, s</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RBCs</td>
</tr>
<tr>
<td>I</td>
<td>13</td>
<td>27 ± 4</td>
<td>0.41 ± 0.03</td>
<td>1.7 ± 0.6</td>
<td>11.3 ± 1.9</td>
<td>7.2 ± 3.4</td>
</tr>
<tr>
<td>II</td>
<td>11</td>
<td>0.41 ± 0.05</td>
<td>1.2 ± 0.6</td>
<td>9.9 ± 1.7</td>
<td>9.8 ± 5.6</td>
<td>10.0 ± 5.8</td>
</tr>
</tbody>
</table>

Values are means ± SD. Hct, hematocrit; RBCs, red blood cells. *P < 0.05 compared with experiment I.

Changes in the other fitted parameters were less pronounced. When recirculation was considered to be absent ($k_{s0} = \infty$), values of $k_{c-m}$ were reduced by 3 ± 6% and values of $k_{m-c}$ were reduced by 14 ± 10% compared with the case of complete recirculation. No significant difference was found for $t_0$ or $v_{rel}$.

At higher cardiac outputs (12 experiments with cardiac output values between 1.01 and 2.80 ml·s⁻¹·kg⁻¹), it is not possible to determine whether quasi-irreversible pulmonary retention occurs, because the returning component of the curve is obscured by the recirculating component. If this is the case, a fit with $k_{s0}$ set to 0 will yield an upper bound for $k_{m-s}$ (0.014 ± 0.010 s⁻¹), whereas a fit with $k_{m-s}$ set to 0 will yield an upper bound for $k_{s0}$ (0.052 ± 0.047 s⁻¹). No significant change of $k_{c-m}$, $t_0$, or $v_{rel}$ was found between upper bound and no recirculation, and $k_{m-c}$ was moderately affected (11 ± 7% reduction with no recirculation).

**Truncation analysis.** In general, previous multiple-indicator-dilution experiments have been evaluated by using curves that were truncated at the time when recirculation first became apparent, with data containing recirculating cells discarded. Because no data were available at longer times, it was not possible to identify parameters for quasi-irreversible pulmonary retention of marginalized labeled neutrophils. The optimized parameters thus were $v_{rel}$, $t_0$, $k_{c-m}$, and $k_{m-c}$, with $k_{m-s}$ set to 0.

Truncated and recirculation analysis provided good-quality fits. For the truncated analysis, Pearson’s $r$ is 0.9957 ± 0.0041 (worse-fit $r = 0.9842$). For the recirculation analysis, it is 0.9925 ± 0.0053 (worse-fit $r = 0.9792$). In the same way as with albumin, the aortic neutrophil curves were composed of first-pass and recirculating cells. Values for $k_{m-c}$ were not significantly different between truncation and recirculation analysis. Values for $k_{c-m}$ could be reduced or increased in truncation analysis compared with recirculation anal-

Fig. 2. Typical set of multiple-indicator-dilution curves demonstrating the pulmonary outflow profile for labeled red blood cells, albumin, and neutrophils. A–C: 3 experiments with approximate doubling of cardiac output. Recirculation of tracers becomes more evident with increasing cardiac output.
ysis but were, on average, reduced \((-14 \pm 36\%, P < 0.05\)). The extreme variations are a reduction of \(-85\%\) and an increase of \(41\%\) in truncation analysis. For each experiment, the values of \(k_{m-c}\) were obtained from the model fit, with considerably less uncertainty with the truncated analysis than with the recirculation analysis [the average coefficient of variation obtained from the information matrix (30) was 0.09 with recirculation vs. 0.29 with truncated curves]. The transfer coefficient for quasi-irreversible retention \(k_{m-s}\) could only be obtained by using recirculation analysis.

As a general conclusion of these considerations, parameters for the margination and demargination of neutrophils could be identified with reasonable accuracy, whereas this is not the case for quasi-irreversible retention, because it is partially obscured by recirculation analysis. However, an upper bound for \(k_{m-s}\) can be determined by assuming complete recirculation in the absence of quasi-irreversible systemic retention. The average upper bound of \(k_{m-s}\) (all experiments except the 3 where no tail was observed) was found to be \(0.025 \pm 0.016 \text{ s}^{-1}\). Because a finite lower bound (within the same order of magnitude) was found in nine experiments, it may safely be assumed that the true value of these parameters is of this order of magnitude in all cases.

The model parameters obtained from the optimized fit of the neutrophil outflow profiles are summarized in Tables 4 and 5. Those experiments where recirculation does not appear within the time of the experiment were evaluated with no truncation of the data and with regard for recirculation. The results of the latter experiments were regarded as comparable to evaluations of the other experiments obtained by recirculation analysis.

### Table 3. Parameters obtained from fit of albumin profiles

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(n)</th>
<th>(P_F) mean</th>
<th>(SD_A)</th>
<th>(SD_B)</th>
<th>(V_F, \text{ml/kg}) mean</th>
<th>(SD_A)</th>
<th>(SD_B)</th>
<th>(V_S, \text{ml/kg}) mean</th>
<th>(SD_A)</th>
<th>(SD_B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td>12</td>
<td>0.21</td>
<td>0.02</td>
<td>0.10</td>
<td>3.0</td>
<td>0.4</td>
<td>1.9</td>
<td>46</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Experiment II</td>
<td>9</td>
<td>0.24</td>
<td>0.02</td>
<td>0.09</td>
<td>2.8</td>
<td>0.3</td>
<td>1.6</td>
<td>46</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

\(P_F\), proportion of cardiac output entering fast compartments; \(V_F\), volume of fast compartments; \(V_S\), volume of slow compartments; \(SD_A\), average individual SD from fitting procedure, reflecting uncertainty of optimization; \(SD_B\), SD for parameters from different experiments, representing interindividual variability.

Fig. 3. Calculated systemic functions for medium- \((A)\) and high-flow \((B)\) representative experiments. \(h_{Alb}\), Systemic function for albumin.

Fig. 4. Outflow profile of tracer albumin with separation into its theoretical first-pass outflow and recirculating components using pharmacokinetic recirculation modeling with the assumption of complete recirculation \([\text{retention coefficient } (k_{co}) = 0]\).
The model enables separation of the neutrophil outflow profile into its different components [shown in Fig. 5 for the case of maximal recirculation ($k_{s0} = 0$)]. The throughput component, comprising $30 \pm 5\%$ of the injected neutrophils, consists of neutrophils that remain in the circulating pool throughout their pulmonary transit. The returning component, which consists of neutrophils that have marginated and then returned to the circulating pool, appears later in time and substantially contributes to the tail portion of each curve. The sum of these first two components constitutes the first-pass outflow of neutrophils. The third and later component is that of recirculating neutrophils.

The transfer coefficients for margination, $k_{c-m}$, were approximately proportional to cardiac output (Fig. 6). This relation was similar whether $k_{c-m}$ is computed with the truncated or the recirculation analysis. The other transfer coefficients as well as the proportion of the throughput component did not correlate significantly with cardiac output. The values of $t_0$ decreased with increasing cardiac output (not shown), whereas there was no flow dependence of the volume of the nonexchanging part of the central circulation with a mean value of $4.7 \pm 1.9$ ml/kg. Values of $t_0$ were also obtained by linear superposition of the Evans blue (albumin) and RBC curves, but with $4.1 \pm 2.1$ s they were not significantly different from the values obtained from the neutrophil data.

**DISCUSSION**

The multiple-indicator-dilution technique has previously been used with labeled neutrophils to evaluate their pulmonary retention. Martin et al. (36, 37) used the multiple-indicator-dilution technique to compare the outflow profiles of labeled neutrophils with those of labeled RBCs in dogs. The shapes of the outflow profiles reported by these authors were essentially identi-
Table 5. Rate constants obtained from different analyses of neutrophil profiles

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Fit Assuming Complete Recirculation ($k_{c-o}=0$)</th>
<th>Fit Assuming No Quasi-Irreversible Pulmonary Retention ($k_{m-o}=0$)</th>
<th>Fit Assuming No Recirculation ($k_{c-o}=$)</th>
<th>Truncation Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{c-o}$</td>
<td>$k_{m-o}$</td>
<td>$k_{m-o}$</td>
<td>$k_{c-o}$</td>
</tr>
<tr>
<td>1</td>
<td>0.216</td>
<td>0.049</td>
<td>0.011</td>
<td>0.212</td>
</tr>
<tr>
<td>2</td>
<td>0.142</td>
<td>0.125</td>
<td>0.066</td>
<td>0.117</td>
</tr>
<tr>
<td>3</td>
<td>0.200</td>
<td>0.035</td>
<td>0.030</td>
<td>0.196</td>
</tr>
<tr>
<td>4</td>
<td>0.192</td>
<td>0.044</td>
<td>0.034</td>
<td>0.189</td>
</tr>
<tr>
<td>5</td>
<td>0.117</td>
<td>0.051</td>
<td>0.015</td>
<td>0.114</td>
</tr>
<tr>
<td>6</td>
<td>0.109</td>
<td>0.027</td>
<td>0.001</td>
<td>0.110</td>
</tr>
<tr>
<td>7</td>
<td>0.218</td>
<td>0.046</td>
<td>0.008</td>
<td>0.216</td>
</tr>
<tr>
<td>8</td>
<td>0.180</td>
<td>0.059</td>
<td>0.031</td>
<td>0.170</td>
</tr>
<tr>
<td>9</td>
<td>0.352</td>
<td>0.039</td>
<td>0.032</td>
<td>0.349</td>
</tr>
<tr>
<td>10</td>
<td>0.353</td>
<td>0.075</td>
<td>0.027</td>
<td>0.350</td>
</tr>
<tr>
<td>11</td>
<td>0.109</td>
<td>0.020</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.206</td>
<td>0.076</td>
<td>0.008</td>
<td>0.200</td>
</tr>
<tr>
<td>13</td>
<td>0.205</td>
<td>0.061</td>
<td>0.008</td>
<td>0.135</td>
</tr>
<tr>
<td>Avg</td>
<td>0.195</td>
<td>0.054</td>
<td>0.023</td>
<td>0.165</td>
</tr>
<tr>
<td></td>
<td>±SDs</td>
<td>±SDs</td>
<td>±SDs</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>±SDs</td>
<td>±SDs</td>
<td>±SDs</td>
<td>±SDs</td>
</tr>
</tbody>
</table>

$k_{c-o}$: Transfer coefficient for neutrophil margination; $k_{m-o}$: transfer coefficient for transfer of neutrophils from the rapid to the slow marginated pool; $k_{c-o}$: coefficient for transfer of neutrophils from the rapid to the slow marginated pool; $k_{m-o}$: transfer coefficient for quasi-irreversible systemic retention; ±SDs, average individual SD from fitting procedure, reflecting uncertainty of the optimization; ±SDs, SD for parameters from different experiments, representing interindividual variability. *Only first run data are available for this experiment. †Neutrophil outflow profile has no identifiable returning or recirculating component.

certal to those observed in the present study, exhibiting an initially high instantaneous extraction. The neutrophil curves crossed over those of the RBCs on the downslope, indicating a later return of labeled neutrophils to the circulation. Martin et al. determined first-pass pulmonary extraction of neutrophils of 70–90% by using a model-free approach based on a comparison of the integrals of the outflow profiles up the peak of the reference curve. These calculations depended on the assumption that cells that have been immobilized within the capillaries do not return to the vasculature before this time and, therefore, entail a possible under-estimation of the actual extraction rate. Our modeling procedure takes into account possible early return of immobilized neutrophils by separating the neutrophils into a throughput (neutrophils that do not participate during their transit) and a returning component (neutrophils that marginate and later return to the circulating pools). According to our calculations, the upslope part of the neutrophil outflow profiles consists mainly of throughput material at all flows, thus corroborating the model-free approach mentioned above. At low flows, the part immediately following the peak is partly shaped by cells that have been temporarily immobilized (Fig. 5).

The pulmonary capillary transit time of neutrophils has been elegantly studied by Gebb et al. (15) using high-magnification video microscopy of subpleural microvessels in dogs. It was found that 46 ± 6% of unlabeled native leukocytes transited through the field of view of the microscope without stopping. Our results are consistent with these observations, given the fact that the field of view comprised only a fraction of the distance between arterioles and venules. Using the same technique with labeled neutrophils, Lien et al.
demonstrated that neutrophils stopped exclusively in capillaries for periods of 0.2–200 s.

Modeling of multiple-indicator-dilution experiments according to the barrier-limited model of Goresky and Nadeau (18) implies that the probability of return of an immobilized particle to the vascular space is constant; therefore, demargination obeys Poisson statistics with monoexponential decay (48). In contrast, the present view of neutrophil margination implies that the time span of immobilization of a neutrophil depends mainly on the ratio of diameters between cell and capillary (23). In keeping with this view, pulmonary transit times of neutrophils observed by Lien et al. (34) showed a higher incidence of longer transit times (>6 s) than predicted by a Poisson distribution. Repploting of these data reveals a transit time distribution following a power law, resulting in a straight line on a log-log plot. Similar transit time distributions were also inferred for the human lung (21).

In the present work, we did not attempt to build a model to describe this behavior in detail. Instead, we used an approximate model that supposes that an immobilized cell may return to the circulation with a high probability that is independent of the time spent in the marginated pool or may become immobilized quasi-permanently with a probability that also does not depend on time. This procedure is based on the idea that, with the experimental method used, return of immobilized neutrophils to the vasculature can be observed only if it occurs very soon after immobilization. The average value of 0.05 s$^{-1}$ for the transfer coefficient of demargination and the ratio $k_{m-c}/(k_{m-c} + k_{m-s}) = 0.7$ obtained in this study can be interpreted in the sense that 70% of immobilized neutrophils have a mean time of immobilization of 20 s, whereas the other 30% have very long immobilization times. This interpretation is in line with previous observations by gamma camera scintigraphy of bimodal disappearance of labeled granulocytes from the human lungs after intravenous injection (47). The time constant of the fast component was ~25 s, a value compatible with a $k_{c-m}$ of 0.05, as reported here, whereas the second time

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**Fig. 5.** Pulmonary outflow profile of labeled neutrophils with theoretical resolution into the throughput (neutrophils that do not marginate), returning (neutrophils that marginate and demarginated during the first transit time), and recirculating components (neutrophils that transit through the systemic circulation and exit the lungs for the 2nd time), with the assumption of complete recirculation ($k_{s0} = 0$). First-pass outflow consists of the sum of the throughput and returning components. A–C: 3 experiments with various cardiac outputs demonstrate the more evident recirculation with higher cardiac outputs.

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constant with ~120 s would fall outside our observation window.

The usual kinetic analysis of multiple-indicator-dilution data has been applied to the handling of molecules or ions that can be immobilized at any point within the capillary bed. This implies that the probability that a cell is immobilized is proportional to the time spent within the capillary bed. With this assumption, the transfer coefficients for immobilization are flow independent, whereas the extraction ratio will decrease with increasing flow. In contrast, the present view of the mechanism of pulmonary margination of neutrophils by mechanical constraint within capillary segments implies that a neutrophil is immobilized with high probability whenever it enters a capillary segment with a diameter that is smaller than the diameter of the cell itself. In this case, the probability that a neutrophil is immobilized before leaving the capillary bed will depend on the number of such obstacles it may encounter within the capillaries and will be independent of the time spent within the capillary bed. In this case, first-pass extraction will be flow independent, whereas the transfer coefficients for immobilization will increase with increasing flow. Indeed, the observations that \( k_{e-m} \) depends on cardiac output, whereas the proportion of throughput does not, corroborates the latter hypothesis.

Our investigations yielded transfer coefficients for immobilization of neutrophils from the capillary space between 0.08 and 0.35 s\(^{-1}\). The probability that a neutrophil that enters the lung is immobilized depends on the transit time of a neutrophil through the capillary bed and can be estimated, with the use of a single-tube model, as \( 1 - \exp[-k_{e-m}(t_{alb} - t_0)/v_{rel}] \), where \( t_{alb} \) is the mean transit time of albumin. This approximate calculation yields an average probability of 0.8 ± 0.1, a value similar to 0.7 ± 0.05 obtained with the model of Goresky and Nadeau (18) (1 – the proportion of the throughput component). With the assumption of 50 capillary segments in a single capillary path (23), the probability for a single segment is \( 1 - \exp[-k_{e-m}(t_{alb} - t_0)/v_{rel}] = 0.034 ± 0.014 \). This means that 1 in every 30 capillary segments will be narrow enough to immobilize arriving neutrophils.

The peaks of the neutrophil curves generally lie below those for albumin and RBCs or are slightly delayed (Fig. 2). Accordingly, the model predicts a slower migration of neutrophils than of RBCs, in agreement with previous observations (27, 28). This may be explained by slower movement of blood in segments containing neutrophils that are not immobilized than in segments that contain no white blood cells.

The proportion of the transit time through the non-exchanging part of central circulation, \( t_0 \), was, at 3.8 s, larger than the average of 0.45 s, found by superposition analysis of octanediol (11), and ~2 s longer than the estimated transit time of the left heart (1.6 s). It was similar to pulmonary arterial transit times (between the pulmonary artery and subpleural arterioles) of ~2 s measured in anesthetized dogs, despite the fact that the venous part of the pulmonary transit times was not measured in these experiments. Again, this discrepancy might occur, because the subpleural part of the lung as visualized through a transparent window in the chest wall of an anesthetized animal might not be representative of the whole lung. However, theoretical considerations (11) suggest that the assumption of a simple delay within the nonexchanging part of the circulation entails that the calculated value of \( t_0 \) underestimates the true transit time of the nonexchanging vessels.

The second peak observed in neutrophil outflow profiles consists partly of recirculating cells. Because these cells have gone through the lung twice, the maximal possible number of these cells is rather low, and a considerable proportion of the tail part of the outflow profile must therefore be attributed to neutrophils that return to the vasculature after having been immobilized transiently. This opens the way for a rough estimation of the initial rate of release of immobilized neutrophils.

We observed no difference in the counts of unlabeled neutrophils between the pulmonary artery and the aorta, in agreement with previous observations (37, 45). This absence of an arteriovenous difference in the presence of ~50% single-pass neutrophil quasi-irreversible retention by the lungs demonstrates a quasi-steady state with simultaneous uptake and release of a quantitatively similar amount of neutrophils during the short time of the experiment.

Limitations of the Method

Although the model provided very good fits of the experimental data, it must be viewed as a first and simplified attempt of the description of first-pass neutrophil microcirculatory kinetics in vivo. The considerable variation of the fitted values of transfer coefficients precludes their use as indicators for putative alterations in neutrophil behavior in cross-sectional studies with varying physiological or pathological conditions. However, the results were fairly reproducible with repetition in the same animal, opening the possibility of assessing the effects of acute interventions in longitudinal studies where baseline conditions in the same animal serve as internal control.

One major difficulty in the application of this method is the occurrence of recirculation. Our approach was to estimate and eliminate the influence of recirculating labeled neutrophils. Direct assessment of neutrophil recirculation by sampling venous blood was, however, not included in the experimental protocol. Instead, we made the simplified assumption that neutrophils show a distribution of recirculation times similar to that of albumin, in the expectation that the true impact of recirculation will not exceed that calculated by using complete and rapid recirculation represented by the albumin curves. The appropriateness of this assumption is supported by right ventricular concentration-time profiles obtained by scintigraphy after intravenous injection in humans. Profiles obtained with \(^{99m}\)Tc-labeled neutrophils (46) were qualitatively similar in
shape to those obtained in a separate study with 99mTc-labeled albumin (3), both showing a flat recirculation response ~15 s after the main peak. An extension of the experimental protocol to venous sampling would certainly be useful for confirming or refuting this assumption.

A further limitation of the method is the use of neutrophils that have been manipulated in vitro by using procedures that cannot exclude partial activation. It has been reported that activation enhances immobilization of neutrophils due to stiffening of the cytoskeleton (14). This could cause increased marginalization with higher values of $k_{cm}$ and explain some of the variability of the data.

Conclusions

The major advantage of this approach is that study of the whole pulmonary circulation in vivo is possible. The results provided an adequate description of the microcirculatory behavior of neutrophils that was consistent with observations using video microscopy and with the hypothesis of mechanical entrapment of neutrophils in narrow capillary segments. It will be interesting to apply the analysis in future studies aimed at determining whether it could be a useful research tool to investigate the interactions between the pulmonary endothelium and neutrophils in physiological and diseased states.

The authors thank Nathalie Ruel and Sonia Lefèvre for technical assistance. This work was supported by the Canadian Institutes for Health Research, the Quebec Heart and Stroke Foundation, and the Fonds de recherche de l’Institut de Cardiologie de Montreal. J. Dupuis is a senior scholar from the Fonds de la recherche en santé du Québec.

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