Mapping 3-Dimensional Neovessel Organization Steps Using Micro-Computed Tomography in a Murine Model of Hindlimb Ischemia—Brief Report

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Objectives—Studying the mechanisms of neovascularization and evaluating the effects of proangiogenic strategies require accurate analysis of the neovascular network. We sought to evaluate the contribution of the microcomputed tomography (mCT) providing high-resolution 3-dimensional (3D) structural data, to a better comprehension of the well-studied mouse hindlimb posts ischemic neovascularization.

Methods and Results—We showed a predominant arteriogenesis process in the thigh and a predominant angiogenesis-related process in the tibiofibular region, in response to ischemia during the first 15 days. After 15 days, mCT quantitative analysis reveals a remodeling of arterial neovessels and a regression depending on the restoration of the blood flow. We provided also new mCT data on the rapid and potent angiogenic effects of mesenchymal stem cell therapy on vessel formation and organization. We discussed the contribution of this technique compared with or in addition to data generated by the more conventional approaches.

Conclusion—This study demonstrated that optimized mCT is a robust method for providing new insights into the 3D understanding of posts ischemic vessel formation. (Arterioscler Thromb Vasc Biol. 2009;29:2090-2092.)

Key Words: computerized tomography • peripheral vascular disease • angiogenesis • mouse model of human disease
All the 3D acquired images were recorded and the different parameters as vessel number, diameter, connectivity, vessel volume, and intervessel space were quantified. To assess the role of MSC in vessel formation, 500,000 MSCs were injected into the thigh and into the anterior tibial muscle 48 hours after surgery and the hindlimb vascular network was analyzed by microCT 8 days after surgery.

### Results and Discussion

#### Use of Neoprene Latex Allows Selective Arterial Filling

We developed and validated a new combination of micronised barium as contrast agent and Neoprene latex as suitable vehicle able to fill only arterial network within a diameter of 20 μm. The viscosity property distinguishes latex vehicle from other contrast agent vectors which more easily fill the whole vascular network (supplemental Results and Discussion section and supplemental Figure I).

#### 3D Quantification of Postischemic Arterial Vascular Growth in the Hindlimb

For the first time, we demonstrated a significant difference in ischemia-induced vascular growth mechanisms between the thigh and the tibiofibular regions as reported on Figure 1. mCT analysis demonstrated that the vascular response in the thigh is mostly attributable to arteriogenesis mechanism as described. At D15, the number of vessel and connectivity increased slightly, their diameter decreasing by two times, with no modification of the total arterial vessel volume in ischemic compared to non ischemic muscle. All these parameters return close to the baseline values by D28 when the perfusion defect was compensated (Figure 1A and 1B). In summary, mCT uncovered novel arterial remodeling data in the thigh. Other approaches as microangiography were limited by the low spatial resolution and the absence of quantitative volumetric analysis. Histological examination is rarely carried out in the thigh,

![Image](http://atvb.ahajournals.org/)

Figure 1. A, mCT imaging of neovascularization over time in mice after hindlimb ischemia on D0, D7, D14, D21, and D28 postoperatively. B, Quantification of mCT 3D images. Parameters are described in supplemental data. The results are the mean of n=8 observations for each time point. Data are mean±SEM. *P<0.05 and **P<0.01, ***P<0.001 vs NI. C, Arterial density in the tibiofibular region quantified by mCT was compared to the kinetics of blood flow recovery as evaluated by laser Doppler imaging and to capillary density as quantified by immunohistochemistry using the CD31 antibody. The three analyses were performed on the same animal; data are mean±SEM. *P<0.05 and **P<0.001 vs D7.

![Image](http://atvb.ahajournals.org/)

Figure 2. mCT images (A) and quantification (B) of the effect of mesenchymal stem cell (MSC) therapy on vessel formation. The results are the mean of n=6 observations for each condition. *P<0.05 vs control.
because of the variability in the degree of ischemia from one muscle to another, of the variations in diameter of medium-calibre arteries detectable only on mCT but not on immunohistochemistry images. Finally, measuring blood flow by laser Doppler is not applicable in this region.

In contrast to the thigh, angiogenesis predominated in the tibiofibular region. In the first 21 days, we showed a dramatic increase in arterial network density (9 fold), volume (6 fold), and vessel connectivity or branching (7 fold) with a decrease of the vessel mean diameter. All of these parameters are the hallmark for a dynamic angiogenic related process with a very dense and divided arterial network. For the first time, we evidenced a complex arterial vascular remodeling between D15 and D28 in the tibiofibular region, less pronounced in the thigh. The number, connectivity, and occupied volume of arterial vessels dropped considerably after D21 (Figure 1B), whereas CD31-positive capillary number and the blood flow gradually improved after 21 days (Figure 1C).

Thus, we propose that the arterial network adapts to tissue perfusion; the precocious development of large- and medium-calibre vessels in the thigh would favor the underlying arterial perfusion and angiogenesis on the tibiofibular region. We then reported after D21, in the tibiofibular region, an arterial neovessel regression whereas venous and lymphatic capillaries develop as evidenced by immunohistolabeling.

Application to the Quantification of an Angiogenesis-Focused Cell Therapy
To dissect the mechanisms of mesenchymal stem cell based angiogenic therapy,9 we examined and quantified by mCT the vascular network in MSC injected versus saline injected hindlimb. MSC graft induced a burst of neovessels as soon as D8, predominant in the tibiofibular region with an increase in the number, volume, and the connectivity of the arterial vessels but with no modification of the mean vessel diameter (Figure 2A and 2B). Blood flow increased significantly in the mice treated with MSCs (0.205 ± 0.10 versus 0.280 ± 0.17 for the ischemic/normal limb ratio in control versus MSC-injected animals at D8, respectively, P<0.05; not shown). In summary, these observations uncovered a precocious and potent angiogenic role of MSC as soon as D8, showing that MSC therapy acts essentially on angiogenesis.

Conclusions
See supplemental materials for a discussion of the findings in this report in relationship to relevant articles in the literature.

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Disclosures
None.

References
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Supplementary data

**Supplementary Materials and Methods section:**

**Animals**

For the post-ischaemia kinetics study, we used male C57Bl/6 mice from Charles River Laboratories. The mice were between 10 and 12-week old, and were housed 4 to 6 per cage. Food and water were available *ad libitum*, with a 12-hour dark/light cycle. This study was conducted in accordance with both institutional guidelines and the guiding principles in force in the European community for the use of experimental animals (L358-86/609/EEC).

**Model of ischemia – Preparation of the animals**

In order to study the formation of neovessels, we used the mouse hindlimb ischemia model as previously described \(^1\), involving ligation of the proximal portion of the femoral artery and of the distal portion of the saphenous artery, and resection of the artery and its branches. Two hours before injection of the contrast medium, the mice received an intraperitoneal injection of a mixture of antiplatelet drugs (acetylsalicylic acid, 75 mg), vasodilators (molsidomin, 1 mg), and anticoagulants (heparin, 300 IU/kg).
**Mesenchymal stem cells culture and characterisation**

Bone marrow cells were harvested and cultured as previously described. 500,000 MSCs were injected into the thigh and into the anterior tibial muscle 48 hours after surgery. The control group is the group that received only MSC’s conditioned medium instead of MSC. Laser Doppler analysis was performed 8 days after injury and then the animals were sacrificed, the contrast medium injected, and the scan performed 8 days after inducing the ischemia.

**Histological examination and immunohistochemistry**

After acquisition of the images using the mCT scanner, the anterior tibial muscle and the quadriceps were removed and embedded in paraffin. Immunohistochemistry was performed as previously described using a rat monoclonal antibody, anti-CD31 (BMA).

**Laser Doppler**

The laser Doppler apparatus used was a LDI device from Moor Instruments. The mice were anaesthetised by intraperitoneal injection of a combination of Rompun® (xylazine) and Imalgene® (ketamine), and the examination was carried out on D7, D14, D21, and D28, as previously described. Image acquisition was performed at a speed of 10 msec/pixel. Mean limb perfusion was calculated automatically by the Moor LDI program, after manually producing a contour of the region of interest on the foot. Perfusion was expressed as the ratio of perfusion on the ischemic side to perfusion on the non-ischemic side.

**mCT Sample preparation**

The mice euthanized by a lethal injection of pentobarbital, were perfused via the brachiocephalic arterial trunk first with a heparinized isotonic solution and then with a 1% paraformaldehyde solution, followed by a mixture of 80% Neoprene latex (Neoprene Latex Dispersion 671 A, Dupont, France) and barium sulphate powdered to 1 µm (3 g/mL, MicrOpaque® oral solution, Guerbet, France). At the end, the mouse was put into acid
solution for the latex to harden, then, after dissection, the organs were fixed overnight in 4% PFA at 4°C. To study the progression of the contrast medium through the arterioles and capillaries during histology, an inert blue dye was mixed with the Neoprene latex and barium sulphate

**mCT scanner data processing and analysis**

*mCT scanner and acquisition technique:* we used the eXplore Locus Micro-CT scanner from General Electric Healthcare® with spatial resolutions of 36 to 7 µm, with the Scan Control® and Reconstruction Utility® programs. We optimised the voltage parameter to 40 kVp with a mean current of 80 µA for a good signal-to-noise ratio compromise. Our acquisition protocol consisted of 360 views and 4 images per position, with a voxel volume of 16 µm³. The quantification parameters were obtained using the Microview ABA® program. We then delimited and digitally deleted the bones of the hind limb and used the density of our contrast medium as a reference for the program. This technique proved to be more effective than using formic acid to decalcify the bone structure since acid baths tend to deform the tissues. This technique also allows a lower threshold to be used which is constant from one animal to another, given the maximum contrast provided by the barium sulphate, and therefore allows higher quality images to be obtained. The variability introduced by the micro-CT method was assessed by performing repeated scans, reconstructions, and quantitative analyses of iso-intensity surfaces on the same arterial specimen. Using an overlay, we showed the reproducibility of the injections (data not shown). We repeated mCT analysis protocols on a large number of hindlimb samples obtained after ischemia at different time points (n=15/group). All the 3D acquired images were recorded and the different parameters as vessel number, diameter, connectivity, vessel volume and inter-vessel space were quantified. We observed very similar results with a very low variability of the measurements in each group (Figure 1B).
Accuracy and reproducibility of mCT: the flexibility of mCT allows comparative studies to be carried out between vascular beds as well as between mutant mouse lines. When used in combination with software for automatic detection and measurement of vessels, the overall pattern of a vascular bed can be characterised in a statistical manner. This method will therefore provide a powerful tool for detailed study of the changes in structure of mouse hind limbs in response to ischaemia. The method was found to generate data with sufficient accuracy and reproducibility to reveal new quantitative information on pathophysiology and therapeutic neovessel formation.

Quantification parameters: the following parameters were obtained using the Microview ABA® program.

1. Vascular density (number of vessels/$\mu m^3$): the number of vessels was calculated using an independent modelling method in order to assign a thickness to the 3-D images. To calculate the number of vessels, the ROI was skeletonized by separating the voxels, passing through the median axis of the vessels. The number of vessels was thus defined as the inverse of the mean space between the median axes of the vascular structure of the ROI.

2. Mean diameter of the vessels (in $\mu m$): in order to calculate the diameter, the program defined a local thickness at each point in the ROI, calculated as the diameter of the largest sphere both containing the point and occurring completely within the structure of the same density. The mean of these data was then obtained in the three spatial planes. The mean diameter of the vessels was calculated as the ratio of the sum of the diameters of all the vessels in a given area to the number of vessels. In our study, density is expressed in $\text{number/} \mu m^3$, and mean diameter in $\mu m$.

3. Connectivity (Euler's number, in Arbitrary Units): connectivity was determined using Odgaard and Gundersen’s method, which is based on the Euler characteristic for studying the edges and vertices of 3-D structures. By definition, connectivity is the maximum number of sides or branches which can be broken within a structure before it is divided into two
separate parts. In our study, connectivity reflects vascular collateralility, with the higher the value the greater the number of collaterals.

4. The mean space between the vessels (in µm): is defined as the inverse of the mean spacing

5. The area occupied by the vessels to the total area (in %)

These parameters are commonly analysed when studying the microstructure of trabecular bone. For these parameters, we defined “regions of interest” (ROI) in both the thigh and tibiofibular areas, where the analysis was conducted to study the responses to ischemia. The thigh region was from the proximal suture to the superior border of the patella, while the tibiofibular region was from the superior border of the head of the fibula to the union between the middle third and distal third of the tibia.

Statistical analysis

All data were expressed as means ± SEM. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with a post-hoc Fisher’s test. The statistical difference between the two groups was examined using the Student’s unpaired t-test after confirming that the variance of data was not heterogeneous or Mann/Whitney test for small samples. Differences were considered to be significant for p values of less than 0.05.

Supplemental results and discussion section:

Latex is a suitable vehicle to define selectively arterial network

Above all, latex has suitable viscosity properties for filling the arterial vascular beds, precisely demarcating the architecture. Because of its viscosity (50 centipoises), only the arterial system is injected when latex is perfused at physiological pressure into the arterial network.
(brachiocephalic arterial trunk). The arteries and arterioles are filled with the latex while the veins and venules are never filled. We used an inert dye mixed with the latex to color the arterial network (blue color). First, a macroscopic observation was done to verify that only arteries were filled but not veins and was completed by microscopic observations (supplementary Fig1). We labeled CD31 positive vessels and rigorously checked on 50 serial sections/samples (n=4) that CD31 positive vessels of more than 20 m were filled.

Simply changing the pH allows it to harden permanently. Latex is easy to handle and inject into small animals, it is difficult to saturate with contrast medium, and avoids the presence of sediments once homogenised. Barium has been used as a contrast agent with good radiopacity. By using the micronised solution, we avoided the contrast agent being deposited in the vessels, so that the vessels could be filled uniformly. There was no diffusion out of the vessels after the death of the animal, and no vessel trauma was observed. An important point concerned the preservation of injected vessel structure. We set up a standardized protocol to avoid alterations in vessel structures described in details in mCT sample preparation paragraph. Before the injection of the contrast agent, the mice received vasodilators and anticoagulants. After euthanasia, we injected in the vasculature a solution of 1% paraformaldehyde. Then, the mixture of latex and barium was gradually injected under a pressure of 1 meter of water in the vascular network. Furthermore, we conducted parallel experiments injecting the vascular network with more diluted latex solution and found that the vessel diameter were not different from whose injected with a 80% solution (not shown).

There was no shrinkage of the latex upon fixing, and consequently no deformation of the vessel, which renders quantification possible, as demonstrated by the reproducibility of the results.

The viscosity property distinguishes latex vehicle from other contrast agent vectors, which more easily fill the whole vascular network

By using an inert dye, it was possible to identify and locate – both macroscopically and microscopically – the injected vessels in tissue sections. The macroscopic photos show
selective arterial filling of all large- and medium-calibre arteries (supplementary Figure 1A). Because of its inert properties, a lead-containing radiopaque silicone rubber called Microfil has been largely reported in vascular mCT studies\(^5\). However, due to its low viscosity, this compound completely fills the arterial vasculature and flows freely from the veins\(^9\)\(^-\)\(^12\). Gelatin at 5-10% has also been used as a vehicle compound with contrast agents, but it needs to be warmed (42°C) before injection and kept at this temperature while injected, and needs to be chilled on ice to allow solidification throughout the organism\(^10\)\(^,\)\(^13\). No arterial and/or vein specific filling has been reported with gelatin. In parallel to the demonstration of an agent specifically labelling the arterial network, we had to demonstrate that the latex also filled small-calibre arterial vessels. The histological sections show that most arteries and arterioles down to a diameter of 20 µm are filled with the contrast medium, and this degree of filling is constant from one animal to another at each of the post-ischaemia kinetic time points (Supplementary Figure 1B). Capillaries with diameters less than 10 µm are only rarely filled with the contrast agent, as demonstrated on CD-31 immunolabelling sections (Supplementary Figure 1B).

In order to quantify the post-ischemic neovascular growth, we removed the bone mineral structure, by computer delimitation, on the 2-dimensional images. This technique proved to be more effective than using formic acid to decalcify the bone structure since acid baths tend to deform the tissues\(^5\). This technique also allows a lower threshold to be used which is constant from one animal to another, given the maximum contrast provided by the barium sulphate, and therefore allows higher quality images to be obtained. The variability introduced by the mCT method was assessed by performing repeated scans, reconstructions, and quantitative analyses of iso-intensity surfaces on the same arterial specimen. Using an overlay, we showed the reproducibility of the injections (data not shown).

### 3D quantification of post-ischemic arterial vascular growth in the hindlimb

The purpose of this study was to gain a better understanding of the dynamic formation of the arterial network on a well-known hindlimb ischemic model of angiogenesis. Many groups as
Dr W. Shaper pioneer group have made tremendous effort to clarify the term arteriogenesis in comparison to angiogenesis, two types of processes that occur in response to ischemia. Duvall CL and coworkers analysed also extensively arteriogenesis process from D0 to D14 after ischemia in a murine hindlimb model using mCT data. Angiogenesis is defined as the growth of new capillaries and should be used to describe the process whereby preexisting capillaries proliferate and sprout to form new capillary networks. Arteriogenesis would be an ischemia-independent mechanism to compensate for the occlusion of a major artery. Our study employed an extensive time course and a combination of 3D mCT acquired images, immunohistochemistry and hemodynamic measurements to allow for a complete representation of the arterial network formation in response to hindlimb ischemia. One of the most innovative aspects of mCT analysis has been the evidence of complex arterial vascular remodelling between days 15 and 28, affecting the density and connectivity of the arterial capillaries with a great increase of branching in the tibiofibular region after ischemia at D15 (Fig 1B). These features could be related to an angiogenic related phenomenon. Here we showed that the tremendous increase of smaller vessels (diameter decrease) at D15 did not correlate with the improvement of blood flow that occurs from D15 to D28. In fact, the number of arterial vessels, the volume occupied by the arterial network and their connectivity in the tibiofibular region decreased considerably after 21 days. These data support and reinforce the concept that capillary sprouting support the local perfusion of ischemic tissue although arterioles or large arteries are necessary to provide enough amounts of blood flow to the ischemic limb.

This arterial remodelling, which was greater in the tibiofibular region than in the thigh, occurred in parallel with the improvement in blood flow shown by laser Doppler. The kinetics of the recovery of blood flow in the ischemic limb conformed to data published by our laboratory and others, with a gradual improvement in perfusion over 21 days, reaching a recovery plateau between days 21 and 28 (Figure 1A). The development of large- and medium-calibre vessels in the thigh gives rise to a significant increase in blood flow and tissue perfusion, favouring the underlying angiogenesis, which indicates that the arterial
network adapts to tissue perfusion. The perfusion studied by laser Doppler focused on the tibiofibular region depends in part on the development of neovascularisation in the thigh. Formation of medium-size collaterals has been shown to be a major factor to recovery blood flow after femoral artery ligation \(^5,^{18}\). Delayed arteriogenesis has been evaluated in different pathological conditions as hypercholesterolemia. Demarcation of the thigh to the calf using mCT 3D images allowed us to discriminate arteriogenesis from angiogenesis process during vascular injury reparation \(^{13}\). Duvall and colleagues obtained very interesting clues on limb revascularization elucidating a role for osteopontin in the development of functional collateral blood flow \(^{19}\). All these pioneer studies provided morphological parameters, quantitative data, volumetric vessel analyses along the 2 weeks after injury. Despite these significant results on the comprehension of vessel formation, these studies have not been able to discriminate arterial vs venous network, thigh vs calf arterial network evolution and to report the arterial remodelling that occurs after D21. Here we showed that the arterial remodelling shown by mCT after 21 days developed in parallel with an increase in capillary density shown by immunolabelling. CD31 labels arterial, venous, and lymphatic capillaries (Figure 1) and allows the capillary density to be calculated. We propose that the increase in capillary density after D21 occurred through an increase in the number of venous and lymphatic capillaries (evidenced by CD31 immunolabelling), whereas arterial vessels regressed as shown by mCT.

**Hallmarks of mesenchymal stem cells on angiogenesis process**

The angiogenic properties of mesenchymal stem cells (MSCs) have been largely reported \(^2,^{20-22}\). A large study has been conducted showing that multipotent progenitor cells can treat ischemic damage in peripheral ischemic disease \(^{23}\). Here we wanted to qualify MSC-induced vascular growth. In the tibiofibular region, the number of arterial vessels increased by 50% in 6 days, compared with animals injected with saline. In parallel, the capillary density calculated by immunohistology increased by 36% in the muscles treated with MSCs compared with control animals (355 ± 202 vs 482 ± 256 CD31-positive vessels/mm\(^2\)) (Figure
2A and 2B). The mean vessel diameter, measured using the mCT technique, did not differ significantly in the mice injected with MSCs. The volume filled by the arterial vessels in the tibiofibular region increased twofold in 6 days in the mice treated with MSCs compared with control animals. Neovessel connectivity increased by 42% in 6 days after injection of the MSCs. (see the 3D movies of ischemic hindlimb treated with MSCs or w/o cells, available online). The increase in capillary density is more gradual when analysed by histology compared with the mCT analysis of arterial capillaries, although the same animals were used in this experiment, and the histological capillary density was assessed after mCT analysis (Figure 2). Histology in random, limited sections only provides a two-dimensional analysis and is not necessarily representative of neovascularisation throughout the arterial vascular tree.

**Conclusion**

The flexibility of mCT allows comparative studies to be carried out between vascular beds as well as between mutant mouse lines. When used in combination with software for automatic detection and measurement of vessels, the overall pattern of a vascular bed can be characterised in a statistical manner. This method will therefore provide a powerful tool for detailed study of the changes in structure and hemodynamics of mouse hind limbs in response to ischemia. The method was found to generate data with sufficient accuracy and reproducibility to reveal new quantitative information on pathophysiology and therapeutic neovessel formation.

**Acknowledgment**

mCT images were generated at the *Plateforme d’Innovation Biotechnologique* (Biotechnology Innovation Platform) of Xavier Arnozan Hospital in Pessac, France.

**References**


Supplementary legend

Suppl Figure 1: Latex has suitable viscosity properties for filling the arterial vascular beds, down to small-calibre arterial vessels. The arteries and arterioles are filled with the latex while the veins and venules are never filled.

By using an inert blue dye mixed with the latex, it was possible to identify and locate both macroscopically (A) and microscopically (B) the injected vessels in tissue sections.

(A) Injection of the coloured latex solution without washing the vasculature with saline in order to visualise the blood remaining in the veins. Arterial-specific filling was demonstrated.
(B) The histological sections show that most arteries and arterioles down to a diameter of 20 \( \mu \text{m} \) are filled with the contrast medium and that the filling degree is constant from one animal to another.

**Supplemental data:**

3D movies of ischemic hindlimb at D8 after ischemia, treated either with MSCs (ischemia + MSC) or w/o cells (ischemia without cells).