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# **Mapping 3-dimensional neovessel organization steps using micro-computed tomography in a murine model of hindlimb ischemia-brief report**

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## **Abstract**

Studying the mechanisms of neo vascularisation and evaluating the effects of proangiogenic strategies require accurate analysis of the neovascular network. We sought to evaluate the contribution of the micro-computed tomography (mCT) providing high-resolution three-dimensional (3D) structural data, to a better comprehension of the well-studied mouse hindlimb post ischemic neovascularisation. We showed a predominant arteriogenesis process in the thigh, and a predominant angiogenesis process in the tibiofibular region, in response to ischemia during the first 15 days. After 15 days, mCT quantitative analysis reveal a remodelling 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 organisation. We discussed the contribution of this technique compared with or in addition to data generated by the more conventional approaches. This study demonstrated that optimised microCT is a robust method for providing new insights into the 3D understanding of post-ischemic vessel formation.

**MESH Keywords** Animals ; Barium ; Contrast Media ; Disease Models, Animal ; Hindlimb ; blood supply ; Imaging, Three-Dimensional ; Ischemia ; pathology ; surgery ; Mesenchymal Stem Cell Transplantation ; Mice ; Neoprene ; Neovascularization, Physiologic ; Peripheral Vascular Diseases ; pathology ; surgery ; Tomography, X-Ray Computed ; methods

## **INTRODUCTION**

As mouse models are largely used to study mechanisms of neovascularisation, vascular repair and growth of collateral vessels<sup>1</sup>, a throughout analysis of the vascular network has gained of interest. Understanding neovessel formation mechanisms requires a complete view of the vascular architecture over the whole of the tissue analysed: size, orientation, branching and organisation of collaterality. However, the classical methods of assessment are not always quantitative, restricted to a limited area of view, evaluate capillary density in 2D sections or report superficial blood flow data<sup>1</sup>. Hence, micro-computed tomography (microCT) can, after the injection of a radiopaque contrast agent, image the vascular network in 3D in an entire organ<sup>2, 3</sup> and give quantitative data. Several studies have investigated the microvascularisation of the kidney, heart, and liver in the rat<sup>4-6</sup>. Only a few teams have used quantitative tools for studying post-ischemic angiogenesis in the mouse<sup>2, 3</sup>.

Here, we used a contrast agent and microCT acquisition procedure dedicated to analyse post-ischemic kinetics of vessel formation and remodelling restricted to arterial network in the mouse hindlimb. We discussed the contribution of these images compared with or in addition to data produced by the conventional laser Doppler and immunolabelling approaches. Our results revealed a predominant arteriogenesis process in the thigh and in contrast a predominant angiogenesis in the tibiofibular region. We showed that neovessel remodelling depends on the restoration of the blood flow. We then provided new data on the mesenchymal stem cell therapy on the feature of vessel formation and organisation.

## **MATERIALS AND METHODS**

Materials and methods related to MSC culture, animal care, model of ischemia, micro-CT data processing and analysis, histological vascularisation quantification appear in the supplementary materials and methods section.

## **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 (supplementary results and discussion section and supplementary Fig 1).

### 3D quantification of post-ischaemic arterial vascular growth in the hind limb

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 Fig 1 . mCT analysis demonstrated that the vascular response in the thigh is mostly due to arteriogenesis mechanism as described<sup>2, 3</sup> . 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, microCT uncovered novel arterial remodelling data in the thigh. Other approaches as microangiography were limited by the low spatial resolution and the absence of quantitative volumetric analysis<sup>7, 8</sup> . Histological examination is rarely carried out in the thigh, 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 microCT 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 (5 fold). All of these parameters are the hallmark for a dynamic angiogenic process with a very dense and divided arterial network. For the first time, we evidenced a complex arterial vascular remodelling 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 (Fig 1B ) while CD31-positive capillary number and the blood flow gradually improved after 21 days (Fig 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 favour the underlying arterial perfusion and angiogenesis on the tibiofibular region. We then reported after D21, in the tibiofibular region, an arterial neovessel regression while venous and lymphatic capillaries develop as evidenced by immunohistolabelling.

#### Application to the quantification of an angiogenesis-focused cell therapy

To dissect the mechanisms of mesenchymal stem cell based angiogenic therapy<sup>9</sup> , we examined and quantified by microCT the vascular network in MSC injected vs saline injected hindlimb. MSC graft induced a burst of neo vessels 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 ( $0.205 \pm 0.10$  vs  $0.280 \pm 0.17$  for the ischemic/normal limb ratio in control vs 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

#### Acknowledgements:

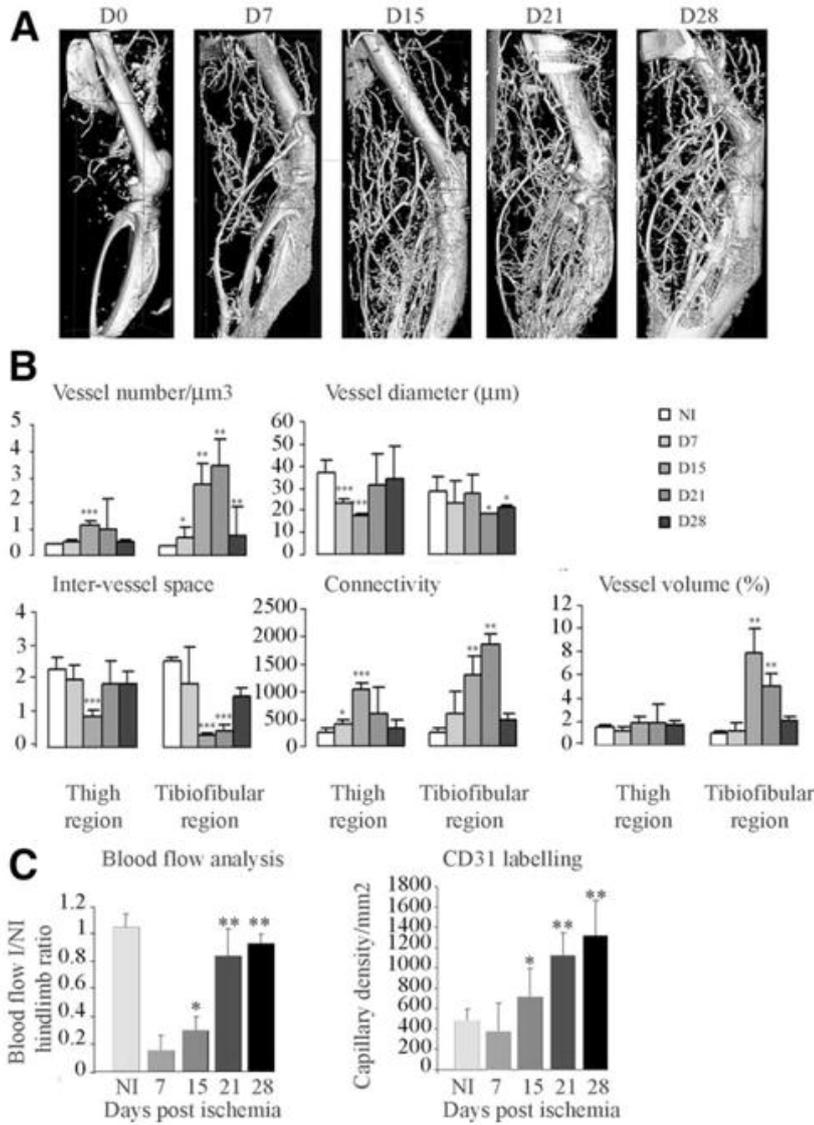
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**Figure 1**

(A) Micro-scanner imaging of neovascularisation over time in mice after hindlimb ischaemia on D0, D7, D14, D21, and D28 post-operatively. (B) Quantification of microCT 3D images. Parameters are described in supplemental data . The results are the mean of n=8 observations for each time point. (C) Arterial density in the tibiofibular region quantified by microCT 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 animals.



**Figure 2**

MicroCT 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.

