

Cross-sectional Vascularization Pattern of the Adipofascial Anterolateral Thigh Flap for Application in Tissue-engineered Bone Grafts

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Background: As part of the engineering of bone grafts, wrapping constructs in well-vascularized tissue, such as fascial flaps, improves bone formation. Our aim was to understand the cross-sectional vascularization pattern of human adipofascial flaps for this application.

Methods: Seven adipofascial anterolateral thigh (ALT) flaps were harvested from five human cadaveric specimens. Axial vessel density was analyzed by immunohistochemistry and quantitative histology.

Results: We found a high density of blood vessels directly superficial to and close to the fascia. A secondary plexus in between this first suprafascial plexus and the subdermal plexus was also identified. In all specimens, this second plexus showed less vascular density, and appeared to be at a constant level within the suprafascial fat throughout the flaps. The peak measurements for this secondary plexus varied between 1.2 and 2 mm above the deep fascia, depending on the donor's body mass index.

Conclusions: Quantitative immunohistochemistry is a reliable method to quantify and locate vessel density in an adipofascial flap. This is vital information before wrapping nonvascularized material into such a flap to estimate the inosculation potential of these vessels and likelihood of survival of the tissue. To profit from both suprafascial vascular plexuses, a correlation between subcutaneous tissue thickness and distance of the second plexus to the fascia should be further investigated. For the moment, we recommend maintaining at least 2–3 mm of subcutaneous fatty tissue on the fascia, to profit from both plexuses. Engineered constructs should be wrapped on the superficial medial side of the fascial flap to enhance vascularization. (*Plast Reconstr Surg Glob Open* 2022;10:e4136; doi: 10.1097/GOX.0000000000004136; Published online 22 February 2022.)

INTRODUCTION

A critical size bone defect is that at which the bone is unable to fill a void by healing itself without importing additional bone from other areas. These defects occur

after trauma, tumor resection or infection. Commonly, the problem is the absence of sufficient bone to be supported by the local microenvironment. The most reliable treatment approach is the transfer of autologous vascularized bone grafts by microsurgical transfer.¹

However, vascularized autologous bone grafts are associated with complications like stress fractures, pseudoarthrosis, longtime immobilization, and donor site morbidity.² The quantity of available bone for transfer is therefore often insufficient and appropriate treatment alternatives are necessary. A promising solution is the tissue engineering of bone grafts.

One of the main challenges of bone tissue engineering is achieving sufficient vascularization at the defect site.³ Scheufler et al have shown that bone engineering is improved by increasing vascular supply to the implanted grafts. In their study in rabbits, an improved vascular supply was established by wrapping cell-seeded ceramic scaffolds

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in a panniculus carnosus flap. This is a thin striated muscular layer underneath the skin of most mammals, similar to the platysma on the human neck.⁴ According to their promising results, identifying similar flaps in homo sapiens for future translation of the technique into a human being would be necessary.

Such a flap has several requirements: low donor morbidity, comfortable location, sufficient size, and stable, well-defined vascularization. The distance between vessel in the flap and cell-seeded construct must be minimized to enhance nutrition of the cells by diffusion. Fascial flaps seem therefore ideal, as they allow wrapping the engineered construct in maximally vascularized, thin and pliable tissue.

We considered the adipofascial flap of the anterolateral thigh (ALT) an ideal candidate as a neoperiosteum for future tissue-engineered bone construct implantations. The ALT flap is well described to cover large soft-tissue defects in head, neck, extremities, and trunk.⁴

Even though the axial vascular anatomy and angiosomes of the thigh are well elucidated,⁵ only a few studies detail the cross-sectional vascular structure of this adipofascial flap. For wrapping cell-seeded constructs, the cross-sectional distribution of the vascular network in the fat surrounding the fascia is of greater importance to decide on the thickness of the flap and how to wrap the construct best.

The aim of this study was to gain insights into the vascularization pattern of the adipofascial ALT flap for the future use of vascularizing tissue-engineered bone constructs. To achieve this we searched a new method to quantify the cross-sectional vascularization density in cadavers more accurately.

MATERIALS AND METHODS

Plastic surgery fellows dissected fresh human cadaveric specimens for this anatomical study. We included deceased adult patients from our university hospital within 3 days, who provided consent for the use of their bodies for science. No restrictions for sex, age, or comorbidities were made. Limbs with prior surgery or trauma involving the soft tissue or the arterial vessels of the thigh were excluded. To document the soft tissue mass, the body mass index (BMI) was recorded. The study protocol was approved by the local ethics committee.

Flap Dissection

The dissection largely followed the known techniques for fasciocutaneous ALT flaps.^{4,6} The main difference was, that the flap was harvested through an anterolateral skin incision instead of a skin excision. The skin was elevated in a subdermal plane. The adipofascial flap was outlined to include the maximum size of fat and fascial tissue of the anterolateral thigh reaching from the lateral aspect of the vastus lateralis to the medial border of the rectus muscle. The tissue was carefully elevated toward the intermuscular septum from these edges. Under loupe magnification, the vessels, either musculocutaneous or septocutaneous perforators, running toward and into the fascia were analyzed.

Takeaways

Question: Is the adipofascial anterolateral flap suitable to wrap and vascularize tissue-engineered bone constructs?

Findings: We found that constructs will be wrapped best in a flap with at least 2–3mm of subcutaneous fatty tissue on the fascia and should be wrapped on the superficial side of the medial part of the flap.

Meaning: The ALT adipofascial flap is providing enough vessels to be a suitable candidate to vascularize tissue-engineered constructs.

The clinically relevant ones for perfusion of the flap were preserved, and side branches into the muscle were ligated. The vascular pedicle was identified, followed, and liberated from its origin on the lateral circumflex artery. The flaps were conserved in a formalin bath and stored in the fridge. We analyzed the cross-sectional vascularization pattern by quantitative histological analysis to gain insights on vessel density and the level of the vascular plexus within the flap.

Immunohistochemistry

Five evenly distributed biopsies were taken of each flap after 2–4 weeks in the formalin bath. A distal medial and lateral, a proximal medial and lateral, and a central full thickness biopsy of 3 cm² were cut out of the flaps by LW, to include the middle, the most likely best perfused area, and the more peripheral areas. They were numbered as shown in Figure 1 and coded.

After dehydration, the samples were embedded in paraffin and sectioned. Then, the fully automated immunohistochemistry process for vessel identification started. Coloring was changed until digital analysis showed consistent results without disintegration of the histological structure. The final process is shown below:

- Deparaffinization of the slides; enzymatic slide preparation by proteases (enzyme-kit by Leica Microsystems GmbH Wetzlar, Germany: Enzyme1);
- Application of the CD31 Antibody (antibody clone: JC70A, by Dako, Baar, Switzerland.);
- Application of a polymer (polymer: Novocastra Bond Polymer Refine Red Detection, based on an alkaline phosphatase by Leica Microsystems GmbH Wetzlar, Germany).
- The reaction caused by adding substratechromogen (Fast Red by Dako, Baar, Switzerland) visualizes the complex as reddish precipitate.
- Fast dehydration and covering of the slides with the mounting medium Pertex concluded the procedure.

Quantitative Histology

To ensure data quality, we defined three standards as inclusion criteria for quantitative graphical analysis of the slices:

1. Intact lamination after the immunohistochemical processes,
2. Fascia providing a straight line of at least 4mm length on the slide, and

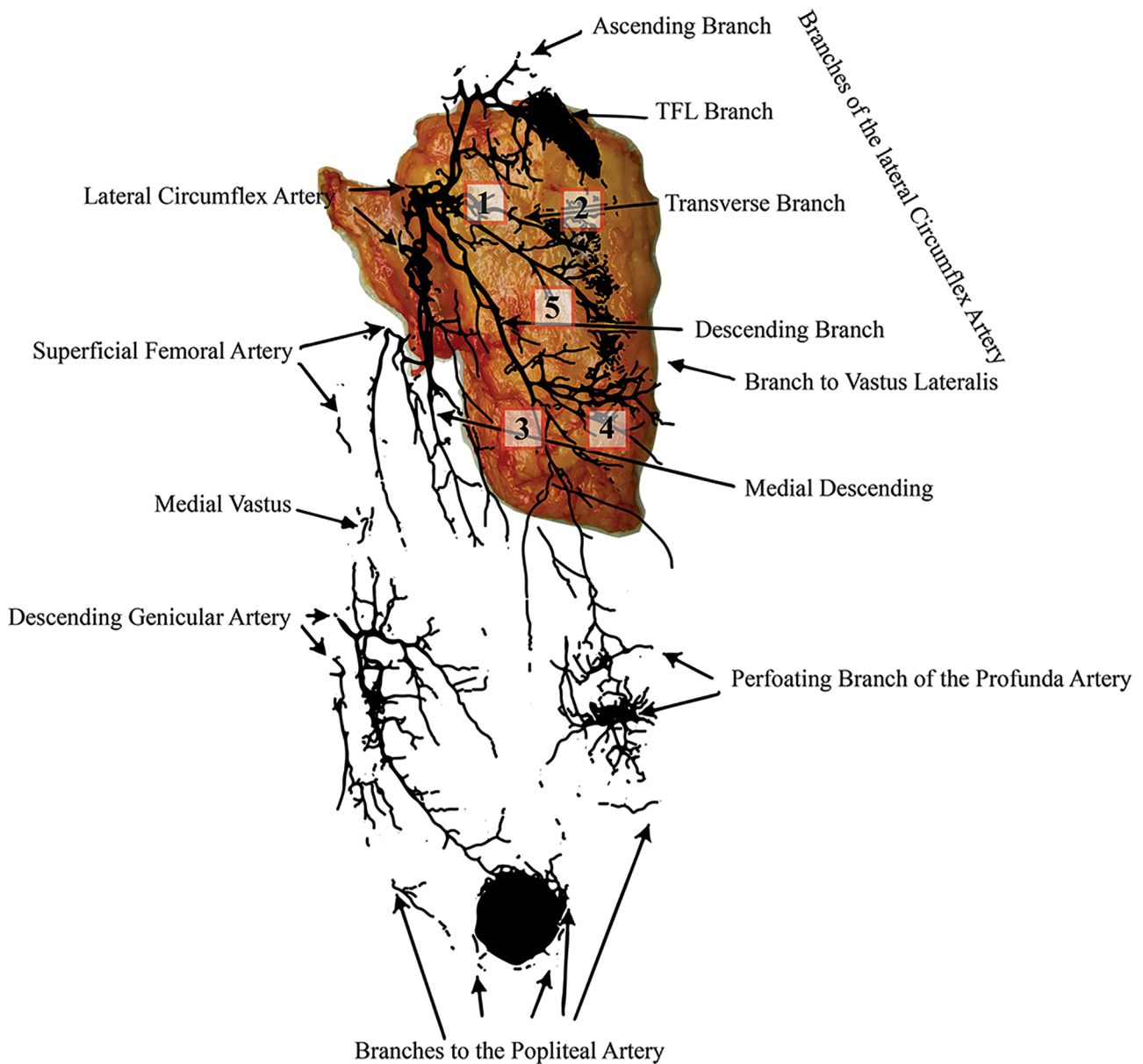


Fig. 1. Superposed angiosome of the anterolateral thigh onto a harvested adipofascial ALT flap with indicated locations of biopsies.

3. Vivid coloring, homogenously spread.

We used a Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany) linked to the program CellP (Olympus Hamburg, Germany) with attached digital photograph camera to digitalize the slides. Pictures of each slide were taken under twofold magnification, and afterward joined together with the CellP merge function. Using Photoshop (ADOBE San Jose, Calif.), red-colored pixels were separated from the surrounding pixels. The selection was then inversed and cut out, resulting in a picture reduced to the red-colored endothelium in cross section on a white background. Tracing of the fascial rim was performed with the Pixelmator graphics software (ver. 2.0.2; Pixelmator Team Ltd., London, UK), and then combined with the picture of the vasculature. This procedure

enabled the identification of the fascia on the sheer-vasculature-image and served as reference level for measuring distances.

Slammer (Ringce, Chromepet, Southern India) was used to subdivide the picture into rectangles of a predefined size with a grid. With ImageJ (<http://rsbweb.nih.gov/ij/>), the thresholding function was used to select all the pixels, except the pixels depicting the grid. A measuring field with the size of 0.2mm height × 0.8mm width was created according to the previously defined grid, and the percentage of pixels below the threshold was set. Thus, all red-colored pixels of each box were measured. Due to the undulating course of the fascia and the common distortion of the tissue layers caused by the processes of immunohistochemistry, we decided to measure a small range of 4mm in width. In height, the

slices were analyzed as far as the staining reached. Excel (Microsoft Office, Microsoft, Seattle, USA) was used for data collection and analysis.

RESULTS

Seven flaps from five cadaveric specimens were dissected in this study (Table 1). The mean age of the cadavers was 80.8 years and the mean BMI 24.1 kg per m². Five biopsies were taken of each flap, and one slice (the qualitative best) was taken from each biopsy; this resulted in 35 stained slices (Fig. 2).

The immunohistochemical staining with the blood vessel-specific endothelial antigen CD31 enhanced the contrast, and it highlights vascular tissue against the surrounding tissue. This was essential for digitalization and analysis.

In a first step, CD31 antigens were colored brownish and combined with a blue colorization of the cell cores as counterstain. This provided too little contrast of vessel to surrounding tissue for standardized graphical analysis. Therefore, staining with a red dye without counterstaining was performed, achieving maximum contrast.

Further, we observed a qualitative failure of the staining if the flaps were preserved in formaldehyde for 4 weeks. Flaps only conserved for 2 weeks had a good contrast and colorization.

In the end, 17 of 35 slices, provided by four flaps harvested from two donors, were of sufficient staining quality, displaying sufficient information for graphical analysis while preserving their original lamination. In terms of vascularization pattern, all four flaps showed a peak value between the fascial edge and 0.5 mm, and in 13 of the 17 biopsies, a second peak of dense vasculature was present more superficially (1–3 mm above the fascial rim)

Figure 3 displays differences of vascularization density above the fascia on the different biopsy locations.^{1–3,6} The proximal and the distal medial mean values show almost identical first peak values. The lateral biopsies show lower but also similar first peaks. The mean value of the central biopsies shows a much higher vessel density than the average of the peripheral biopsies (Fig. 4).

Figure 5 displays the summarized mean values of each flap, and it thus demonstrates the inter-individual variation of the vascularization patterns. Specimen 5 has higher first peak levels and the second peaks occur later compared with the levels of specimen 4, even though both patients are thin male adults.

Figure 6 shows a comparison of the average of all the slides per donor. It reveals the peripheral vascular plexus plane at a peak 2 mm above the fascia in specimen 5 at a BMI of 22.3 kg per m². The flaps of specimen 4 show the

vascular plexus plane at 1.2 mm above the fascia, in a body with a BMI of 18.3 kg per m². We found the values of specimen 4 to be generally lower.

DISCUSSION

With our newly established method for vessel density measurement within flap tissue, we were able to detect a high density of CD31-positive cells in the biopsies directly above the fascia, and a second density-peak a few millimeters further above. These findings correspond to the already known vascular networks within ALT flaps.⁷ We consider our method therefore as reliable to quantify vascular networks and to locate them in an adipofascial flap.

To our knowledge, it is the first time quantitative immunohistochemistry is used to examine vascular density patterns in a flap. Compared with perfusion measurements by CT scans or MRI,⁷ this method gives a more detailed estimate of the number of vessels at a certain level within the cross-sectional anatomy of the flap. This is important to evaluate the inosculation potential of these vessels into adjacent avascular tissue in a clinical setting, or when wrapping tissue-engineered constructs. It is less precise to define the perfused area of a certain angiosome which is highly dependent on blood pressure and local regulation.

Our immunohistochemical vessel quantification shows higher vessel density in the vascular plexus closer to the fascia than in the more superficial one. The measurements for the more superficial vascular plexus vary in density of vascularization and level above the fascia, in terms of biopsy location and also between specimens. This could be associated with the donors' subcutaneous fatty tissue mass and therefore we correlated the BMI to the finding. The more superficial vascular plexus density is associated with a higher BMI and inversely proportional to the age of the donor. This more superficial vascular plexus probably represents connecting vessels from the fascia to the

Table 1. Demographic Characteristics of the Specimen

	Age (y)	Gender	BMI (kg/m ²)	Flaps (n)
1	76	Man	27.7	1
2	82	Man	25.4	1
3	89	Man	27.0	1
4	69	Man	18.3	2
5	88	Man	22.3	2

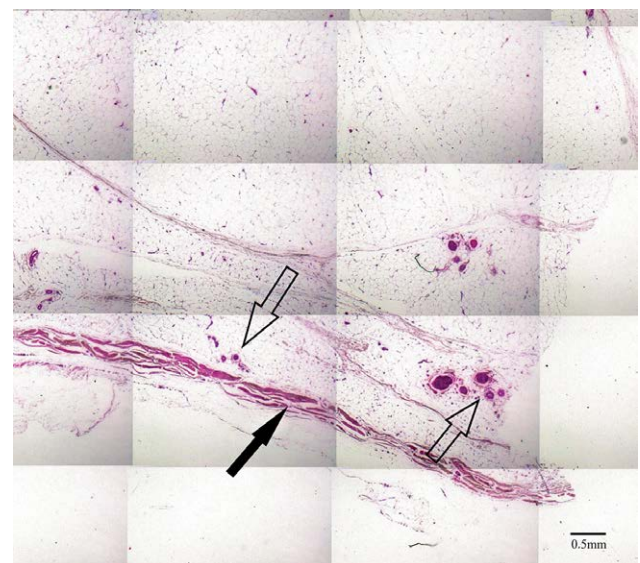


Fig. 2. Histology for quantitative measurement of stained vessels (fascia—black arrow, big vessels close to the fascia and further off—transparent arrows).

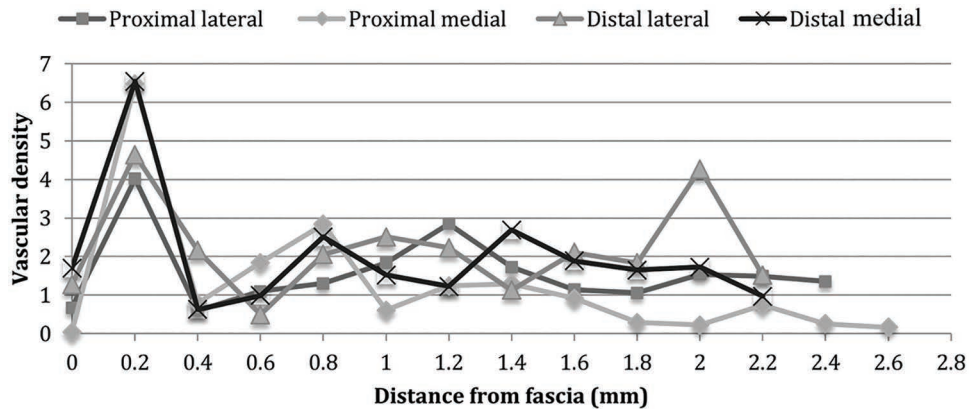


Fig. 3. Comparison of vascularization pattern above the fascia on different locations.

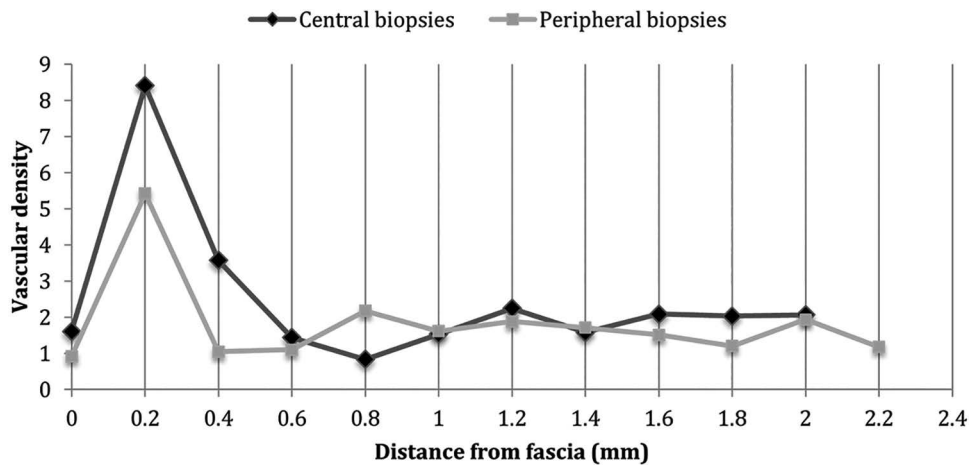


Fig. 4. Peripheral locations summarized and compared with the summarized central biopsies.

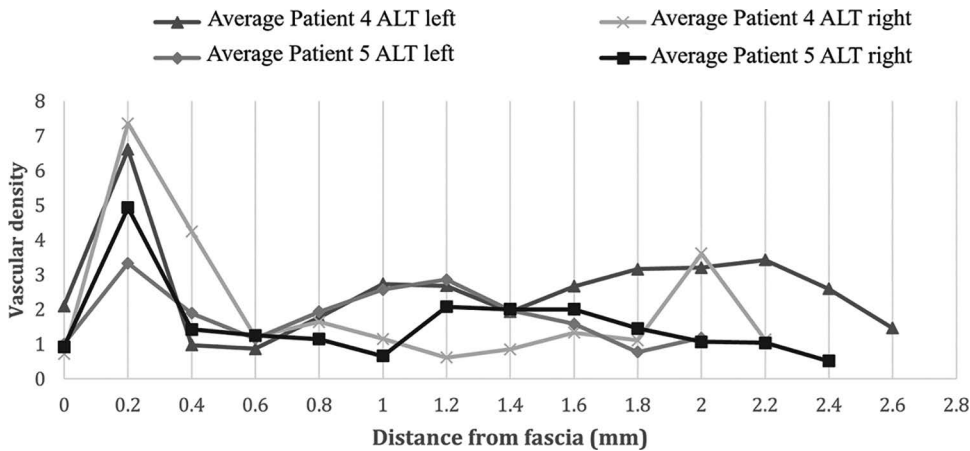


Fig. 5. Comparison of the four flaps.

dermal plexus, and its location is dependent on the soft tissue mass and location within the flap. Due to a low number of suitable cases, these findings remain an observation. However, this could be of clinical relevance, when harvesting a thicker adipofascial flap, or when reducing the bulk in a fasciocutaneous flap, to increase robustness of their respective vascularization.

The central biopsy in every flap corresponded to the location of the most perforating vessels. Not surprisingly, maximal vascularization above the fascia was found in the center of the ALT flap, decreasing towards the fascial rims. However, the medial periphery was showing higher vascular density than the lateral biopsies. This needs to be confirmed or revised in further series, but for the moment we

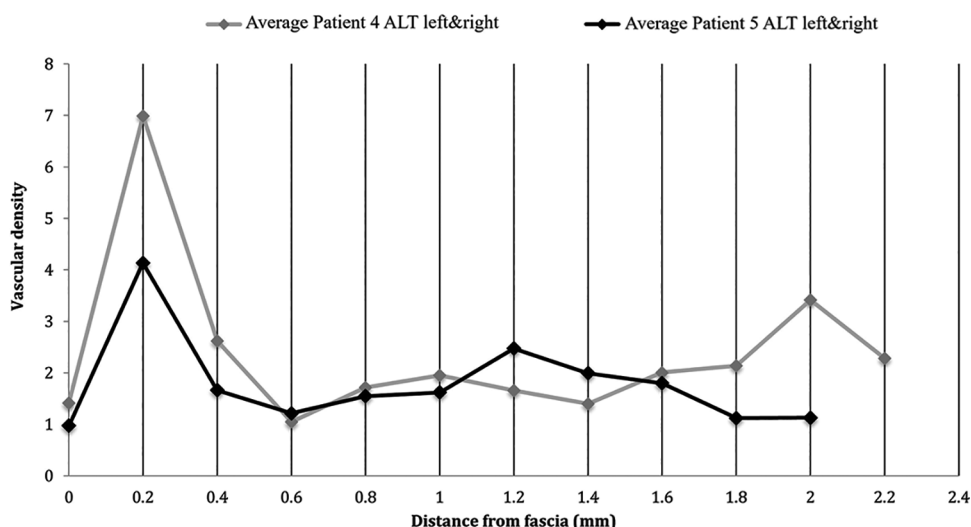


Fig. 6. Comparison of two donors.

would consider the ALT flap to be more robust medial to the descending branch from the lateral femoral circumflex artery for clinical application. When wrapping avascular engineered grafts, the medial ALT adipofascial flap is clearly preferable to ensure maximal vessel density close to the construct.

Ching-Hua Hsieh et al⁷ recommend preserving a layer of fatty tissue of 2–3 mm thickness to increase viability of the adipofascial flap. Our results confirm the safety of this margin to include the majority of vessels.

However, the second peak between 1 and 3 mm above the fascia in our series probably only represents linking vessels between the subdermal plexus and the epifascial plexus, which are not crucial for vascularization of a fascial flap. According to our measurements, a thinner adipofascial flap with only 1–2 mm of fat on top of the fascia should be safe. This should be investigated further before clinical application.

Also, this second peak may be of interest for clinicians seeking a thicker adipofascial ALT flap. If a correlation between the donor's BMI and the depth of the second vasculature density peak can be confirmed in further series, the implantation depth could be chosen individually based on the patient's BMI. For now, we recommend maintaining at least 2–3 mm of subcutaneous fatty tissue on the fascia in patients with normal weight, to profit from both vasculature peak layers.

The consistency of the axial and cross-sectional vascularization of the ALT flap describes it as a useful tool for bringing the needed vascularization into a tissue-engineered construct, such as tissue-engineered bone grafts. Our findings support clinical translation of the preclinical trial from Scheufler et al,⁶ who was wrapping cell-seeded ceramic scaffolds in a panniculus carnosus muscle flap in rabbits. In comparison with the study of Ismail et al⁸ where an internal approach was used to allow vascularization of a bone graft from the inside only, by inserting an arteriovenous bundle inside the scaffold, the wrapping with an ALT flap could be used alone for smaller constructs or in

combination with an internal AV bundle in larger cases, to minimize diffusion distance and nutrition of cells on seeded constructs.

Due to a small number of donors and the initial problems developing sufficient immunohistochemical staining and preservation of the histopathological structure, our results are of little statistical power and allow no generalization. To confirm the trends, findings, and statements made in this study, a series of flaps (also of different anatomical origin) processed in the same way needs to follow. Now the staining and analysis method is established, this can be performed for various flaps depending on the clinical and translational scenario.

Just recently, the platysma has been re-described as a free flap option for blink reconstruction.⁹ This flap could be an option to wrap constructs. In humans, it is anatomically the closest flap available to a panniculus carnosus flap. As a muscular flap, it would probably provide an even richer vascular network than the ALT flap. However, the platysma would probably be only suitable for small- to medium-sized defects and technically even more demanding to harvest. Furthermore, its suitability should be checked for cross-sectional vascular anatomy.

We confirm that a consistent perfusion of tissue-engineered bone grafts should be provided at any position of the anterolateral adipofascial thigh flap. When wrapping a bone construct with this flap to enhance vascularization, one must consider accommodating extra space for the soft tissue sleeve of at least 4 mm in diameter at the implantation site.

With minimal donor site morbidity, dense and consistent vascularization and the thin nature of the flap, we can validate that the ALT flap qualifies as an optimal choice for the next step in bringing tissue-engineered bone grafts into the clinic.

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