Perfusion territories subtended by penetrating coronary arteries increase in size and decrease in number toward the subendocardium

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van Horssen P, van den Wijngaard JP, Brandt MJ, Hoefer IE, Spaan JA, Siebes M. Perfusion territories subtended by penetrating coronary arteries increase in size and decrease in number toward the subendocardium. Am J Physiol Heart Circ Physiol 306: H496–H504, 2014. First published December 20, 2013; doi:10.1152/ajpheart.00584.2013.—Blood flow distribution within the myocardium and the location and extent of areas at risk in case of coronary artery disease are dependent on the distribution and morphology of intramural vascular crowns. Knowledge of the intramural vasculature is essential in novel multiscale and multiphysics modeling of the heart. For this study, eight canine hearts were analyzed with an imaging cryomicrotome, developed to acquire high-resolution spatial data on three-dimensional vascular structures. The obtained vasculature was skeletonized, and for each penetrating artery starting from the epicardium, the dependent vascular crown was defined. Three-dimensional Voronoi tessellation was applied with the end points of the terminal segments as center points. The centroid of end points in each branch allowed classification of the corresponding perfusion territories in subendocardial, midmyocardial, and subepicardial. Subendocardial regions have relatively few territories of about 0.5 ml in volume having their own penetrating artery at the epicardium, whereas the subepicardium is perfused by a multitude of small perfusion territories, in the order of 0.01 ml. Vascular volume density of small arteries up till 400 μm was 3.2% at the subendocardium territories but only 0.8% in the subepicardium territories. Their higher volume density corresponds to compensation for flow impeding forces by cardiac contraction. These density differences result in different scaling law properties of vascular volume and tissue mass per territory type. This novel three-dimensional quantitative analysis may form the basis for patient-specific computational models on coronary perfusion and aid the interpretation of image-based clinical methods for assessing the transmural perfusion distribution.

intramural coronary arteries; transmural vascular vessel density; imaging cryomicrotome; multiscale and multiphysics modeling; vascular scaling laws

While the coordinated control of vascular smooth muscle tone is essential for metabolic adaptation and autoregulation of coronary blood flow, the structure and spatial organization of the intramural myocardial vascular bed are important determinants in the distribution of coronary blood flow especially at full vasodilation. It is well known that the subendocardium is the most vulnerable for ischemia (13, 15, 37) which relates to the high level of contraction related compressive forces in that region. This contraction thus reduces subendocardial perfusion as function of diastolic time fraction and perfusion pressure (2, 7, 14, 40). The vascular bed compensates for that by a higher volume of small vessels in the subendocardial region (47).

From two-dimensional (2-D) imaging of the intramural vasculature in slabs of heart tissue it was recognized that some penetrating transmural arteries reach the subendocardium and others not (8). However, quantitative analysis of the morphology of these intramural crowns is not available but necessary for understanding myocardial perfusion distribution.

Currently, clinical techniques for perfusion measurements are being developed based on distribution of contrast agents by positron emission tomography (PET) and magnetic resonance imaging (MRI) (5, 16, 29). Interpretation of contrast distribution imaging can be improved by quantitative knowledge of the intramural branching of the coronary arterial tree (31). Since ischemia is occurring predominantly at the subendocardium, especially knowledge on the organization of the vessels at the subendocardium is required (5, 14, 15).

With present computer power it has become possible to analyze in silico perfusion distribution in relation to a multitude of physical and physiological processes at different scales (25, 26, 30). Essential for such state of the art modeling is detailed and accurate quantitative knowledge of the anatomy of the intramural coronary arterial tree. Until now especially results from corrosion cast techniques were used. These techniques yielded detailed vascular morphology in hearts of humans and large animals and resulted in the formulation of branching rules, but information on the three-dimensional (3-D) aspects of the coronary tree was lost (22, 45). These branching rules were used to stochastically fill the 3-D space within the myocardial wall resulting in a virtual vascular tree, which served as the basis to relate structure to physiology by flow simulations in the vasodilated network of a non-beating heart (1, 4, 36). Hence, these perfusion models are inherently oversimplified and fail to predict some important aspects such as transmural differences in flow distribution (4).

Currently, with the recent technique of imaging cryomicrotome analysis, a fluorescent replica cast can be fully reconstructed, leaving the 3-D vascular structure intact (38, 42). Hence, for each vessel segment the distal vascular structure and thereby perfusion territory can be determined (44). This technique is therefore well suited to determine the flow path of blood and contrast agents through the coronary vascular bed.

This study aimed at identifying vascular crowns subtended by penetrating arteries by their dominant transmural location, volume and vessel diameter distribution. Transmural location is classified based on the centroid of the crowns with respect to 3 tissue layers of equal thickness: subendocardium, midmyocardium and subepicardium. We hypothesized that crowns
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classified as subendocardial are largest with the highest small artery density and that scaling laws are layer-dependent.

MATERIALS AND METHODS

Canine Data Set

A series of acute physiological experiments with the objective of quantifying collateral flow were carried out in 8 canines and provided the data sets for this study. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Utrecht Medical Center. After premedication with dorimtor (0.03 mg/kg im), ketamine (0.03 mg/kg im), and atropine (0.5 mg im) anesthesia was induced by Sufenta forte (10 μg·kg⁻¹·h⁻¹ iv) the animals were intubated, and a surgical level of anesthesia was maintained by mechanical ventilation with a mixture of propofol (24 mg·kg⁻¹·h⁻¹ iv), Sufenta forte (3 μg·kg⁻¹·h⁻¹ iv), and oxygen. The hearts were exposed through vena cava puncture. Coordinated heart contraction was reported here), heparin was administered and the animals were euthanized by mechanical ventilation with a mixture of propofol (24 mg·kg⁻¹·h⁻¹ iv), Sufenta forte (3 μg·kg⁻¹·h⁻¹ iv), and oxygen. The hearts were exposed by left thoracotomy via the 4th intercostal space. After a number of physiological measurements were carried out (not reported here), heparin was administered and the animals were euthanized through vena cava puncture. Coordinated heart contraction was stopped by inducing fibrillation using a 9-V battery. The heart was then excised and the coronary arteries were cannulated and flushed retrograde from the aortic root with calcium-free 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer containing 4.7 mmol/l potassium chloride and adenosine for vasodilation of the microcirculation. In this way the heart was brought into a diastolic state. When blood was no longer visible in the buffer dripping from the heart the coronaries were filled with cast replica material (Batson no. 17, Polysciences) consisting of a monomer base solution, a catalyst and a promoter as described previously (38, 43). Potomac Yellow (440/490) (Radiant Colour, Belgium) and UV Blue (Vaspec, Switzerland) (365/505) provided the fluorescent base for the replica plastic. The cast material was allowed to harden for 24 h at ambient temperature. The fully prepared heart was embedded in carboxymethylcellulose sodium solvent 5% (Brunschwig Chemie, The Netherlands) and Indian ink 5% (Royal Talens, The Netherlands) and frozen at −20°C.

Cryomicrotome Processing

The imaging cryomicrotome uses the episcopic reflectance technique to create digital volume data from multispectral images of the embedded tissue block surface after successive removal of imaged sections. The bulk surface can be illuminated by a choice of clusters of power light emitting diodes (Luxeon V, Star, Royal Blue, Lumileds Lighting), each cluster emitting light at a different central wavelength. The excitation and emission bandwidth is tuned to the sample fluorescence with selectable filters (Chroma Rockingham VT). The frozen specimen was cut at 25 μm slice thickness. After each slice, the block face of the remaining bulk material was imaged using a digital camera (Apogee Alta U-16) equipped with a variable focus lens (70–180 mm, Nikon), achieving an in-plane image resolution of 25 μm. Registered stacks of bright light and fluorescent images were obtained with excitation and emission light at 440/20 nm (UV Blue) and 480/20 nm (Potomac Yellow), respectively. After in-plane downsampling and combining slices, stacks were digitally processed at a spatial resolution of 50 μm. The black and white photos delivered an outline representation of the heart, whereas the fluorescent images yielded a 3-D reconstruction of the vasculature at 50 × 50 × 50 μm³ voxel resolution.

Image Preprocessing and Segmentation

The following sections briefly discuss the image processing steps needed to arrive at a segmented representation of the vascular tree. Deconvolution. Image degradation due to light scatter, transparency of the tissue and blurring of the lens was corrected by an image deconvolution with a system-specific point spread function. The deconvolution process enhances the vessel borders by removing the halo around the vessels caused by tissue transparency. Without deconvolution, the connectivity of some of the smaller vessels is lost and thus the level of detail of the reconstructed vascular tree. The earlier established point spread function for the cryomicrotome setup was used (33). We use a custom made 3-D iterative deconvolution program that was written in CUDA (NVIDIA), which uses 4 graphics cards in a parallel setup to deblur the images.

After deconvolution, a binary representation of the vessels was skeletonized using a topology-preserving thinning algorithm (32, 41, 43). The threshold for this process was manually chosen to be just below the intensity of the smallest visible vessels. The pruned result was stored as a text file containing the xyz coordinates of the center points of the vascular tree. Custom software was written in Lazarus (www.lazarus.freepascal.org/) to automatically read these xyz coordinates of the vascular tree and to subsequently reconstruct the topological representative vascular tree where the center points of the vessel segments along the entire tree were classified according to nearest neighbor connections into end points with only one neighbor, midpoints with two neighbors, and bifurcation points with three neighbors and so on. Branch segments were defined by a set of sequential neighboring midpoints between two non-mid points (43). The automatic segmentation of the entire vascular tree was accomplished within several hours on a high end PC and yielded a detailed 3-D representation of the vascular network morphology for further processing. Diameter measurement. Diameter measurements were performed for each mid-point from the cross-sectional intensity profile along a set of 64 vectors perpendicular to the vessels local longitudinal axis. With the vessel center as the maximum intensity, each vector was extended while the intensity was measured until the full width at half maximum of the obtained intensity profile was reached. The intensity profile was interpolated to get subpixel measurement of the vector lengths. The diameter at each mid-point follows from averaging of the length of all vectors. The cross-section of the segment was assumed to be circular with constant diameter defined by averaging over its mid-point diameters.

Tessellation and classification. The outline images were segmented by thresholding and provided a binary representation of the cardiac tissue. The epicardial surface was extracted from this data set, by using ImageJ (35). Stem segments of all penetrating arteries that coincided with the extracted epicardial surface were selected for processing. Stem segments in the septum were identified as branching of the septal artery. The corresponding crown vasculature was morphometrically analyzed using the stem segments as seeding point and linking all downstream vasculature to its corresponding stem segment. With all the crowns determined, the whole coronary tree was decomposed into 3-D crown defined perfusion territories by Voronoi tessellation (20) with the end points per stem as center points (Fig. 1A). Segments with a diameter of 100 μm were selected as terminal segments in this tessellation. The myocardial boundaries, as estimated from the outline images, formed the enclosed 3-D subspace for the 3-D tessellation process. All Voronoi cells originating from the same penetrating artery were grouped together to form a well-defined perfusion territory. A centroid was assigned to each penetrating branch based on the average position in 3-D space of its end-points. The myocardial wall was subdivided into three layers of equal radial thickness (Fig. 1A) and will be further referenced to as the first, second, and third layer. Territories were classified based on the position of their centroid in the layers and were grouped into three layer-classified, perfusion regions; an endocardial region, a midmyocardial region and an epicardial region (Fig. 1B). Layer-classified perfusion regions belong to either left ventricle free wall (LV), right ventricle free wall (RV), or septum (SEP), as defined in Fig. 3C. Note that the epicardial layer of the SEP could also function as the endocardial layer of the RV, but will be assigned to the septum throughout this study.
Statistical Methods

A D’Agostino and Pearson test were used to test the normality of the data. Normally distributed data were compared using an unpaired Student’s t-test or a one-way analysis of variance followed by post hoc Tukey’s multiple comparison, as appropriate. For nonnormal data a Kruskal-Wallis test followed by Dunn’s multiple comparison was used. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed in Graphpad Prism (GraphPad Software, La Jolla, CA).

RESULTS

A typical result of the cryomicrotome-obtained vascular data is shown in Fig. 2. Figure 2A depicts a maximal intensity projection of a series of fluorescent cast images representing a slice of tissue with thickness of 8 mm halfway between base and apex. Note that some crowns with the stem penetrating from the epicardium reach the endocardium and others not. Figure 2B depicts a zoom-in image of a selection of this slice as indicated in Fig. 2A and shows the microvasculature within the myocardial wall. One crown at the bottom right reaches not farther than the midmyocardium and is enclosed by crowns that reach the subendocardium. Fig. 2C demonstrates the segmented result of Fig. 2B, with cylinders as the vessel segments. These segmented vessels are used for quantitative analysis.

Figure 1 illustrates that regions composed of crowns assigned to a specific layer by their centroid are not confined within the equidistant transmural tissue layers. The boundaries separating each crown region follow a rather irregular path. Figure 1B demonstrates the disparity between the actual morphology of perfusion territories resulting from collation of adjacent crown regions with the same layer assignment and the equidistant layers. The resulting overlap of crown-based territories across more than one layer is also clear from the example of a complete transverse section shown in Fig. 3, B and C. The distribution of volumes derived from the perfusion regions assigned to each layer is quantified in Fig. 4A. For the LV, the average tissue volumes of the transmural layers were 26, 47, and 70 cm$^3$ from endocardium to epicardium. In contrast, the average volume of crown-based perfusion regions was 35, 59, and 48 cm$^3$ from endo- to epicardium. The fractional volume overlap of crown-based perfusion regions within each layer is demonstrated in Fig. 4B. For the LV, layer 1 (innermost layer) is comprised for 65% of the crown-based endocardial perfusion region, for 34% of the mid-myocardial region, and only for 1% of the subepicardial-classified perfusion region. For layer 2, (middle layer), the overlap with crown-based perfusion regions is 28, 62, and 10% from endo- to epicardium, and for layer 3 (outer layer), 8, 31, and 61%, respectively. Hence, up to a variable degree, crowns classified to all three layers contribute to the perfusion of a particular layer, but there is a dominant overlap with corresponding crowns assigned to that layer. A similar trend is seen in the RV. For SEP, the

Fig. 2. A: maximum intensity projection (200 slices = 8 mm) of the coronary arteries at a transverse section of the heart halfway between base and apex. B: expanded view of area indicated in A. Note that not all detected vessels are shown in this image. C: fully segmented binary tree of the vessels shown in B.
midmyocardial region is dominant in all layers (Fig. 3C). This is due to the course of the septal artery, and therefore stem segments lie within the second layer as opposed to the left and right ventricle where the stem segments originate from the outermost layer.

The territories of crowns classified by region type differed significantly in volume and number as depicted in Fig. 5, A and B, respectively. From endo- to epicardial classification, the territorial volume becomes smaller, but the number of territories normalized to cardiac tissue volume is more than proportional higher. The territory volumes differed between regions for LV and RV ($P < 0.001$). The endo- and the midmyocardial perfusion regions for the SEP were similar in volume, but both were significantly larger than the epicardial region ($P < 0.05$ and $P < 0.001$). Territories in the subepicardial region significantly outnumbered those in the subendocardial regions for LV ($P < 0.001$) and RV ($P < 0.01$), but not for the SEP. In general, the endocardial territories were largest in size but smaller in number.

In agreement with the larger territorial volumes, the stem segments of subendocardial classified territories had a larger diameter. As illustrated in Fig. 6A for an example of a single heart, the log-log relationship between the stem segment radius and perfused tissue volume for each separate territory type in the LV free wall follows the form $Y = kX^b$ (6). Endocardial territories had a larger stem segment diameter compared with the mid- and epicardial territories (Fig. 6B), corresponding to the increased volume for the individual endocardial territories as shown in Fig. 5A. Stem segment diameter was significantly different between all territory types ($P < 0.001$) for LV and RV, with increasing radius toward the endocardium. In the septum, only the radius of the midmyocardial stem segments was significantly larger than that of the epicardial stem segments, which is the result of the position of the septal branch perfusing the SEP area as noted above.

The distribution of vascular volume density, expressed as vascular volume divided by tissue volume, is shown in Fig. 7 per vessel segment radius in bins of 50-μm size for the different regions in LV, RV, and SEP. For the endocardial classified territories, the volume density of the smaller arteries ($<150 \mu m$) was largest in the first layer but decreased for the second and third (outermost) layer. For the midmyocardial regions, these differences were smaller and the vascular density of the smaller vessels was actually largest in the second layer. Total volume density was highest for the endocardial regions for LV and RV followed by the mid- and epicardial
The vascular volume density distribution (Fig. 7) suggests a difference in scaling law behavior for vascular volume versus tissue mass for the different regions. The vascular volume-tissue mass relationship is plotted in Fig. 8A for a single heart to illustrate the differences in slope for the different regions. The lower vascular density of particularly the smaller vessels in the epicardial region results in a less steep slope for the scaling law relationship and, therefore, a lower exponent value. A less pronounced difference exists between the midmyocardial and subendocardial region. The scaling law was investigated for all hearts and the exponent value plotted as a function of region type (Fig. 8B). A decline in exponent was found for the midmyocardial regions ($P < 0.001$), followed by the subepicardial regions ($P < 0.01$) compared with the subendocardial regions.

**DISCUSSION**

To our knowledge this study provides the first analysis of the intramural structure of the entire canine arterial vasculature in high detail while maintaining its original 3-D structure. In this study, we divided the myocardium into territories as defined by the crowns of transmural branches penetrating from the epicardium into the myocardial wall. Independently of the territories, the myocardium of the LV free wall, RV free wall, and septum was divided into three tissue layers as is often done in perfusion distribution studies. Territories were classified into endo-, mid-, and epicardial regions, depending on the position of the end points in the crown of the underlying branch with respect to the tissue layers. Most territories cover more than one layer. However, the largest part of each layer is perfused by territories classified to that layer. The size of the territories decreases from subendocardium to mid- and subepicardium. This effect was most pronounced for the LV free wall.

The vascular volume density in the LV free wall, defined as the volume of the small arteries divided by the territory volume, is larger for subendocardial territories than epicardial territories. For small arteries with radii in the range of 50–100 μm, this density was more than three times larger. The variation of volume density of these small arteries over the septal territories was less than in the LV and RV free walls. The regional differences in vascular density are also apparent by the relation that exists between cumulative vascular volume and the tissue mass perfused by it. This relationship, formulated as a scaling law, is therefore different for the three territory types.

Fig. 5. A: territory volume for each region type. The median volume of the Endo territories is largest, followed by the Mid territories. Layer-specific distribution of volumes represents pooled data over all animals per region type. B: number of territories normalized for total cardiac tissue volume (in cm$^3$). Only few territories are present within the Endo region, whereas almost 10-fold more territories are found at the Epi region. Probability cross bars indicate 10–90th percentile range. *$P < 0.05$; ***$P < 0.01$; ***$P < 0.001$. NS, not significant.

Fig. 6. A: stem segment radius vs. perfused tissue volume as illustrated for a single heart. Endo territories are larger and have a stem segment with larger diameter. More spread in the perfused tissue volume can be seen for the Epi territories at the same stem radius. B: stem segment radius for the LV, RV, and SEP for each region type. The distribution of stem segment radii follows the distribution of territory volumes per region (see Fig. 5A). Endo territories at the LV have a larger median radius compared with the Endo territories at the RV. *$P < 0.05$; ***$P < 0.001$. 

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The regional differences in morphological findings correspond to the previously experimentally found higher perfusion at the subendocardium than subepicardium in the diastolic heart (47).

**Data Interpretation**

It may be assumed that ischemic and infarcted areas will follow the external contours of territories distal of the ischemic front. In previous studies, it was found that the subendocardium is more vulnerable for ischemia and infarctions than the outer layers of the myocardium (9, 40). The tissue areas classified as subendocardial therefore delineate the most vulnerable territories. In addition, ischemia and resulting necrosis do not only depend on the structure of the vascular bed but also on the compressive forces exerted by cardiac contraction on the intramural vessels. These flow-impeding forces are related to the mechanical stress field generated in the myocardium, which follows the organization of muscle fibers rather than that of the vascular bed. This organization results in a gradient of compressive forces increasing toward the endocardium in the radial direction within all territories. These compressive forces will result in a perfusion reduction at the endocardial positioned vessels by increasing the contraction related extravascular component of local coronary resistance. The compressive forces during systole are therefore highest for the inner layer of the myocardium, which is closest to the LV cavity. It is therefore expected that ischemia and infarction follow the contour of the endocardial territories.

From branch anatomy, it can be inferred that impediment of perfusion in the more endocardial territory will result in a salvaging effect of tissue in the more epicardial situated area within such a territory. Hence, a reduced flow toward the endocardial area within a territory as resulting from infarction causes a reduced flow through the proximal territorial main artery and therefore a smaller pressure drop to the more proximal areas within the territory, which will improve local perfusion as a result. This effect can be denoted as reversed steal. Apart from interaction in perfusion through transmural arteries, there is also interaction between perfusion territories via the epicardial conduit arteries.

The higher density of small artery volume at the subendocardium corresponds to the earlier study of Wusten et al. (47) where intramural blood volume was measured by γ-ray spectrometry of slices of the left ventricle. In that study, the coronary system was filled with a mixture of gelatin and...
Barium sulfate to which Sr-85 labeled microspheres were added. The filling pressure was set at 130 mmHg and the gelatin mixture did not enter the capillaries. The larger transmural arteries were excluded from analysis. It was found that total small arterial volume fraction of vascular filling was about 4.8% at the subendocardium, 3.4% at the midmyocardium, and 2.2% at the subepicardium. This corresponds reasonably well with the vascular density calculated from this study for vessels smaller than 400 μm in the LV free wall, which is 3.2% for the subendocardial regions, 2.6% for the midmyocardial regions, and 0.8% for the subepicardial regions.

Our results indicate a smaller vascular density than found in the study of Wusten et al. (47). where a similar method of filling was used as previously described by Schlesinger et al. (34). In these studies filling reached about 50% of the 20 μm diameter vessels and therefore includes more vascular volume than presented in this study.

Branching Characteristic and Modeling of Myocardial Perfusion

Branching characteristics of the coronary tree in healthy hearts have been described based on 2-D imaging of transversal and/or longitudinal slices of myocardium (10, 23, 47). However, transversal and longitudinal slices per definition cannot capture the same vessel or crown. Such 2-D observations have led Estes et al. (8) to classify two types of crowns, those that reach the endocardium with terminal segments and those that do not. Kato introduced a modified classification with 3 types, none of which focus on the midmyocardium (23). Two types distinguish crowns with terminal segments at the near endocardium and with terminal segments in the trabeculae carneae or papillary muscle. The third type defines crowns with terminal segments in the subepicardium. Apart from the lack of midmyocardial crowns in the classification of Kato, another major difference between his and the present classification is the sharing of stems by the different crown types, which is not allowed in the present classification where the stems of crowns with perfusion preference in different myocardial layers are unique. Quantitative comparison of our data with earlier data on morphology is not possible due to experimental limitations related to the slice-confined 2-D imaging, the absence of quantitative data in these earlier studies on crown volume and vessel diameters, and differences in the definition of crown classification.

The lack of 3-D morphological data stimulated several model approaches (17, 18) where the intramural tree structure is generated based on one-dimensional morphological relationships derived from corrosion cast measurements (21, 45). These model studies are valuable in the analysis of some aspects of the coronary circulation such as heterogeneous distribution of flow or the effect of vascular structure on regional differences of vascular compression. These models may benefit from incorporating our findings on vascular branch heterogeneity and the different types of perfusion territories.

The one-dimensional morphological approach has led to the derivation of scaling laws between several vascular characteristics (48). For a collection of 3-D data, the scaling law behavior has been confirmed between tissue mass and the radius of the stem segment feeding the corresponding crown (6). This relationship was validated in our data for the stem segments at the base of the penetrating arteries and their corresponding territory size. Separated on region type, the implications of the increased tissue volume toward the endocardium suggest an increased stem segment diameter for the endocardial territories. This was also observed from the example data in Fig. 6A and further shown in Fig. 6B as the median stem diameter for all hearts increased from the subepi- to midto subendocardial region.

Scaling laws are applied to trees or subtrees. This study demonstrates that vessel density depends on myocardial depth within territories. Since territories extend over varying muscle depth scaling rules must be different per territory type.

Study Limitations

Vascular reconstruction from cryomicrotome imaging is limited by the filling of the arteries and by the optical resolution of the setup. In previous experiments (24, 38), we established that the cast material does not penetrate vessels smaller than 10 μm in diameter, and hence capillaries and veins were not filled which simplified the image analysis. At the cost of reducing the diameter resolution of 25 μm, but for computational reasons the 3-D images were downsampled to 50 μm³ voxels in this study. Voronoi tessellation was then performed using a 100-μm diameter cut-off as criterion for the terminal segments to stay well above the detection limit of 50 μm and to avoid variability between crowns based on the smallest diameter detected. This may have impacted on the estimation
of crown-based related tissue volume and even on the classification of the crowns to layers. However, the excluded vessel segments are <100 μm and only would extend perfusion territories in the order of their length. Length and diameter of vessel segments are related (45), and on average, the length of a segment with diameter of 100 μm is 0.1 mm, which is about 5% of the thickness of the classification layers. Moreover, potential errors in estimating regional boundaries occur at both sides of territorial interfaces, and resulting truncation errors will likely cancel out. Obviously, the course of the boundary planes would become more precise when smaller vessels were included in the tessellation. However, this would also induce additional noise introduced by variable degree of filling of the smallest vessels between hearts and between regions within hearts, which is circumvented by using a threshold that can be determined with the same accuracy in all regions.

Loss of a single vascular branch may occur when air bubbles are introduced during the filling procedure. Extra care was taken when filling the hearts and the data were manually inspected afterward to ensure adequate filling throughout the tree. The main finding of distinguishable perfusion territories therefore does not change.

Spurious branches may occur as an artifact from pruning algorithms (32). These branches would influence the Voronoi tessellation, since they add false end-points and obviously would influence the vascular volume measurements as well. Therefore, the data were cleaned from spurious branches by automated inspection of the diameter measurement of each terminal segment.

The data set was obtained from experiments on dogs directed to the analysis of collateral flow. For this physiological study, the dog was used as experimental animal since it possesses innate collaterals in contrast to, e.g., the pig (27, 41). With respect to presence of innate collateral vessels, the dog’s heart resembles the human heart (3). It seems important to repeat the present study on healthy human hearts, but these obviously are more difficult to obtain for such a study. However, there is certainly qualitative similarity between the images obtained from slices of the healthy human heart (11, 23) and dog heart obtained by radiography as reported before (47) and as obtained from the present study (Fig. 2A).

It is important that the resistance vessels are fully vasodilated to attain a maximal diameter at the pressures imposed. Muscle contraction results in compression of intramural vessels (19, 28, 39), which was prevented by the absence of calcium in the perfusate before casting. Absence of calcium should also prevent smooth muscle contraction. Additionally, adenosine was administered, which was demonstrated to be functional in hearts perfused according to Langendorff using crystalloid buffer media (46).

In some images the larger arteries may be overexposed complicating the diameter estimation of these vessels (12). However, the diameters of these large epicardial arteries are not relevant since the present analysis starts with the penetrating vessels that were not overexposed.

**Conclusion**

Penetrating arteries starting from the epicardium define separate perfusion territories that likely relate to boundaries of ischemic and infarcted tissue. Classification of their crowns into subendocardial, midmyocardial, and subepicardial layers demonstrate that the perfusion territories increase in size and decrease in number from epicardium to endocardium. The subendocardium is therefore perfused by a small subset of penetrating arteries. Layer-specific vascular volume densities resulted in different scaling law properties of vascular volume and tissue mass. Our observations may play an important role in models of vascular tree generation and aid the interpretation of noninvasive image-based clinical methods for assessing transmural perfusion distribution.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


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