In Vivo Assessment of Retinal Vascular Wall Dimensions

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PURPOSE. Retinal blood vessel diameter and arteriovenous ratio (AVR) are commonly used diagnostic parameters. Because vascular walls are typically not visible in funduscopy, clinical AVR estimation is based on the lumen rather than the entire vessel diameter. Here the authors used a transgenic mouse model to quantify AVR in vivo based on total vessel dimensions (wall and lumen).

METHODS. Confocal scanning laser ophthalmoscopy (cSLO) and indocyanine green angiography of the retinal vasculature were performed in wild-type and transgenic mice expressing green fluorescent protein (GFP) under the transcriptional control of the smooth muscle type α-actin (αSMA) promoter. Spectral-domain-OCT and ERG were performed to control for integrity of retinal structure and function in vivo and histology to demonstrate the location of GFP expression.

RESULTS. Native cSLO imaging and angiography yielded only inner vessel diameters similar to those observed through clinical funduscopy. In αSMA-GFP mice, autofluorescence imaging of the GFP-marked vascular walls also allowed the determination of outer vessel diameters. The mean AVR based on either inner diameter (AVRid = 0.72 ± 0.08) or outer diameter (AVRod = 0.97 ± 0.09) measurements were significantly different (P < 0.01).

CONCLUSIONS. Transgenic αSMA-GFP expression in murine vessel wall components allowed quantification of retinal vessel outer diameters in vivo. Although arteries and veins differ in lumen and vessel wall width, they share a common outer diameter, leading to an AVR close to unity. Because vessel walls are primary targets in common hypertensive and metabolic diseases, αSMA-GFP transgenic mice may prove valuable in the detailed assessment of such disorders in vivo. (Invest Ophthalmol Vis Sci. 2010;51:5254–5259) DOI:10.1167/iovs.09-5139

The retinal vasculature is often regarded as a window to the general circulation, providing exemplary information about changes in vascular morphology and function. Given that retinal, cerebral, and coronary blood vessels feature similar anatomy and physiology,1–3 funduscopic evaluation of retinal blood vessels has long been part of the routine clinical investigation, especially in hypertensive and metabolic disorders that lead to systemic small vessel disease.

In funduscopy, diameters of first- and second-order retinal arterioles and venules approximate a 2:3 ratio. Assessment of this arteriovenous ratio (AVR), the ratio of arteriolar diameter to venule diameter, and the general appearance of retinal vessels are based on the normal light reflex of the retinal vasculature, which is formed by the reflection from the interface between the blood column and the vessel wall.4–5 In the thin-walled veins, the strong absorbance of green light by hemoglobin leads to the characteristic dark appearance.6 Retinal arteries have a more silvery look, indicating that light is reflected before it reaches the blood itself. Although these arteries have a strong muscular wall, they appear smaller than the veins, suggesting that the main site of reflection is close to the bloodstream and not near the outside of the vessel.7 In conclusion, AVR based on funduscopic evaluation closely reflects the relationship between the arterial and the venous inner diameter and does not include the outer diameter (i.e., the wall) of the vessel.

To make the outer diameter of the retinal vessels accessible in vivo, we used a transgenic animal model that expresses green fluorescent protein (GFP) under the transcriptional control of the smooth muscle type α-actin promoter (αSMA). Only cells with intrinsic expression of smooth muscle type α-actin also express GFP, resulting in specific GFP labeling of the vessel wall by the fluorescence of smooth muscle cells and a subgroup of pericytes with smooth muscle type α-actin expression. Because capillary pericytes do not feature contractile properties, only the precapillary and postcapillary vessels are stained, and the expression pattern as such mirrors the auto-regulative/contractile capacity of the retinal vasculature (Tsai YJ, et al. IOVS 2002;43:E-Abstract 1929).8 GFP is excitable at the wavelength of the autofluorescence mode (488 nm) of a standard cSLO device; therefore, we were able to visualize smooth muscle cells and contractile pericytes within the vessel walls of retinal arterioles and venules.9–10 We used this method to investigate the mean difference between the inner and outer diameters of arterial and venous blood vessels and to determine the respective AVR in αSMA-GFP mice. Furthermore, given that
transgenic GFP expression in retinal cells may affect the structure and function of the retina,\textsuperscript{11} we investigated the structural and functional integrity of the transgenic mouse retina in vivo to exclude confounding effects on the AVR from retinal pathology in the transgenic mouse line.

**Materials and Methods**

**Animals**

In this experimental study, six wild-type (C57/BL6) and six transgenic mice with GFP expression under transcriptional control of the \(\alpha\)SMA promoter were used at 8 weeks of age (Tsai JY, et al. IOVS 2002;43: E-Abstract 1929). The regulatory sequence of the \(\alpha\)SMA gene used contains 1074 bp of the 5’ flanking region, the transcription start site, 48 bp of exon 1, the 2.5-kb intron 1, and the 15-bp exon 2 of mouse \(\alpha\)SMA.\textsuperscript{12} In these animals, GFP is specifically expressed in both vascular and nonvascular smooth muscle cells and pericytes with contractile properties.\textsuperscript{13} All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures involving animals were reviewed and approved by the appropriate local governmental and academic committees at the University of Tübingen.

**Confocal Scanning Laser Ophthalmoscopy**

For retinal imaging, we used a commercially available confocal scanning laser microscope (HRA I SLO; Heidelberg Engineering, Dossenheim, Germany) featuring two argon wavelengths (488 nm and 514 nm) in the short-wavelength range and two infrared wavelengths (795-nm and 830-nm diode lasers) in the long-wavelength range. The 488-nm argon laser was used for measuring autofluorescence (AF; in this case, detecting the GFP signal) and the 795-nm diode laser for indocyanine green angiography (ICGA). Barrier filters were used at 500 and 800 nm, respectively. A detailed protocol for anesthesia and imaging is described elsewhere.\textsuperscript{14} Briefly, mice were anesthetized by subcutaneous injection of ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg), and their pupils were dilated with tropicamide eyedrops (Mydriaticum Stullin; Pharma Stullin, Stullin, Germany) before image acquisition.

**Spectral Domain Optical Coherence Tomography**

Spectral domain optical coherence tomography (SD-OCT) was performed in the same session as cSLO, as previously described, to control for the structural integrity of the transgenic mouse retina.\textsuperscript{14,15} Briefly, mouse eyes were subjected to SD-OCT using a commercially available device (Spectralis HRA+OCT; Heidelberg Engineering). For acquisition of circular scans, a mean of 16 images was calculated with automated alignment of iterative recordings using the automated real-time mode, thereby increasing the signal-to-noise ratio by a factor of 4.\textsuperscript{16,17} Resultant data were exported as 8-bit color bitmap files and processed in a graphics editing program (Photoshop CS3; Adobe Systems, San Jose, CA). For quantification of retinal thickness, we used the proprietary software package version 3.1 from Heidelberg Engineering. Briefly, each volume scan consisted of 121 parallel line scans with an interscan step of 1 pixel of radius) of the illumination area and a 4 to 1.5 log cd · s/m\(^2\) divided into 10 steps. Ten responses were averaged with an interstimulus interval of either 5 seconds or 17 seconds (for 0, 0.5, 1, and 1.5 log cd · s/m\(^2\)).

**Quantification of Vessel Diameter and AVR**

To improve the reproducibility of the quantification results, a vessel detection algorithm was implemented (MATLAB; The MathWorks, Inc., Natick, MA) that allows stretching a circle centered on the optic disc used as the vessel detection line. Along this line, the luminescence differences (mean of circle line ± 5 pixels of radius) of the illumination-equalized grayscale images were calculated to find vessel boundaries. We used a polar coordinate resolution of 1° to obtain 360 grayscale pixels. In AF images, only boundaries of arteries were identified in the first iteration because they produce a much stronger signal in this mode than do boundaries of veins. In the next iteration, the identified arteries were omitted from the detection process to find the boundaries of veins. A similar protocol was applied to ICGA images using the vessel information from the previous AF image analysis. The resultant boundary information was used to quantify the inner and outer vessel diameters and to calculate the respective AVR. Briefly, all vessels crossing the circular detection line with diameters of 3.45 mm were quantified. At an eccentricity of 1.725 mm, all vessels were quantified before their bifurcation (i.e., as first-order vessels). To avoid bias in manually pairing vessels (see Fig. 4), the average of the arteriolar diameters was divided by that of the venules. Each vessel diameter was quantified using five iterative measurements at the five neighboring pixels along the length of the vessel at the circular ring scan. The average of those values was used to quantify the AVR.

**Histology**

To verify the localization of GFP expression in \(\alpha\)SMA-GFP mice, eyes were enucleated after in vivo imaging and immediately fixed in 4% paraformaldehyde for 4 hours. After washing with PBS, retinas were permeabilized in 0.5% Triton-100 and 1% BSA for 30 minutes and then incubated with Bandeiraea simplicifolia isofloctol conjugate to tetramethyl rhodamine (TRITC, 1:50; Sigma-Aldrich, Munich, Germany) at 4°C overnight. After washing with PBS, retinas were flat mounted on objective slides with 50% glycerol. Photos were taken using a fluorescence microscope connected to a video camera (Leica, Wetzlar, Germany).

**Human Data and Donor Tissue**

All experiments involving human subjects adhered to the tenets of the Declaration of Helsinki. The fundus picture of a patient with characteristic Gunn sign was taken after written informed consent was obtained and was processed as anonymous clinical data (Photoshop CS3; Adobe). The human donor eye was obtained after proper written informed consent was obtained. Tissue was fixed in 4% formalin and incubated in aqua bidest for 30 minutes. Then the retina was digested in 3% trypsin resolved in 0.2 M Tris-HCl (pH 7.4) for 3 hours at 37°C. Subsequently, the isolated retinal vasculature was dried on objective slides and stained with periodic-acid Schiff and hematoxylin. Quantitative analysis was achieved by measuring arteriolar and venular diameters in the corresponding areas of the inner third and the middle third of the retina using an image analysis system (CUE2; Olympus Opticals, Hamburg, Germany).

**Results**

**Confocal Scanning Laser Ophthalmoscopy**

En face imaging of retinal vessels using cSLO showed no apparent difference in the native red-free mode (514 nm) between wild-type and \(\alpha\)SMA-GFP mice (Figs. 1A, 1B). When using the AF mode (488 nm), \(\alpha\)SMA-GFP mice displayed a...
strong GFP signal outlining retinal vessels, though there was only the physiological background signal in wild-type mice (Figs. 1C, 1D). Although the strength of the GFP signal correlated with the number of smooth muscle cells and contractile pericytes, the greater width of the muscular walls of arteries and arterioles produced a stronger signal than that of veins and venules (Fig. 1D; see Figs. 3C, 3D, 4B). ICGA allowed visualization of both retinal and choroidal structures as the infrared light penetrated pigmented tissue such as the RPE and choriocapillaris. Because ICG featured high affinity to plasma proteins (>98% was bound), it remained within the vascular lumen for a considerable time even if the vessels were fenestrated. Thus, we used ICG angiography to determine the inner diameter of the retinal vessels in wild-type and αSMA-GFP mice and found no apparent difference in the vasculature pattern in any of the investigated animals (representative data are shown in Figs. 1E, 1F).

**Spectral Domain Optical Coherence Tomography**

In αSMA-GFP transgenic and wild-type mice, SD-OCT was performed in addition to cSLO imaging analysis to exclude any abnormalities potentially associated with the transgene expression. No structural differences were found between the retinas of wild-type and αSMA-GFP mice at the site of AVR measurement and beyond with both cSLO and SD-OCT imaging (Figs. 1G, 1H). Retinal thickness in wild-type mice in the central 3 mm measured 238.3 μm ± 4.9 μm (mean ± SD) compared with 238.6 μm ± 3.5 μm in αSMA-GFP mice (P = 0.4; n = 6).

**Electroretinography**

To determine whether transgenic αSMA-GFP and wild-type mice differed in terms of retinal function, flash ERGs were recorded under scotopic conditions. The flash ERG responses in αSMA-GFP mice (n = 5) were comparable to those in wild-type mice (n = 5) even at 14 months of age (Fig. 2), indicating that the vasculature was able to maintain its physiological role for the inner retinal layers at least up to the age tested.

**In Vivo AVR Assessment**

Although ICGA allowed assessment of the inner vessel diameter (Figs. 3A, 3B), the vessel wall and thus the outer vessel diameter was visible in AF mode images of αSMA-GFP mice (Figs. 3C, 3D). Because ICGA uses IR stimulation at 795 nm and the AF mode blue light stimulation at 488 nm, both channels could be used simultaneously. We quantified the arterial and venous vessel diameters in ICGA (inner diameter) and AF (outer diameter) recordings at the same eccentricity from the optic nerve head, which allowed us to compare the respective AVR (Fig. 4). The mean AVR based on inner diameters was AVRid = 0.72 ± 0.08 (mean ± SD), and that based on outer diameters was AVRod = 0.97 ± 0.09. The difference between AVRid and AVRod was statistically significant (P < 0.01; n = 6).

**Histology**

Light microscopic analysis of retinal wholemount preparations was performed to verify the localization and distribution of GFP expression in the αSMA-GFP mice. As shown in Figure 5, retinal vasculature was marked by lectin labeled with TRITC. Transgenic GFP in αSMA-GFP mice was strongly expressed in smooth muscle cells (SMCs), and contractile pericytes were expressed in retinal arterioles and in the first- and second-order branches of the retinal arterioles. In addition, GFP was weakly expressed in the few SMCs and residual contractile pericytes in retinal venules. No GFP expression was detected in retinal capillaries or in nonvascular cells. Thus, the expression pattern of GFP also indicated the site of peripheral control of perfusion pressure and mirrored the hemodynamic force distribution in the retinal vasculature.

**Human Samples**

The human retina was digested with trypsin to display the entire retinal microvascular network (Figs. 6A–C). This allowed us to quantify the dimensions of the first- and second-order retinal arterioles and venules ex vivo. Given that the vessel...
wall, including the smooth muscle cells, endothelial cells, and pericytes, remained intact in the trypsin digestion, the result-
ant AVR could be regarded as based on the outer diameter. Calculated AVR in first-order (AVR_{trypsin 1°} = 0.90 ± 0.11; n = 10) and second-order (AVR_{trypsin 2°} = 0.89 ± 0.06; n = 10) vessels did not differ significantly.

**DISCUSSION**

Ophthalmoscopic examination of retinal circulation provides valuable information on the integrity of the retinal vascular system. Given that retinal vessels share anatomic and physiological aspects with cerebral and coronary blood vessels, qualitative aspects and quantitative measures such as the retinal AVR can help to assess the state of the vasculature in general. This has implications for some of the major causes of morbidity and mortality (e.g., diabetes and systemic hypertension).

Because of the low optical density of the normal vessel wall, AVR estimation is based on the inner vessel diameter (the blood column), and a ratio of 2:3 is regarded as normal.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932962/)

**Figure 2.** Electroretinographic evaluation in 14-month-old αSMA-GFP (n = 3) versus wildtype (n = 5) mice. (A) Dark-adapted (scotopic) single-flash ERGs of a wildtype (left) and an αSMA-GFP mouse (right). Vertical line crossing each trace shows the timing of the light flash. (B) Scotopic b-wave amplitudes from wildtype and αSMA-GFP mice as a function of the logarithm of the flash intensity. Boxes: 25% and 75% quantiles ranges. Whiskers: 5% and 95% quantiles. Solid line connects the medians of the αSMA-GFP data. Dotted lines: 5% and 95% quantiles of the wildtype data.

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932962/)

**Figure 3.** In vivo visualization of inner versus outer vessel diameter in an αSMA-GFP mouse. (A, B) ICGA displays inner diameter of retinal vessels. (C, D) GFP signal in contractile elements of the vessel wall in αSMA-GFP mice is detected in the AF modus. Difference between inner (ICGA; A, B) and outer (AF; C, D) diameters of retinal vessels can be appreciated.

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932962/)

**Figure 4.** Quantification of inner diameter based on (A) ICGA and outer diameter based on (B) GFP signal detection in contractile elements of the vessel walls in the αSMA-GFP mouse using the AF mode. (C) Arteriovenous ratios based on ICGA/inner diameter (mean ± SD; 0.72 ± 0.08) and AF/outer diameter (mean ± SD; 0.97 ± 0.09) are presented for each mouse (n = 6).
expression in some retinal cells, the transgenic expression evidence for a potentially toxic effect of transgenic GFP because of its low optical density, is not— or at least is not 

This, we argue, is the hypertrophic vessel wall, which, an invisible circumferential structure is causing the compression. Indeed, it seems that appear to impinge directly on the venule. Indeed, it seems that 

FIGURE 5. Histologic analysis of GFP expression pattern in an αSMA-GFP mouse. (A, B) Lectin-TRITC staining of the vascular endothelium equally displays all retinal vessels in the wholemount preparation. (C, D) Transgenic expression of GFP in the contractile components of the vessel wall displays the spatial distribution of hemodynamic regulatory elements within the retinal vasculature (i.e., smooth muscle cells and contractile pericytes). (E, F) Overlay of lectin-TRITC and GFP signals. GFP signal is most prominent in first-order arterioles (solid arrowhead) and decreases subsequently in second-order arterioles (solid arrow) and third-order arterioles (open arrow). Few GFP-positive cells are located at venules (open arrowhead). Original magnifications, ×25 (left), ×200 (right).

ever, there are clinical signs that indicate structural changes in the vessel wall secondary to for example, arterial hypertensive disease, in which persistently elevated blood pressure leads to atherosclerosis in retinal blood vessels. This process is characterized by intimal hyalinization, medial hypertrophy, and endothelial hyperplasia, leading to increased overall thickness and altered optical qualities of the formerly transparent vessel wall. One exemplary clinical finding is the crossing phenomenon (Gunn sign, Fig. 6D), which is thought to arise from venous compression by a thickened arteriole within the shared sheath causing hourglass constrictions of the vein on both sides of the crossing. However, in routine funduscopy, the visible inner diameter of the arteriole does not appear to impinge directly on the venule. Indeed, it seems that an invisible circumferential structure is causing the compression. This, we argue, is the hypertrophic vessel wall, which, because of its low optical density, is not—or at least is not easily—detectable in routine funduscopy.

The αSMA-GFP mouse model allows in vivo examination of the vessel wall and quantification of the AVR based on both inner and outer diameters as the GFP expression demarcates the contractile components of the vessel walls because of its expression under the αSMA promoter (Tsai JY, et al. IOVS 2002;43:E-Abstract 1929). Although there is evidence for a potentially toxic effect of transgenic GFP expression in some retinal cells, the transgenic expression of GFP in the αSMA-GFP mouse did not lead to any perceivable abnormality. Indeed, our data demonstrate normal anatomy, including the retinal vasculature and laminar composition, and normal retinal function of both the inner and the outer retina in the αSMA-GFP mouse compared with wild-type control animals (Figs. 1–5). Hence, there is no indication that the AVR as determined in the αSMA-GFP mouse would differ from wild-type animals.

Although structural differences between arterioles and venules with regard to vessel wall composition are well known, ex vivo studies are hampered by the confounding effects of histologic preparation on tissue composition and dimension. Nevertheless, the calculated AVR based on the quantification of vessel diameter in the trypsin-digested human specimen was remarkably close to the AVR in vivo based on the in vivo approach in the αSMA-GFP mice. The remaining difference might well be explained by interspecies difference or the lack of systemic blood pressure on vessels ex vivo, which is expected to affect arterial and venous vessels differently because of the discrepancy in vessel wall composition (e.g., number of smooth muscle cells and pericytes).

In conclusion, we were able to visualize and quantify retinal vessel outer diameters in vivo in an αSMA-GFP transgenic mouse. We found that though arterioles and venules differ substantially in lumen and vessel wall width, they share a common outer diameter, leading to an AVR close to unity. This setup allows the detection of dynamic changes in a noninvasive time line analysis to explore, for example, the effect of pathologic stimuli or therapeutic interventions on the retinal vascular system. Thereby, it may provide new and exciting insights into disease dynamics in common ophthalmologic disorders such as diabetic retinopathy.

FIGURE 6. Human retinal microvasculature ex vivo and in vivo. (A–C) Trypsin-digested human specimen displays the outer vessel diameter of both arterioles and venules. Although the fixation and digestion protocol is a potential source of differential tissue alteration, the AVR base on repetitive measurements (C) of first-order (AVRexp 1° = 0.90 ± 0.11; n = 10) and second-order (AVRexp 2° = 0.89 ± 0.06; n = 10) vessels were close to the AVR measured in vivo in the mouse (0.97 ± 0.09). (D) In vivo fundus picture taken in a patient with systemic hypertension and classic presentation of a positive Gunn sign. The hypertrophic arteriolar vessel wall, with its characteristic low optical density, impinges on the venule and causes the classic hourglass constriction, whereas the visible inner diameter seems only indirectly involved.
References