In Vivo Characterization of Spontaneous Retinal Neovascularization in the Mouse Eye by Multifunctional Optical Coherence Tomography

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PURPOSE. To investigate the early development of spontaneous retinal neovascularization in the murine retina by a multifunctional optical coherence tomography approach. To characterize involved tissue changes in vivo and describe structural and functional changes over time.

METHODS. A multifunctional optical coherence tomography (OCT) system providing 3-fold contrast comprising reflectivity, polarization sensitivity, and OCT angiography (OCTA) was utilized to image very-low-density lipoprotein receptor (VLDLR) knockout mice. Baseline measurements were acquired as early as postnatal day 14 and a follow-up of neovascularization development was performed until the age of 3 months. Control mice were imaged accordingly and a multiparametric image analysis was performed to characterize different stages of pathologic vascular growth. Histology was conducted at the endpoint of the experiment. An interventional pilot experiment was conducted to investigate the effect of the anti-vascular endothelial growth factor (VEGF) agent aflibercept on the development of retinal neovascularization.

RESULTS. Onset of neovascularization was imaged at baseline, and significant changes were encountered in the retina over time, including reduced retinal thickness, increase of lesion volume, migration of pigmented structures, and presence of abnormal blood flow in the outer retina. Multifunctional image contrast was correlated to ex vivo histology. Microscopic analysis of retinal flat mounts and cross-sectional samples confirmed the changes observed in vivo structural and functional OCT images. Administration of an anti-VEGF agent resulted in a significantly reduced lesion volume.

CONCLUSIONS. Longitudinal, multifunctional OCT imaging of infant VLDLR−/− mouse retinas enabled a multiparametric, in vivo staging of neovascularization formation from before lesion onset until their manifestation.

Keywords: optical coherence tomography, retinal neovascularization, retinal pigment epithelium, imaging/image analysis: nonclinical, OCT/OCT angiography
and hence may not provide a full picture of a disease. Preclinical research can potentially fill this gap by enabling prospective studies under controlled conditions and thus plays a crucial role in basic ophthalmic research focusing on ocular diseases such as AMD \(^9\) or glaucoma. \(^10\) The recent success of pharmaceutical clinical trials to prevent vision loss in AMD has led to an upswing of ophthalmic drug development. Hereby animal models of neovascular diseases have been playing an important role in the assessment of the antiangiogenic activity and the ability to reduce vascular growth. \(^11\) Rodents are among the most popular model organisms due to their small size, cost efficiency, and the variety of strains available. Although animal models will not exactly reflect the pathophysiology of human ocular diseases, models were established that imitate specific pathologic aspects in high detail and that allow identification of potential molecular players in AMD. \(^9\) The very-low-density lipoprotein receptor (VLDLR) knockout mouse model is a popular model of wet AMD. \(^12\) VLDLR \(^/-\) mice develop neovascularization with retinal origin and show very similar traits as seen in type 3 neovascularization. \(^13\) Analogously to the human eye, there is a need to precisely characterize the phenotype of animal models in order to understand the pathophysiology such that these models can reliably be used for comparative studies of the human eye and the development of novel molecular approaches preventing disease progression. \(^15\) \(^17\)

Multimodal imaging proved to be a valuable approach for the differentiation between different AMD types in the human eye. \(^4\) Especially SD-OCT pushed diagnosis and has recently been combined with OCT angiography (OCTA) \(^16\) \(^15\) in order to enable a better understanding of vascular patterns associated with neovascularizations on a capillary level in the human eye \(^20\) \(^21\) but also in the rodent retina. \(^22\) \(^25\) Polarization-sensitive (PS)-OCT \(^26\) \(^28\) is another functional extension that has recently been used in clinical studies to advance tissue differentiation of macular diseases, for example, by contrasting migrated RPE or by visualizing subretinal fibrosis. \(^29\) \(^35\) Combining OCTA and PS-OCT into a multifunctional OCT imaging approach was in the past demonstrated for skin tissue but also for the human eye. \(^36\) \(^38\) Our group adapted PS-OCT for preclinical imaging of the rodent eye \(^39\) \(^40\) and recently extended the method to a multifunctional OCT retinal imaging approach. \(^41\) \(^42\) In our past study of the VLDLR knockout mouse model, we introduced a multifunctional OCT approach enabling a description of long-term retinal changes occurring between 1 and 11 months of age. \(^43\) One limitation of that study was the rather late baseline measurement only after neovascularizations, which in the mutant mice usually form between postnatal day (P)14 and P21 \(^41\) \(^42\) and had already been well developed.

In this work we tackled the characterization of early spontaneous neovascularization processes. Hence, the baseline measurement was scheduled at the age of just 2 weeks, followed by three measurements at the ages of 1, 2, and 3 months, respectively. Thus, we established (1) an in vivo characterization of the spontaneous neovascularization forming in the VLDLR \(^/-\) retina, thereby differentiating involved structures, particularly blood vessels and RPE, based on their intrinsic scattering properties; see Figure 1a. These multifunctional contrast parameters were (2) correlated to histology and could precisely confirm motion contrast and PS contrast as well as the conventional OCT contrast. Furthermore, (3) a proof-of-concept experiment demonstrated the suitability of the imaging approach for interventional studies based on the administration of an anti-vascular endothelial growth factor (VEGF) agent into the mouse eye. Hence, the presented results may foster the use of OCT in pharmaceutical preclinical studies to develop new molecular approaches for vision-threatening diseases such as AMD.

**METHODS**

**Animals**

Heterozygous breeding of VLDLR mice (Vldr\(^{+/+}\)heter. The Jackson Laboratory, Bar Harbor, ME, USA; Stock No: 002529) was established at the Division of Biomedical Research, Medical University of Vienna, and animals were kept under controlled lighting conditions (12 hours dark, 12 hours light). Three groups, namely knockout (KO), control (CO), and interventional knockout (IKO), of each five animals \((N = 5)\) were bred by time-mating. Homozygous VLDLR \(^/-\) were used for the KO and IKO group, and their heterozygous littermates were used for the CO group.

All mice in the KO and CO groups were initially imaged at P17 \((\pm 2.0)\), which served as a baseline (BL) measurement. Three follow-up measurements (F1, F2, and F3) were acquired at P31, P59, and P85, respectively. \((\pm 0.0)\). Mice in the IKO group were imaged at P14 \((\pm 0.0)\), P28, P57, P85, and P122 \((\pm 0.0)\). Mice in the KO and CO groups were female, and mice of the IKO group were male. The acquisition timeline and study design are shown in Figure 1b. Mice were anesthetized with a dose of 133 mg/kg body weight ketamine (Ketavet; Intervet GmbH, Vienna, Austria) and 5.3 mg/kg body weight xylazine (Rompun; Bayer Austria GmbH, Vienna, Austria) and additional dosing if necessary. The dose at the BL and F1 measurements were administered with a Hamilton (Reno, NV, USA) microsyringe, owing to the small size and weight of the mice at these stages; see Figure 1c. For the BL and F1 experiments, the mice were anesthetized subcutaneously \((s.c.)\) while for all other follow-up measurements the anesthetics were administered intraperitoneally. Mice were antagonized with 0.2 mg/kg atipamezole (Antisedan; Orion Corporation, Espoo, Finland) \(s.c.\) if necessary after 1 hour. Both eyes of the KO and CO groups were imaged at every time point. Both eyes of IKO mice were imaged at F1 through F4 while only the interventional eye was imaged at the BL to reduce the time under anesthesia required for the experiment.

The pupils were dilated using one drop of each tropicamide (Mydriaticum, topical; Agepha Pharmaceuticals, Vienna, Austria) and phenylephrine (2.5%, topical). Eyes of the animals were kept moistened by artificial eye drops (Oculotect; Novartis Pharma GmbH, Vienna, Austria) under anesthesia required for the experiment. Anticoagulation was performed by intraperitoneal administration of 0.9% NaCl (0.1–0.2 ml. s.c.). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under a protocol approved by the ethics committee of the Medical University of Vienna and the Austrian Federal Ministry of Science, Research and Economy (GZ:BMWFU-66.009/0121-WF/V/3b/2010).

**Multifunctional Optical Coherence Tomography**

In this work we utilized a multifunctional OCT imaging system that was described in detail by Fialová et al. \(^35\) and has been recently used to image mouse \(^41\) and rat models \(^12\) \(^44\) of ophthalmic diseases. In short, the system is based on a SD, PS-OCT unit enabling high-speed retinal imaging at 83-kHz A-scan rate with an axial resolution of 3.8 μm in retinal tissue. A repeated raster scanning protocol was used to image the posterior eye and covered a field of view of approximately 1×1 mm\(^2\) on the retina. The acquisition time for the 2000 B-scans containing 512 A-scans at 400 distinct locations was approximately 15 seconds. The signal-to-noise ratio was improved by averaging repeated B-scans, and OCTA images were calculated.
based on the complex differences of consecutive B-scans at the same location.\textsuperscript{19,41} The degree of polarization uniformity (DOPU) parameter\textsuperscript{45} was determined based on the PS-OCT contrast and enabled the identification of depolarizing tissue such as melanin pigments in the RPE or the choroid.\textsuperscript{40} Using the DOPU parameter, the polarization-preserving retina can be distinguished from tissues exhibiting polarization scrambling as it is caused by melanin in the RPE and choroid. DOPU images shown in this work map polarization-preserving tissue in red (DOPU > 1); depolarizing tissue in yellowish to bluish hues (DOPU ≤ 0); and background regions with low reflectivity, which do not allow reliable DOPU calculation, in black. The threshold for the OCTA and DOPU images was defined as the mean signal plus three times the standard deviation of the background determined in the vitreous. The imaging concept provides coregistered, 3-fold contrast, which enables differentiation of static from moving scatterers (blood flow) and pigmented from nonpigmented tissue (via depolarization). A sketch of the multifunctional OCT imaging concept and contrast mechanisms for the characterization of neovascularizations is shown in Figure 1a.

**Multimodal Image Analysis**

A detailed description of the postprocessing steps can be found in our previous study,\textsuperscript{41} and thus only a brief description of the quantitative parameters and additionally introduced analysis is given in the following. Postprocessing of the raw data was performed by custom-made software written in MATLAB (R2015b; The MathWorks, Inc., Natick, MA, USA). Fiji (ImageJ v.1.51p)\textsuperscript{46} was used for three-dimensional (3D) rendering and visualization.

**Thickness Evaluation.** The total (inner limiting membrane [ILM] to RPE) and outer retinal (outer plexiform layer [OPL] to RPE) thickness were calculated as the average values in a circumpapillary annulus with an outer radius of 225 pixels (~450 μm). Hereby, a central circular region with a radius of 125 pixels (~250 μm) was excluded.

![Figure 1](http://arvojournals.org/)
Lesion Segmentation. Retinal lesions that were present as hyperreflective structures in the reflectivity B-scan images were segmented manually by two graders (G1, G2). The data were anonymized beforehand, such that the graders did not know which eye at which time point was currently being evaluated, to ensure an objective interpretation of the image data. The task of manual segmentation was performed using ITK-SNAP (version 3.6.0). The number of lesions for each animal was determined by an en face projection of the binary segmentation data, and connected voxels were assigned as one lesion. 

Depolarization Analysis. Each DOPU volume was segmented by Otsu’s adaptive thresholding method. The resulting binary image was used to differentiate polarization-preserving (0) from depolarizing tissue (1). 

Lesion Model. To characterize the lesions at the different ages, a descriptive lesion model was developed based on the manual segmentation in the reflectivity images. The centroid of each lesion was used to coregister all lesions for the four different datasets that did not fulfill the validation were removed and disregarded in the following quantitative analysis. The reduced numbers of samples (N) are given in the corresponding Results section. 

Validation. All datasets were validated and inspected for proper retinal layer segmentation and motion correction. Datasets that did not fulfill the validation were removed and disregarded in the following quantitative analysis. The reduced numbers of samples (N) are given in the corresponding Results section. 

Histology
For histologic analysis, two mice of each group, KO and CO, were euthanized after the last OCT measurement on P85. Eyes were enucleated immediately, opened, and fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 2 hours at room temperature (RT). Left eyes were dehydrated in ascending concentrations of ethanol and embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin/eosin (HE) or Heidenhain’s azan stain. Right eyes were prepared for blood vessel labeling in retinal whole mounts. 

Labeling of Blood Vessels in Retinal Whole Mounts.
Retinas were dissected free, washed in PBS, and cryoprotected in ascending concentrations of sucrose. After three freeze/thaw cycles, retinas were blocked in 3% normal donkey serum in PBS vehicle (L PBS vehicle; Bayer Pharma AG, Berlin, Germany) at P14. Administration of Anti-VEGF
Mice of the IKO group received a unilateral intravitreal injection of the anti-VEGF agent afilibercept (Eylea; 2 μg in 0.5 μL PBS vehicle; Bayer Pharma AG, Berlin, Germany) at P14. The concentration of the anti-VEGF was calculated to resemble the dose usually used for human patients. Mice were anesthetized as described above and additionally received 200 mg/kg metamizole (Novalgin; Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany) and 5 mg/kg meloxicam (Metacam; Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany). The right eye was locally anesthetized by a drop of Oxybuprocain (1%; AGEPA GmbH, Vienna, Austria) and NaCl solution on the cornea prior to the intervention. Three investigators ensured the stabilization of the animal and monitored the injection. The mice were restrained in lateral recumbency, eyes were protruded by applying subtle pressure around the eye cup, and the bolus was injected through the sclera into the vitreous. A 35-gauge Hamilton needle was used to inject the anti-VEGF solution intravitreally.

Statistical Analysis
The quantitative parameters investigated in this work were tested for significant changes over time and between groups. To determine significant changes over time, Friedman’s test was used with post hoc Mann-Whitney U tests. To compare differences between the groups, a Mann-Whitney U test was performed. To conclude on the interpretability of the acquired image data and to analyze the interobserver variability, a 2-way mixed, agreement, single-measures intraclass correlation coefficient (ICC) was determined for the manual segmentation of the two graders. All statistical analysis was done using R (The R Foundation, v.3.3.1). All values are given as mean values (±standard deviation) unless otherwise stated. For parameters evaluated by both graders, only values for G1 are given, and plotted. P values < 0.05 were considered as statistically significant.

Results
Lesion Onset and Development
Retinal lesions manifest as hyperreflective structures in the outer retina of VDLIR−/− mice. Figure 2a shows 3D reflectivity images (top) along with the manual segmentation including the segmented chorioid (middle) and a reflectivity projection of the outer retina (bottom). Some hyperreflective spots were visible in the outer retina at P15 (yellow arrow). In comparison, the outer retinas of the control mice did not show such spots at any time; see Figure 2b. The number of retinal lesions increased from P15 to P29 and a substantial growth of the hyperreflective area in the outer retina was observed at P57 and P83. In the following sections, different tissue structures causing an increased reflectivity in the outer retina, such as translocated melanin pigments or blood vessels, are differentiated. To quantify the development of retinal lesions, the hyperreflective sites were segmented manually and the number of lesions, as well as their volume, was determined. The average number of lesions per animal is plotted in Figure 2c, where each thin line represents the development per mouse eye and the thick line shows the average number of lesions for all investigated eyes (N = 10). The average number of lesions was determined as 7.6 (±5.5), 10.0 (±3.4), 7.7 (±1.9), and 6.8 (±1.7) for the BL, F1, F2, and F3 measurements, respectively. Figure 2d shows the lesion volume development per eye (thin lines) and their average (thick line). The average lesion volume was quantified as 0.0009 (±0.0008), 0.0062 (±0.0026), 0.0104 (±0.0042), and 0.0112 (±0.0034) mm3 for the BL, F1, F2, and F3 measurements, respectively. A significant change in the lesion volume was determined (P < 0.001). Post hoc tests revealed significant changes between BL and F1, BL and F2, BL and F3, F1 and F2, and F1 and F3 (all P < 0.05). Furthermore, the image data were analyzed regarding thinning of the total retina and outer retina and compared to the CO group; see Figures 2e and 2f. While thinning of the retina was observed for all mice between BL and F1, the thickness continuously decreased also at older age only for the KO group. A change of the total retinal thickness was determined for the KO group (P < 0.001) and revealed
FIGURE 2. Structural changes assessed by OCT reflectivity contrast. (a) 3D reflectivity images along with their corresponding lesion and choroid segmentation and outer retinal reflectivity projection. A time series between P15 and P83 shows the significant increase in lesion volume. At BL only few lesions were determined and are indicated by yellow arrows. (b) En face projections in the outer retina of control mice did not show lesion development. (c) The number of retinal lesions peaked at F1. (d) The lesion volume increased significantly and peaked with 0.0112 mm³ at F3. (e) Total and (f) outer retinal thickness changes in the KO and CO groups over time. A significant decrease was determined for the total and outer retinal thickness for the KO over the CO group.
significant thinning between BL and F2, BL and F3, and F1 and F3 (all \( P < 0.05 \)). A significant change (\( P < 0.001 \)) was also observed regarding the outer retinal thickness over time, where a significant decrease was observed between F1 and F2 and between F1 and F3 (all \( P < 0.05 \)). No significant changes were determined for the CO group regarding the total retinal thickness (\( P = 0.127 \)) and outer retinal thickness (\( P = 0.212 \)). Furthermore, a significant total retinal thickness difference between the KO and CO groups was determined at F2 (\( P = 0.034 \)). The outer retina was significantly reduced in the KO group at BL, F2, and F3 (all \( P < 0.05 \)). While for the KO group complete datasets were available for nine cases, the number of complete datasets of the CO group was five. A comparison between the manually segmented lesions of G1 and G2 resulted in an ICC of 0.94, which shows an excellent agreement between the two raters and suggests easy interpretability of the OCT image data.

**Changes in Depolarization Properties Due to Melanin Movement**

Development of neovascularization causes severe retinal changes including dislocation of melanin granules from the RPE or choroid. Melanin pigments cause depolarization of the defined incident polarization state in PS-OCT. This effect results in low DOPU values and is indicated by a yellowish to bluish appearance in the false-color representation of the DOPU images. Polarization-preserving tissue is shown in red, and parts undercutting an intensity threshold are shown in black. Figure 3a shows en face minimum DOPU projections of the outer retina in a VLDLR \(^{-/-} \) mouse. DOPU maps show the presence of depolarizing intraretinal deposits at F2 (PS7) and F3 (PS8). For comparison, the same projection type for the control mouse data does not reveal depolarizing intraretinal deposits; see Figure 3b. Corresponding reflectivity and DOPU B-scans are shown in Figures 3c through 3f while their positions within the volume are indicated by a white line in Figures 3a and 3b. DOPU and reflectivity changes occurring at a lesion site can be observed very clearly over time. For comparison, Figures 3d and 3e show a healthy retina at F1 and F3. A clear distinction between the retina and RPE is possible. Moreover, in some parts the RPE can be distinguished from the choroid based on the DOPU changes (RPE and choroid are indicated by white arrows). Outer retinal depolarization was encountered only from F1 onward. The volumetric increase of depolarizing tissue is shown in Figure 3g for the individual eyes (thin lines) and in average (thick lines). The analysis revealed no migration of depolarizing pixels in the CO group. Figure 3h shows a depth distribution of the depolarizing granules, taking the upper RPE border segmentation as a reference (\( z = 0 \)). A bimodal distribution was hereby observed at BL and F1 for the KO and CO groups, which indicates two clusters of depolarizing voxels—one representing the RPE and one the choroid. While no longitudinal changes were observed for the CO group (only minor changes in the choroid), the distribution in the KO group changed substantially and a migration of some depolarizing tissue was observed along with a blurring of the two peaks (see zoom-in). While the Friedman’s test indicated significant changes in migrated depolarizing voxels (number) over time (\( P < 0.01 \)), the post hoc tests revealed no significant changes, most likely due to the small sample size (complete datasets, \( N = 5 \)) and ties that did not allow an exact calculation of the \( P \) value.

**Imaging Spontaneous Neovascularization by OCTA and Correlation to Histology**

Neovascularization forms in the VLDLR \(^{-/-} \) mouse from the retinal plexuses, similar to the type 3 neovascularization in AMD patients. OCTA was used to identify changes in the vascular plexuses and to monitor the development of spontaneous neovascularization over time. Figure 4a shows the vascular plexuses from BL to F3 as depth color-coded vascular maps between the ILM (cyan) and RPE (red). In addition, the deeper capillary plexus (DCP) in the outer plexiform layers is shown as this structure is believed to be the origin of the neovascularizations. The DCP map shows the repeatability of the OCTA measurements over time, although signal fluctuations can be observed and interpretation must therefore be made with care. The presence of blood flow in the subsensory retina can be seen in the OCTA maps (stars) and is visible as bright spots in the DCP projections with yellow circles. Bright spots within and in the proximity of the optic nerve head (ONH), indicated with red circles, are not subject to new vessel growth and are mainly caused by the en face projection, where connective vessels are cut and usually occur as larger spots. A 3D rendering of the OCTA volume is shown in Figure 4b for F1 and F3. While one lesion with blood flow was observed at F1 (yellow star), newly developed neovascularizations can be seen in the OCTA rendering at F3 (green stars). A zoom-in into the lesion reveals the connectivity of the lesion to the DCP and is shown for different perspectives in Figures 4c and 4d. The OCTA map at F3 was finally correlated to the isoelectric-labeled histologic flat-mounted retina: see Figure 4e. A check-pattern overlay of the DCP map on the corresponding projection in the whole mount shows the good correlation between the two modalities by the continuity of blood vessels. Figures 4f and 4g show maps of the histologic preparation and the OCTA, respectively. Exemplary lesion sites, showing the excellent agreement between the two modalities, are highlighted with a yellowish dotted line.

**Lesion Model**

To establish a lesion model and characterize the lesion development, all regions of retinal lesions were clustered by date (BL, F1, F2, and F3). The individual lesion traits were analyzed for each date. Figure 5a shows the spatial distribution over time for an individual lesion in the en face view as well as in a cross section. The measurement at BL shows a vertical, densely packed lesion with limited spatial (lateral) expansion. At F1 the lesion spreads out and merges with nearby lesions at F2 and F3, resulting in a larger spatial distribution. An exemplary lesion for the different ages and 3-fold contrast channels is shown in Figure 5b. The change in depth position of the lesion centroids over time is shown in Figure 5c and reveals a movement toward the subsensory retina between BL and F1. With F1, the centroids remain in the lower 20% of the retina. The individual lesion volume over time is shown in Figure 5d. The lesion volume was determined as 0.0002 (±0.0002), 0.0006 (±0.0008), 0.0015 (±0.0027), and 0.0012 (±0.0028) mm\(^3\) for the BL, F1, F2, and F3 measurements, respectively. To estimate the distribution of the retinal lesions in the field of view of the OCT images, the minimum distance to the neighboring lesion was determined. The minimum average distance was quantified as 187.46 (±110.97), 184.04 (±93.90), 197.49 (±113.92), and 188.75 (±105.81) µm for the BL, F1, F2, and F3 measurements, respectively; see Figure 5e.

**Histologic Analysis**

Histologic analysis was performed for two mice each of the KO and the CO groups. Figure 6a shows a flat-mounted, isoelectric-labeled VLDLR \(^{-/-} \) retina revealing the vasculature of the entire mouse retina. The area scanned by OCT is indicated by a white dashed rectangle. Figure 6b shows a retinal flat mount of an age-matched control mouse. A zoom-in and detailed visualiza-
Figure 3. Polarization changes assessed by PS-OCT. (a) Minimum DOPU projection in the outer retina shows increased depolarizing deposits in the retina at F2 and F3. (b) Minimum DOPU projection in the outer retina of a control mouse does not show presence of depolarization. (c, d) Reflectivity and DOPU B-scans of the same dataset as indicated by white lines in the en face maps. Increased depolarization in the lesions in the subsensory retina can be noticed at F3. Scale bar: 150 μm. (e, f) Reflectivity and DOPU B-scans of CO mouse retina. Clear separation between retina (polarization-preserving) and RPE (depolarization) can be noticed. Polarization changes reveal a border between RPE and choroid at locations indicated with white arrows. (g) Depolarizing tissue increased over time. No migration of depolarizing tissue was found for the CO group. (h) Axial distribution of depolarizing voxels with the RPE taken as a reference. While a bimodal distribution can be observed at BL and F1 for the KO and CO groups, the distribution blurred with time and a migration of depolarizing pixels into the retina was observed as indicated in the zoom-in.
tion of the capillary plexuses are shown in Figures 6c and 6d for the KO and CO mouse retina, indicated by a blue and orange dashed rectangle in Figures 6a and 6b, respectively. Neovascularizations in the outer retina of the VLDLR−/− mouse are visualized in yellow. A correlation to the OCTA images of the dataset is shown in Figures 4e through 4g. HE-stained cross sections show accumulation of melanin granules within the retina in the proximity of emerged blood vessels. A correlation between a DOPU B-scan image and the corresponding HE-stained tissue section is shown in Figure 6e. Hereby, both lesion and connective vessels (indicated by green stars) show excellent correlation to OCT reflectivity and DOPU images. Other landmarks used to correlate the cross-sectional positions are indicated by yellow stars (1, blood vessel; 2, pigmentation of hyaloid remnant; 3, ONH). Figure 6f shows an azan-stained cross section through a retinal lesion site that reveals migrated melanin (see zoom-in Fig. 6g), and collagen tissue (blue) between RPE and choroid as well as in the sclera. The melanin pigment granules show a different morphology in the RPE (Fig. 6h) and the choroid (Fig. 6i). While in the RPE, melanin granules have an elongated, ellipsoid shape, melanin granules in the choroid are smaller and primarily exhibit a circular shape. Microscopic analysis revealed that melanin granules at the lesion sites distinctly showed an elongated shape and therefore may have migrated from the RPE. For comparison, part of the retina of a KO mouse is shown in Figure 6j. It reveals an intact structure in the proximity of the ONH.

Effect of Anti-VEGF on the Development of Retinal Lesion Sites in VLDLR−/− Mice

To investigate the effect of an anti-VEGF agent on the development of spontaneous neovascularization in VLDLR−/− mouse retinas, the retinal lesions in the interventional eye as well as in the fellow eye were segmented. Three-dimensional reflectivity and lesion renderings together with en face reflectivity projections of an (interventional) eye and its corresponding fellow eye are shown in Figures 7a and 7b, respectively. While no substantial lesion development was observed in the scanned area of the eye that had received the anti-VEGF agent, the fellow eye developed lesions (one lesion is indicated by yellow arrows). A cross section of a lesion site is
FIGURE 5. Retinal lesions and characterization in the VLDLR−/− mice. (a) The spatial distribution around its centroid for the different stages of development. Merging of lesions and spreading out was noticed at F2 and F3. (b) Exemplary lesion development by multifunctional OCT. Progressive outer retinal destruction can be seen in the reflectivity images. Blood flow in the neovascularization is visible in the OCTA images, and migration of pigmented (depolarizing) tissue is shown in the DOPU cross sections through the lesion. (c) Vertical distribution of retinal lesions over time. (d) Individual lesion volume over time. (e) Minimum distance to closest neighboring lesion. Scale bar: 150 μm.
shown for F2 (P56) and F3 (P85). Furthermore, a minimum DOPU projection of the fellow eye shows presence of depolarization in the outer retina, while no depolarization was found in the outer retina of the treated eye (compare Figs. 7b, 7c). The overall evaluation of the lesion volume showed a reduced lesion volume for the IKO group compared to the KO group and the fellow eyes of the IKO group (IKO-FE). The lesion volume at BL, F1, F2, and F3 was significantly reduced
FIGURE 7. Anti-VEGF therapy and effect on retinal lesion development. (a) 3D reflectivity and lesion segmentation renderings, and outer reflectivity maps of a VLDLR<sup>−/−</sup> mouse treated with anti-VEGF (b) The fellow eye (FE) shows the presence of lesions (indicated by yellow arrows in the en face projections). 3D rendering of choroid a lesion segmentation shows lesion formation in the outer retina with the age of 2 and 3 months. The reflectivity cross sections indicated by the orange and green line in en face projections of P56 and P85 show the presence of a retinal lesion. Depolarization maps indicate the disruption of the retina at the respective follow-up measurements. (c) No substantial melanin migration was detected for the treated eye shown in (a). (d) The number of lesions was reduced for the IKO group and showed an increase that peaked at F2 for IKO-FE and F3 for the IKO group. (e) Lesion volume was significantly reduced for the IKO group. Lesion volume was also reduced for the IKO-FE group but did not reveal any significant difference. (f) Analysis of the depolarization volume revealed a delay and reduction of depolarization in the outer retina for the IKO and IKO-FE groups.
between the IKO and KO groups (all P values < 0.05), but not between the other groups; see Figure 7c. Also a reduction of the number of retinal lesion sites was determined for the IKO eyes and was on average 0.3 (±0.5), 3 (±1.7), 3.4 (±3.3), 5.6 (±5.9), and 3.8 (±3.4) for BL; F1, F2, F3, F4, and F5, respectively (see Fig. 7f). Analysis of the depolarization volume revealed a delay and reduction of depolarizing pixels in the eyes of the IKO and the IKO fellow eyes; see Figure 7f.

**DISCUSSION**

A multifunctional OCT imaging approach featuring a 3-fold contrast based on reflectivity, polarization sensitivity, and angiography was used for the classification of different structures during the development of spontaneous neovascularizations in the VLDLR<sup>−/−</sup> mouse model. Neovascularizations in the VLDLR<sup>−/−</sup> mouse model have retinal origin and resemble type 3 neovascularization in human neovascular AMD. Lesions form between P14 and P21, which was therefore set as the BL measurement time point for this study. Lesions at this early stage were observed as small bright spots in the en face projection and mainly were identified as connective vessels growing outward. Only approximately half of the lesions at that time reached the subretinal sensory (see Fig. 5c), and thus confirm the retinal origin of the vessels. At the first follow-up measurement (at approximately 1 month of age), larger hyperreflective areas were visible in the outer retina (see Fig. 2a) and the external limiting membrane was already progressively affected by lesions. At F1, the average number of lesions peaked before the individual lesion sites connected with each other by anastomosis. This resulted in a lower number of retinal lesions sites at F2 and F3; see Figure 2c. Analysis of the depolarization contrast, particularly the DopU parameter, showed no detectable RPE disruption or choroidal involvement in the neovascularization process at BL and F1. This qualitative observation was confirmed by an evaluation of the axial distribution of highly depolarizing voxels (see Fig. 3h). At the age of 2 and 3 months (F2 and F3), areas of increased depolarization were visible in the subretinal retina. Migration of clusters of melanin granules indicated a disruption of the RPE and choroid at this stage. The depolarization lesion volume continuously increased from F1 onward; see Figure 3f. Quantification of depolarizing tissue changes proved capable of identifying pathophysiological processes. A comparison of Figures 2d and 3g reveals, at early stages, a more pronounced increase of hyperreflective lesion volume as compared to the increase in lesion volume determined using low DopU values, suggesting that lesions were earliest identified using reflectivity information. Nevertheless, the presented approach based on depolarization segmentation and quantification shows that disruption of the pigmented layers can be identified by PS-OCT and hence be used for a characterization of pathologic processes therein. We believe that the DopU parameter could potentially be used as an early marker for neovascularization development in mouse models mimicking type 1 or 2 neovascularization. Sprouting of new vessels and presence of blood flow were identified using OCTA during the longitudinal study. A connectivity of newly formed vessels to the DCP was determined in vivo (see Figs. 4b–d) and was confirmed by precisely matching the in vivo OCTA data with ex vivo isolectin-labeled histology flat mounts (see Figs. 4e–g). OCT angiograms of the DCP show bright spots at the location of neovascular growth. Similar bright spots were previously also described in OCTA images of human patients suffering from wet AMD with neovascularization type 3, where neovascularizations form a dense mesh of tiny vessels. Depending on the lateral resolution of the OCTA system, vessels may appear broader than their true anatomic extension. Thus, in cases where newly formed vessels leave the DCP in close proximity to each other, bright spots rather than individual vessels may be observed by OCTA; see Figures ia and ig. Correlation with histology revealed the presence of multiple tiny vessels in locations of bright spots in the OCT angiograms. Using the intrinsic contrast in multifunctional OCT images, lesions appearing hyperreflective in conventional OCT images were further labeled into lesions containing migrated melanin and blood flow. Furthermore, disruption and degeneration of photoreceptors, as seen in the histologic images (Figs. 6e–j), may affect the appearance of that layer in OCT reflectivity images since photoreceptors have directional scattering properties.

Moreover, the analysis of histologic data enabled a more detailed interpretation of the acquired data and suggests the beneficial complementary use of OCT and histology. Whole mounts allowed an analysis of the whole retina rather than the limited field of view of the OCT image data. The retinal lesions were similarly distributed over the whole retina and thus the region around the ONH seems an appropriate location to investigate lesion development. Furthermore, histologic analysis of the melanin pigments found in the retina at the stage of 2 and 3 months of age showed a morphologic structure (ellipsoid shape) similar to those found in the RPE, which suggests the origin of the granules to be the RPE rather than the choroid. This suggests that the amount of choroidal involvement in the neovascularization development at this stage is rather low.

The results presented in this work were in excellent agreement with our previous study on the long-term retinal changes in the VLDLR<sup>−/−</sup> mouse model that shares the month 2 (F2) and 3 (F3) investigation. The average retinal thicknesses of the overlapping investigations are within the standard deviation of each other. Also, the numbers of retinal lesions is in good agreement: 6.5 (±3.1) at the age of 3 months (previous study) compares well with 6.8 (±1.7) in this work. In this work, 20 longitudinal datasets of mutant mice were acquired already before the age of 2 months. In comparison, in our previous study, only five eyes of mice younger than 2 months were investigated, and as a consequence, the quantitative evaluation was performed only between the ages of 2 and 10 months. Adapting the experimental setting in the present work allowed a considerably earlier investigation of neovascularization onset. Therefore, we here show a more complete characterization of the lesion development and long-term retinal changes of the VLDLR<sup>−/−</sup> mouse model using OCT. Furthermore, imaging the mouse retina as early as P14 allowed a classification and staging of retinal neovascularization from before lesion onset until manifestation of lesion sites. The following stages were observed in vivo: (1) intraretinal sprouting from the DCP between 2 and 3 weeks of age; (2) disruption of the subretinal retina during 1 and 2 months of age; (3) manifestation of neovascularization and anastomosis of retinal–retinal and retinal–choroidal vascularization at the endpoint of this experiment with an age of 3 months. The in vivo classification in this work is in agreement with a description mainly based on ex vivo histology by Hu et al. This characterization reflects well the currently established staging systems for type 3 neovascularizations. In our previous work on the VLDLR<sup>−/−</sup> mouse model, structural changes in the retina and the PS-OCT contrast were correlated to histology. In this work, we not only precisely matched the PS-OCT contrast to ex vivo histology (see Fig. 6e), but we furthermore demonstrated a close match between in vivo OCTA and ex vivo whole mounts. Thus, the 3-fold contrast approach was entirely confirmed by ex vivo histology (see Figs. 4, 6). In contrast to the recent understanding of the
development of this neovascularization subtype in neovascular AMD, we did not find evidence of drusenoid pigment epithelial detachments (PED) prior to the formation of retinal neovascularizations in the VLDLR−/− mouse retina. In patients suffering from type 3 neovascularization, a retinal thickening due to accumulation of intraretinal fluid/cysts in the proximity of the neovascularization sites can be observed.3,21 We did not note intraretinal fluid, nor was the retina found to be thicker around lesion sites. In contrast, a decrease of the outer retina suggests a deformation of the retina without the accumulation of fluid in the VLDLR−/− mouse.

Besides the pathophysiological gain of knowledge by prospective preclinical studies under controlled conditions, preclinical studies are used in pharmacological studies. In this work, a proof-of-concept interventional study was conducted and the effect of aflibercept as an anti-VEGF agent was investigated. Intravitreal administration of aflibercept in one eye of the VLDLR−/− mice at P14 resulted in a significant reduction of lesion development. This effect was observed for the whole (extended) time of 4 months. The number of emerged lesions peaked at an age of 3 months (rather than an age of 1 month in the KO group; see Fig. 7d). This may indicate a slowdown of the neovascularization process by the treatment with an anti-VEGF agent. Evaluation of the fellow-eye lesion development also revealed a decrease in lesion volume and number of lesions, although the reduction was observed to be not statistically significant. This bilateral effect after unilateral injection was also reported in clinical studies and may be attributed to the systemic pharmacokinetics of the administered agent.53,54 Recent work using popular anti-VEGF agents demonstrated that aflibercept and bevacizumab have a stronger systemic exposure than ranibizumab.55 This result is also in agreement with the findings published by Miki et al.,56 who observed a systemic effect of bevacizumab in a transgenic mouse model, but not with ranibizumab. They found that both anti-VEGF agents led to a significant reduction in neovascularization area of the injected eye.56 Furthermore, a comparison of that study and the pilot study presented in this work reveals the benefits and drawbacks of OCT for interventional studies. For histologic analysis, tissue preparation is tedious and time-consuming, and the longitudinal comparison of different mice is required; the in vivo approach offers the opportunity to study the exact same eye at different time points. Also, neovascularization features can be extracted directly and rapidly from the image data. The major drawback of the OCT setup used is the limited field of view whereas the study of retinal flat mounts enables the investigation of the entire retina. A comparison of the histologic whole-mount area to the scanned area by OCT is shown in Figures 6a through 6d.

A limitation in the conducted experiments is the low number of animals used for the longitudinal interpretation. Especially the number of eyes for the interventional group (N = 5) was rather low; hence, the interpretation of the results should be made with care. Nevertheless, significant differences were found and suggest the great potential of in vivo OCT studies in preclinical ophthalmic research. Also, the reproducibility of the results, compared to our previous study, is excellent and underscores the comparability and interpretability of the acquired image data. While in our preceding work only the number of lesions and their location were analyzed, the detailed segmentation in this work allowed a more versatile analysis and interpretation of the multifunctional image data. Segmenting lesions manually is tedious and time-consuming. Hence, a large-scale study, as would be demanded for pharmacological investigations, would require the development of an automated analysis of the image data and could potentially be combined with treatment prediction models as recently shown for clinical studies.57,58 Combining the multifunctional OCT imaging concept with novel machine learning techniques thus could potentially foster the development of new molecular approaches targeting the formation of neovascularization in AMD but also could potentially reveal new biomarkers that could be translated to the human eye.

In conclusion, a multifunctional OCT imaging approach was used to characterize different stages of spontaneous retinal neovascularization formation in the VLDLR−/− retinas from before lesion onset until the manifestation of lesions and associated retinal degeneration. The 3-fold contrast, comprising conventional OCT contrast based on reflectivity, PS-OCT contrast, and OCTA, was used to differentiate various structures playing key roles in neovascularization such as pigmented structures and retinal vasculature. The longitudinal evaluation ranging from 2 weeks postnatally to 4 months of age revealed a significant impairment within the study period. While the retinas appeared almost normal at the BL measurement, significant changes were observed by the multiparametric analysis during follow-up. An interventional pilot study using aflibercept demonstrated the inhibition of neovascularization. Histologic analysis confirmed the ability of the different functional OCT extensions to identify different retinal structures.

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