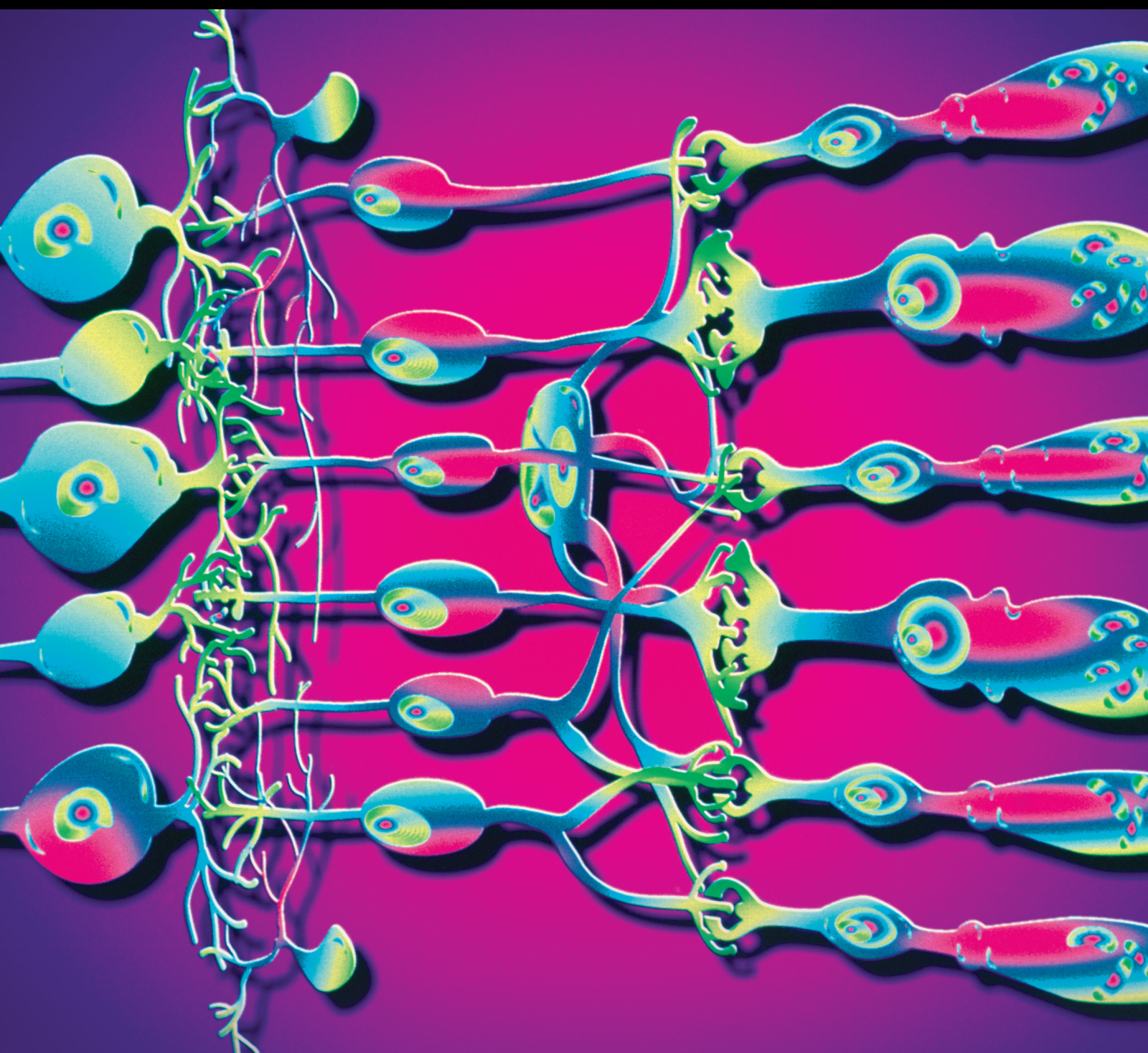
Journal of Ophthalmology

Anti-VEGF and Beyond

Guest Editors: Thomas Bertelmann, Hakan Kaymak, Michael J. Koss, and Florian Kretz



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Guest Editors: Kazuo Toda, Jorge L. Zeredo, Sae Uchida, and Vitaly Napadow

Editorial Board

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Editorial
Anti-VEGF and Beyond

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Almost one decade ago, the first anti-VEGF drugs to treat neovascular eye disorders were approved by the respective health authorities and brought into routine clinical care. This "revolution" in the field of ophthalmology offered the treating physicians the chance to sustainably save vision for patients affected for the first time. Since these days, the understanding of the efficacy and safety profiles of the anti-VEGF drugs available has constantly grown. However, many open questions remain. Furthermore, new alternative and/or additional substances targeting VEGF and other factors such as PDGF and PIGF are on the horizon to alter and further improve our treatment strategies for neovascular eye diseases. This special issue focused on both topics, and many research groups shared their valuable research results to better understand the mode of action of various anti-VEGF as well as of new molecules to counteract neovascular eye diseases.

Illuminating the traditional indications, neovascular age-related macular degeneration (nAMD), diabetic macular edema (DME), and macular edema due to retinal vein occlusions (RVO), the questions advancing into the focus of interest for some time have been when and how to switch or switch back different anti-VEGF drugs, if at all switching these substances is favorable in comparison to maintaining the anti-VEGF in use to ensure the best clinical and anatomical outcome for the individual patient. In this respect, T. H. C. Tran et al. investigated the two-year outcome of Aflibercept in the treatment of pigment epithelium detachments (PED) refractory to Ranibizumab. They could show that in the short term, this switch was successful, whereas in the long term, no significant improvements were obtained and thus switching in this scenario appears to be questionable. A. Herbaut et al. share their results of eyes suffering from DME which were switched from Ranibizumab and/or Dexamethasone treatment to intravitreal Aflibercept injections. Their results show a significant functional and anatomical improvement after switching in the short term after 6 months. It would be interesting to experience, if these differences persist in the longer term though. A. Pielen et al. investigated the effect of switching to Dexamethasone implants in RVO-affected eyes refractory to anti-VEGF injections, previous Dexamethasone implants, or treatment-naive eyes. The results displayed herein were inconsistent: despite a significant reduction when switching from anti-VEGF to Dexamethasone, a significant improvement in BCVA failed. Overall, there were three more puzzle pieces of the signification of switching (anti-VEGF) drugs in the treatment strategy of neovascular eye disorders. The data precisely show that further research is essential to attain the best treatment option for the individual patient.

Y. Subhi and T. L. Sørensen investigated different aspects of nAMD patients older than 90 years of age and showed that

overall 7% of nAMD patients treated belong to this group. In this subpopulation, treatment is oftentimes discontinued by death or various treatment burdens. Aflibercept showed superior visual as well as anatomical outcomes in comparison to Ranibizumab after 2 years. The authors conclude that new strategies regarding treatment burdens and the use of specific anti-VEGF substances might be needed for the future, as patients are getting older and older.

Beside efficacy, possible side effects of any anti-VEGF therapy emerged into the focus of vitreoretinal research. Specifically, the progression rate of retinal pigment epithelium (RPE) loss during intravitreal anti-VEGF treatment is an important aspect, because the induction of RPE atrophy might hamper visual recovery. J. Wons et al. compared the atrophy progression rates between Ranibizumab and Aflibercept in eyes suffering from nAMD and could show that no significant differences between both treatment modalities exist.

Another focus of this special issue turned out to be the effect of anti-VEGF therapy on eyes with retinopathy of prematurity (ROP). Since the ongoing RAINBOW extension study (ClinicalTrials.gov Identifier: NCT02640664) is evaluating the effect of Ranibizumab on functional and anatomical outcomes in ROP-affected eyes in a large, worldwide, clinical trial, this might become an approved therapeutic treatment option in the future and is therefore of immanent interest. In this regard, Q. Huang et al. underlined with their results published herein that eyes of infants treated bilaterally with intravitreally injected Ranibizumab can react differently and that reactivation after previous injections in ROP-affected eyes is an important issue ophthalmologists should be aware of. Furthermore, J. J. Tan et al. showed in a neonatal rat model that exposure of intermittent hypoxia-induced injured retinal microvasculature to anti-VEGF substances can result in vascular leakage and adverse effects in the developing neonate. This aspect is of clinical importance, because anti-VEGF use in the treatment of ROP infants is increasing by the day and caution regarding systemic side effects is warranted.

Beside these traditional indications for anti-VEGF treatment, new fields have arisen, among these was (neovascular) glaucoma. In many cases, especially in neovascular glaucoma, therapeutic approaches can be challenging. J. Kwon and K. R. Sung showed in their retrospective report of eyes suffering from neovascular glaucoma that preoperatively injected Bevacizumab before Ahmed glaucoma valve implantation can enhance overall success rates but conclude that subsequent prospective studies are needed to confirm this possible beneficial effect. M. Slabaugh and S. Salim not only report in their nicely written overview on the use of anti-VEGF substances in glaucoma surgery the potential benefit of these drugs but also claim that a precise role needs to be defined in the future.

Beside anti-VEGF agents, a bunch of new substances treating neovascular eye disorders are being developed and brought into phase I to III clinical trials. Before administered in first-in-man investigations, experimental approaches are needed to evaluate the potential efficacy and side effects. In this regard, C. Ren et al. reported an oral tyrosine kinase inhibitor, CM082, to treat experimental choroidal neovascularizations in rats. They could show that CM082 passed the blood-retina barrier and was detectable within the eyes in a reasonable concentration which in turn leads to significantly less neovascularization in comparison to controls. Thus, the idea of an oral application of drugs for the treatment of neovascular eye disorders seems to be viable. The future will tell if this molecule will find its way into clinical trials. Finally, D. Ning et al. demonstrated a novel pathway (Wnt/beta-catenin/COX-2/VEGF) to play a pathogenetic role in the development of retinopathies, and thus, this novel pathway might be a new target for future therapeutic approaches.

Overall, this special issue provides a variety of up-to-date basic and clinical research results of anti-VEGF and other substances targeting neovascular eye disorders. In light of the fact that different anti-PDGF molecules that were deemed to be the next step in the treatment of neovascular eye diseases and especially of nAMD-affected eyes recently failed in phase III clinical trials, it seems obvious that further intensive research is necessary to improve overall treatment outcomes for our patients.

Acknowledgments

We want to thank the contributing authors for submitting their work to this special issue as well as all the reviewers involved for spending their time and reasonable input.

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Research Article

The Effect of CM082, an Oral Tyrosine Kinase Inhibitor, on Experimental Choroidal Neovascularization in Rats

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The aims of this study were to evaluate the effects of CM082 on the development of choroidal neovascularization (CNV) in a laser-induced CNV rat model and to determine the drug concentration in the ocular tissues. After the laser-induced CNV model was established in rats, CM082 was orally administered. The effects of CM082 on the CNV lesions were assessed using fundus fluorescein angiography (FFA), CNV histology, and retinal pigment epithelium- (RPE-) choroid-sclera eyecup analysis. The concentrations of CM082 in the plasma and eye tissues were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results of FFA, histology, and RPE-choroid-sclera eyecup analysis demonstrated that the CM082-treated (10 mg/kg/d or 30 mg/kg/d) rats exhibited significantly less neovascularization than did the control group. The total concentration of CM082 in the eyes (172.86 ± 57.11 ng/g) was similar to that in the plasma (196.87 ± 73.13 ng/ml). Within the eye, the concentrations of CM082 and its metabolites were highest in the retina-sclera. The orally administered CM082 thus effectively passed through the blood-retina barrier (BRB) to reach the retina in the Brown Norway rats. Therefore, at both 10 mg/kg/d and 30 mg/kg/d, CM082 was able to reduce CNV lesions in the laser-induced CNV rat model.

1. Introduction

Age-related macular degeneration (AMD) is one of the most common causes of irreversible central visual loss in people over 65 years of age in Europe and North America [1, 2]. There are two different subgroups of AMD: the geographic atrophy, or dry, form and the chronically neovascularized, wet form. Although the wet form accounts for only 10% of AMD cases, it is the main cause of visual loss in 60–80% of AMD patients [3, 4]. Therefore, it is necessary to understand the pathology of choroidal neovascularization (CNV) and to identify efficient therapies.

A large number of studies have indicated that neovascularization is caused by the overexpression of vascular endothelial growth factor (VEGF), a growth factor that plays a major role both in the development of normal blood vessels and in abnormal angiogenesis [5–7]. There are five VEGF isoforms (VEGF-A, B, C, D, and E) as well as placental growth factor (PIGF) in the VEGF family [8, 9]. VEGF has to bind to one or more VEGF receptors (VEGFRs) to exert its function [10]. When bound to one or more VEGFs, VEGFR autophosphorylates and dimerizes to phosphorylate the specific intracellular tyrosine residue that activates the signaling pathway, which then specifically promotes the

mitosis and proliferation of vascular endothelial cells and regulates their migration and survival [11, 12]. Anti-VEGF therapies that reduce the interaction of VEGF with its receptors, such as ranibizumab [13], aflibercept [14], and bevacizumab [15], are widely used to treat patients with CNV secondary to AMD and other pathological conditions. Reducing VEGF-A binding to VEGFRs, and especially VEGFR-2, is the main target of ranibizumab and bevacizumab [16]. Although these therapeutic drugs are relatively effective for treating AMD and related eye diseases, not all patients respond to them and many exhibit decreased drug susceptibility during treatment [17]. Additionally, repeated intravitreal injection can cause rare but serious side effects such as ocular pain, infection, or hemorrhage. To avoid the intravitreal injection-related complications and relapse, it has been necessary to develop a less invasive treatment.

In addition to VEGF, a growing body of evidence indicates that platelet-derived growth factor (PDGF) contributes to neovascularization in AMD [18]. PDGF plays a role in angiogenesis by recruiting pericytes to the newly formed blood vessels and maintaining the stabilization and maturation of blood vessels. Furthermore, pericyte-derived VEGF and cell-cell contacts may participate in promoting endothelial survival and may guide migration. The previously established endothelial/pericyte associations and vessel stabilization are disrupted when PDGF/PDGF receptor (PDGFR) signaling is inhibited [19]. Considering the synergistic effects of VEGF and PDGF signaling, therapeutic methods of inhibiting both the VEGF and the PDGF pathways using two biologics (e.g., ranibizumab and Fovista) are being actively investigated [20, 21]. Nevertheless, phase 3 clinical trial demonstrated that the addition of Fovista to a monthly Lucentis regimen did not result in benefit as measured by the mean change in visual acuity at the 12 month time point. It is necessary to develop a better understanding in anti-PDGF therapies for AMD.

CM082 is a multitarget tyrosine kinase inhibitor that can suppress neovascularization by inhibiting the VEGF, PDGF, c-kit, and Flt-3 receptor tyrosine kinases. CM082 is a novel derivative of sunitinib that has been approved for the treatment of cancers and that was designed to have a more favorable toxicity profile than sunitinib. The oral administration of CM082 is more convenient than intravitreal injection, and its inhibition of both VEGFR and PDGFR might be more effective than anti-VEGF injections alone. Tyrogenex has completed a phase 1 clinical study of X-82 (CM082) in the USA and is currently conducting a randomized phase 2b study in patients with exudative AMD [22] (NCT02348359). Meanwhile, AnewPharma is conducting a phase 1 study in China (NCT02452385). Here, we report the effect of orally administered CM082 on CNV lesions in a rat model and describe the concentration of CM082 in the ocular tissues.

2. Materials and Methods

2.1. Compound. CM082 (lot 20100111-B) was provided by Tyrogenex, Inc. (Palm Beach Gardens, FL, USA). It was formulated as a suspension in 0.5% HPMC-K4 M and 0.2% SLS in double-distilled water. The concentration was 2 or 6 mg/ml, and it was stored at 4°C. A dose of 10 mg/kg/d or 30 mg/kg/d was administered by oral gavage, with a dosing volume of 5 ml/kg.

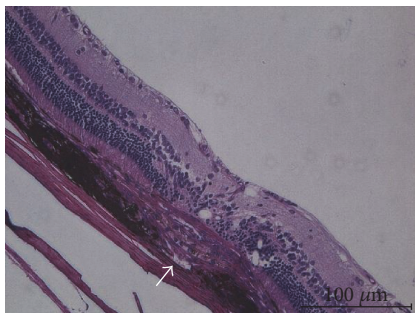
2.2. Animals. A total of 70 male Brown Norway (BN) rats (age, 10 weeks; weight, 200 ± 20 g) were used. All rats were handled in compliance with the ARRIVE guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee of the College of Medicine, Tongji University, Shanghai, China.

2.3. Laser-Induced CNV in Rats. Laser photocoagulation-induced CNV was established as previously described [23]. Preoperative preparation included general anesthesia, which was induced with an intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). The rats' pupils were dilated using 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P, Santen Pharmaceutical Co., Osaka, Japan). Surface anesthesia was induced with 0.5% Alcaine (Alcon (China) Ophthalmic Product Co., Beijing, China). Bruch's membrane of the right eye was injured using the following laser parameters: 532 nm wavelength, 360 mW intensity, 0.1 s duration, and 50 μm spot size. Eight to ten laser spots were applied to the major retinal vessels at approximately the same distance to the optic disc. A laser-induced cavitation bubble or slight hemorrhage indicated a rupture in Bruch's membrane. Fundus photography was taken immediately after laser photocoagulation to check for fundus hemorrhage.

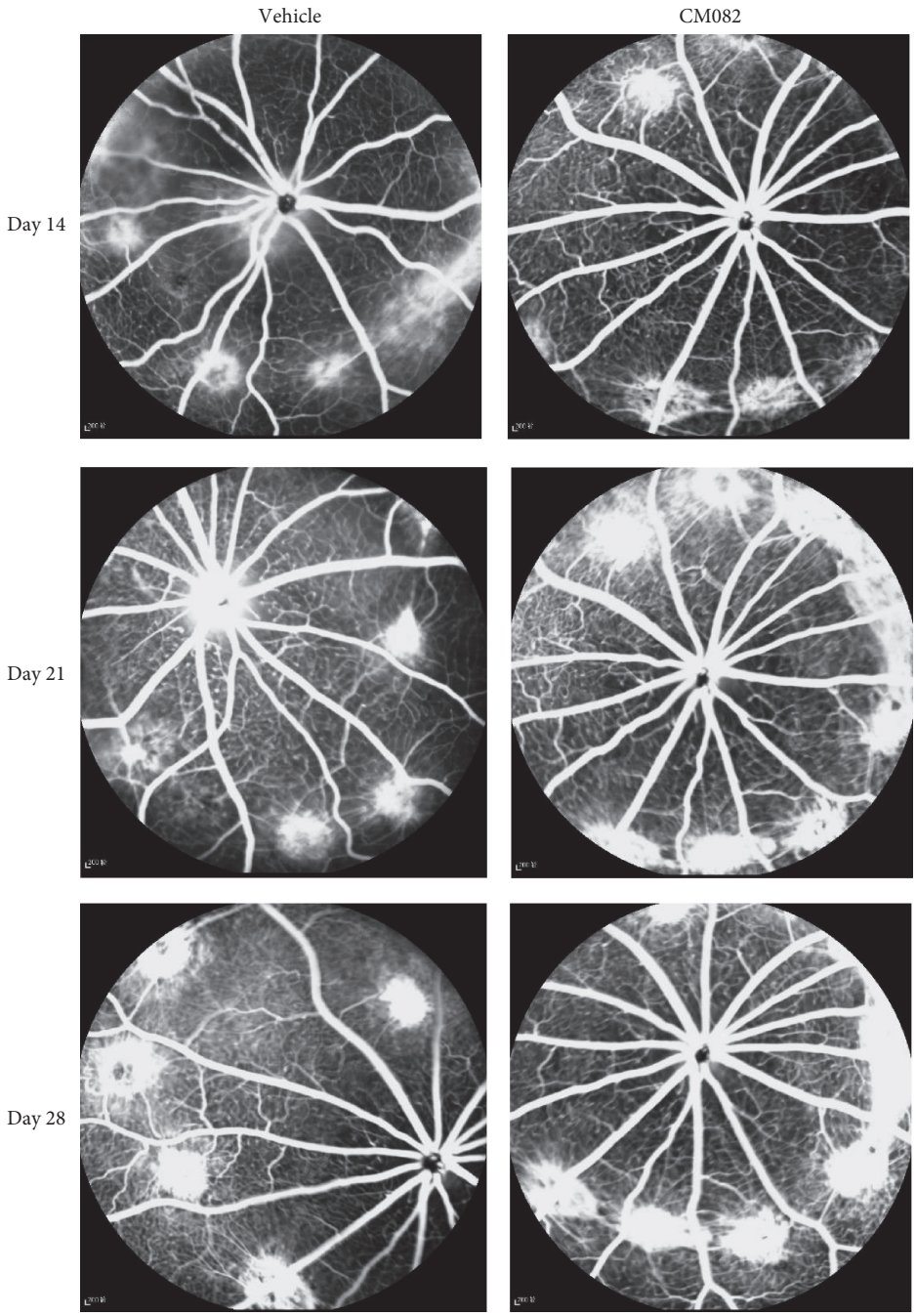
2.4. CM082 Treatment of Experimental CNV. To assess the CM082 distribution and the inhibitory effects of CM082 on CNV development, the 70 rats were randomly divided into 2 groups: 10 rats for evaluating the CNV rat model and to detect the distribution of CM082 and its metabolites and 60 rats for assessing the effects of CM082 on the experimental CNV. CM082 was administered orally at dose of 10 mg/kg/d or 30 mg/kg/d, while the vehicle (5 mg/kg/d) was used as a negative control.

2.5. LC-MS/MC Analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to detect the concentrations of CM082 and its metabolites (X-297 ($C_{22}H_{24}FN_5O_3$) and X-471 ($C_{23}H_{28}FN_5O_4$)) in the plasma and ocular tissues. An API-4000 triple-quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA) coupled with a Shimadzu liquid chromatography system (Shimadzu Cooperation, Kyoto, Japan) was used for the analysis. CM082, its metabolites, and tolbutamide were dissolved in DMSO to generate 1.0 mg/ml stock solutions stored at 4°C. A total of 200 μl of the tolbutamide stock solution was dissolved in acetonitrile to produce internal-standard working solutions. The plasma and ocular tissue samples were analyzed along with the standard and quality-control samples. The data were analyzed with Analyst 1.6.1 (AB Sciex, Framingham, MA, USA).

2.6. Fluorescence Angiography. Fundus fluorescence angiography (FFA) was performed on days 7, 14, and 21 after laser

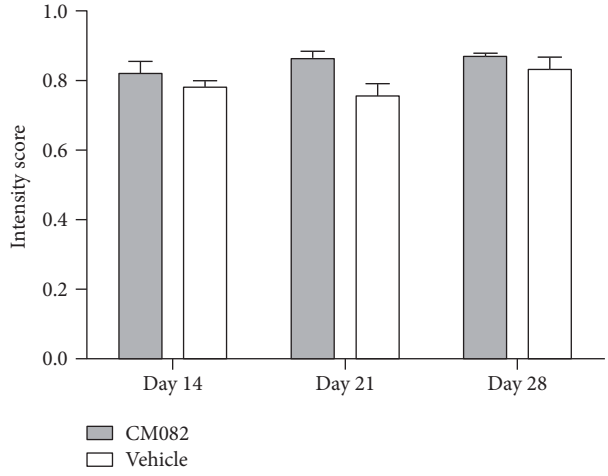


(a)



(b)

Figure 1: Continued.



(c)

Figure 1: Histology and FFA results of CNV modeling. (a) Histology of CNV lesions stained with HE shows that CNV formation and broke the Brunch's membrane at 21 days after photocoagulation. Dome-like CNV complexes, consisting of fibrovascular tissue, retinal pigment epithelial cells, and pigment clumps, are shown and white arrow indicates the vessel lumina of neovascular. (b) FFA results showed fluorescein leakage of CNV in each group at day 14, 21, and 28 (7 days after CM082 treatment) separately. The signal intensity of capillary in background region was defined as "0" whereas the signal intensity of the main branch of the retinal vein was defined as "1". (c) There is no significant difference between the intensity score of leakage in each group at day 14, 21, and 28 ($p > 0.05$). Scale bar, 100 μm.

photocoagulation. The preoperative preparations were the same as those used to establish the CNV model. Approximately 0.2 ml of 10% sodium fluorescein (Alcon Japan, Tokyo, Japan) was injected intraperitoneally, and the leakage of fluorescein from the laser lesions was monitored dynamically for up to 15 min. The images were obtained using confocal scanning laser ophthalmoscopy (Spectralis, Heidelberg Engineering Inc., Heidelberg, Germany) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The signal intensities (brightness of the CNV lesions) defined the degree of leakage. As a reference, the intensity of the capillaries in the normal regions was defined as 0, whereas the intensity at the major branch of the retinal vein was defined as 1.

2.7. CNV Histology and Immunohistochemistry. The rats were euthanized after the FFA examination on day 14 or 21 after laser injury, and a histological examination was performed on 4 rats per group at each time point. The animals were enucleated, and the eyes were fixed in paraformaldehyde for 24 h (the eyelashes were also removed). After fixation, the eyes were embedded in paraffin and serially sectioned at 5 μm. Routine hematoxylin-eosin (HE) staining was performed on selected slides, and the serial sections were examined using a light microscope. Immunohistochemical staining was performed on the histological sections from the CM082-treated and vehicle-treated groups 14 and 21 days after laser administration. Routine immunolocalization procedures were used to dewax and rehydrate the slides. The slides were incubated with 3% hydrogen peroxidase for 25 min and washed in phosphate-buffered saline (PBS) for 5 min. The slides were then blocked in goat serum for 15 min. Next, the sections were incubated with a phosphorylated anti-VEGFR-2 antibody (Nanjing Bioworld Biotech Co., Jiangsu, China) overnight at 4°C, followed by incubation with Goat Anti-Rabbit/Rat IgG antibody (Dako Denmark A/S, Denmark) for 30 min. The sections were subsequently incubated with horseradish peroxidase-conjugated streptavidin (Thermo Fisher Scientific China, Shanghai, China) for 30 min and then counterstained with hematoxylin for 2 min. Finally, the slides were mounted in aqueous mounting medium and examined using ScanScope.

2.8. Measurement of the CNV Area. To assess the inhibitory effects of CM082 on CNV development, the photocoagulated rats were randomly divided into 2 groups: (1) a CM082-treated group ($n = 9$) and (2) a vehicle-treated group ($n = 9$). CM082 (30 mg/kg/d or 5 ml/kg/d) or vehicle (5 ml/kg/d) was administered beginning on the 7th day after laser injury. On the 7th, 14th, or 21st day, 3 rats from each group were anesthetized, and the left ventricle of the heart was perfused with 2 ml of PBS containing 50 mg of fluorescein isothiocyanate- (FITC-) dextran (2×10^6 average molecular weight; Sigma-Aldrich). The enucleated eyes were fixed in 4% paraformaldehyde for 1 h. Retinal pigment epithelium- (RPE-) choroid-sclera flat mounts were then produced by hemisecting the eye, and the neural retina was peeled away from the underlying RPE. Radial cuts were performed to permit the tissue to be flattened onto a microscope slide, with the RPE side facing up, after which the CNV lesions in the flat mounts were examined by scanning laser confocal microscopy (Zeiss, Jena, Germany). The CNV area was measured by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.9. Statistical Analysis. All statistical graphs were generated in GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). The intensity of leakage detected by FFA and the

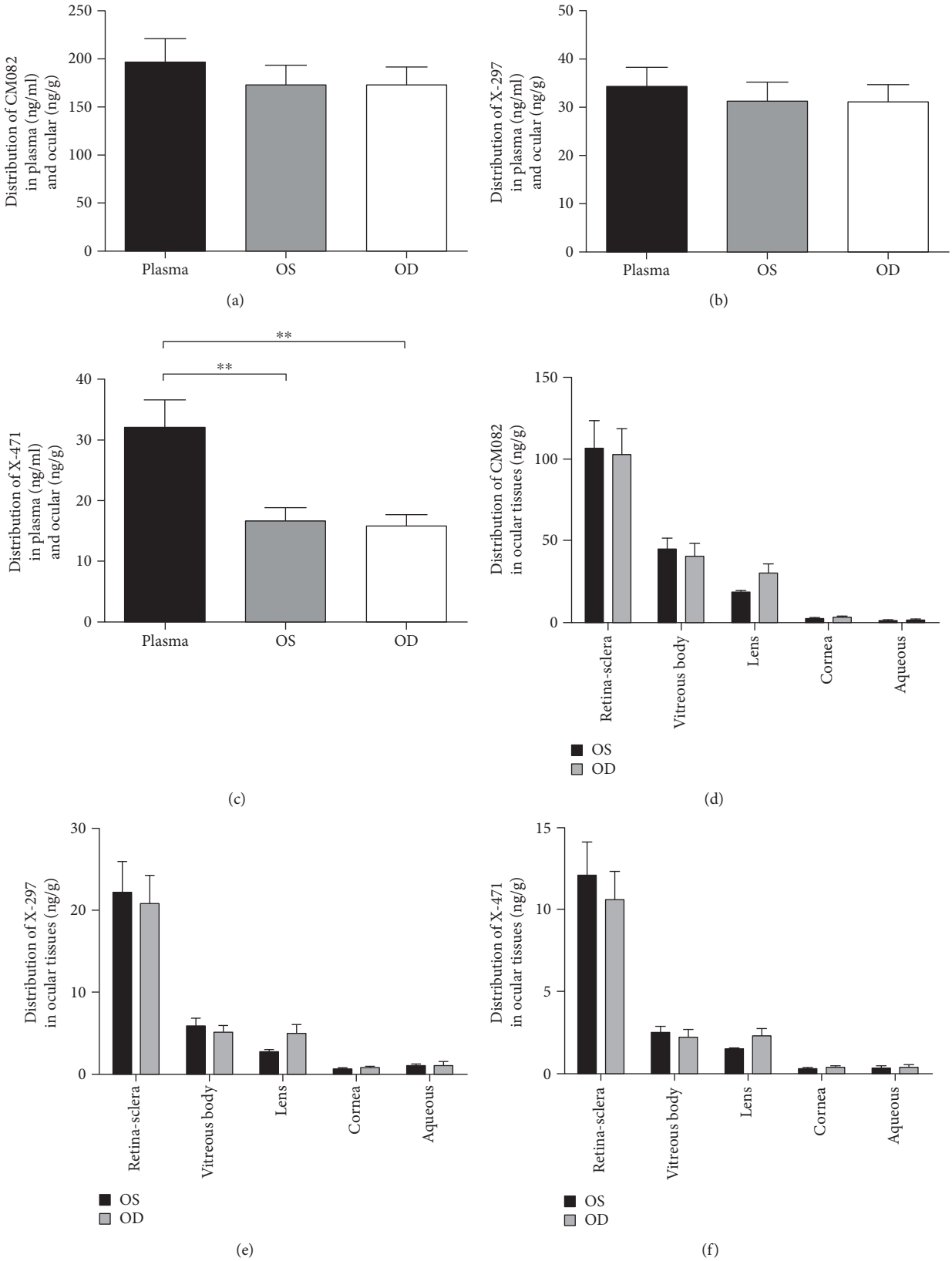


Figure 2: Distribution of CM082 and its metabolites in plasma and eye tissues. (a, b, and c) The concentrations (ng/ml or ng/g) of CM082, X-297, and X-471 in plasma and ocular tissues ($n = 9$). There is no significant difference between the concentration of CM082 in plasma and eyes. The same is true for X-297. However, the concentration of X471 in eyes (OS: 16.68 ± 2.18, OD: 15.82 ± 1.85) is obviously lower than that in plasma (32.04 ± 4.57) ($^{**}p < 0.01$). (d, e, and f) The concentration of CM082, X-297, and X-471 was detected in different ocular tissues ($n = 9$). There is no significant difference in concentration of CM082, X-297, and X-471 between OS and OD.

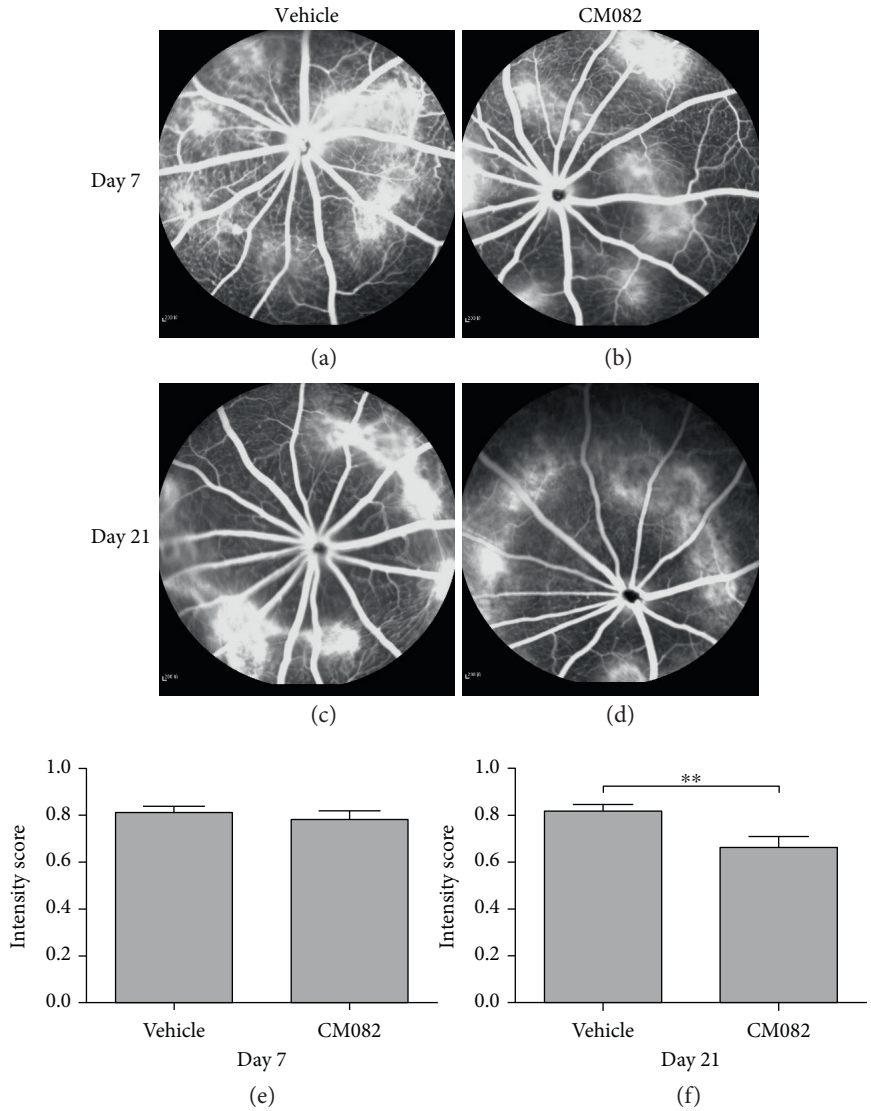


Figure 3: FFA results of the CM082 (10 mg/kg/d) group and the corresponding vehicle-treated group at different time points. (a, b; $n = 10$) FFA showed that the leakage of fluorescein at laser spots are at nearly same extent in vehicle-treated and CM082 group 7 days after laser injury ($p > 0.05$, $n = 9$). (c, d) Leakage of fluorescein in the CM082-treated group was reduced at 21 days after laser injury ($^{**}p < 0.01$, $n = 9$).

CNV lesion areas detected following the FITC-dextran perfusion were assessed with ImageJ (National Institutes of Health, Bethesda, MD, USA) and evaluated with one-way analysis of variance (ANOVA) and Scheffe's multiple comparison tests using SPSS (SPSS version 20.0, Chicago, IL, USA). The results are presented as the mean ± SEM, unless otherwise stated, and box plots are used to graphically display the data from the different groups. In this study, $p \leq 0.05$ was considered statistically significant.

3. Results

Sixty-eight BN rats were used in this study. Two of them were discarded because of large areas of fundus hemorrhage after laser injury, and another one had a slight subretinal hemorrhage, although the hemorrhage was absorbed at 7 days after the laser injury. None of the rats in our research exhibited conjunctival hemorrhage, corneal opacity, cataracts, retinal detachment, or an anesthesia accident. All rats tolerated the CM082 treatment well, and there was no behavioral change, death, or body weight loss during treatment.

3.1. CNV Rat Model Establishment and Distribution of CM082 in the Eye. Ten BN rats were photocoagulated in the oculus dexter (OD) to build the CNV rat model. Histological examination was performed at day 21, while FFA was conducted at days 14 and 21 to ensure that the CNV model was established successfully and to provide evidence that neovascularization was induced (Figures 1(a), 1(b), and 1(c)). After modeling CNV successfully, to investigate whether CM082 and its metabolites (X-297 and X-471) can reach the retina effectively, the rest of the nine rats were orally treated with CM082 at 10 mg/kg/d for 9 days, starting from day 22. FFA was performed after 7 days of CM082

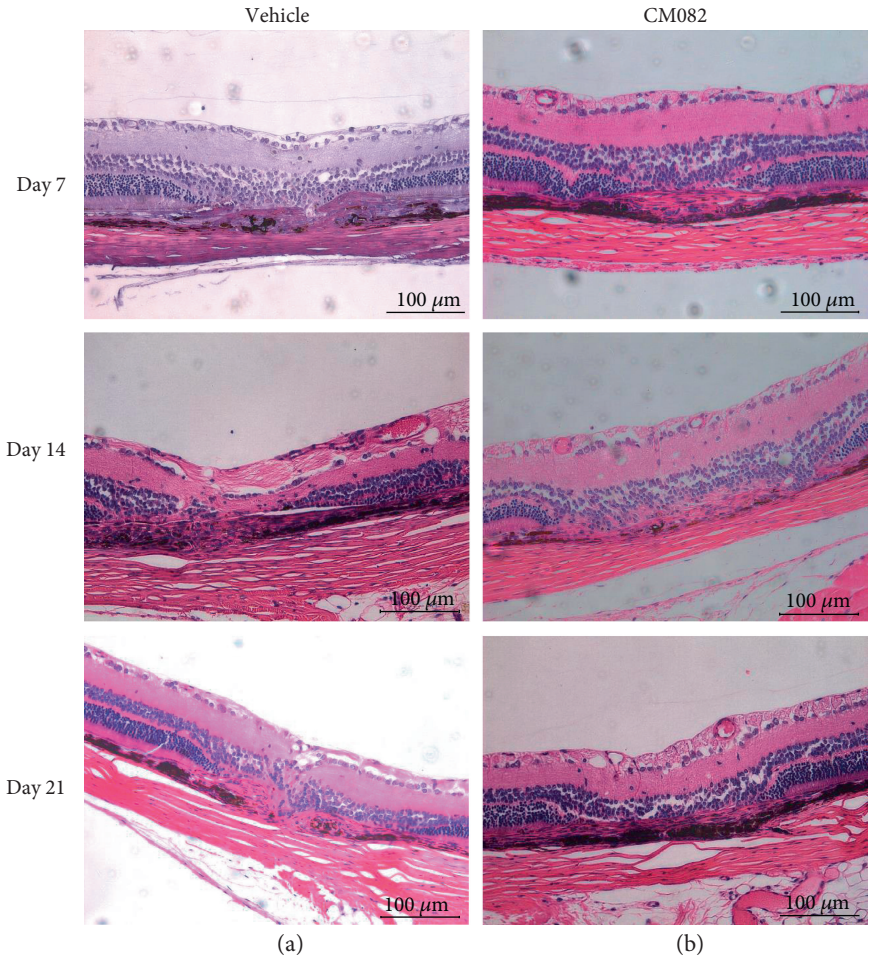


Figure 4: Histology examine stained with HE of the CM082 (10 mg/kg/d) group ($n = 2$) and the corresponding vehicle group ($n = 2$) was obtained after FFA examination at day 7, 14, and 21. (a, b) Results showed a smaller CNV size of the CM082-treated group at day14 and 21. CNV complexes consist of retinal pigment epithelial cells, pigment clumps, and vascular tufts were observed. The CNV under CM082 treated was thinner in the center compared with that in the vehicle-treated group. Scale bar, 100 μm.

administration, but there was no significant reduction in fluorescein leakage compared to leakage at days 14 and 21 (Figures 1(d) and 1(e), $p > 0.05$). This result may have been due to the fact that CM082 was administered at day 22, when the CNV had formed completely and irreversibly. Two days after FFA, the rats were sacrificed, and we determined the concentrations of CM082 in the plasma (ng/ml) and the ocular tissues (ng/g) 2 h after CM082 treatment. The concentrations of CM082 in the plasma, the oculus sinister (OS), and the OD were 196.87 ± 24.38 ng/ml, 172.74 ± 20.83 ng/g, and 172.97 ± 18.33 ng/g, respectively. The corresponding concentrations of X-297 were 34.42 ± 3.86 ng/ml, 31.28 ± 4.00 ng/g, and 31.11 ± 3.56 ng/g, while those of X-471 were 32.04 ± 4.57 ng/ml, 16.68 ± 2.18 ng/g, and 15.82 ± 1.85 ng/g. Although the concentrations of CM082 and X-297 in the plasma were slightly higher than those in the ocular tissues, the difference was not significant, demonstrating that CM082 and X-297 can both enter the ocular tissues effectively (Figures 2(a) and 2(b), $p > 0.05$). However, the concentration of X-471 in the plasma was significantly higher than that in the ocular tissues (Figure 2(c)), suggesting that X-471 may be less effective at passing through the blood-retina barrier (BRB). Within the ocular tissues, the distributions of CM082, X-297, and X-471 were highest in the retina-sclera 2 h after CM082 administration. There was no significant difference in the drug concentrations between the OS and the OD (Figures 2(d), 2(e), and 2(f)), indicating that the laser injury did not affect the drug distribution in the eyes. These results showed that CM082, X-297, and X-471 (to a lesser extent) can successfully pass through the BRB and reach the retina.

3.2. Regression of Established CNV after CM082 Application. To determine whether CM082 inhibited CNV progression, 60 BN rats were divided into 4 groups and randomized by weight, with 10 rats receiving CM082 at 10 mg/kg/d, 10 receiving CM082 at 30 mg/kg/d, 20 receiving vehicle treatment, and 20 undergoing the RPE-choroid-sclera preparation. Either CM082 or vehicle was administered beginning on the 7th day after laser injury. The CNV analysis was performed on day 14 or 21 after laser photocoagulation using FFA, histological examinations, and immunohistochemistry.

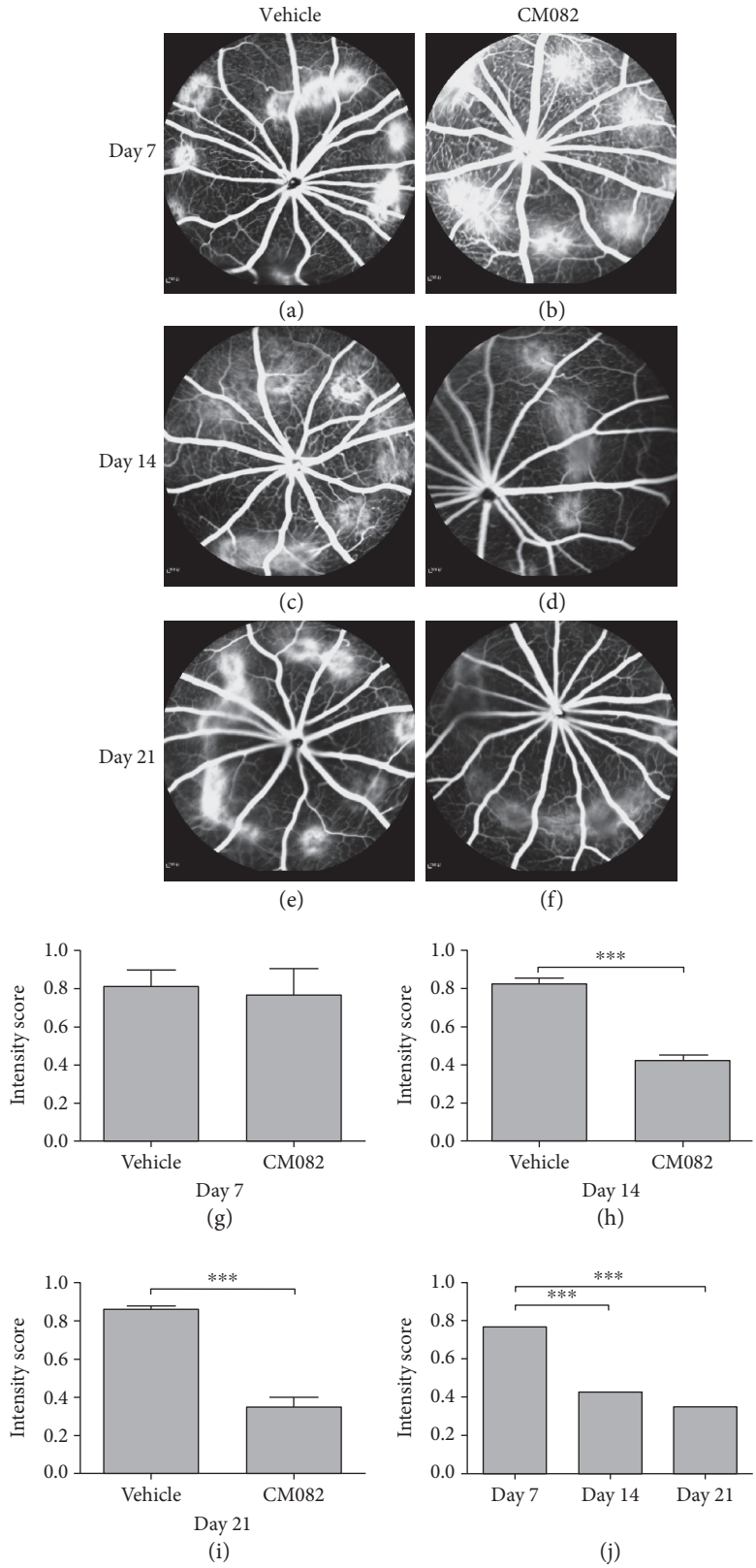


Figure 5: FFA results of the CM082 (30 mg/kg/d)-treated group and the corresponding dose of the vehicle-treated group at different time points. Results of FFA showed the leakage of fluorescein at laser spots in vehicle-treated and CM082 group 7 (a, b; $n = 10$), 14 (c, d; $n = 9$), and 21 (e, f; $n = 8$) days after laser injury. (g, h, and i) The fluorescence signal intensity of CM082-treated group was statistically lower than the vehicle-treated group at 14 days and 21 days ($^{***}p < 0.001$). (j) The signal intensity at 14 and 21 days was obviously lower than that at 7 days in the CM082 group ($^{***}p < 0.001$).

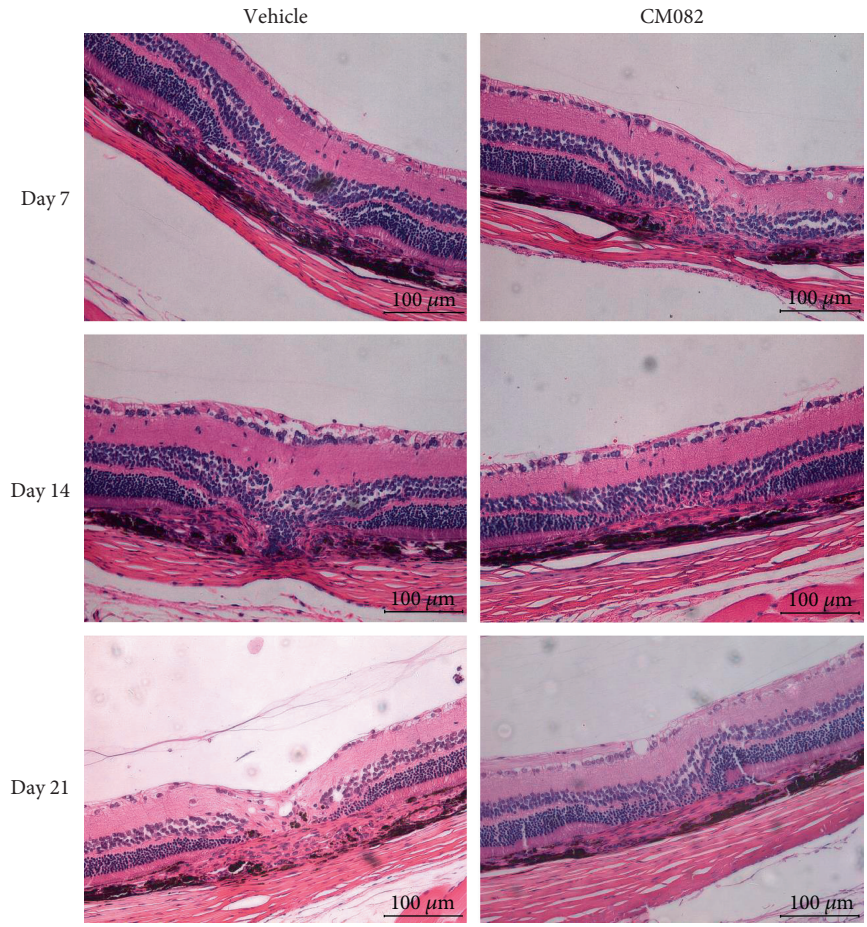


Figure 6: Histology of CNV lesions stained with hematoxylin-eosin of the CM082 (30 mg/kg) group ($n = 6$) and the corresponding dose of the vehicle group ($n = 6$) was obtained after FFA examination. Results showed that administration of CM082 reduces laser-induced CNV lesions. (a) In the vehicle-treated group, CNV formed and broke the Brunch's membrane. The depigmentation in RPE, aggregation of macrophage, and neovascularization were obtained 14 days and 21 days after photocoagulation. (b) In the CM082 group, the edema, depigmentation, and CNV areas significantly decreased comparing to vehicle-treated group at the same time point. Scale bar, 100 μm.

3.2.1. Effects of CM082 on CNV at a Dose of 10 mg/kg/d. The group receiving 10 mg/kg/d CM082 was analyzed by FFA before CM082 administration. The results on days 7 and 21 after laser injury are shown in Figure 3. The FFA results on day 7 were similar between the ultimately vehicle-treated and CM082-treated groups (vehicle: 0.81 ± 0.03, CM082: 0.78 ± 0.04; Figures 3(a), 3(b), and 3(e)). We then began to treat the rats with CM082 at 10 mg/kg/d, and FFA was again performed at day 21 (14 days after CM082 dosing). The leakage in the CM082-treated group was significantly lower than that in the vehicle-treated group (vehicle: 0.82 ± 0.03, CM082: 0.66 ± 0.05; Figures 3(c), 3(d), and 3(f)), which indicated that oral administration of CM082 at 10 mg/kg/d can reduce CNV leakage.

We further demonstrated the effects of CM082 using histological examination of the retina of BN rats. At day 21, the CNV lesions were smaller, and there was less CNV complex (Figure 4). The results showed not only an inhibitory but also a regressive effect of CM082 on CNV development and suggested that orally treating BN rats with CM082 at 10 mg/kg/d can reverse CNV without significant toxicity.

3.2.2. Effects of CM082 on CNV at a Dose of 30 mg/kg/d. To investigate whether CM082 can be administered at a higher dose, we treated BN rats with CM082 at 30 mg/kg/d 7 days after photocoagulation. Before dosing, we performed FFA to ensure that CNV leakage was occurring in the two groups at the approximate baseline (vehicle: 0.81 ± 0.02, CM082: 0.77 ± 0.04; Figures 5(a), 5(b), and 5(g); $p > 0.05$). After CM082 administration, the results on days 14 and 21 showed that the fluorescein leakage in the CM082-treated group (day 14: 0.42 ± 0.03, day 21: 0.35 ± 0.05) was significantly reduced compared to that in the vehicle-treated group (day 14: 0.83 ± 0.03, day 21: 0.86 ± 0.02; Figures 5(c), 5(d), 5(e), 5(f), 5(h), and 5(i); $^{***}p < 0.001$). More importantly, while the intensities in the vehicle-treated group increased slightly over time (indicating disease progression), the intensities in the CM082-treated group decreased over time, suggesting that CM082 can not only inhibit CNV progression but also cause regression of CNV lesions (Figure 5(j)).

The CNV lesions were stained with HE 14 and 21 days after laser photocoagulation, as shown in Figure 6. In the photocoagulation lesions of the vehicle-treated group,

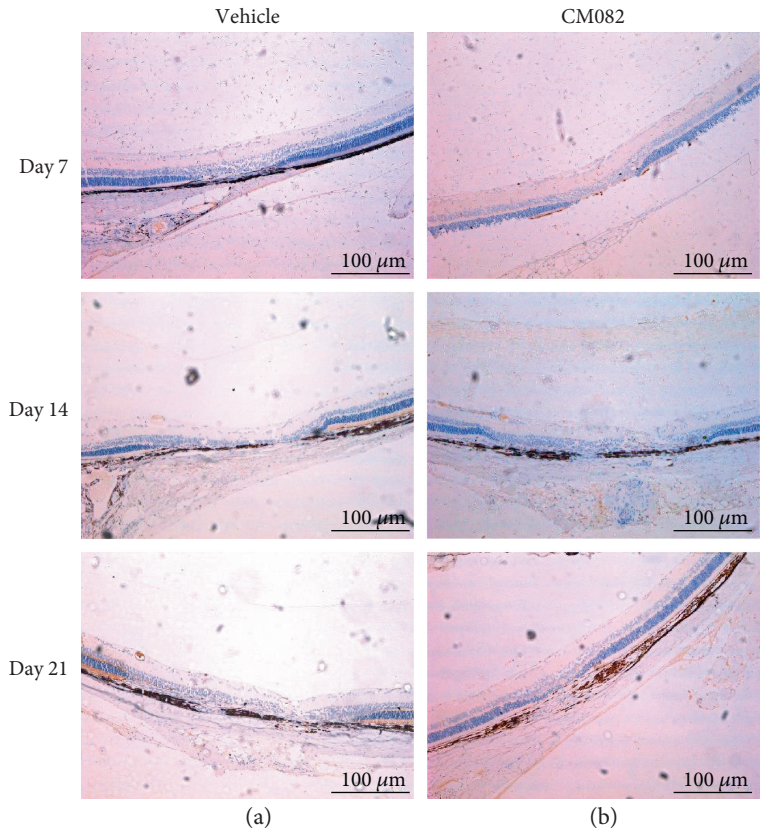


Figure 7: Immunohistochemical analysis of the CM082 (30 mg/kg) group and the corresponding dose of the vehicle group. (a, $n = 3$) The result of immunohistochemical stain with p-VEGFR-2 in the vehicle-treated group at 7, 14 and 21, days after laser photocoagulated. There are large numbers of yellow granules distributed in vessels, outer plexiform layer, and RPE layer. (b, $n = 3$) The expression of p-VEGFR-2 in each structure in the CM082-treated group was significantly lower than the control group at the same time point. Scale bar, 100 μm.

depigmentation was observed in the RPE, and CNV had formed in the retinal neuroepithelial layer (RNL), which disrupted Bruch's membrane. Macrophage aggregation and neovascularization between the retinal layers were also observed (Figures 6(a) and 6(c)). The edema, depigmentation, and CNV areas in the CM082-treated group (Figures 6(b) and 6(d)) were significantly decreased compared to those in the vehicle-treated group. The results indicated that CM082 can reduce neovascularization and arrest CNV formation.

Figure 7 shows that 14 and 21 days after laser injury, phosphorylated VEGFR-2 (p-VEGFR-2) was distributed in the vessels, outer plexiform layer (OPL), and RPE layer in the vehicle-treated group (Figures 7(a) and 7(c)), whereas CM082 administration successfully reduced the aggregation of p-VEGFR-2 in the RPE and OPL (Figures 7(b) and 7(d)). These results suggested that suppressing VEGFR-2 phosphorylation is one of the mechanisms by which CM082 inhibits CNV.

3.2.3. Results of the RPE-Choroid-Sclera Preparations. To further confirm the reverse effect of CM082 at a dose of 30 mg/kg/d on the CNV area, we prepared RPE-choroid-sclera by perfusion with FITC-dextran. For this purpose, another 20 rats were randomly divided into two groups. Seven days after laser photocoagulation, we examined the neovascularization area in each group to confirm that the groups exhibited similar levels. In particular, the areas of the CNV lesions in the vehicle-treated group were similar to those in the CM082-treated group (vehicle: $4.84 \pm 0.72\,\mu m^2 \times 10^4$, CM082: $4.45 \pm 0.90\,\mu m^2 \times 10^4$; Figures 8(a), 8(b), and 8(g)). The vehicle or CM082 was then administered to each group until 14 or 21 days after laser injury. We demonstrated that the areas of the CNV lesions in the CM082-treated group (14 days: $1.48 \pm 0.24\,\mu m^2 \times 10^4$, 21 days: $1.03 \pm 0.27\,\mu m^2 \times 10^4$) were significantly decreased compared to those in the vehicle-treated group (14 days: $9.60 \pm 1.68\,\mu m^2 \times 10^4$, 21 days: 19.61×10^4; Figures 8(c) – 8(i); $^{**}p < 0.01$, $^{***}p < 0.001$). In addition to comparing the CM082-treated group to the vehicle-treated group, we analyzed the CNV area in the CM082-treated group at different time points. The results indicated that the CNV lesions had significantly regressed following CM082 administration (Figure 8(j); $^{*}p < 0.05$). All these results confirmed the FFA results and demonstrated the regression of CNV lesions following CM082 treatment.

4. Discussion

In our study, we investigated the effect of a novel receptor tyrosine kinase inhibitor (CM082) on CNV and determined the concentration of CM082 in the eyes of BN rats. The

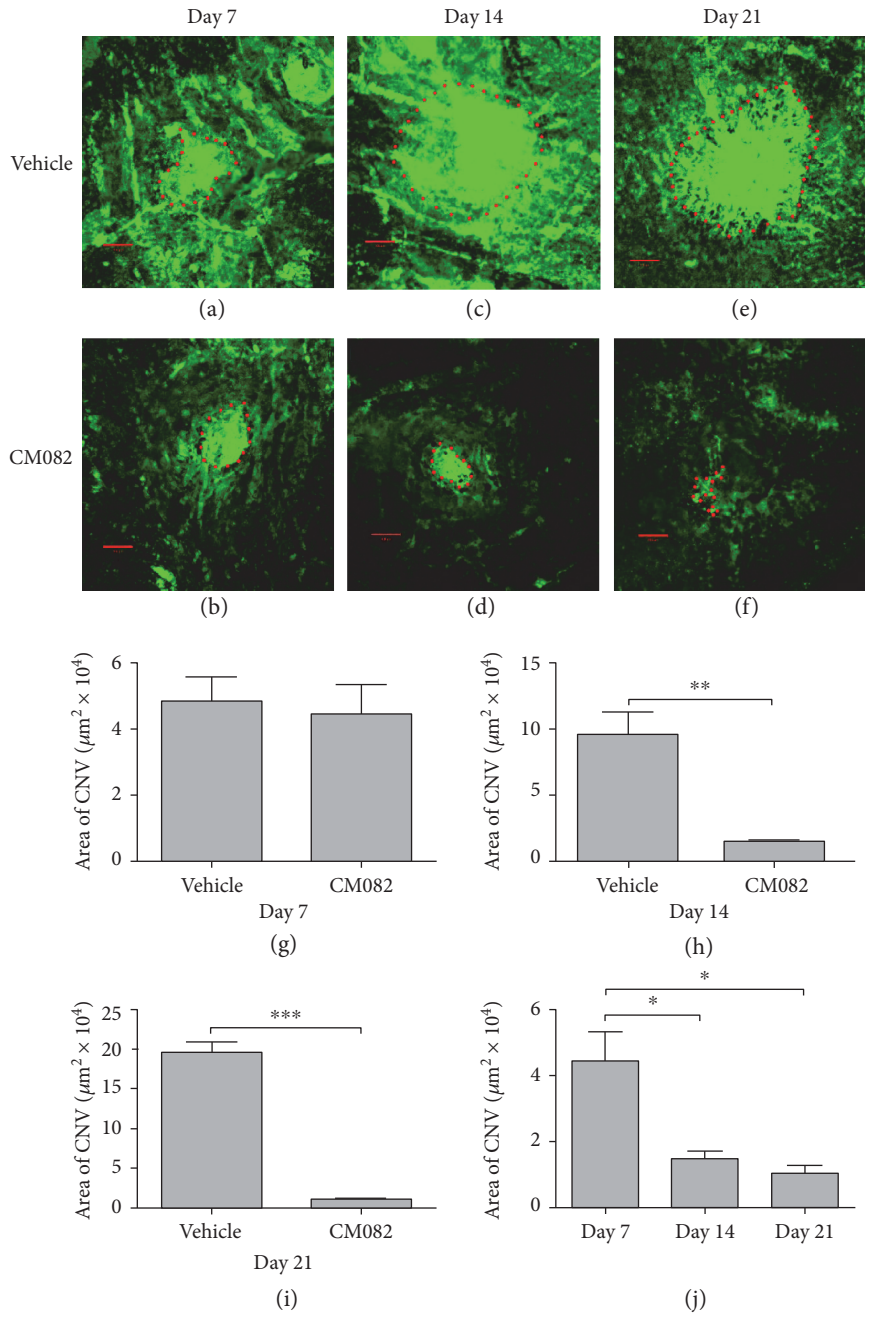


Figure 8: Representative CNV lesions in RPE-choroid-sclera flat mounts by perfusion of fluorescein isothiocyanate-dextrcan were obtained 7, 14, and 21 days after photocoagulation. (a, b) The areas of CNV lesions in the vehicle-treated and CM082 group at day 7 were shown and without statistical difference. CM082 (or vehicle) was delivered orally from day 7 ($n = 3$). The area of CNV lesions was significantly reduced under CM082 treatment at day 14 (c, d; $n = 3$) and 21 (e, f; $n = 3$). (h, i) There is a significantly inhibition of CNV area in CM082 group compared with that in vehicle-treated group ($^{**}p < 0.01$, $^{***}p < 0.001$). (j) The CNV area in the CM082 group showed a regression at day 14 and 21 compared with that in day 7 ($^{*}p < 0.05$). Scale bar, 100 μm.

results for both the extent and the areas of the CNV lesions in the CM082-treated group were significantly decreased and demonstrated regression compared to the results observed for the vehicle-treated group. The reduced expression of p-VEGFR-2 in the CM082-treated group compared to the vehicle-treated group indicated that VEGF signaling was inhibited by CM082 in the retina-choroid tissues of the CNV rat model. As previously reported, the most common function of VEGF is promotion of neovascularization, more so than maintaining existing vessels. Thus, we found a regressive effect of CM082 on early established CNV that may not be due to VEGF blockade. Because CM082 plays a role in inhibiting PDGF signaling, we conjecture that CNV regression is a result of PDGFR inhibition and pericyte dysfunction. However, Figure 1(e) showed that CM082 treatment started from day 22 exerted little efficiency. It indicated that

CM082 should be treated at an early stage of CNV formation. Subsequent research will be needed to determine the importance of the mechanism of PDGF signaling inhibition by CM082 in neovascular regression and normal vessel stabilization. The pharmacokinetics and distribution results showed that there was no significant difference in the CM082 concentrations in the OS, OD, and plasma. It demonstrates that CM082 was absorbed rapidly and passed the BRB to affect the retina following oral administration. The high concentrations of CM082 in the retina and choroid could be therapeutically beneficial for exudative AMD, whereas the low concentration in the aqueous fluid may be explained by the low solubility of CM082 in tears and other liquid contained in the aqueous fluid. The results of our study demonstrated that CM082 can inhibit CNV formation and effectively induce regression of established CNV following oral administration. After oral administration, CM082 is equally distributed to the eyes and is efficiently absorbed. This is the first report of the effects of this treatment in a CNV animal model.

As previously mentioned, the pathology of CNV is not completely understood and the therapeutic methods are limiting. Certain recent clinical trials have reported that intravitreal injections of a VEGF inhibitor can arrest type 1 CNV progression, which reduces the central thickness of the retina and efficiently prevents vision loss in patients with wet AMD [24, 25]. Currently, anti-VEGF therapies, such as ranibizumab and bevacizumab, have become the main treatment for exudative AMD in the clinic. However, these therapies cannot induce regression of established, type 2 CNV, and repeated injections of anti-VEGF treatments may cause a number of complications, including vision impairment, media opacification, and intraocular inflammation [26]. Moreover, some individuals do not respond to intravitreal anti-VEGF drugs, so their vision is not improved over baseline [27]. The underlying mechanism may be due to the complex interaction of VEGFs and VEGFRs. Ranibizumab and bevacizumab are antibodies that target VEGF-A. However, VEGF-D and VEGF-E also play roles in neovascularization by binding VEGF receptors [28, 29]. Besides, studies have noticed PDGF, another critical factor in angiogenesis. It is a crucial molecule in vessel progression and stabilization that binds to PDGFR [19]. Previous studies indicated that treatments that simultaneously inhibit VEGFRs and PDGFRs may not only suppress neovascularization but also cause the regression of established vessels [20, 30]. Consequently, simultaneous inhibition of VEGF and PDGF may contribute to reducing nonresponsiveness.

CM082 is an orally bioavailable small-molecule inhibitor of all isoforms of VEGFRs and PDGFRs, with antiangiogenic and antineoplastic effects. Its design was based on sunitinib. Preclinical studies have indicated that sunitinib can inhibit corneal neovascularization [31]. Vatalanib, pazopanib, and sorafenib are tyrosine kinase inhibitors similar to sunitinib. Vatalanib can inhibit PDGFR and c-kit and has been confirmed to inhibit neovascularization [32]. Pazopanib can inhibit VEGFR, PDGFR, and c-kit. A phase 1/2 clinical trial has demonstrated that this drug exhibits promising antitumor activity and has a favorable toxicity profile [33]. This therapy has also been tried in the form of eye drops or as an oral medicine to treat exudative AMD [34, 35]. These studies indicate that this type of multitarget receptor tyrosine kinase inhibitor can significantly suppress CNV development. Tyrogenex has completed a phase 1 clinical trial of CM082 and is conducting a phase 2b study in patients with exudative AMD. In a model of oxidative-induced retinopathy, the inhibition rate in a CM082-treated group reached 71.1% compared to that in a control group. Results from another CNV model produced by subretinal injection of Matrigel also indicated that CM082 reduced the areas of CNV lesions.

In conclusion, oral administration of 10 mg/kg/d or 30 mg/kg/d CM082 reduces the area of the CNV lesions and pathological neovascularization in a laser-induced CNV model in BN rats. CM082 treatment could reduce the necessity of intravitreal injections and decrease side effects, as oral administration and long-term application are permitted. More thorough pharmacological and therapeutic analyses of CM082 will be required to illustrate the mechanism of PDGF signaling inhibition and the safety and cost.

5. Conclusion

CM082 can reach the retina successfully by oral treatment in BN rats. Oral administration of 10 mg/kg/d or 30 mg/kg/d CM082 reduces the area of the CNV lesions and pathological neovascularization in a laser-induced CNV model in BN rats.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Chengda Ren and Hui Shi contributed equally to this work.

Acknowledgments

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Research Article

The Involvement of β-Catenin/COX-2/VEGF Axis in NMDA-Caused Retinopathy

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NMDA, a molecule that is capable of producing the loss of retinal ganglia cells (RGCs), has been widely studied; however, the detailed mechanism is not yet clarified. Previously, Wnt/β-catenin signaling has been suggested to be involved in the NMDA-induced retinopathy. In addition, previous investigations in our group demonstrated the presence of a Wnt/β-catenin/COX-2 axis in dorsal root ganglions (DRGs). Therefore, here in this paper, we tested whether there is an association of such axis with NMDA-induced RGC loss. Rat retinal damage models generated by intravitreal injection of NMDA were used to measure the expression levels of β-catenin, COX-2, and VEGF in retinas, and the neuron numbers of the retinal GCL of rats were counted. Then, pharmacological tools (MK801, a NMDA receptor inhibitor; Dickkopf homolog 1, a specific inhibitor of the Wnt pathway; NS-398, a COX-2 inhibitor; and bevacizumab, IVB, a VEGF inhibitor) were introduced to evaluate the detailed roles of Wnt/β-catenin, COX-2, and VEGF in retinopathy of rats. Results demonstrated that all three factors in sequence are positively regulated neuronal loss induced by NMDA. These observations indicated that the Wnt pathway/COX-2/VEGF axis plays a pathogenic role in retinopathy and represented novel therapeutic targets.

1. Introduction

Retinal ganglion cell death is a characteristic of many ophthalmological diseases, such as glaucoma, proliferative diabetic retinopathy, and retinal vein occlusion [1], but the underlying mechanism is not completely clarified, while glutamate-induced neurotoxicity is confirmed [2, 3]. The N-methyl-d-aspartic acid (NMDA) receptor, a glutamate receptor subtype [4, 5], being activated can lead to a large Ca^{2+} influx. This excess intracellular Ca^{2+} causes predominant neuronal excitotoxicity mechanisms and is thought to be an underlying mechanism of glaucoma-induced neuronal cell death [6].

Recent evidence indicates that the canonical Wnt pathway was activated with the NMDA receptor activation [7, 8]. β-Catenin is an essential downstream effector in the canonical Wnt/β-catenin signaling pathway. In the absence of Wnt ligands, β-catenin is phosphorylated by a protein complex containing glycogen synthase kinase-3β (GSK-3β) and is constantly degraded to prevent its accumulation [9]. Upon exposure to an appropriate stimulus, β-catenin is translocated from the cytoplasm to the nucleus, where it interacts with members of the T-cell factor for DNA binding and regulates the expression of target genes, including inflammatory factors, such as COX-2 [10]. Retinal inflammation is believed to play a causative role in vascular leakage,

which can lead to diabetic macular edema, and in retinal neovascularization. Leukostasis is believed to contribute to capillary nonperfusion and local ischemia, which subsequently induces the overexpression of vascular endothelial growth factor (VEGF) [11–14]. Increased VEGF levels are responsible for the retinal vascular leakage.

In the present study, we used NMDA-treated rats to examine the possible role of β-catenin accumulation on the loss of neurons in the damaged retinas of model animals. The Wnt/β-catenin pathway in the rat retina was further mimicked by treatment with TWS119 (a GSK-3β inhibitor), which caused abnormal β-catenin accumulation [15]. We subsequently investigated whether MK801 (a NMDA receptor inhibitor), Dickkopf-1 (DKK-1, an inhibitor of the Wnt/β-catenin pathway), NS-398 (a COX-2 inhibitor), and bevacizumab (IVB, a VEGF inhibitor) can modulate β-catenin, COX-2, and VEGF expression levels and the neuronal loss in the retinas of the rats.

2. Materials and Methods

2.1. Animals. Experimental male Sprague-Dawley rats (weighing 180–200 g) were provided by Hubei Center for Disease Control and Prevention (China). The animals were acclimatized to the laboratories for one week prior to manipulation and were group housed (except for during the experimental procedures) in a controlled environment (temperature $20 \pm 2^{\circ}$C and humidity 70%) under a 12-hour light-dark cycle. Water and food were supplied ad libitum. All experiments followed the WHO Guidance of Humane Care and Use of Laboratory Animals. The protocols were approved by the Committee on the Ethics of Animal Experiments of the South-Central University for Nationalities, China (permit number: 2013-SCUEC-AEC-006). Every effort was made to minimize the number of animals used and their suffering.

2.2. Treatments of Rats. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Sigma Chemical, St Louis, MO, USA; 40 mg/kg). NMDA (Sigma Chemical; 2 μl, 40 nM) was intravitreally injected with a 33 gauge needle connected to a microsyringe after dilation of the rat pupil with tropicamide (Sigma Chemical) in both eyes of each animal. MK801 (Sigma Chemical, St Louis, MO, USA; 0.01, 0.1, 1 μM), a NMDA receptor antagonist; NS-398 (1, 10, and 100 μM), a COX-2 selective inhibitor; DKK-1 (R&D Systems; 10, 50, 100 ng/ml), a Wnt/β-catenin signaling inhibitor; and bevacizumab (0.25, 2.5, and 25 mg/ml), an anti-VEGF antibody were intravitreally injected with the 80 nmol NMDA in some rats. Besides, TWS119 (Cayman Chemical, Ann Arbor, MI; 2 μl, 1 μM) was intravitreally injected after dilation of the rat pupil with tropicamide. IVB (0.25–25 mg/ml) was intravitreally injected with 1 μM TWS119 at the same rate. NS398 (a COX-2 selective inhibitor; 1–100 μM) was intravitreally injected with 1 μM TWS119 in the other rats [16]. Control rats ($n = 4$) were injected with phosphate-buffered saline (PBS). The tip of the needle was inserted through the dorsal limbus of the eye [17], and the total injection volume was 2 μl. Animals with lens damage or vitreal haemorrhage were excluded from the study. The rats were sacrificed on the seventh day after treatments. The eyes were enucleated, formalin-fixed for 24 h, and paraffin-embedded for sectioning.

2.3. Slide Sectioning and HE Staining to Count the Number of Neurons. The slides made from the eyes of the rats were stained with hematoxylin and eosin (H&E). The number of neurons in the RGC layer within an area (20 μm, parallel to the retinal GCL) of the central retina (1-2 mm from the optic disc) was counted in individual sections. A total of six sections from every five serial sections were examined for each retina. The morphological characteristics of adjacent tissue sections were assessed using a Nikon Eclipse Ti fluorescence microscope (Osaka, Japan).

2.4. Immunohistochemistry Analysis for the Expressions of COX-2 and VEGF in Eyes. Sections of the eyes from the rats were prepared for immunohistochemical staining according to the methods described previously (with slight modifications). Samples were treated according to a simple immunohistochemical staining method using a Histofine rat MAX-PO (MULTI) kit (Nichirei Corp, Tokyo, Japan) [18]. Briefly, the sections were immersed in the dimethylbenzene for 30 min to remove paraffin, washed step elution by aqueous ethanol for 5 min each time. Then, the endogenous peroxidases were inhibited with 0.3% hydrogen peroxidase for 15 min and washed with ultrapure water. After that, the sections were treated by microwaving them in 0.01 M citrate acid buffer (pH 6.0) at 700 W for 10 min. After washing thoroughly, the sections were incubated with an anti-VEGF polyclonal antibody (Boster, Wuhan, China; 1 : 100 dilution) and anti-COX-2 polyclonal antibody (Cayman Chemical, Ann Arbor, MI; 1 : 200 dilution) overnight at 4°C. Then, the sections were immersed in DAB (Histofine, Nichirei Corp, Tokyo, Japan) and quantified by a multispectral image analysis.

2.5. Immunofluorescence Analysis for the Expressions of β-Catenin in Eyes from Rats. The immunofluorescence analysis of β-catenin expression was performed with an anti-β-catenin polyclonal antibody (Cayman; 1 : 100 dilution). The tissue sections were then incubated for 1 h at room temperature with Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; 1 : 1000 dilution), washed three times with PBS, and visualized under a Nikon Eclipse Ti fluorescence microscope [18].

2.6. Statistical Analysis. In the immunohistochemical analysis, the retinal layers within an area (20 μm, parallel to the retinal GCL) of the central retina (1-2 mm from the optic disc) in individual sections were observed. A total of six sections from every five serial sections were examined for each retina. The data are presented as the means ± SEM. All statistical analyses were performed using GraphPad Prism 5.0 software package. Comparisons between the mean variables of two groups were made by one-way ANOVA of Bonferroni. $^{\dagger}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control $^{\ddagger}P > 0.05$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus model.

3. Results

3.1. Wnt/β-Catenin, COX-2, and VEGF Were Involved in MK801-Induced Protection of Retinal Neuron Cells from Damage in NMDA-Treated Rats. The neurons in the retinas of normal untreated rats were compact and clear. However, there was an obvious loss of neurons in the retinal ganglion cell layer (GCL) of rats treated with NMDA. The number of neurons in the retinal GCL of the NMDA-treated rats was decreased significantly (49 ± 4% of control) in comparison to that of normal rats (100 ± 5%; Figures 1(a) and 1(b)), indicating that the retinal neurons were damaged by NMDA treatment. In comparison to that of NMDA-treated rats (49 ± 4% of control), the retinal GCL neuron number in the rats treated with NMDA plus MK801 increased in a dose-dependent manner (72 ± 3%, 78 ± 5%, and 79 ± 4% of control, Figure 1(b)), demonstrating that inhibiting the NMDA receptor has a protective effect on the neurons.

Then, we investigated whether the Wnt/β-catenin signaling pathway plays a role in the rats' retinal damage induced by NMDA. As shown in Figures 1(a) and 1(c), compared with the retinas of normal rats (100 ± 16%), the retinas of the rats treated with NMDA displayed more abundant β-catenin expression in the GCL (278 ± 9% of the control). These data suggest that β-catenin accumulation might participate in the NMDA-induced loss of retinal neurons. Then, the effect of MK801 on the expression of β-catenin in the rats' retinas was investigated. After the treatment with different doses of MK801 (0.01, 0.1, and 1 μM) in the presence of NMDA, the expression of β-catenin in the GCL was decreased significantly (138 ± 12%, 146 ± 4%, and 122 ± 11%, resp.).

As we have indicated that our previous works demonstrated that COX-2 could be an important downstream of β-catenin, we detected the expression of COX-2 in retinopathy caused by NMDA. COX-2 expression of the rats' retinas examined by immunohistochemical assays was shown in Figure 1(d). Compared with the normal control rats (100 ± 8%), COX-2 expression of NMDA-treated rats was overexpressed (228 ± 18% of the control). After the treatment with MK801 (0.01, 0.1, and 1 μM) plus NMDA, the overexpressions of COX-2 were reversed (137 ± 7%, 130 ± 12%, and 98 ± 9% of the control, resp.; Figure 1(d)) as compared with those in NMDA-treated rats.

The VEGF expression level was examined by immunohistochemical assays and quantified by a multispectral image analysis. As shown in Figure 1(e), the VEGF expression in the retinal GCL of the NMDA-treated rats was increased in comparison to normal rats. The expressions of VEGF in the NMDA-treated rats' retinas were 276 ± 7% of the control in GCL. In the retinas of the rats treated with MK801 (0.01, 0.1, and 1 μM) plus NMDA, the VEGF expression was lower in the GCL (122 ± 7%, 122 ± 10%, and 101 ± 10% of the control, resp.) than that of the NMDA-treated rats. These results showed that VEGF overexpression could be attenuated by blocking NMDA receptors.

3.2. The Wnt/β-Catenin Pathway Was Activated in the Retinopathy Caused by NMDA. To further characterized the role of Wnt pathway, or more specifically β-catenin, we treated some other rats with intravitreal injections of NMDA plus DKK-1, which is a widely used and effective inhibitor of Wnt/β-catenin signal pathway. Immunofluorescent images showed that DKK-1 can decrease the expression of β-catenin in retina tissue (Figure 2(a)). The neuron numbers in the retinal GCL of the rats treated with DKK-1 (10, 50, and 100 ng/ml) plus NMDA elevated significantly (81 ± 4%, 86 ± 6%, and 86 ± 4% of control, resp.) compared with those in the NMDA group (49 ± 4% of control; Figure 2(b)), indicating that the inhibition of Wnt/β-catenin could block the NMDA-induced loss of neurons. To further investigate the role of β-catenin signal in neuronal loss, we utilized TWS119 (a GSK-3β inhibitor which inhibited the degradation of β-catenin) to induce abnormal β-catenin accumulation which mimicked the activation of the canonical Wnt/β-catenin signaling pathway [18]. The neuron number in the retinal GCL of TWS119-treated rats was counted, and it was obviously decreased (52 ± 3% of the control), thus indicating that the overexpression of β-catenin induced by TWS119 leads to neuronal loss.

The immunofluorescence analyses showed that β-catenin was overexpressed in the retinal GCL of NMDA-treated rats (278 ± 9% of the control) and TWS119-treated rats (372 ± 13% of the control). Meanwhile, DKK-1 (10, 50, and 100 ng/ml) plus NMDA attenuated the overexpression of β-catenin (122 ± 11%, 124 ± 10%, and 106 ± 13% of the control, resp.; Figure 2(c)). As shown in Figures 2(d) and 2(e), COX-2 was decreased (160 ± 12%, 124 ± 12%, and 128 ± 12% of control, resp.) compared with NMDA-induced rats (228 ± 18% of control), and VEGF was decreased (133 ± 14%, 133 ± 16%, and 127 ± 20% of control, resp.) compared with NMDA-induced rats (228 ± 18% of control; Figure 2(e)), accompanying with low expression of β-catenin in the presence of DKK-1 (10, 50, and 100 ng/ml). Meanwhile, with high expression of β-catenin (372 ± 13% of control) caused by TWS119, COX-2 was increased (169 ± 14% of control), and VEGF was increased (277 ± 9% of control) as well.

3.3. COX-2 Was Involved in the Loss of Neuron Cells Caused by NMDA-Induced Activation of β-Catenin. To further assess the relationship between β-catenin and COX-2, we treated some rats with an intravitreal injection of NMDA plus NS-398, an inhibitor of COX-2. The representative image of the rats treated with 10 μM NS-398 plus NMDA was shown in Figure 3(a). The neuron numbers in the retinal GCL of the rats treated with NMDA plus NS-398 (10, 50, and 100 ng/ml) were elevated (78 ± 5%, 86 ± 2%, and 96 ± 4% of control, resp.) compared with those in the NMDA-treated group (49 ± 4% of control). This demonstrated that the inhibition of COX-2 could block the NMDA-induced loss of neurons, which may also contribute to retinal protection (Figure 3(b)).

In addition, the neuron numbers in the retinal GCL of the rats treated with NS-398 (10, 50, and 100 ng/ml) plus TWS119 elevated (73 ± 6%, 80 ± 6%, and 80 ± 4% of control, resp.) compared with those in the TWS119 group (52 ± 3% of control). This indicated that the inhibition of COX-2 could block the TWS119-induced loss of neurons, which may contribute to retinal protection (Figure 4(b)).

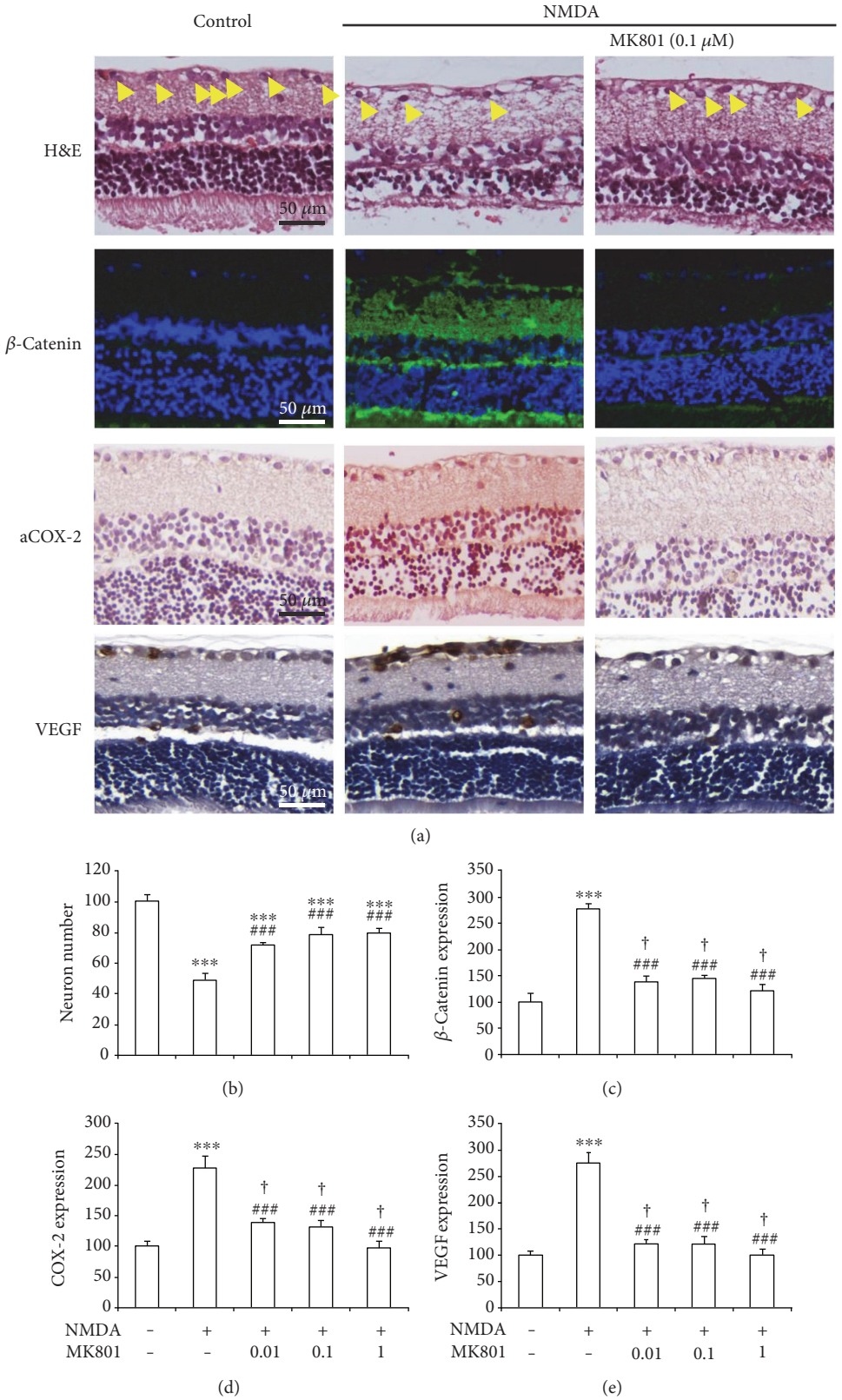

Figure 1: The protection of MK801 on retinal neuron cells in NMDA-treated rats. (a) Representative images (H&E staining, expression of β-catenin, COX-2, and VEGF) of the rats. (b) Neuron numbers of RGCs with respect to control (untreated), in NMDA-injected retinas of animals treated with MK801. (c) β-Catenin expression of retinas with respect to control in NMDA-injected retinas of animals treated with MK801. (d) COX-2 expression of retinas with respect to control in NMDA-injected retinas of animals treated with MK801. (e) VEGF expression of retinas with respect to control in NMDA-injected retinas of animals treated with MK801.

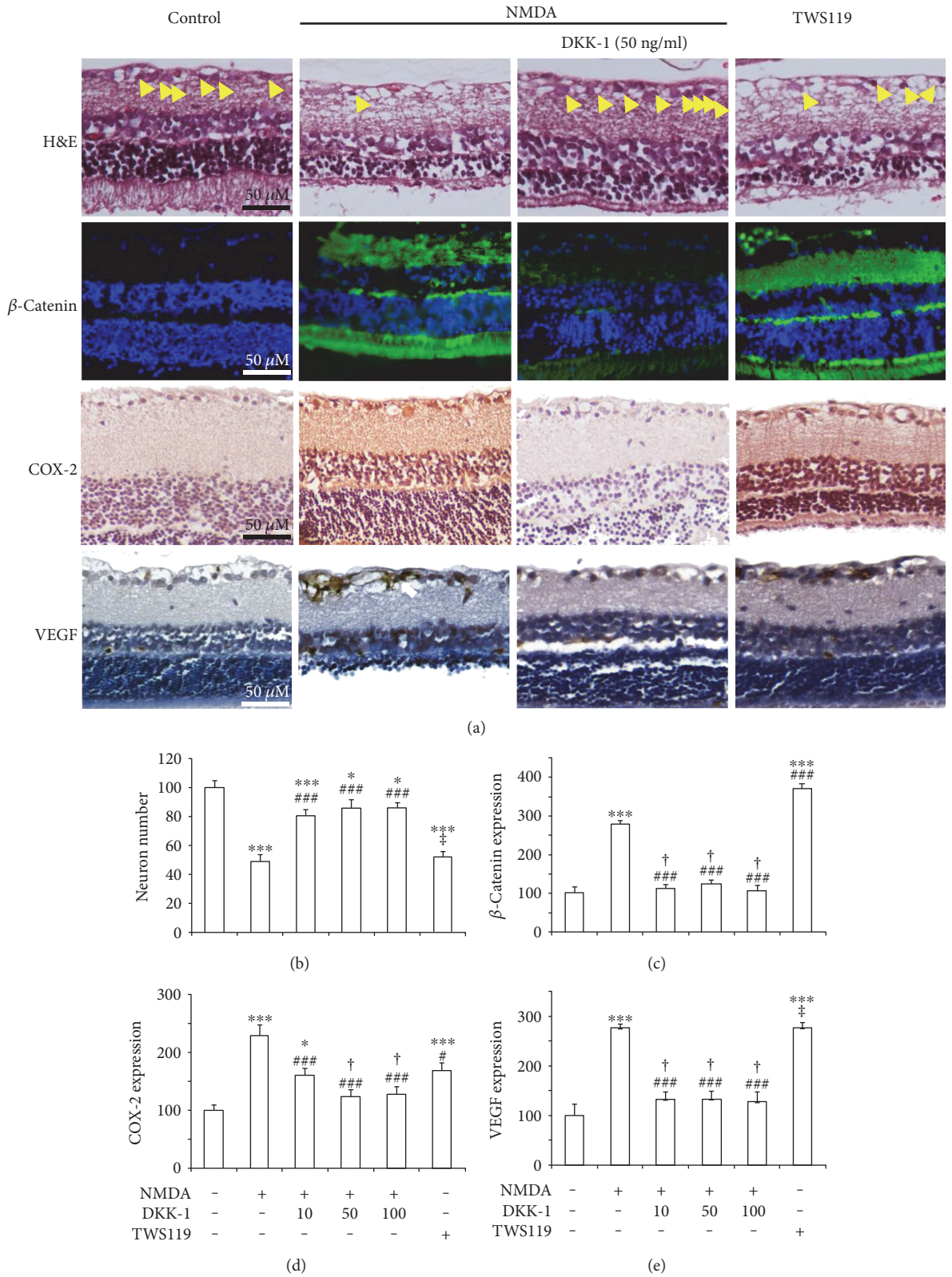

Figure 2: The Wnt/β-catenin pathway was activated in the retinopathy caused by NMDA. (a) Representative images (H&E staining, expression of β-catenin, COX-2, and VEGF) of the rats. (b) Neuron numbers of RGCs with respect to control (untreated), in NMDA-injected retinas of animals treated with DKK-1. (c) β-Catenin expression of retinas with respect to control (untreated), in NMDA-injected retinas of animals treated with DKK-1. (d) COX-2 expression of retinas with respect to control, in NMDA-injected retinas of animals treated with DKK-1. (e) VEGF expression of retinas with respect to control, in NMDA-injected retinas of animals treated with DKK-1.

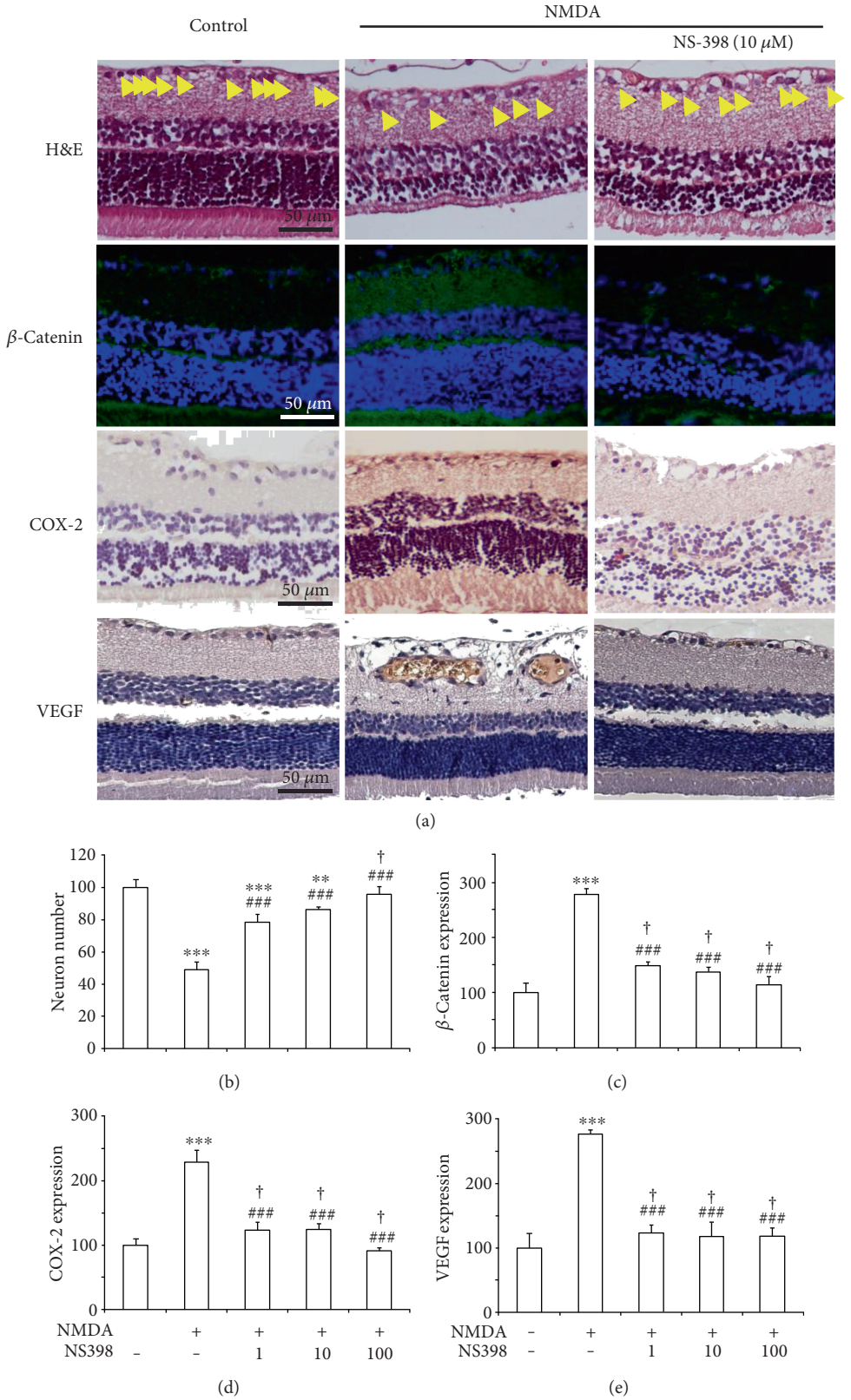

Figure 3: NS-398 could attenuate the NMDA-induced activation of β-catenin causing neuron cell loss. (a) Representative images (H&E staining, expression of β-catenin, COX-2, and VEGF) of the rats. (b) Neuron number of RGCs with respect to control (untreated), in NMDA-injected retinas of animals treated with NS-398. (c) β-Catenin expression of retinas with respect to control in NMDA-injected retinas of animals treated with NS-398. (d) COX-2 expression of retinas with respect to control in NMDA-injected retinas of animals treated with NS-398. (e) VEGF expression of retinas with respect to control in NMDA-injected retinas of animals treated with NS-398.

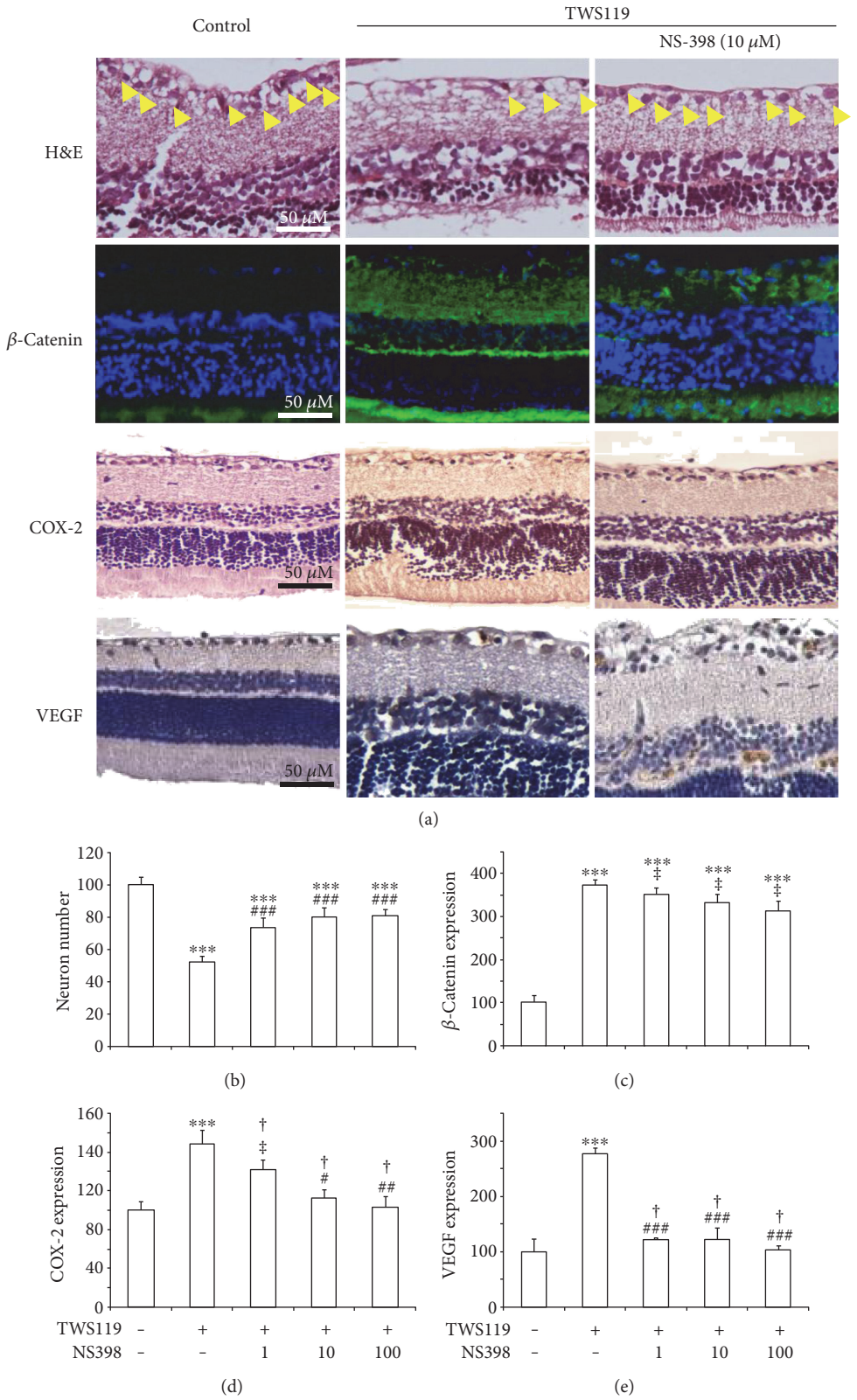

Figure 4: NS-398 could attenuate the TWS119-induced activation of β-catenin causing neuron cell loss. (a) Representative images (H&E staining, expression of β-catenin, COX-2, and VEGF) of the rats. (b) Neuron number of RGCs with respect to control (untreated), in TWS119-injected retinas of animals treated with NS-398. (c) β-Catenin expression of retinas with respect to control in TWS119-injected retinas of animals treated with NS-398. (d) COX-2 expression of retinas with respect to control in TWS119-injected retinas of animals treated with NS-398. (e) VEGF expression of retinas with respect to control in TWS119-injected retinas of animals treated with NS-398.

Then, we detected the expression of β-catenin with NS398 (1, 10, and 100 μM) plus NMDA, and we found that the expression of COX-2 in NMDA plus NS-398 ($124 \pm 11\%$, $124 \pm 8\%$, and $91 \pm 4\%$ of control, resp.) was lower compared with the NMDA group ($228 \pm 18\%$ of control; Figure 3(d)). In the same trend, β-catenin was decreased ($148 \pm 7\%$, $137 \pm 8\%$, and $114 \pm 13\%$ of control, resp.; Figure 3(c)), compared with the NMDA group ($278 \pm 9\%$ of control); VEGF was decreased ($122 \pm 18\%$, $117 \pm 22\%$, and $118 \pm 12\%$ of control, resp.; Figure 3(e)), compared with the NMDA group ($276 \pm 7\%$ of control).

While the expression of β-catenin ($350 \pm 16\%$, $350 \pm 16\%$, and $332 \pm 18\%$ of control, resp.) was unchanged in TWS119 plus NS-398 compared with TWS119 group ($372 \pm 13\%$; Figure 4(c)), COX-2 was attenuated ($141 \pm 10\%$, $113 \pm 8\%$, and $102 \pm 12\%$ of control, resp.) in TWS119 plus NS-398 (1, 10, and 100 μM; Figure 4(d)), and VEGF was decreased ($121 \pm 3\%$, $122 \pm 20\%$, and $102 \pm 7\%$ of control, resp.; Figure 4(e)).

3.4. VEGF Was the Downstream of Wnt/β-Catenin Signaling and COX-2 in Retinopathy Caused by NMDA. Bevacizumab (IVB), an anti-VEGF antibody that could inhibit VEGF, was used to further characterize the retinopathy caused by NMDA. As shown in Figures 5(a) and 5(b), the neuron numbers in the retinal GCL of the rats treated with IVB (0.25, 2.5, and 25 mg/ml) plus NMDA elevated ($64 \pm 3\%$, $68 \pm 8\%$, and $70 \pm 5\%$ of control, resp.) compared with those in the NMDA group ($49 \pm 4\%$ of control). This indicated that the inhibition of VEGF could at least partially prevent the NMDA-induced loss of neurons, which may contribute to retinal protection. In accordance, Figure 5(e) presented that the IVB (0.25, 2.5, and 25 mg/ml) treatment decreased the expression of VEGF in the retinas of rats treated with NMDA ($168 \pm 11\%$, $137 \pm 11\%$, and $140 \pm 18\%$ of control, resp.) compared with NMDA-treated rats ($276 \pm 7\%$). In the contrary, Figures 5(c) and 5(d) demonstrated that IVB (0.25, 2.5, and 25 mg/ml) treatment could affect the expression of neither β-catenin ($280 \pm 9\%$, $278 \pm 17\%$, and $283 \pm 19\%$ of control, resp.) nor COX-2 ($205 \pm 17\%$, $219 \pm 13\%$, and $213 \pm 16\%$ of control, resp.) in the retinas of rats treated with NMDA ($278 \pm 9\%$ and $220 \pm 18\%$). These findings demonstrate that the inhibition of VEGF did not modulate the β-catenin and COX-2 overexpression induced by NMDA, although the inhibition of VEGF helped attenuate the loss of neurons.

To evaluate the relationship between activation of Wnt pathway and VEGF, the VEGF expression level of TWS119-treated retinas in rats was examined by immunohistochemical assays. We could see from Figure 6(b) that an intravitreal injection of TWS119 plus IVB (0.25, 2.5, and 25 mg/ml) increased the number of retinal GCL neurons ($70 \pm 3\%$, $75 \pm 4\%$, and $78 \pm 6\%$ of control, resp.) in the rats treated with TWS119 ($52 \pm 3\%$ of the control). Statistical analyses also showed that the IVB (0.25, 2.5, and 25 mg/ml) treatment decreased the expression of VEGF in the retinas of rats treated with TWS119 ($151 \pm 14\%$, $138 \pm 22\%$, and $125 \pm 12\%$ of control, resp.) compared with TWS119-treated rats ($277 \pm 23\%$), as shown in Figure 6(e). Moreover, IVB failed to inhibit β-catenin expression levels in the retinal GCL of TWS119-treated rats ($364 \pm 12\%$, $375 \pm 7\%$, and $379 \pm 6\%$ of control, resp.). The expression of COX-2 in the retinas of TWS119-treated rats was also unchanged after treatment with IVB (Figure 6(d)). Statistical analyses show that the IVB (0.25, 2.5, and 25 mg/ml) treatment decreased the expression of COX-2 in the retinas of rats treated with TWS119 ($167 \pm 16\%$, $169 \pm 19\%$, and $163 \pm 9\%$ of control, resp.) compared with TWS119-treated rats ($169 \pm 14\%$ of control).

3.5. Discussion. The present study demonstrates that the β-catenin signaling pathway is activated by NMDA in the retinas of rats and for the first time that the activation of the β-catenin signaling pathway is involved in the overexpression of COX-2 and VEGF, which leads to retinal ganglion cell loss in the retinal GCL of rats treated with NMDA. Furthermore, we have shown that DKK-1, NS-398, and IVB can ameliorate retinal ganglion cell loss, suggesting that the β-catenin/COX-2/VEGF axis plays a causative role in retinopathy treated with NMDA. Therefore, these observations have established a new mechanism for the NMDA-induced retinal ganglion cell loss (Figure 7).

Excessive activation of NMDA receptors *in vitro* and *in vivo* might directly cause the degeneration of retinal neurons which due to the large amount of NMDA receptors present on the membranes of retinal GCL neurons [19–21]. Our present study revealed that there was extensive neuronal loss after intravitreal injection of NMDA. The activation of the NMDA receptor was thought to be insufficient to explain the neuronal loss [22, 23]. In the present study, we found that cotreatment with MK801 (100 nM or 1 μM) could decrease the NMDA-induced neuron loss. The activation of the Wnt/β-catenin signaling pathway was previously found in injured retinal tissues *in vitro* [24]. In addition, an inhibitor (Dickkopf-1) of the Wnt/β-catenin signaling pathway could alleviate diabetic retinopathies [18, 25]. To evaluate the activation of the Wnt/β-catenin pathway in NMDA-induced retinal damage in rats, we examined retinal β-catenin levels in the NMDA-damaged retinas of rats. The immunofluorescence analyses demonstrated abnormal accumulation of β-catenin in the retinal GCL of the NMDA-treated rats in comparison to that of normal rats. COX-2 is one of the target genes and a modulating factor of the Wnt/β-catenin pathways, we checked the COX-2 expression and found that COX-2 increased after NMDA treatment. In addition, VEGF was increased after NMDA treatment. Notably, the inhibition of the NMDA receptor by MK801 could decrease the expression levels of β-catenin, COX-2, and VEGF (Figure 1). These findings have shown that the inhibitors of NMDA can protect retinal ganglion cells through decreasing the expression of β-catenin, COX-2, and VEGF.

Since Wnt pathway is known to be activated under retinopathy conditions [24], we further demonstrated the causative role of activated Wnt signaling in retinal caused by NMDA. We blocked the Wnt pathway by using DKK-1, a specific peptide inhibitor of the Wnt pathway. Intravitreal injection of NMDA plus DKK-1 is sufficient to mitigate retinal inflammation as it blocks the overexpression of

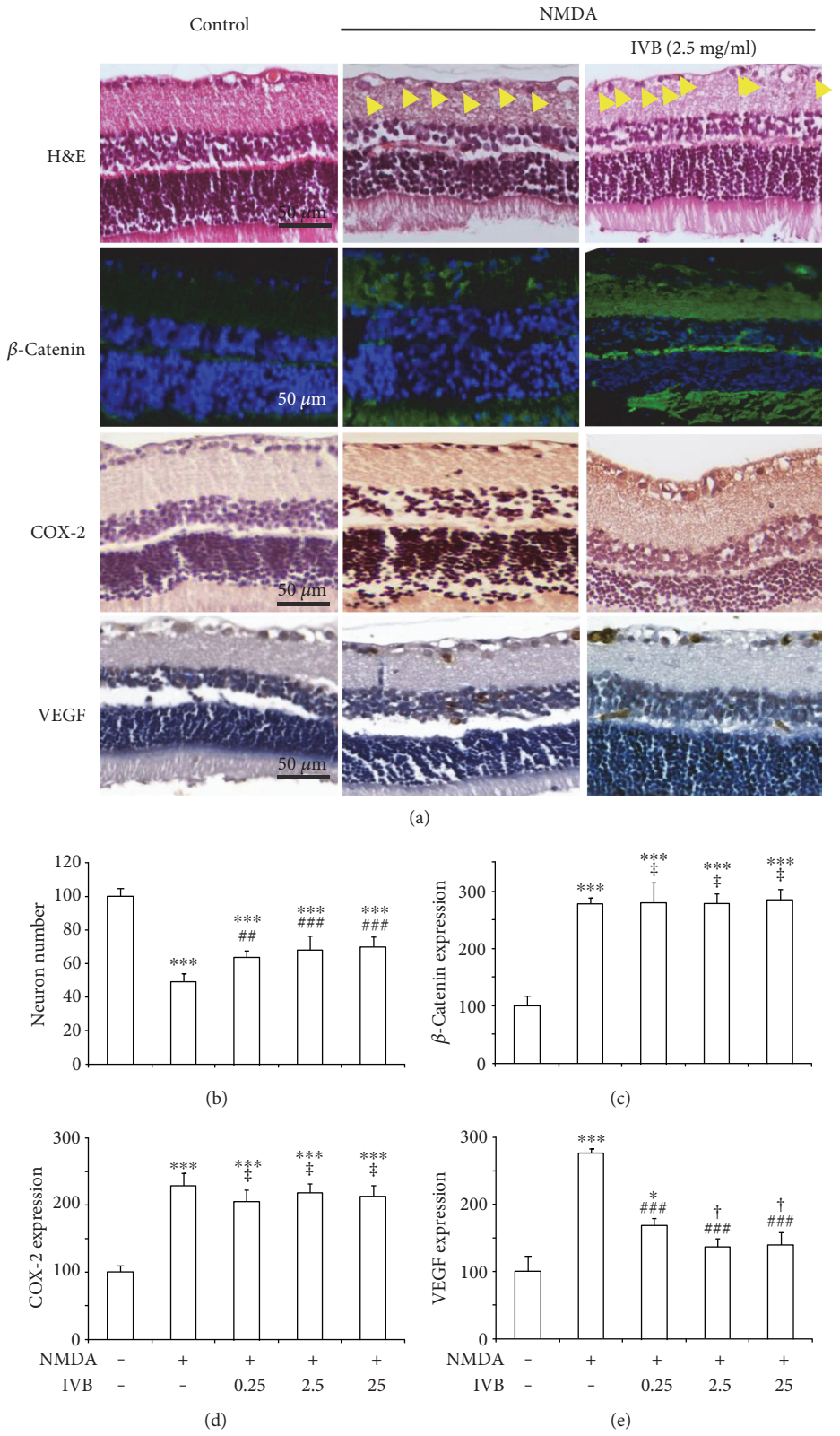

Figure 5: IVB could ameliorate retinopathy caused by NMDA through reducing VEGF expression (a) Representative images (H&E staining, expression of β-catenin, COX-2, and VEGF) of the rats. (b) Neuron number of RGCs with respect to control (untreated), in NMDA-injected retinas of animals treated with IVB. (c) β-Catenin expression of retinas with respect to control in NMDA-injected retinas of animals treated with IVB. (d) COX-2 expression of retinas with respect to control in NMDA-injected retinas of animals treated with IVB. (e) VEGF expression of retinas with respect to control in NMDA-injected retinas of animals treated with NMDA.

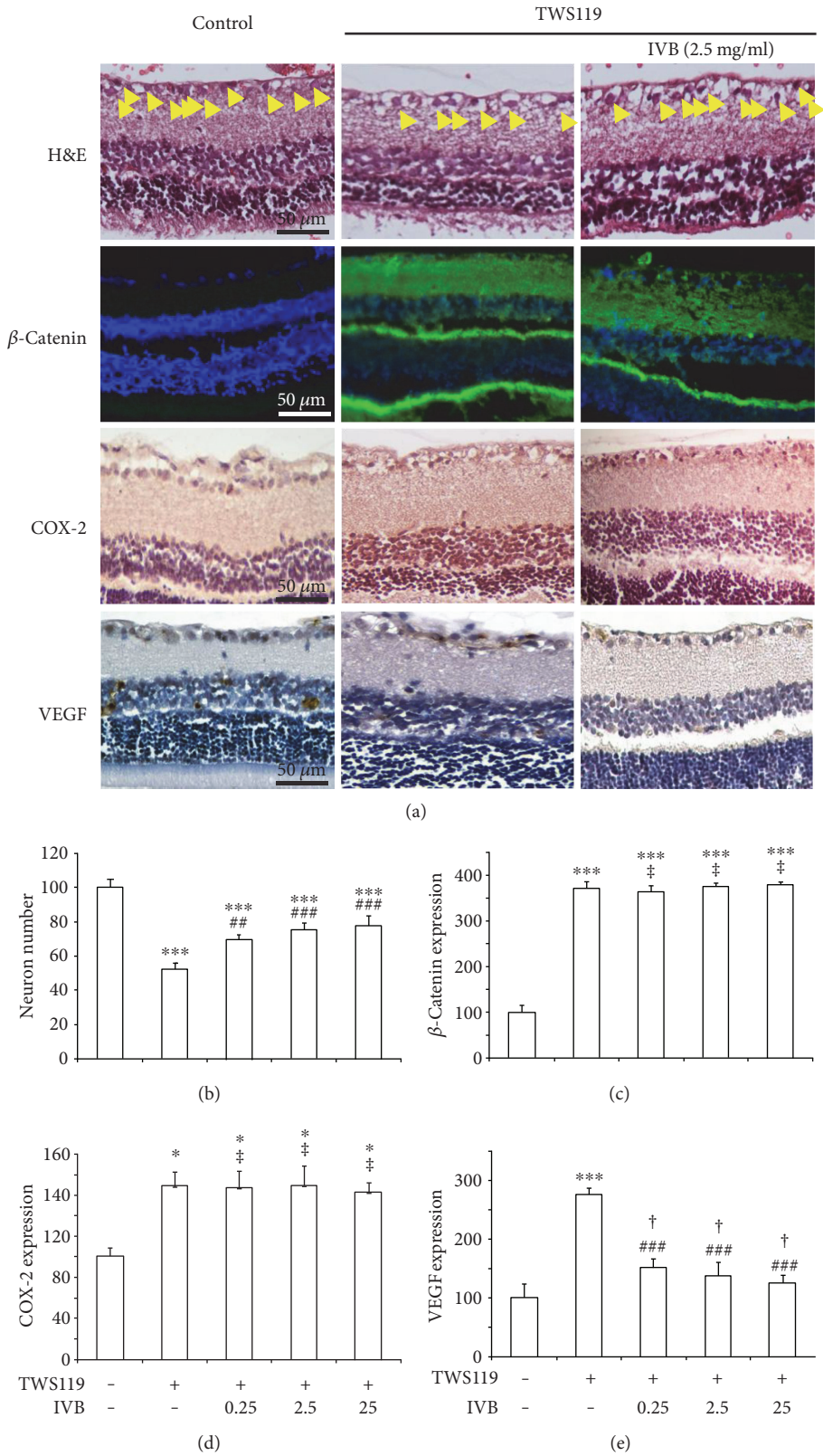

Figure 6: IVB could ameliorate retinopathy caused by TWS119 through reducing VEGF expression (a) Representative images (H&E staining, expression of β-catenin, COX-2, and VEGF) of the rats. (b) Neuron number of RGCs with respect to control (untreated), in TWS119-injected retinas of animals treated with IVB. (c) β-Catenin expression of retinas with respect to control in TWS119-injected retinas of animals treated with IVB. (d) COX-2 expression of retinas with respect to control in TWS119-injected retinas of animals treated with IVB. (e) VEGF expression of retinas with respect to control in TWS119-injected retinas of animals treated with TWS119.

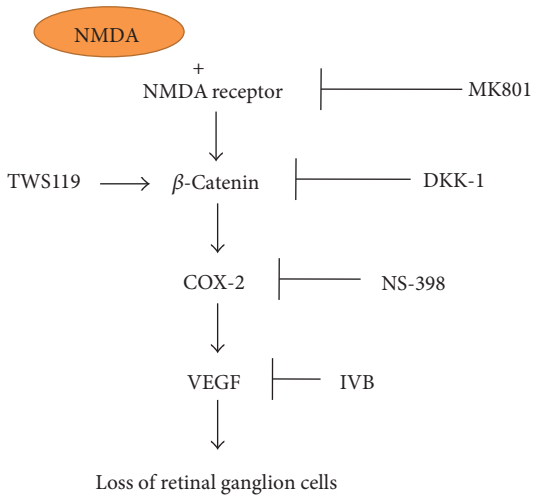

Figure 7: Schematic graph illustrating the involvement of Wnt/β-catenin, COX-2, and VEGF in retinopathy induced by NMDA.

β-catenin, which is the upstream of COX-2. Similarly, DKK-1 also reduced the ischemia-induced retinal neovascularization, the VEGF expression. These results indicate that blockade of the Wnt pathway is sufficient to ameliorate the damage caused by NMDA. Further, we mimicked the activation of the β-catenin pathway using TWS119. By inhibiting GSK-3β activation, TWS119 leads to the accumulation of β-catenin in the cytoplasm, which subsequently translocates to the nucleus [15]. Our immunofluorescence data demonstrated that either VEGF or COX-2 was overexpressed in the GCL of the TWS119-treated rats. Moreover, we found that the TWS119-treated rats suffered more serious neuronal loss than the control rats did (Figure 2). These results indicate that stimulation of the β-catenin signaling pathway could induce neuronal loss. Further, activation of the β-catenin signaling pathway, without NMDA treatment, was sufficient to induce COX-2, VEGF expression, and neuronal loss.

In the present study, the expression levels of both COX-2 and VEGF increased after the administration of either NMDA or TWS119. Therefore, COX-2 and VEGF might be regulated by the activation of the β-catenin pathway in damaged retinas. We used specific inhibitors to examine the interaction between these various molecules.

NS-398, an inhibitor of COX-2, could attenuate the loss of retinal ganglion cells. Interestingly, inhibiting COX-2 by NS398 could decrease the NMDA-induced β-catenin overexpression that shows that COX-2 maybe upstream of β-catenin, which was confirmed by Li T that COX-2 can promote hepatocarcinogenesis through activation of β-catenin signaling pathway [17]. Similarly, inhibiting COX-2 by NS398 could attenuate the NMDA-induced VEGF overexpression (Figure 3). However, in TWS119-treated rats, NS398 failed to inhibit the activation of β-catenin. This is in accordance with our previous work [10] that there could be a feedback loop between β-catenin and COX-2.

These findings have shown that COX-2 is upstream of β-catenin and maybe upstream of VEGF. We are continuing our investigations to further elucidate how COX-2 signaling regulates β-catenin and VEGF. We found that NS398 was administered with TWS119 which could protect retinal ganglion cell from losing. However, NS398 could not reverse the TWS119-induced β-catenin overexpression but could attenuate VEGF overexpression caused by TWS119 (Figure 4). This further demonstrated that VEGF maybe downstream of COX-2.

IVB is an anti-VEGF agent used to treat diabetic macular edema. In this study, we found that treatment with IVB could not reverse the NMDA- or TWS119-induced COX-2 and β-catenin overexpression (Figures 5 and 6). But IVB can protect retinal ganglion cells treated with NMDA or TWS119. These results demonstrate inhibiting VEGF did not interfere with the β-catenin and COX-2 expression suggesting that VEGF is downstream of COX-2, which was proved in gastric cancer [16].

In summary, the present study provides the first evidence showing that activation of the β-catenin/COX-2/VEGF signaling pathway is an important pathogenic mechanism underlying the NMDA-induced retinal damage in animal models. Thus, the β-catenin/COX-2/VEGF pathway might represent a new target for pharmaceutical intervention for retinal diseases.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Dan Ning and Wei Kevin Zhang contributed equally to this work.

Acknowledgments

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Clinical Study

Two-Year Outcome of Aflibercept in Patients with Pigment Epithelial Detachment due to Neovascular Age-Related Macular Degeneration (nAMD) Refractory to Ranibizumab

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Purpose. To evaluate the response of intravitreal aflibercept injection (IAI) in eyes with detachment of retinal pigment epithelium (DEP) secondary to nAMD refractory to monthly ranibizumab. *Patients and Methods*. This is a retrospective, multicenter study. All patients received 3 IAI then treated as needed every 4 weeks for 12 months. During the second year, the eyes were treated with a treat- and-extend regimen. *Results*. Forty-four eyes were included. Best-corrected visual acuity improved significantly after the loading phase (3.1 ± 6.4 letters) and at 6 months (2.8 ± 6.4 letters), but change was not significant at 1 year and 2 years. The height of the DEP was significantly decreased at 3 months and 6 months, but the difference did not reach statistical difference at 1 and 2 years. Rate of eyes with complete resolution of exudation was 59% after the loading phase and 34.3% at 2 years. Mean interval of anti-VEGF injection was extended from 31 ± 2.6 days to 61 ± 5 days after conversion. *Conclusions*. Aflibercept intravitreal injection in patients with fibrovascular DEP due to nAMD who respond poorly to monthly ranibizumab led to short-term functional and anatomical improvement. Reduction of intravitreal injection frequency was obtained until 2 years of follow-up.

1. Introduction

Neovascular age-related macular degeneration is characterized by choroidal neovascularization (CNV), which lead to the accumulation of intraretinal fluid (IRF), subretinal fluid (SRF), and pigment epithelium detachment (PED). The prognosis of nAMD has improved considerably with intravitreal (IVT) injections of anti-VEGF [1]. Ranibizumab, a fragment binding to the monoclonal antigen VEGF-A without an Fc fragment, neutralizes all the active isoforms of VEGF-A [1]. The efficacy and safety of ranibizumab has been demonstrated in the MARINA, ANCHOR, and CATT studies, and this product was approved for the treatment of nAMD in Europe in January 2007 [1–4]. Aflibercept, approved in Europe in November 2012, was available and reimbursed in exudative AMD in France since November 2013. Aflibercept is a fusion protein that combines VEGF receptor 1 and 2 fragments (VEGFR1, VEGFR2) with an Fc fragment. It binds to VEGF-A, VEGF-B, and placental growth factor (PIGF). After the induction period, bimonthly intravitreal aflibercept injection (IAI) has been shown to be safe and effective as ranibizumab monthly injection in the treatment of nAMD in phase III of VIEW 1 and VIEW 2 studies [5].

Pigment epithelial detachments (PEDs) have been identified in up to 66.5% eyes in nAMD and are generally associated with poor visual prognosis, with loss of more than 3 lines in approximately 50% of patients within 1 year [6]. Clinical trials in nAMD have either excluded eyes with PED or have not performed subanalysis of PED response to

treatment [2–4, 7]. Although anti-VEGF is the standard care of nAMD [2–4, 8, 9], some cases are refractory with persistent fluid, and others develop a tolerance or tachyphylaxis defined by a decrease in anatomical response over time while they respond initially to treatment [10].

Pharmacological studies have shown that aflibercept differs from bevacizumab and ranibizumab by its higher affinity and additionally inhibits placental growth factor (PIGF) [11]. These differences led to several studies on the advantages of switching from ranibizumab and/or bevacizumab to aflibercept in refractory nAMD cases and its potential for the treatment of PED-related nAMD [12–17]. With the exception of some studies [18–21], most studies showed anatomical [15, 17, 22–28] but usually no functional benefit.

The purpose of the study was to evaluate the intravitreal aflibercept for the treatment of type 1 choroidal neovascularization-related PEDs with persistent exudation despite monthly ranibizumab during the 12 months preceding conversion with at least 2 years of follow-up.

2. Patients and Method

This is a retrospective, multicenter, nonrandomized study which recruited patients with PEDs due to type 1 choroidal neovascularization (CNV) who had been treated with monthly ranibizumab for at least 12 months in 3 centers (Centre Ophtalmologique de l'Odéon, Paris, France; Clinique de la Louvière, Lille, France; and the Ophthalmology Department, Catholic University of Lille, France). Informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki, and all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. This study named "OPENN" (a noninterventional Observational retrosPective study to access the Efficacy of intravitreal injections of aflibercept in NonNaïve patients with fibrovascular retinal pigment epithelium detachment secondary to wet AMD) had obtained governmental regulations with the reference MMS/SBA/AR149432.

Inclusion criteria were as follows: (1) eyes with PEDs related to type 1 CNV refractory to monthly ranibizumab (defined as the presence of intraretinal fluid (IRF) or subretinal fluid (SRF) at each visit 1 month after injection) during the 12 months leading to the switch from ranibizumab to aflibercept between November 2013 to April 2014, (2) baseline best-corrected visual acuity (BCVA) score between 20/25 and 20/250, and (3) last ranibizumab injection less than 3 months prior to the initiation of aflibercept injections (2 mg/0.05 ml). The PEDs secondary to exudative AMD were defined by the presence of subfoveolar occult CNV and vascularized DEP on fluorescein angiography (pinpoint leakage), hypofluorescence of DEP and choroidal neovascular visualization on indocyanine green angiography, and hyporeflective DEP on optical coherence tomography (SD-OCT) [6].

Exclusion criteria were the history of intraocular surgery, intraocular inflammation during the 3 months prior to initiation of aflibercept therapy, history of triamcinolone acetonide IVT therapy, macular laser photocoagulation or photodynamic therapy, PED due to a cause other than AMD, and occult neovascular lesions without DEP or any other active retinal disease.

Measurement of Early Treatment Diabetic Retinopathy Study (ETDRS), best-corrected visual acuity (BCVA), intraocular pressure assessment, spectral-domain optical coherence tomography (SD-OCT), fluorescein angiography, and indocyanine green angiography using a confocal laser scanning ophthalmoscope (HRA2; Heidelberg Engineering GmbH, Heidelberg, Germany) were performed at baseline. Visual acuity, adverse event monitoring, and SD-OCT were recorded at each visit using 49 line cube examination of Spectralis. The SD-OCT-derived images had been obtained by using an eye-tracking system. Inverted images had also been routinely obtained by enhanced depth imaging technique (EDI) [29]. Central retinal thickness (CRT) and macular (MT) volume were computed automatically by the software (Heidelberg Eye Explorer, Heidelberg, Germany). Maximum PED height was measured on SD-OCT imaging using the built-in caliper tool. Maximum height was defined as the distance from underneath the hyperreflective pigment epithelium band perpendicular to the Bruch's membrane on SD-OCT on a 1 : 1 scale, including extrafoveal location. Subfoveal choroidal thickness (CT) was defined as the vertical distance between the Bruch's membrane and the chorioscleral scale using EDI-OCT through the center of the fovea. PED height and subfoveal choroidal thickness were manually measured. Analyses of OCT scans and variables measurements were conducted by an ophthalmologist (AB) masked to the patient's characteristics. The presence of IRF, SRF, hyperreflective subretinal exudation (HSE), and disruption of inner segment/outer segment (IS/OS) was defined as previously [30, 31].

All patients received 3 monthly intravitreal of aflibercept at baseline (2 mg/0.05 ml) and then were scheduled for monthly monitoring visits including an ETDRS score measurement and SD-OCT [32]. After the loading phase, patients were treated with pro re nata (PRN) regimen during the first year and "treat and extend" regimen during the second year. Additional imaging was planned upon the physician's discretion. Reinjection criteria were the presence of IRF or SRF on SD-OCT but not fluid immediately underneath the PED. During the second year, treatment interval was extended in case of absence of IRF or SRF.

Data such as demographic characteristics, history of disease, history of ranibizumab treatment, and follow-up duration before and after the switch were collected from medical records and entered into an electronic file. ETDRS score, central macular thickness (CMT), macular volume, subfoveal choroidal thickness, the maximum height of the PED (from the Bruch's membrane to the posterior surface of the pigmentary epithelium), the presence of intraretinal fluid (IRF), subretinal fluid (SRF), subretinal hyperreflective exudations (SHE), and disruption of the IS/OS (Inner/Outer segment) zone were collected. Data were collected monthly from baseline to 6 months, at 12 months and at 24 months.

The statistical analysis was performed as paired comparisons between different time points using SPSS for Windows

(version 17.0/SPSS Inc., Chicago, IL). The paired t-test and Wilcoxon were used for comparison between paired continuous variables, and t-test and Mann–Whitney U test were used for comparison between subgroups. Statistical significance was set as $p < 0.05$.

3. Results

A total of 44 eyes of 44 patients with PEDs due to nAMD previously treated with at least 12 intravitreal ranibizumab injections during the last 12 months were switched to aflibercept therapy. Visual acuity from the 12-month period before enrollment was available for all patients. Mean BCVA was 67 ± 12.2 12 months before switching therapy and 64.4 ± 13 at baseline. There was a trend for worsening of BCVA (-2.6 ± 9.4, $p = 0.07$) during the 12-month preinclusion period.

Baseline (time of switch from ranibizumab to aflibercept) characteristics of patient cohort are summarized in Table 1. Thirty patients were female (68.2%), and the mean age was 78.5 ± 9.5 years. Duration of PED history was 43 ± 3.6 months at time of switch, and 27.8 ranibizumab injections have been given during this period. During the year preceding the medication switch, mean of 12 ± 1 injections had been given. Distribution of fluid was as followed: IRF in 22 eyes (50%), SRF in 15 eyes (11.4%), and both intra- and subretinal fluid in 15 eyes (34%). Eighteen eyes (40.9%) displayed hyperreflective subretinal exudation, and 19 eyes (43.2%) had IS/OS segment zone disruption.

Table 1: Characteristics of the patient cohort at baseline.

Study eye, n	44
Mean age, mean ± SD, range, years	78.5 ± 9.5 (54–98)
Gender distribution, male/female	14/30 (31.8/68.2%)
Mean follow-up before switch ± SD, months	43 ± 3.6
Mean number of ranibizumab injections in the 12 months before enrollment, mean ± SD	12 ± 1
BCVA, ETDRS letters, mean ± SD (range)	64.5 ± 13 (35–80)
Central retinal thickness, μm, mean ± SD, (range)	313 ± 85 (185–508)
PED height, μm, mean ± SD, range	221 ± 120 (38–518)
Intraretinal fluid, n (%)	22 (50%)
Subretinal Fluid, n (%)	26 (59%)
Intra- and subretinal fluid	15 (34%)
Hyperreflective subretinal exudation	18 (40.9%)
IS/OS segment disruption	19 (43.2%)

BCVA: best-corrected visual acuity; ETDRS: Early Treatment Diabetic Retinopathy Scale; PED: pigment epithelial detachment.

3.1. Adverse Events. Adverse events were reported in 2 patients. A transient ischemic attack occurred at month 5 in one patient. Multiple myelomas were discovered in another patient at month 6. Three patients were lost to follow-up from month 6 to month 12. Two eyes had evidence of macular atrophy at 1 year. Three patients were dead and 3 others were lost to follow-up from month 12 to month 24. Three eyes developed progression of cataract during the second year. At the end of the study, 38 patients reached the end point of 2 years.

3.2. Functional Response to Aflibercept. Change in visual acuity and OCT parameters were summarized in Table 2.

A statistically significant improvement in BCVA was reported at month 3 (+3.2 letters) (from 64.4 ± 13 to 67.6 ± 12, $p = 0.002$) and at month 6 (+2.84 letters) (67.3 ± 11.2, $p = 0.005$). At month 12 and month 24, visual change was not significant compared to baseline (64.8 ± 13.3, $p = 0.6$ and 61.4 ± 13.9, $p = 0.18$). After 2 years of aflibercept treatment, 2 eyes (4.8%) displayed ≥15 letters gain, no patient had 10–14 letters gain, 3 patients (13.7%) had 5–9 letters gain, 3 patients (13.7%) had 0–4 letters gain, and 4 (24.3%) patients had experienced ≥15 letters loss. Visual acuity evolution was illustrated in Figure 1.

3.3. Anatomical Response to Aflibercept

3.3.1. Central Retinal Thickness (CRT) and Macular Volume (MV). CRT was $313 \pm 85\,\mu$m at baseline decreased to 301 ± 77.5 at M3 after the loading phase ($-12 \pm 67\,\mu$m, NS). Change of CRT did not reach the significant difference at any time point of the study (M6: $312 \pm 91\,\mu$m, NS; M12: $312 \pm 84\,\mu$m, NS; M24: 312 ± 12.3, NS). MV did not change from baseline to any time point during the 2 years of follow-up (7.7 ± 1.4 at baseline; 7.75 ± 1.1 at month 3; 7.8 ± 1.11 at month 6; 7.8 ± 1.2 at month 12; and 7.6 ± 1.44, NS).

3.3.2. Distribution of Fluid and Qualitative SD-OCT Analysis. Distribution of fluid on SD-OCT was summarized in Table 3. IRF and/or SRF were present in all eyes at the beginning of the study. At month 3, 26/44 eyes (59%) displayed complete resolution on SD-OCT and 12/35 eyes (34.3%) had complete resolution of exudation at 2 years end- point. SHE was present in 18 eyes (43.2%) at baseline, in 2 eyes (4.5%) at month 3 and in 5 eyes (13.1%) at 2 years. IS/OS disruption was observed in 19/44 eyes (43.2%) at baseline, in 17/44 eyes (38.6%) at month 3 and month 6, then the rate increased to 29/44 (65.9%) eyes at one year and in 30/38 (78.9%) at 2 years. Visual change was −1.4 letters in 8 eyes without IS/OS disruption and −2.2 letters in eyes with IS/OS disruption.

3.3.3. Pigment Epithelium Detachment Response to Aflibercept. A decrease in mean PED height compared to baseline was observed at month 3 (from a preaflibercept mean of $224 \pm 18.5\,\mu$m to $198 \pm 19.5\,\mu$m $-26.6\,\mu$m, $p = 0.025$, after the loading phase) and to $190.4 \pm 17.4\,\mu$m at month 6 ($-28.1\,\mu$m). However, change in PED height was not significant at 1 year ($216 \pm 17.9\,\mu$m) and 2 years ($200 \pm 19.5\,\mu$m). A PED height reduction of at least 20% was achieved in 13/44 (29.5%) eyes after the loading phase, in 9/36 (25%) eyes at 1 year and in 9/37 (24.3%) eyes at 2 years. In three eyes, the PED was flattened at the last follow-up; however, visual acuity decreased in these eyes (−8 to −25 letters) because of macular atrophy. PED height evolution was illustrated in Figure 2.

Table 2: Comparison of functional and morphologic changes from time of switch to 24 months.

Central macular thickness (μm)	Baseline $N = 44$	Month 3 $N = 44$	p	Month 6 $N = 44$	p	Month 12 $N = 44$	p	Month 24 $N = 38$	p
ETDRS	64.4 ± 13.1	67.6 ± 12.3	0.002	67.3 ± 11	0.005	64.8 ± 13	0.6	61.4 ± 13.8	0.2
Central macular thickness (μm)	313 ± 13	312 ± 91	0.2	312 ± 14.2	0.9	313 ± 13.9	0.9	305 ± 76	0.6
Macular volume (mm^3)	7.70 ± 2.2	7.75 ± 0.16	0.8	7.8 ± 0.16	0.6	7.85 ± 0.16	0.4	7.8 ± 0.65	0.3
PED height (μm)	224.7 ± 18.5	198.5 ± 19.5	0.25	190 ± 17.3	0.5	224 ± 21	0.27	200 ± 19	0.14
Subfoveal choroidal thickness (μm)	179 ± 68					158 ± 67	0.2	174 ± 56	0.5

Data are mean ± SD unless indicated otherwise. p: continuous variables compared by independent samples t-test from baseline; BCVA: best-corrected visual acuity; ETDRS: Early Treatment Diabetic Retinopathy Scale; PED: pigment epithelial detachment.

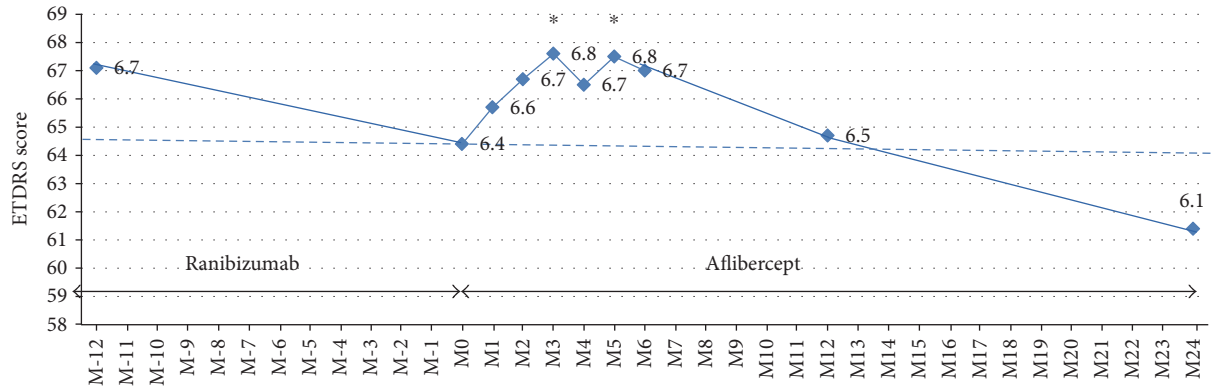

Figure 1: Visual acuity change over visits. ETDRS: Early Treatment Diabetic Retinopathy Study scale for visual acuity; M-12: 12 months before switching from ranibizumab to aflibercept; M0: time of switch from ranibizumab to aflibercept; M3: 3 months after switch; M6: 6 months after switch; and M12: 12 months after switch. $^*p < 0.05$.

Table 3: Qualitative analysis of SD-OCT at different time points.

Number of eyes	Month 0 $N = 44$	Month 4 $N = 44$	Month 6 $N = 43$	Month 12 $N = 38$	Month 24 $N = 38$
SRF	26 (59%)	13 (29.5%)	16 (37.2%)	18 (47.3%)	17 (44.7%)
IRF	22 (50%)	9 (20.4%)	9 (20.9%)	20 (52.6%)	16 (42.1%)
No fluid	0 (0%)	18 (59%)	23 (53.4%)	10 (26.3%)	13 (34.3%)
SHE	18 (40.9%)	2 (4.5%)	1 (2.3%)	5 (13.1%)	5 (13.1%)
IS/OS disruptions	19 (43.2%)	17 (38.6%)	16 (37.2%)	17 (44.7%)	30 (78.9%)

SRF: subretinal fluid; IRF: intraretinal fluid; SHE: subretinal hyperreflective exudation; IS/OS: inner segment/outer segment.

3.3.4. Subfoveal Choroidal Thickness. Subfoveal choroidal thickness was available at baseline, 12 months, and 24 months. It decreased from 179 ± 68 μm at baseline to 158 ± 67 μm at month 12 with a mean change of −25 μm, then increased to 174 ± 56 μm (NS from baseline, $p = 0.5$).

3.4. Frequency of Anti-VEGF Intravitreal Injection. Mean time between the last injection of ranibizumab and the first injection of aflibercept was 2.0 ± 1.2 months. Mean interval of ranibizumab injections during the year before medication switch was 31 ± 2.6 days. Mean number of intravitreal aflibercept injection was 7.6 ± 0.6 during the first year (including the 3 monthly injections at the loading phase) and 6.9 ± 0.6 during the second year. Mean interval of intravitreal aflibercept injection, from the end of the loading phase and 1 year time point, was 60.9 ± 5 days (ranging from 28–135) and 61.5 ± 5 (ranging from 31.8 ± 155) days during the second year, which was longer than the mean interval injection before medication switch ($p < 0.0001$). Two eyes needed monthly aflibercept injection, and stepwise from Q8W to Q4W occurred between 9 months and 1 year after medication change. The average number of injections was reduced approximately by 0.6 compared with the 12 months before the switch.

4. Discussion

This retrospective, observational study was designed to evaluate the efficacy and safety of switch from ranibizumab to aflibercept in patients with PEDs related to nAMD with persistent exudation despite monthly ranibizumab injection during the 12-month period preceding inclusion. The results

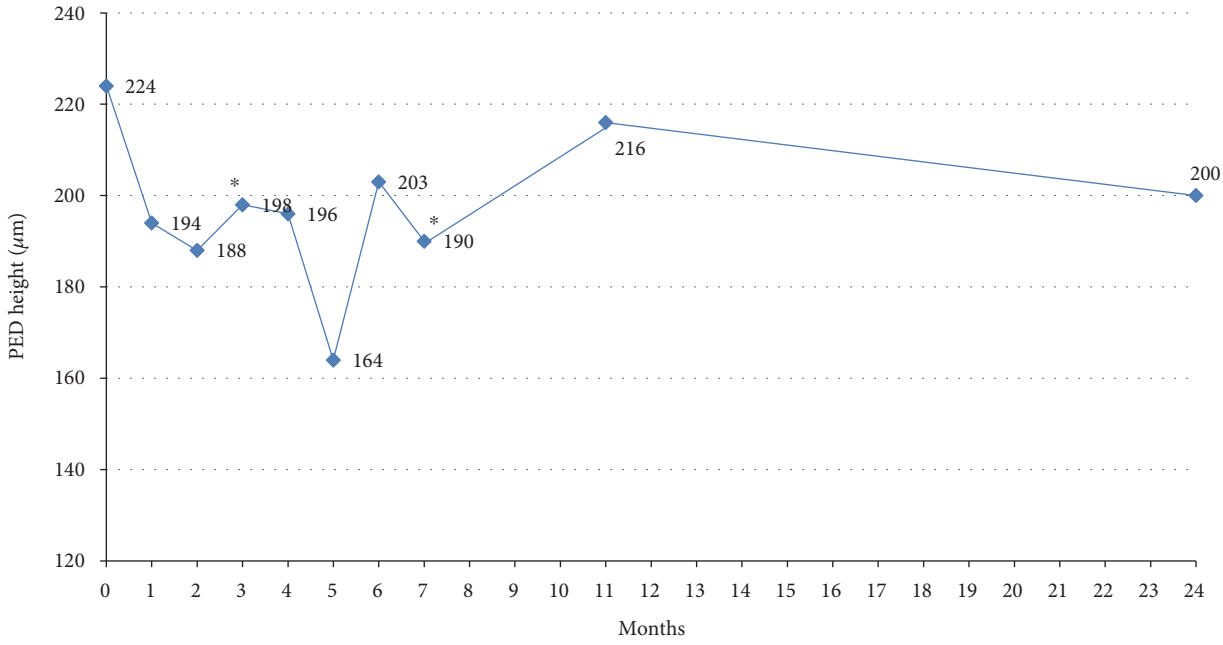

Figure 2: PED height change over visits from month 0 to month 24. M0: time of switch from ranibizumab to aflibercept. Significant decrease of mean PED height was found at 3 months and 6 months after switch ($^*p < 0.05$). Change was not significant at 12 months and 24 months.

showed that medication switch, even conducted after 43 month duration of PEDs, led to a short-term improvement in both functional and anatomical response: significant moderate visual gain at 3 months (+3 letters) and at 6 months (+2.6 letters), decrease of maximum PED height at 3 months (−26.6 μm) and at 6 months (−28.1 μm), and decreased rate of eyes with IRF, SRF, and SHE until 6 months after switch. The benefit was then less significant with time course at 1 and 2 years. At the end of 2 year follow-up, visual acuity remained stable compared to baseline, and 34.4% eyes achieved a dry macula. Choroidal thickness decreased at one year, but this effect disappeared at 2 years. The frequency of anti-VEGF injections dropped from 12 ± 1 the year preceding medication switch to 7.6 ± 0.6 during the first year and 6.9 ± 0.6 during the second year. Treatment interval was lengthened from 31 ± 2.6 days to 61 ± 5 days after the loading phase then remained unchanged with time course. Overall, these patients could have anatomic improvement, and the injection intervals could be extended.

Various studies have examined the efficacy of aflibercept in patients with PED refractory of ranibizumab and/or bevacizumab treatment [17, 18, 20, 23, 33–35]. However, comparisons are difficult due to many differences: duration of anti-VEGF varied from 3 months to 12 months before switch, inclusion of vascularized PEDs [17, 20, 33] and/or serous PEDs; [15] visual acuity was expressed in ETDRS and logMAR; and PED improvement expressed in maximum height, volume [21], and diameter [20]. Mean follow-up varied from 3 months to 1 year. In these reports, switching from ranibizumab to aflibercept result to unchanged visual acuity in some retrospective studies [15, 18, 26, 28], whereas visual gain had been demonstrated at 6 months or 12 months end point in other prospective studies [17, 19, 35] or retrospective studies with short-term results at 3 months or 6 months [20, 21]. Our study reports results at different time points from baseline to 2 years of aflibercept treatment for PEDs related to nAMD with persistent exudation despite monthly ranibizumab the year prior conversion. We found that functional response under anti-VEGF therapy may change with time course: indeed, in this population with 44 months PEDs history duration, there was a trend toward worse visual acuity from 12 months prior to time of switch (−2.6, $p = 0.07$), and slight visual gain was obtained during 6 months after conversion to aflibercept (+3.2 at month 3 and +2.8 at month 6). Visual acuity decreased after this period and became unchanged at 1 year compared to baseline, and there was a nonsignificant loss of −2.2 letters at 2 years. Visual loss with time course may be explained by retinal structure damage due to chronicity of disease (mean duration of PEDs to switch was 44 months, IS/OS segment disruption found in 78.9% at 2 years in our study) or recurrence of exudation with time with increase rate of eyes with IRF and SRF. This could also indicate a newly developing tachyphylaxis.

Significant improvement in DEP height at month 3 and month 6 in our study is consistent with previously published data showing that treatment with aflibercept led to significant anatomic improvements in patients with persistent exudation under other anti-VEGF [15, 17–20, 23, 28, 33]. Change of PED height was not found at 1 and 2 years, which might be explained by manual measurement of maximum height and absence of inferior limit of PED height in inclusion criteria in our study. Indeed, 1/4 to 1/3 of eyes had reduction of at least 20% over time, which was similar to other reports [15].

The absence of significant changes in the central retinal thickness in our study is surprising. Reduction on CRT thickness varied from −19 μm to −68 μm after a six-month period

of aflibercept therapy in published data [17, 18, 20, 23, 35]. These data may reflect variations in patient populations between studies or the fact that our study did not fully meet the power requirements to demonstrate a statistically significant difference. We also found temporary change in the choroidal thickness under aflibercept at one year, in accordance with most of the publications reporting a decrease in the choroidal thickness under aflibercept in naive and switched eyes with nAMD [36, 37].

Aflibercept treatment frequency is a point that needs to be investigated. In this cohort, all patients were treated with >10 injections of ranibizumab in the year prior to conversion, so there was no under treatment before switch. We achieved stabilization of visual acuity with a mean 7.6 injections in the first year (including 3 monthly aflibercept injection of the loading phase) and 6.9 injections during the second year, and mean interval injections was extended from 31 days to 61 days. We did not observe extended interval injection between the first and the second year. Veritti et al. reported an average of only 0.3 ± 0.1 injections per eye per month using pro re nata regimen without loading phase (3.6 ± 1.7 injections for 12 months) after medication switch in a prospective study including 32 eyes with nAMD-related PED, while these patients were administered a total of 4.5 ± 1.2 ranibizumab in the 6 months prior to changing therapy to aflibercept. The frequency was approximately 0.6 injections per eye per month in our study, which is consistent with Singh et al. who found a frequency of 0.7 after medication change [35]. Messenger et al. observed a decreased injection frequency with aflibercept only in patients who received at least 10 injections in the prior 12 months (three fewer injections per year on average), as well as improvement of anatomical outcomes. This suggests that patients with PEDs who required monthly ranibizumab would benefit from transition with extended interval treatment regimen offered by aflibercept [34, 38]. This switch benefit seemed likely because the higher binding affinity of aflibercept [11] and its theoretically longer ligand-binding activity [39]. However, switching from ranibizumab to aflibercept did not reduce the need for retreatment without a selection of refractory cases [16, 34, 40].

This study has limitations of the retrospective nature; the manual measurement of PED height and choroidal thickness and the absence of the control group continuing monthly ranibizumab. It has the advantage of homogenous lesion characteristics (PEDs due to type 1 choroidal neovascularization), homogenous interval between the last injection of `ranibizumab and first injection of aflibercept which may influence the amount of fluid at the time of medication change, and standardized aflibercept regimen.

5. Conclusions

In patients with choroidal neovascularization type I associated with a fibrovascular DEP with persistent exudation despite monthly ranibizumab, conversion to aflibercept led to short-term functional and anatomic improvement until 6 months and preserved visual function until 2 years. In this particular form of nAMD, transitioning to aflibercept was associated with a reduced injection frequency, suggesting potential cost saving in this population. Further, prospective study with control groups is needed to determine the benefit of switching from ranibizumab to aflibercept.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Clinical Study

Switching to Aflibercept in Diabetic Macular Edema Not Responding to Ranibizumab and/or Intravitreal Dexamethasone Implant

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Purpose. To assess short-term functional and anatomical outcomes of refractory diabetic macular edema (DME) following a switch from ranibizumab or dexamethasone to aflibercept. *Methods*. We included retrospectively eyes with persistent DME after at least 3 ranibizumab and/or one dexamethasone implant intravitreal injections (IVI). The primary endpoint was the mean change in visual acuity (VA) at month 6 (M6) after switching. *Results*. Twenty-five eyes were included. Before switching to aflibercept, 23 eyes received a median of 9.5 ranibizumab, and among them, 6 eyes received one dexamethasone implant after ranibizumab and 2 eyes received only one dexamethasone implant. Baseline VA, before any IVI, was 52.9 ± 16.5 letters, and preswitch VA was 57.1 ± 19.6 letters. The mean VA gain was +8 letters ($p = 0.01$) between preswitch and M6. The mean central retinal thickness was 470.8 ± 129.9 μm before the switch and 303.3 ± 59.1 μm at M6 ($p = 0.001$). *Conclusion*. Switching to aflibercept in refractory DME results in significant functional and anatomical improvement. The study was approved by the France Macula Federation ethical committee (FMF 2017-138).

1. Introduction

Diabetic macular edema (DME) is the leading cause of visual impairment in patients with diabetic retinopathy [1]. The prevalence of diabetes is increasing worldwide. In diabetic patients, the prevalence of DME reaches about 5% [2, 3]. The cost associated with visual disability and treatment is high and makes DME a global health issue.

DME is mainly due to an abnormal vascular permeability involving vascular endothelial growth factor (VEGF). Intravitreal anti-VEGF therapy is currently one of the treatments of DME along with corticosteroids: in 2012, ranibizumab has been approved by the Food & Drug Administration (FDA) following the RISE and RIDE studies [4]; then, aflibercept has been approved for the treatment of DME in 2014 following the VIVID and VISTA phase 3 clinical trials [5]. In France, aflibercept and dexamethasone are reimbursed by the healthcare system, respectively, since September and October 2015, while ranibizumab is reimbursed since 2012.

Thus, the first approved therapy with an intravitreally applied agent used in our practice in DME patients was ranibizumab because of its earlier availability. In our previously published real-life series of patients treated with ranibizumab, 18% did not respond to 3 intravitreal injections and showed a visual gain < 5 letters and an anatomical improvement in central retinal thickness (CRT) < 10% of the initial CRT [6]. Moreover, 26.5% of our patients never achieved a flat retina (<300 μm) during the one-year follow-up. In these particular cases where ranibizumab is not effective, a switch to another treatment is needed. Clinicians may either change

the pharmaceutical class and use dexamethasone implant, also approved in this indication [7], or switch to another anti-VEGF agent. Some studies [8–10] reported results supporting the efficacy of aflibercept after ranibizumab failure.

Unlike ranibizumab, aflibercept binds not only VEGF-A but also VEGF-B and placental growth factor (PlGF) [11]. This additional mechanism of action may also explain the possible efficacy of aflibercept after ranibizumab failure even if they belong to the same therapeutic class. A switch to another pharmaceutical class such as corticosteroids is also possible. Dexamethasone, the only corticosteroid reimbursed for the treatment of DME in France, has a nonspecific anti-VEGF effect associated with an anti-inflammatory effect and allows a continuous delivery over a few months.

The aim of this study was to assess the short-term outcomes following a switch from ranibizumab or dexamethasone therapy to aflibercept in the treatment of refractory DME.

2. Patients and Methods

A retrospective study was conducted in a tertiary center specialized in imaging and treatment of retinal diseases. All consecutive patients diagnosed with DME and treated with ranibizumab (0.5 mg) and/or dexamethasone (0.7 mg) and subsequently switched to aflibercept (2.0 mg) between June 2015 and August 2016 were included. Indications for switching to aflibercept included persistent DME under a previous well-conducted treatment, defined as no reduction, incomplete resolution (<10% improvement of central retinal thickness (CRT)), increase in central subfield thickening, or persistence of central cysts on SD-OCT, considered as significant by the investigator after a loading phase of at least 3 injections. The choice of switching was at the discretion of the investigator. Decision for switching to aflibercept after dexamethasone was taken when these same anatomical criteria were observed at the second visit (8 weeks) after at least one dexamethasone injection.

This study was conducted in accordance with the tenets of the Declaration of Helsinki, and an informed consent was obtained from all patients. Approval was obtained from the France Macula Federation ethical committee (FMF 2017-138).

Inclusion criteria were as follows: patients with type 1 or 2 diabetes, with persistent DME defined by a loss of the foveal pit, and a CRT > 300 μm on SD-OCT (Cirrus 5000, ZEISS, Meditec) responsible for a loss of vision (preswitch visual acuity (VA)). Only patients who received at least the first 3 monthly aflibercept injections were included in the study.

Exclusion criteria were as follows: other ocular conditions impairing vision or complication of diabetic retinopathy (tractional retinal detachment, vitreous hemorrhage), fewer than three ranibizumab injections prior to the switch to aflibercept, and incomplete imaging or clinical data.

All patients underwent a complete baseline ophthalmological examination including VA on ETDRS chart, slit-lamp examination, fundus imaging, and SD-OCT. VA was measured monthly, and SD-OCT scans were assessed 4 weeks after the third injection and at month 6 of follow-up. Baseline VA was defined as the initial VA before any intravitreal treatment and preswitch VA as the VA just before switching to aflibercept. All patients were assessed every 4 weeks after the 3 initial aflibercept injections and treated on an as-needed, pro re nata (PRN), regimen in case of recurrence based on functional (VA < 78 letters or 20/32) and anatomical parameters (CRT > 300 μm). Recurrence was considered as a loss of VA ≥ 5 letters between 2 consecutive visits or CRT > 300 μm or a CRT increase > 10% or significant cyst reappearance at the discretion of the investigator.

SD-OCT scans were obtained using the Cirrus 5000 (ZEISS, Meditec) and were reviewed by the investigator to document the presence of intraretinal/subretinal fluid and decide if the patient needed additional injections.

The primary endpoint was the mean variation in VA between the preswitch time and month 6 (M6) after the switch.

Secondary endpoints were VA after 3 initial intravitreal injections of aflibercept (M3), CRT at 3 and 6 months, and number of injections.

Statistical analysis was performed using a *t*-test with graph pad software in the overall population after verification of a normal distribution. In the subanalyses and in small samples such as the dexamethasone group, for example, a nonparametric Mann–Whitney test was used in case of unpaired values or a Wilcoxon test in case of paired values. The results of VA and CRT are presented as mean ± SD. For small samples, the results were presented as median (min–max).

A p value <0.05 was considered statistically significant. Two subgroup analyses were performed: one analysis dividing final CRT into CRT < 300 μm and CRT > 300 μm and another dividing preswitch VA into VA < 20/40 (70 letters) and VA ≥ 20/40.

3. Results

Twenty-nine eyes were screened but the data at 6 months were not available for 4 eyes that were excluded for the following reasons: patient dropout (2 eyes) and 2 eyes underwent cataract surgery between 3 and 6 months after the first injection of aflibercept. Thus, 25 eyes of 21 patients met the inclusion criteria and were included. Patient mean age was 63.1 ± 10.8 years (range: 33–83 years). There was a slight female predominance with 13 women included (62%).

Baseline characteristics are presented in Table 1.

Before inclusion, 23 eyes received a mean number of 9 ± 4.6 (median: 9.5, range: 3–15 injections) ranibizumab injections, and among them, 6 eyes received a mean number of 1.5 (median: 1, range: 1–3 injections) dexamethasone implants following ranibizumab treatment. Two eyes received only one dexamethasone implant before switching to aflibercept. The mean follow-up duration was 2.2 ± 0.2 (1.9–2.6) months at M3 and 5.7 ± 0.5 (5. 2–7) months at M6 after the first aflibercept injection.

3.1. Visual Outcomes after Switching to Aflibercept. The mean baseline VA before any intravitreal injection was of 52.9 ± 16.5 letters and the mean VA prior to the switch was of

Table 1: Patient baseline demographics and clinical characteristics.

		Patient demographics and clinical characteristics ($n = 21$ patients, 25 eyes)
Gender		13 female/8 male
Age (years)	Median (min–max)	64 (33–83)
Diabetes	Type 1, n (%)	2 (9.6)
	Type 2, n (%)	19 (90.4)
Insulin/ODT/ODT + insulin (n)		12/3/6
HbA1c levels (%)	Median (min–max)	8.3 (7.5–10.7)
High blood pressure, n (%)		18 (78.3)
Pan retinal photocoagulation, n (%)		20 (75), 2 ongoing
Lens Status, n (%)	Phakic	12 (48)
	IOL	13 (52)
Ranibizumab ($n = 23$)	Median number of injections (min–max)	9.5 (3–15)
Ozurdex only ($n = 2$)	Median number of dexamethasone injections	1
Ranibizumab + Ozurdex ($n = 6$)	Median number of dexamethasone injections (min–max)	1 (1–3)
Macular laser history (n)		2

ODT: oral diabetes treatment.

Table 2: Functional outcomes: visual acuity before any intravitreal injection (baseline) and before and after switch to aflibercept at M3 and M6.

	Baseline	Preswitch	M3 postswitch	M6 postswitch
Number of eyes	25	25	25	25
Mean letter score (SD)	52.9 (16.5)	57.1 (19.6)	65.5 (16.4)	65.1 (15.2)
F			2.914	
p value (ANOVA test)			0.04*	
Mean VA change from preswitch (SD)			8.4 (14.1)	8 (15.1)
p value (compared to preswitch VA)			0.006**	0.01*
Mean VA change from baseline (SD)		4.2 (3.3)	12.6 (11.6)	12.2
p value (compared to preswitch VA)		0.34	<0.0001****	0.0003***

F: result of variance test (ordinary one-way ANOVA). Except for ANOVA test, p values were obtained after a paired parametric t-test after verification of the normal distribution. *<0.05; **<0.01; ***<0.001; and ****<0.0001.

57.1 ± 19.6 (+4.2 letters of visual gain). VA improved to 65.5 ± 16.4 letters ($p = 0.006$) and 65.1 ± 15.2 letters ($p = 0.01$) after 3 and 6 months of follow-up, respectively, corresponding to a mean VA change of +8.4 and +8 letters at 3 and 6 months (Table 2, Figure 1).

3.2. Anatomical Outcomes after Switching to Aflibercept. The mean baseline CRT before any intravitreal injection was 532 ± 186.2 μm, the mean CRT prior to the switch was 470.8 ± 129.9 μm (−58.2 μm), and a significant reduction to 315.6 ± 89.7 μm ($p = 0.001$) and 303.3 ± 59.1 μm ($p = 0.001$) was observed at 3 and 6 months, respectively, corresponding to a mean decrease of −155.2 ± 144.7 μm and −167.5 ± 149.3 μm, respectively, at 3 and 6 months (Table 3, Figure 2).

3.3. Subgroup Analysis. A subgroup analysis was performed to determine the percentage of eyes with a CRT < 300 μm after switching to aflibercept and the impact on VA (Table 4).

After 6 months of follow-up, 60% of the eyes had a CRT < 300 μm. Among them, the preswitch VA and CRT were 55.9 ± 20.4 letters and 497.4 ± 139.9 μm, respectively, and they significantly improved to 67 ± 14.8 letters ($p = 0.02$) and 267.9 ± 28.7 μm, corresponding to a mean VA change of +11.1 letters (Table 4).

At 6 months, 40% of the eyes still had a CRT > 300 μm. In this group, the preswitch VA and CRT were 59 ± 19.1 letters and 460.2 ± 120.1 μm and improved to 62.2 ± 16.3 letters ($p = 0.44$) and 356.3 ± 53.1 μm at 6 months, corresponding to a mean VA gain of +3.2 letters.

The second subgroup analysis (Tables 5 and 6) was performed to determine the impact of the preswitch VA on the efficacy of aflibercept. Patients were divided into two groups: one with a preswitch VA < 70 letters (20/40 Snellen equivalent, low VA) and one with a preswitch VA ≥ 70 letters (high VA). The mean VA change was +10.6 ± 17.4 letters ($p = 0.02$) in the low VA group versus +2.4 ± 5.7 letters in the high VA group ($p = 0.27$). There was no

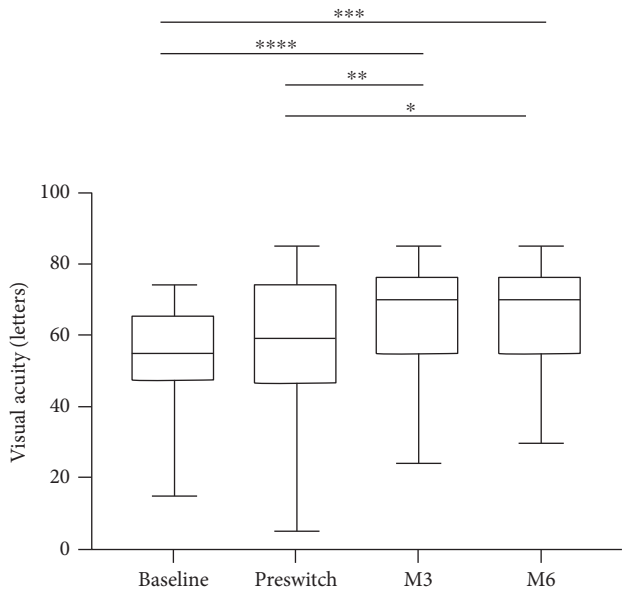

Figure 1: Change in visual acuity (ETDRS chart) over 6 months of follow-up after switch to aflibercept. Box plots representing at each time point the distribution of data from bottom to top: minimum, first quartile, median, third quartile, and maximum. p values were obtained after a paired parametric t-test after verification of the normal distribution. $^{*}<0.05$; $^{**}<0.01$; $^{***}<0.001$; and $^{****}<0.0001$.

significant difference in preswitch, M3, and M6 CRT between both groups (Table 6).

Eight eyes were previously treated with intravitreal dexamethasone implant before switching to aflibercept. Their median baseline VA was 45 (15–54) letters, and their preswitch median VA was 46.5 (5–74) letters. After switching, they improved their median VA to 54.5 (24–85) at M3 ($p = 0.24$ compared to preswitch VA) and 54.5 (24–85) at M6 ($p = 0.34$ compared to preswitch VA). Their mean VA change was $+12.25 \pm 22.4$ (min: −17, max: +54) letters at M6.

Their median baseline CRT was 477 μm (306–1088 μm), and their preswitch median CRT was 390.5 μm (327–696 μm). After switching, they improved their median CRT to 284.5 μm (204–623 μm) at M3 ($p = 0.03$ compared to preswitch CRT) and 298.5 μm (236–591 μm) at M6 ($p = 0.007$ compared to preswitch CRT). Their mean CRT change was -170.5 ± 188 μm (min: +91, max: −431) at M6.

The functional and anatomical outcomes among patients treated by ranibizumab monotherapy, dexamethasone monotherapy, or combined monotherapy before switching to aflibercept are presented in Table 7.

No serious adverse event following intravitreal injections was noted in this study.

4. Discussion

In this study, we showed a rapid anatomical and functional improvement in eyes with persistent DME that poorly responded to ranibizumab and/or dexamethasone after a switch to aflibercept.

Only a few studies have assessed the outcomes of a switch to aflibercept after chronic anti-VEGF therapy for persistent DME. In a prospective study, Wood et al. [9] have shown a significant anatomical improvement in 14 patients who switched to aflibercept after a single injection.

Another recent retrospective study [10] has shown a significant functional and anatomical improvement in 21 eyes after a switch to aflibercept. In this study, no fixed pattern was used for aflibercept treatment after the switch: a median number of 3 aflibercept injections was received during a mean follow-up of 5 months with an interval of 2.4 months between aflibercept injections.

In our study, when a switch was decided, we made the choice to prescribe a complete treatment protocol including 3 monthly aflibercept injections before patient assessment as we considered that the switch required a new loading phase of injections.

A more recent retrospective study [8] on this topic has shown a significant anatomical improvement and an overall trend to functional improvement after switching without reaching significance. However, half of the cohort did not attend the fourth visit after switching. In the 22 patients who attended the fourth visit after the switch, the VA was significantly increased. The authors have suggested that a longer follow-up after switching to aflibercept is necessary for a more accurate assessment of VA outcomes.

In our study, we found a functional and anatomical improvement 6 months after aflibercept switch. We assumed that this result could be due to our strict retreatment criteria mainly based on anatomical features instead of criteria based on the functional improvement only and to the systematic prescription of 3 monthly injections when the switch was decided. Indeed, we considered that a switch could be relevant even when the vision was improved although some fluid was still present in the retina. This assumption was confirmed, in particular in patients with a final CRT < 300 μm; in addition to an initial visual gain of 5.2 letters between the baseline and the preswitch time, their VA improved by 11.1 more letters ($p = 0.02$) after the switch to aflibercept.

A subgroup analysis was performed to determine the impact of the preswitch VA. We found a higher final VA change of +10.6 letters when the preswitch VA was <70 letters, but with a lower final VA of 58 letters at 6 months versus a gain of only +2.4 letters in the group with a preswitch VA ≥ 70 letters, with a much higher final VA of 80.1 letters at 6 months, without any difference in CRT between both groups at preswitch, M3, and M6.

The slight visual gain in the group with the highest preswitch VA could be explained by the ceiling effect [12]. It is known that one of the good predictive factors of DME treatment is the baseline VA: the higher the baseline VA is, the better the final VA will be. However, our study showed that it is also important to switch when the preswitch VA is good, because the better the preswitch VA was, the better the final VA was in our patients.

However, our study was one of the first "real-life" study assessing the switch to aflibercept in DME resistant to ranibizumab at the dose of 0.5 mg. Similarly, for instance, Rahimy et al. [8] have explored the switch to aflibercept after treatment with 0.3 mg ranibizumab or bevacizumab.

Table 3: Anatomical outcomes: central retinal thickness before any intravitreal injection (baseline) and before and after switch to aflibercept at M3 and M6.

	Baseline	Preswitch	M3 postswitch	M6 postswitch
Number of eyes	25	25	25	25
Mean CRT in μm (SD)	532 (186.2)	470.8 (129.9)	315.6 (89.7)	303.3 (59.1)
F			21.47	
p value (ANOVA test)			0.0013**	
Mean CRT change from preswitch in μm (SD)			−155.2 (144.7)	−167.5 (149.3)
p value (compared to preswitch CRT)			<0.0001****	<0.0001****
Mean CRT change from baseline in μm (SD)		−61.2 (176)	−216.4 (226.1)	−228.7 (212.2)
p value (compared to baseline CRT)		0.07	<0.0001****	<0.0001****

CRT: central retinal thickness; F: result of variance test (ordinary one-way ANOVA). Except for ANOVA test, p values were obtained after a paired parametric t-test after verification that the distribution was normal. **<0.01 and ****<0.0001.

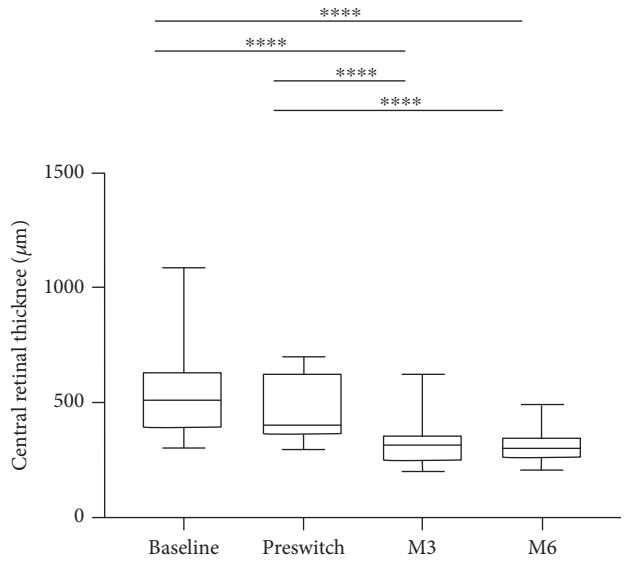

Figure 2: Change in central retinal thickness over 6 months of follow-up after switch to aflibercept. Box plots representing at each time point the distribution of data from bottom to top: minimum, first quartile, median, third quartile, and maximum. p values were obtained after a paired parametric t-test after verification of the normal distribution. ****<0.0001.

Even with a higher dose of ranibizumab, 0.5 mg versus 0.3 mg, DME was still persistent in our series. After the switch to aflibercept and 6 months of follow-up, 60% of the eyes had a CRT < 300 μm. The functional response was particularly important in these patients with a mean VA change of +11.1 letters. This study also confirmed that the treatment switch could improve anatomical and functional outcomes in patients with fluid persistence.

Switching to dexamethasone implant has shown good functional and anatomical outcomes after ranibizumab failure in DME treatment [13–15]. Here, in case of dexamethasone treatment failure (8 eyes), the VA gain was +12.25 letters at 6 months. Our study was the first, to the best of our knowledge, to analyze a switch from dexamethasone to aflibercept.

Our real-life short-term results are consistent with those of the VIVID and VISTA studies [16], with a mean VA before aflibercept injections of 57 letters versus about 59 letters in the VIVID and VISTA studies and a visual gain of +8 letters in our study versus between +8.5 and +11 letters in the VIVID and VISTA studies at 6 months.

In case of drug switch in DME treatment, it is easy to understand that a switch from corticosteroids, when ineffective, to anti-VEGF may improve CRT and VA by restoring the inner blood-retinal barrier through the differential effects of these various treatment classes. However, in cases of switch from ranibizumab to aflibercept, two anti-VEGF agents, the efficacy of aflibercept could be due either to a switch effect in case of autoantibody development to prior anti-VEGF therapy [17, 18] or to the different targets of both drugs. Indeed, ranibizumab only binds free VEGF-A leading to VEGFR2 inhibition only, while aflibercept binds VEGF-A, PlGF, and VEGF-B leading to VEGFR1 and 2 inhibition [11]. This differential pathophysiological effect could explain the effect of aflibercept after ranibizumab failure. However, no study of a switch assessing the opposite pattern is currently available (switch to ranibizumab and/or intravitreal dexamethasone implant in DME not responding to aflibercept) to confirm this assumption.

Our study is limited by its retrospective design, the absence of a control group (ranibizumab monotherapy, dexamethasone monotherapy, or combination of both) to compare outcomes of eyes not switched to aflibercept and receiving their initial treatment for an extended period of time. The follow-up of 6 months was not intended to observe the long-term effect of the treatment but mainly to confirm the efficacy of aflibercept treatment in case of failure of other therapies in DME.

In conclusion, despite its limitations, this study provides a potentially useful clinical insight into DME not responding to ranibizumab and/or dexamethasone in a real-life setting. Our results supports early DME treatment switch before patients experience a severe vision loss when the first therapy is not effective, since 60% of our

Table 4: Subgroup analysis according to CRT < 300 or ≥300 μm at M6.

	Final CRT (M6) CRT < 300 microns (group 1)	CRT > 300 microns (group 2)	p value[a] (comparison between groups 1 and 2)
Number of eyes	15	10	
Mean letter score at baseline prior to any injection (SD)	50.7 (17.3)	55.7 (15.9)	0.48
Mean letter score preswitch (SD)	55.9 (20.4)	59 (19.1)	0.7
Mean letter score postswitch M3 (SD)	67 (14)	63.4 (20.1)	1
Mean letter score postswitch M6 (SD)	67 (14.8)	62.2 (16.3)	0.45
Mean VA change from preswitch (SD)	11.1 (16.2)	3.2 (12.5)	0.2
F	7.38	0.96	
p value (ANOVA test)	0.003**	0.4	
p value[b] (comparison between VA preswitch and M6 within each group)	0.02*	0.44	

ANOVA test was performed to assess significance between VA at baseline, preswitch, M3, and M6 after switch to aflibercept within each group. [a]p values were obtained after an unpaired nonparametric Mann–Whitney test between groups 1 and 2 at each time point. [b]p values were obtained after a paired nonparametric Wilcoxon test between preswitch VA and VA at M6. VA: visual acuity. *<0.05 and **<0.01.

Table 5: Subgroup analysis of the impact of the preswitch VA (< or ≥70 letters) on VA.

	Preswitch visual acuity VA < 70 letters (group 1)	VA ≥ 70 letters (group 2)	p value[a] (comparison between groups 1 and 2)
Number of eyes	17	8	
Mean letter score at baseline prior to any injection (SD)	48.1 (17.2)	63.9 (7.9)	0.007**
Mean letter score preswitch (SD)	47.4 (15.8)	77.7 (5)	0.001***
Mean letter score postswitch M3 (SD)	59 (15.8)	79.4 (5.3)	0.001***
Mean letter score postswitch M6 (SD)	58 (13)	80.1 (5.6)	0.001***
Mean VA change from preswitch (SD)	10.6 (17.4)	2.4 (5.7)	0.2
F	5.6	15.37	
p value (ANOVA test)	0.004**	0.03*	
p value[b] (comparison between VA preswitch and M6 within each group)	0.02*	0.5	

ANOVA test was performed to assess significance between VA at baseline, preswitch, M3, and M6 after switch to aflibercept within each group. [a]p values were obtained after an unpaired nonparametric Mann–Whitney test between groups 1 and 2 at each time point. [b]p values were obtained after an paired nonparametric Wilcoxon test between preswitch VA and VA at M6. VA: visual acuity. *<0.05; **<0.01; and ***<0.001.

Table 6: Subgroup analysis of the impact of the preswitch VA (< or ≥70 letters) on CRT.

	Preswitch visual acuity VA < 70 letters (group 1)	VA ≥ 70 letters (group 2)	p value[a] (comparison between groups 1 and 2)
Number of eyes	17	8	
Mean CRT in μm at baseline prior to any injection (SD)	582.7 (201.3)	430.5 (96.8)	0.03*
Mean CRT in μm preswitch (SD)	495.2 (142.7)	418.9 (82)	0.28
Mean CRT postswitch M3 (SD)	312.7 (106)	321.6 (40.6)	0.47
Mean CRT postswitch M6 (SD)	301.8 (69.1)	306.4 (32.5)	0.62
F	18.21	9.42	
p value (ANOVA test)	<0.0001****	0.003**	
p value[b] (comparison between CRT preswitch and M6 within each group)	0.0002***	0.008**	

ANOVA test was performed to assess significance between CRT at baseline, preswitch, M3, and M6 after switch to aflibercept within each group. [a]p values were obtained after an unpaired nonparametric Mann–Whitney test between groups 1 and 2 at each time point. [b]p values were obtained after a paired nonparametric Wilcoxon test between preswitch CRT and CRT at M6 within each group. VA: visual acuity; CRT: central retinal thickness. *<0.05; **<0.01; ***<0.001; and ****<0.0001.

Table 7: Subanalysis assessing functional and anatomical outcomes depending on treatment received before switch (ranibizumab monotherapy, dexamethasone monotherapy, or combined therapy).

	N	Baseline	Preswitch	M3	M6	*F*	*p* value (ANOVA test)
Visual acuity (letters on ETDRS chart)							
Ranibizumab monotherapy median (min–max)	17	59 (15–74)	65 (37–85)	74 (35–85)	70 (50–85)	9.747	0.0002***
Dexamethasone monotherapy median (min–max)	2	64 (54–74)	60 (46–74)	60 (35–85)	63.5 (44–83)	NA	NA
Combined therapy median (min–max)	6	39.5 (15–50)	43.5 (5–53)	54.5 (24–74)	54.5 (35–74)	2.5	0.15
p value[a] (comparison to baseline VA)			0.09	0.0002***	0.0035**		
p value[b] (comparison to preswitch VA)				0.004**	0.03*		
Central retinal thickness (μm)							
Ranibizumab monotherapy median (min–max)	17	513 (376–831)	476 (315–660)	324 (208–388)	296	24.75	0.0001****
Dexamethasone monotherapy median (min–max)	2	373 (306–440)	531 (366–696)	468 (313–623)	314.5 (299–330)	NA	NA
Combined therapy median (min–max)	6	540 (350–1088)	390.5 (327–677)	251.5 (204–420)	272.5 (208–388)	5.019	0.053
p value[a] (comparison to baseline CRT)			0.08	<0.0001****	<0.0001****		
p value[b] (comparison to preswitch CRT)				<0.0001****	<0.0001****		

F: result of variance test (ordinary one-way ANOVA). ANOVA test was performed to assess significance between VA or CRT at baseline, preswitch, M3, and M6 after switch to aflibercept within each group except for dexamethasone group ($n = 2$). [a]*p* values were obtained after a paired nonparametric Wilcoxon test in comparison to baseline VA or CRT within the ranibizumab group; [b]*p* values were obtained after a paired nonparametric Wilcoxon test in comparison to preswitch VA or CRT within the ranibizumab group. VA: visual acuity; CRT: central retinal thickness. *<0.05; **<0.01; ***<0.001; and ****<0.0001.

patients achieved a complete fluid resolution and a good visual improvement.

Disclosure

This study was presented as a poster at the ARVO annual meeting, Baltimore, May 2017.

Conflicts of Interest

Antoine Herbaut, Lise Qu-Knafo, and Bahram Bodaghi have nothing to disclose. Franck Fajnkuchen and Sylvia Nghiem-Buffet are consultants for Allergan, Bayer, and Novartis outside of the submitted work. Audrey Giocanti-Auregan is a consultant for Allergan, Alimera, Bayer, and Novartis outside of the submitted work.

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Research Article

Switch of Intravitreal Therapy for Macular Edema Secondary to Retinal Vein Occlusion from Anti-VEGF to Dexamethasone Implant and Vice Versa

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Purpose. To evaluate the anatomical and functional outcome of intravitreal dexamethasone implant for macular edema secondary to central (C) or branch (B) retinal vein occlusion (RVO) in patients with persistent macular edema (ME) refractory to intravitreal antivascular endothelial growth factor (VEGF) treatment compared to treatment naïve patients and to dexamethasone-refractory eyes switched to anti-VEGF. *Methods*. Retrospective, observational study including 30 eyes previously treated with anti-VEGF (8 CRVO, 22 BRVO, mean age 69 ± 10 yrs), compared to 11 treatment naïve eyes (6 CRVO, 5 BRVO, 73 ± 11 yrs) and compared to dexamethasone nonresponders (2 CRVO, 4 BRVO, 69 ± 12). Outcome parameters were change in best-corrected visual acuity (BCVA) and central foveal thickness (CFT) measured by spectral-domain optical coherence tomography. *Results*. Mean BCVA improvement after switch to dexamethasone implant was 4 letters ($p = 0.08$), and treatment naïve eyes gained 10 letters ($p = 0.66$), while we noted no change in eyes after switch to anti-VEGF ($p = 0.74$). Median CFT decrease was most pronounced in treatment naïve patients ($-437\,\mu$m, $p = 0.002$) compared to anti-VEGF refractory eyes ($-170\,\mu$m, $p = 0.003$) and dexamethasone-refractory eyes (-157, $p = 0.31$). *Conclusions*. Dexamethasone significantly reduced ME secondary to RVO refractory to anti-VEGF. Functional gain was limited compared to treatment naïve eyes, probably due to worse BCVA and CFT at baseline in treatment naïve eyes.

1. Introduction

Visual impairment secondary to central or branch retinal vein occlusion (CRVO, BRVO) is mostly caused by macular edema. Intravitreal treatment with either anti-VEGF (vascular endothelial growth factor) or corticosteroids is efficacious and safe [1–3]. Anti-VEGF agents that were currently used are ranibizumab (Lucentis, Novartis Pharma, Switzerland) [4–7], aflibercept (Eylea, Bayer AG, Germany) [8–13], and off-label bevacizumab (Avastin, Roche, Germany) [14, 15]. Among intravitreal corticosteroids, dexamethasone implant (Ozurdex, Allergan, Ireland) is a device approved for macular edema secondary to RVO [16, 17] and diabetes. Pivotal trials that led to approval were conducted in parallel so that head-to-head comparison between anti-VEGF agents and dexamethasone implant was missing until very recently. Consequently, evidenced-based recommendations for treatment of macular edema could only be based on indirect comparison, rendering a decision for the first- and second-line therapeutic treatment recommendation almost impossible [1, 2, 18]. Treatment for macular edema could be initiated with both options and should consider the individual ophthalmological disposition and the patients' circumstances (characteristics to consider are, among others, age, lens status, presence of glaucoma, and mobility).

Head-to-head trials comparing dexamethasone and ranibizumab for macular edema due to RVO are COMO (http://clinicaltrials.gov [19]), COMRADE-B [20], COMRADE-C [21], and COMRADE-Extension trials [submitted by Feltgen et al.]. Results of direct comparison show that both treatments lead to significant improvement of best-corrected visual acuity (BCVA) and macular morphology, but continuous treatment with anti-VEGF ranibizumab given on a pro re nata (PRN) regimen is superior compared to dexamethasone implant at six months (given at a minimum of six months, following the European label and COMRADE trials [20, 21]) as well as compared to PRN dexamethasone after 12 months (given at a minimum of 5-month intervals, COMO trial [19]). Retrospective comparative real-life studies suggest a comparable effect of anti-VEGF injections and dexamethasone implant based on PRN regimen for both [22, 23]. Current experts' consensus recommend intravitreal anti-VEGF first line with a minimum of 3 consecutive monthly injections [3, 24–27]. In case of insufficient effect and persistent or recurrent macular edema, a switch between intravitreal treatments is recommended. This could either be a switch between different anti-VEGF agents or a switch to dexamethasone implant. There is no evidence from prospective randomized controlled trials (RCT) investigating such a switch. But results from pivotal trials as well as knowledge on switch of intravitreal therapy in age-related macular degeneration [28, 29] support the supposed approach.

We conducted the present retrospective observational study to investigate the effects of switch between intravitreal therapy on function and morphology in patients who presented with macular edema secondary to BRVO or CRVO and received either initial anti-VEGF treatment or dexamethasone implant.

2. Materials and Methods

This retrospective, observational study was conducted in accordance with the Declaration of Helsinki (1964) and Good Clinical Practice. Before patient recruitment, the study was reviewed and approved by an independent Ethical Committee.

We searched our data for patients with treatment for macular edema secondary to BRVO or CRVO with a switch in intravitreal treatment. Patients had received either (i) anti-VEGF intravitreal injections followed by dexamethasone implant (group: Anti-VEGF_Dexamethasone), (ii) dexamethasone implant followed by anti-VEGF (Dexamethasone_anti-VEGF), or (iii) dexamethasone implant only (treatment naïve group: Dexamethasone). The latter group served as real-life control of treatment effects of dexamethasone implant in RVO. The decision to switch intravitreal therapy was based on clinical findings on examination and could be classified as poor response or no response either in functional (best-corrected visual acuity, subjective visual acuity) or morphological parameters (central foveal thickness (CFT) in spectral-domain optical coherence tomography (SD-OCT)). In the Anti-VEGF_Dexamethasone group, eyes did not respond (sufficiently) to a minimum of three consecutive monthly intravitreal anti-VEGF injections before switch to dexamethasone implant. In the Dexamethasone_anti-VEGF group, eyes did not respond to one or more dexamethasone implants and showed recurrence of macular edema from month two to three onwards after implantation, due to the European label of dexamethasone implant at the time of treatment patients could receive a second implant only after 6 months after the first implant. We recorded the reason for the switch if one of the following classifications was documented as main reason for change of intravitreal therapy: deterioration, stagnation, patient's choice, decompensation of intraocular pressure (IOP), and not known.

Outcome parameters were change in BCVA (logMAR) and CFT (μm) measured by SD-OCT before and after treatment initiation or switch, respectively.

2.1. Statistical Analysis. We longitudinally compared visual acuities at baseline (before change of treatment) to the best visual acuities after change and to the visual acuities on record, respectively. We used paired t-tests to assess statistical significance. These calculations were performed for all three treatment groups separately. We did not compensate for multiple testing due to the explorative nature of this retrospective project. Central retinal thickness was analyzed alongside visual acuity using analogous calculations.

3. Results

The search for patients treated for macular edema secondary to BRVO or CRVO resulted in 47 patients (one eye per patient). Analysis included 30 eyes in the Anti-VEGF_Dexamethasone group (8 CRVO, 22 BRVO, median age 72 years (yrs), mean age 69 ± 10 yrs), compared to 11 treatment naïve eyes (6 CRVO, 5 BRVO, median age 80 yrs, mean age 73 ± 11 yrs) and compared to 6 eyes in the Dexamethasone_anti-VEGF group (2 CRVO, 4 BRVO, median age 69 yrs, mean age 69 ± 12 yrs). The median number of anti-VEGF injections before the switch was 6 (quartiles 3.25; 10), compared to 1.5 dexamethasone implants (quartiles 1; 2) before the switch to anti-VEGF. Patient characteristics and reason for switch are shown in Table 1. The most frequent reasons for a switch were stagnation of BCVA and/or CFT due to macular edema (47% Anti-VEGF_Dexamethasone; 67% Dexamethasone_anti-VEGF) and deterioration (40% and 33%, resp.). Switch to anti-VEGF injections due to IOP increase was only documented for one patient in the Anti-VEGF_Dexamethasone group and none in the Dexamethasone_anti-VEGF group. IOP increase independent of a switch occurred more frequently after intravitreal treatment with dexamethasone implant compared to anti-VEGF. All patients were sufficiently treated with local antiglaucomatous treatment to reduce IOP; none received glaucoma surgery.

3.1. Functional and Morphological Results after Switch of Intravitreal Therapy. Switch from anti-VEGF to dexamethasone after a median of 6 anti-VEGF injections led to BCVA improvement of 4 letters ($p = 0.08$, Figure 1) and decrease

Table 1: Patients' baseline characteristics and reason for switch of intravitreal therapy.

	Anti-VEGF to dexamethasone $n = 30$	Dexamethasone to anti-VEGF $n = 6$	Dexamethasone $n = 11$
BRVO	73% (22)	67% (4)	45% (5)
CRVO	20% (6)	17% (1)	55% (6)
Gender (% female)	50%	50%	45%
Age (at baseline [years])	69 ± 10	70 ± 13	74 ± 12
BCVA before switch (mean ± SD [logMAR])	0.42 ± 0.28	0.55 ± 0.34	1.07 ± 0.69
BCVA before switch (mean ± SD [logMAR])	0.36 ± 0.31	0.52 ± 0.45	0.88 ± 0.50
Number of intravitreal injections before switch (median, quartiles)	6 [3.25; 10]	1.5 [1;2]	
Reason for switch			
(i) Deterioration	40% (12)	33% (2)	
(ii) Stagnation	47% (14)	67% (4)	
(iii) Patients' choice	3% (1)	0	
(iv) IOP decompensation	3% (1)	0	
(v) Not known	7% (2)	0	

BRVO/CRVO: branch/central retinal vein occlusion; BCVA: best-corrected visual acuity.

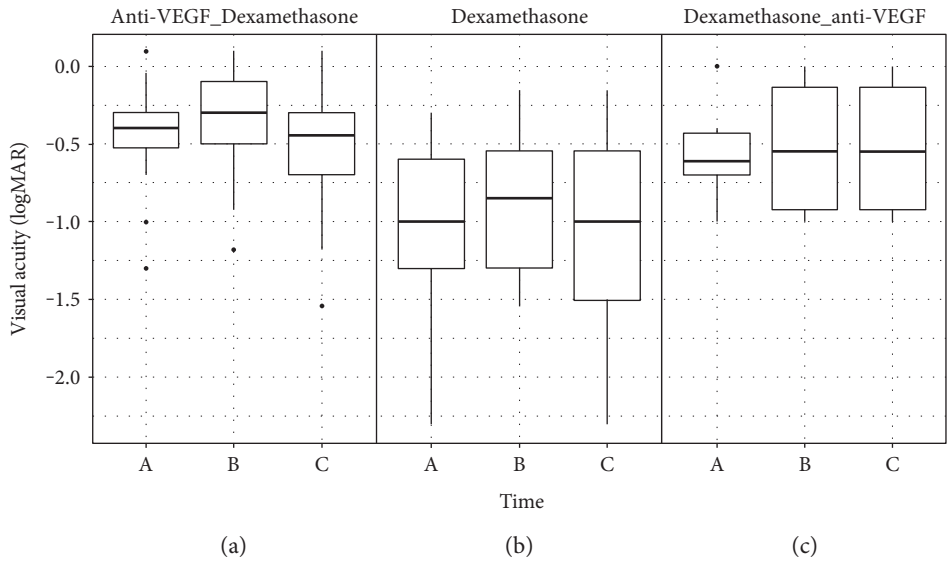

Figure 1: Mean BCVA improvement after dexamethasone implant in anti-VEGF refractory eyes was 4 letters ((a) change in logMAR 0.42 to 0.36, $p = 0.08$); treatment naïve eyes gained 10 letters after dexamethasone implant ((b) change in logMAR 1.07 to 0.82, $p = 0.66$), while we noted no significant change in eyes refractory to dexamethasone implant after switch to anti-VEGF ((c) change in logMAR 0.55 to 0.52, $p = 0.74$). A = BCVA before switch from anti-VEGF to dexamethasone (a) or vice versa (c) or before treatment (b); B = best BCVA after switch/treatment at 88 days [70; 176], 92 days [87; 100], and 123 days [96; 210]; and C = at the end of observation (median follow-up was 4.3 months for the Anti-VEGF_Dexamethasone group, 3.1 months for the Dexamethasone group, and 6.0 months for the Dexamethasone_anti-VEGF group).

of CFT from 455 μm [323; 542] to 285 μm [219; 460] (change −170 μm, $p = 0.003$, Figure 2). Switch from dexamethasone to anti-VEGF after mean 1.5 implants led to a stabilization of BCVA ($p = 0.74$) despite a change in CFT from 555 μm [395; 675] to 398 μm [245; 535] (change −157 μm, $p = 0.31$). The most pronounced improvement in BCVA and CFT was noted in treatment naïve eyes after dexamethasone implant (BCVA +10 letters, $p = 0.66$; CFT from 675 μm [580; 810] to 238 μm [188; 348], change −437 μm, $p = 0.002$). Mean CFT at the end of follow-up remained significantly reduced in the Anti-VEGF_Dexamethasone group (285 μm [219; 460]) as well as in the Dexamethasone group (238 μm [188; 347]), while we noticed a persistent higher CFT at the end of follow-up in the Dexamethasone_anti-VEGF group (398 μm [245; 535]). Notably, there was a difference in follow-up between groups: Median follow-up was 4.3 months (129 days [75; 335]) for the Anti-VEGF_Dexamethasone group, 3.1 months (94 days [87; 135]) for the Dexamethasone

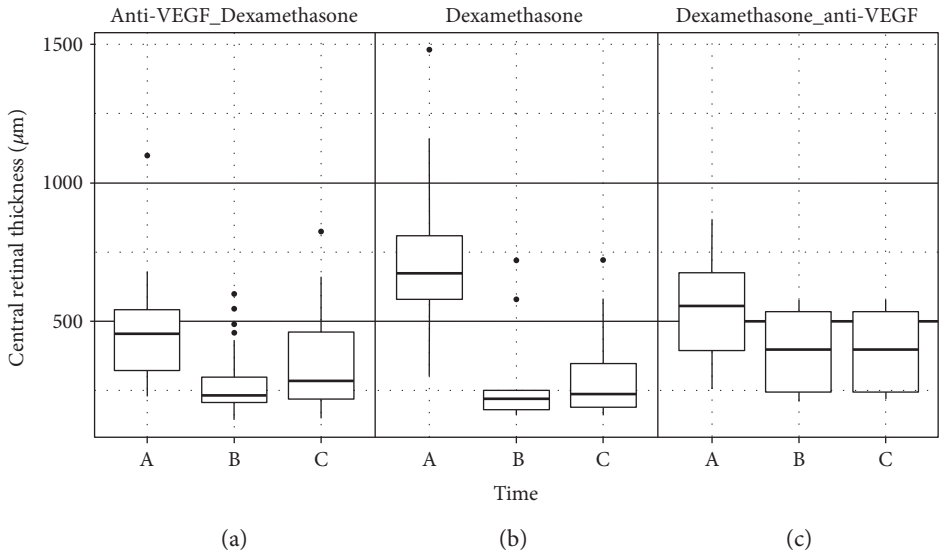

Figure 2: Mean CFT decrease was most pronounced in treatment naïve eyes ((b) from 675 μm [580; 810] to 238 μm [188; 348], change −437 μm, $p = 0.002$) compared to Anti-VEGF_Dexamethasone group ((a) from 455 μm [323; 542] to 285 μm [219; 460], change −170 μm, $p = 0.003$) and Dexamethasone_anti-VEGF treated eyes ((c) from 555 μm [395; 675] to 398 μm [245; 535], change −157 μm, $p = 0.31$). A = CFT before switch from anti-VEGF to dexamethasone (a) or vice versa (c) or before treatment (b); B = CFT at the time of the best BCVA after switch/treatment (at 88 days [70; 176], 92 days [87; 100], and 123 days [96; 210]); and C = at the end of observation (median follow-up was 4.3 months for the Anti-VEGF_Dexamethasone group, 3.1 months for the Dexamethasone group, and 6.0 months for the Dexamethasone_anti-VEGF group).

group, and 6.0 months (181 days [156; 261]) for the Dexamethasone_anti-VEGF group.

The rate of "dry eyes" defined as CFT equal or less than 225 μm was 46% (Anti-VEGF_Dexamethasone), 50% (Dexamethasone), and 17% (Dexamethasone_anti-VEGF) at the time of the best BCVA and 29%, 40%, and 17%, respectively, at the end of follow-up.

The time of the best BCVA was 88 days [70; 176], 92 days [87; 100], and 123 days [96; 210] after switch or initiation of therapy, respectively.

Ischemia and subfoveal atrophy of the retinal pigment epithelium were assessed in all SD-OCT scans and fluorescein angiography (if available), but we did not record any in the eyes of all groups.

4. Discussion

A positive effect on BCVA and CFT is seen in both of our study groups after switch of intravitreal therapy for macular edema secondary to RVO either from anti-VEGF to dexamethasone implant or vice versa. A comparable positive response to switch from anti-VEGF to dexamethasone was seen in a group of 18 patients, who showed visual improvement of 0.25 logMAR and reduction of macular edema by −146 μm [30]. Another study investigated 48 patients and concluded that switch from anti-VEGF to dexamethasone seemed to be more beneficial in short-term visual acuity and long-term morphological results compared to a switch from dexamethasone to anti-VEGF [31]. But results for the latter group were limited by group size (8 versus 40) comparable to the difference in our study. The positive response seems to apply to switch of therapy in recalcitrant or recurrent macular edema secondary to RVO. In contrast, there seems to be no positive or additive effect if dexamethasone is given after an initial upload of 3 anti-VEGF injection as a fixed combination compared to dexamethasone alone in treatment naïve RVO eyes [32].

In our study, BCVA gain after dexamethasone implant was limited in eyes treated before with median 6 anti-VEGF injections compared to treatment naïve eyes. This effect might be due to worse BCVA and higher CFT in treatment naïve eyes. The rate of CRVO was higher in the Dexamethasone group compared to both switch groups. This could well contribute to the worse baseline BCVA in the treatment naïve eyes as well as the limited BCVA at the end of follow-up. ME following CRVO is more pronounced compared to BRVO, and patients often need more frequently a higher number of intravitreal treatment compared to BRVO. Notably, there was a difference in follow-up between groups which could also contribute to the effects seen (3.1 months dexamethasone versus 4.3 months after switch to dexamethasone and 6.0 months after switch to anti-VEGF). The effect seen in treatment naïve eyes after dexamethasone implant was comparable to results in pivotal dexamethasone trials GENEVA [16, 17] and results of head-to-head trials COMRADE-B [20], COMRADE-C [21], and COMO [19]. After the relevant improvement in BCVA and CFT following dexamethasone implant, we noticed the characteristic decrease of both, BCVA and CFT, at the end of observation in the Dexamethasone group.

Similar but less pronounced effects were visible in the Anti-VEGF_Dexamethasone group. After the switch, we noticed an increase in BCVA, which attenuated until the end of observation. The initial decrease in CFT corresponded

well with the improved BCVA, while we did not see a corresponding pronounced increase in CFT at the end of observation. This could be due to limited morphological response in pretreated eyes, which might be more limited the longer or more frequent the previous treatment. Long-term data on anti-VEGF therapy in RVO show that initial BCVA improvement could be stabilized up to 4 years of treatment (RETAIN study [33]). On the other hand, we know that morphological effects appear before functional effects and long-standing macular edema may harm the macula irrevocably, which is a reason to treat macular edema as soon as possible after onset and visual impairment. Results of all pivotal trials on anti-VEGF treatment showed that gain in BCVA was limited initially as well as on the long-term, if treatment was deferred by 6-month sham treatment [4, 5, 8, 11, 14]. There is evidence that early anti-VEGF treatment may reduce the risk and frequency of recurrent macular edema [34].

Results of our group Dexamethasone_anti-VEGF are certainly limited due to the small patient number. Comparable limitations apply to a previous study investigating the switch from anti-VEGF to dexamethasone (40 eyes) and vice versa (8 eyes) [31]. The discrepancy between group size within a real-life setting might be attributable to various reasons including the decision to start more often with anti-VEGF due to preferred practice patterns or possible negative adverse effects of dexamethasone (cataract and IOP), resulting in more eyes in the group of Anti-VEGF_Dexamethasone switch than the other. But results are still valuable, showing that the switch from dexamethasone to anti-VEGF might reduce recalcitrant macular edema (CFT) and stabilize visual acuity. This is the only group in which we noticed no drop in BCVA at the end of observation. Macular edema following RVO needs repetitive intravitreal treatment, and reinjection of anti-VEGF is possible every four weeks allowing for as little morphological and functional fluctuation as possible. On the contrary, dexamethasone implant was approved for use every 6 months. Results of the GENEVA trial [16, 17] as well as COMRADE-B [20], COMRADE-C [21], and COMO [19] show that the effect of dexamethasone implant on BCVA and CFT is most pronounced after 2 months and diminishes from then onwards. Many authors come to the conclusion that a reimplantation is necessary after 3 or 4 months to prevent undulation and stabilize the gain in BCVA.

Current recommendations on treatment of macular edema secondary to RVO recommend initial treatment with multiple anti-VEGF injections as safest option to start with [3, 24, 25, 27]. There is little evidence from head-to-head comparison between different anti-VEGF, but similar effects were shown and it is supposed that the effects of different anti-VEGF agents are noninferior in comparison (MARVEL study [35], SCORE-2 [36]). If macular edema is refractory or recurrent, most experts' consensus recommend a switch within the group of anti-VEGF agents and secondary a switch to intravitreal corticosteroids. Among corticosteroids, dexamethasone implant might be preferred to triamcinolone because of the standardized dosing and less visual disturbance by the implant compared to triamcinolone. Our results add to the knowledge on intravitreal treatment of macular edema due to RVO and support recommendations to switch.

5. Conclusions

Intravitreal dexamethasone significantly reduced macular edema due to RVO that was refractory to anti-VEGF intravitreal treatment. However, gain in function as well as morphological improvement were limited after the switch compared to treatment naïve eyes. This could be attributed to significantly worse BCVA and CFT at baseline in treatment naïve macular edema. Switch from dexamethasone to anti-VEGF could stabilize BCVA and CFT. Factors to predict patients' response to anti-VEGF or dexamethasone intravitreal therapy before treatment initiation remain to be determined.

Disclosure

Data were presented by the corresponding author as a poster at the ARVO annual meeting 2014, Poster 684, ARVO Annual Meeting, 06-Mai-2014, Orlando, Florida.

Conflicts of Interest

Amelie Pielen received honorary for lectures and travel support from Novartis Pharma, Pharm Allergan, Bayer AG, Thea Pharm; she is the principal investigator in trials sponsored by Böhringer Ingelheim, Novartis Pharma, Bayer AG, Roche, bioeq, Nicox. Bernd Junker received honorary for lectures and travel support from Novartis Pharma, Pharm Allergan, Bayer AG. All other authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Ocular Adverse Effects of Intravitreal Bevacizumab Are Potentiated by Intermittent Hypoxia in a Rat Model of Oxygen-Induced Retinopathy

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Intravitreal bevacizumab (Avastin) use in preterm infants with retinopathy of prematurity is associated with severe neurological disabilities, suggesting vascular leakage. We examined the hypothesis that intermittent hypoxia (IH) potentiates intravitreal Avastin leakage. Neonatal rats at birth were exposed to IH from birth (P0)–P14. At P14, the time of eye opening in rats, a single dose of Avastin (0.125 mg) was injected intravitreally into the left eye. Animals were placed in room air (RA) until P23 or P45 for recovery (IHR). Hyperoxia-exposed and RA littermates served as oxygen controls, and equivalent volume saline served as the placebo controls. At P23 and P45 ocular angiogenesis, retinal pathology and ocular and systemic biomarkers of angiogenesis were examined. Retinal flatmounts showed poor peripheral vascularization in Avastin-treated and fellow eyes at P23, with numerous punctate hemorrhages and dilated, tortuous vessels with anastomoses at P45 in the rats exposed to IH. These adverse effects were associated with robust increases in systemic VEGF and in both treated and untreated fellow eyes. Histological analysis showed severe damage in the inner plexiform and inner nuclear layers. Exposure of IH/IHR-induced injured retinal microvasculature to anti-VEGF substances can result in vascular leakage and adverse effects in the developing neonate.

1. Introduction

Retinopathy of prematurity (ROP) has been extensively studied in clinical trials but remains a major cause of severe irreversible blindness worldwide. The exact physiologic mechanisms and optimal treatment of significant clinical disease are poorly elucidated. Extremely low gestational age neonates (ELGANs) requiring excessive supplemental oxygen, and who experience frequent arterial oxygen desaturations or apneas, are at a high risk for pathologic retinal vasculature development [1–3]. This process of "threshold" retinopathy ultimately leads to subnormal anatomic and functional development: permanently underdeveloped retina, macular dragging, retinal detachment, neovascular glaucoma, blindness, and ultimately phthisis bulbi [4]. The incidence of ROP in developed countries is estimated to be 5–8% of preterm infants while in developing countries, it may be as high as 30% [5]. Despite advancements in treatment, the number of individuals with blindness due to ROP is still as high as 50,000 worldwide [6].

Although long-term studies such as Cryotherapy for Retinopathy of Prematurity (CRYO-ROP) showed reduction of disease compared to observation, almost half (44.7%) of cryotherapy-treated eyes still had unfavorable visual acuity outcomes [4]. Laser panretinal photocoagulation has surpassed cryotherapy as the standard of care, as the Early Treatment in Retinopathy of Prematurity Study (ETROP) showed at least equal success to cryotherapy in

all treated eyes and significantly improved outcomes in those with the most posterior and severe disease [7]. Laser photocoagulation ablates the undeveloped retina, does not allow for full vascularization, and potentially exaggerates myopia [8]. On the other hand, its effects are durable and permanent. A myriad of trials have sought to parse out the associated exacerbating and remitting factors to allow improved success with treatment, especially with aggressive posterior ROP (APROP) where functional and anatomic success is low [9–11]. Of the newer treatment modalities, off-label use of intravitreal bevacizumab (Avastin), a recombinant humanized vascular endothelial growth factor inhibitor (anti-VEGF) that binds to all VEGF-A isoforms, has shown the most promise as it does not immediately destroy any retinal tissue as does laser therapy. The landmark BEAT-ROP study demonstrated advantage of intravitreal bevacizumab over laser therapy for zone 1 stage 3+ ROP by improving structural outcomes, decreasing recurrence, and allowing continued development of peripheral retina [12, 13]. However, its use in ROP is somewhat controversial. In the BEAT-ROP study, assessment of local and systemic safety profile could not be determined due to study size [12]. With Avastin, it is also the case that significant, potentially blinding ROP may develop at an altered time course, making close and extended follow-up critical [14]. The occurrence of APROP coincides with crucial stages of development of vital organs such as the brain, heart, lungs, and kidneys. These structures require VEGF for normal development, and any alterations caused by anti-VEGF therapies may cause long-term adverse effects [15]. Avastin is a highly potent anticancer agent, used off-label for ophthalmic conditions, and is not formulated for use within the eye. Recent studies have since tried to address safety of intravitreal Avastin in ROP and demonstrated numerous acute and latent retinal adverse effects [16–20], as well as a higher incidence of severe neurological disabilities [21].

Avastin is considered as a species-specific antibody for use in humans. However, numerous studies have reported Avastin efficacy in rats [22–28]. Avastin was shown to bind murine VEGF in three independent molecular bioassays [24]. Furthermore, toxicology studies conducted in rabbits showed reduced wound healing, decreased maternal and fetal body weights, increased fetal resorptions, and a number of teratogenic effects (Avastin package insert), demonstrating that Avastin effects are not exclusive to humans. Therefore, using an established rat model of oxygen-induced retinopathy (OIR), which closely simulates the frequent, brief intermittent hypoxic (IH) episodes (or apneas) experienced by ELGANs, we tested the hypothesis that the numerous adverse effects of Avastin that are reported in the literature may be due to potentiation by IH. The rationales for this hypothesis were (a) ELGANs experience several hundred episodes of IH during the first weeks of postnatal life, (b) IH causes endothelial impairment and function leading to vascular leakiness, (c) restitution of blood flow through severely injured microvasculature during reoxygenation following an IH episode (IHR) leads to hemorrhage, and (d) leakage into the systemic circulation will result in adverse effects in the developing neonate.

2. Materials and Methods

2.1. Experimental Design. All experiments were approved by the State University of New York (SUNY), Downstate Medical Center Institutional Animal Care and Use Committee, Brooklyn, NY. Certified infection-free, timed-pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) at 17 days gestation. The animals were housed in an animal facility with a 12-hour day/12-hour night cycle and provided standard laboratory diet and water ad libitum until delivery. Within 2-3 hours of birth, newborn rat pups delivering on the same day were pooled and randomly assigned to expanded litters of 18 pups/litter (9 males and 9 females). Gender was determined by the anogenital distance. The expanded litter size was used to simulate relative postnatal malnutrition of extremely low gestational age newborns (ELGANs) who are at increased risk for severe ROP. Each pup was weighed and measured for linear growth (crown to rump length in centimeters) and randomized to either (1) room air (RA), (2) hyperoxia (50% O_2), or (3) intermittent hypoxia (IH, 50/12% O_2 cycling) from P0 to P14. The IH cycling profile consisted of hyperoxia (50% O_2) with brief (1 minute) hypoxia (12% O_2) episodes (3 clusters, 10 minutes apart) for a total of 8 clustered hypoxic episodes per day. This clustering design has been shown to produce a severe form of oxygen-induced retinopathy (OIR) in neonatal rats [29–34] and confirmed in human neonates [3]. At P14 (time of eye opening in rats), animals were anesthetized with halothane/oxygen, povidone iodine was placed on the conjunctiva, and 5 μL of Avastin was injected into the left eye. A volume and dose of Avastin were based on previous studies [23]. Preterm infants receive 0.625 mg Avastin per eye for a total concentration of 1.25 mg. We injected only one eye with a total concentration of 0.125 mg. The right eyes received equivalent volume sterile normal saline and served as placebo controls. Sterile normal saline was used as the placebo control according to the manufacturer's instructions in the package insert to dilute Avastin in 0.9% sodium chloride. Other investigators have used intravitreal balanced salt csolution as placebo controls [23]. The animals were monitored daily for signs of infection. At P23 and P45, the animals were euthanized and blood and eyes collected for the assessment of VEGF, sVEGFR-1, and IGF-I; retinal angiogenesis and pathology; and expression of ocular angiogenesis biomarkers.

2.2. Sample Collection. Both eyes from 9 male and 9 female pups in each group were enucleated and rinsed in ice-cold phosphate-buffered saline (pH 7.4) on ice. The vitreous fluid (VF) samples were aspirated as previously described [29, 31–34] using a sterile 27-gauge needle attached to a 0.5 mL tuberculin syringe. VF samples were pooled to obtain 3 male and 3 female samples in each group. The retinas and choroids were then excised and processed as previously described [29, 31–34]. To obtain enough tissue, samples were pooled and a total of 6 samples (3 males and 3 females) per group were analyzed. Pooled samples were placed in sterile individual tubes containing ceramic beads and homogenized using a Fast-Prep 24 system (MP Biomedicals, Solon, OH,

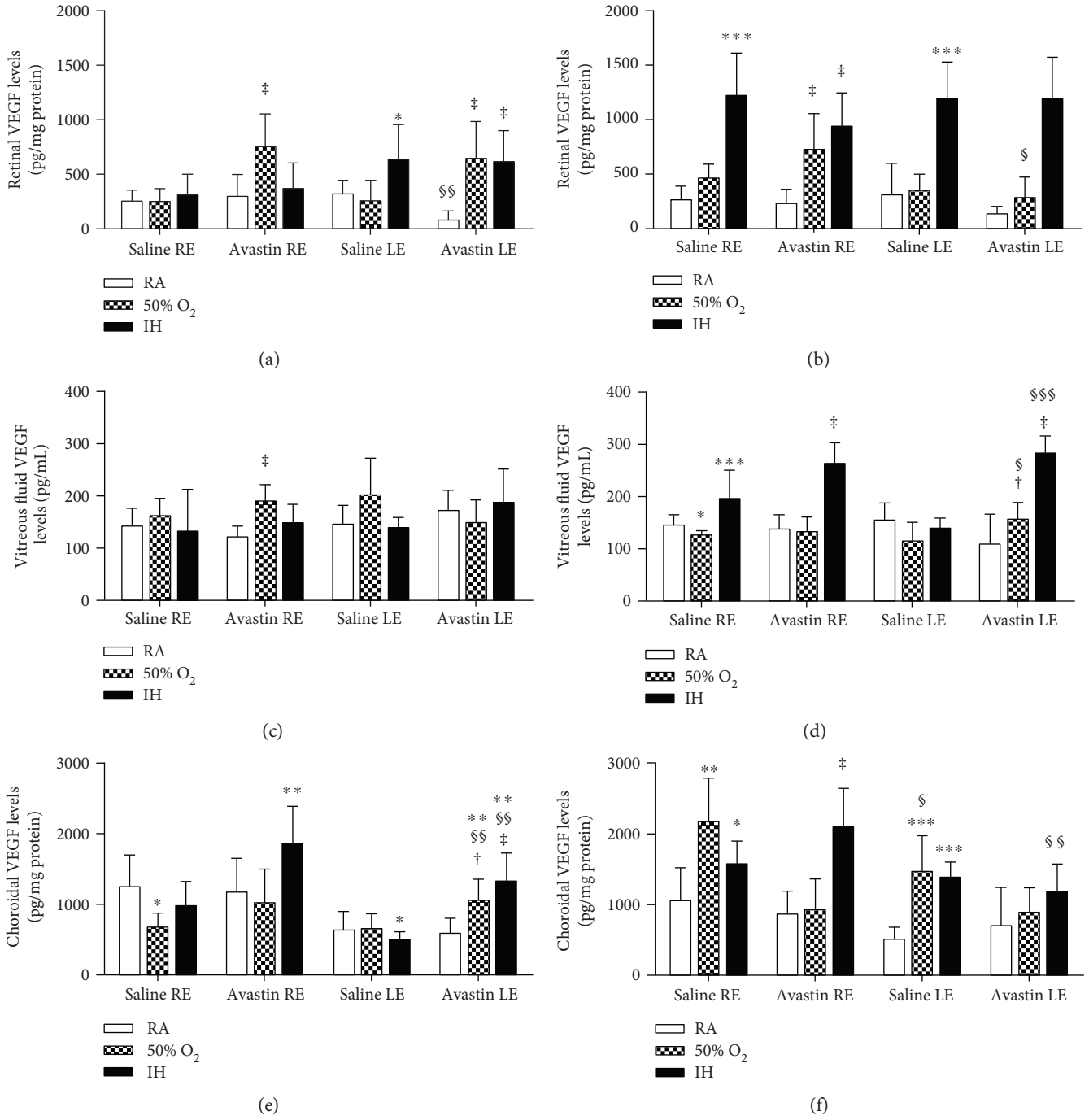

Figure 1: Effects of Avastin on retinal (a, b), vitreous fluid (c, d), and choroidal (e, f) VEGF levels in adolescent 23-day-old (a, c, and e) and pubertal 45-day-old (b, d, and f) rats exposed to room air (RA), hyperoxia (50% O_2), and intermittent hypoxia (IH). Animals exposed to IH received 50% O_2 with brief, clustered hypoxia (12% O_2) episodes for a total of 8 episodes per day from P0 to P14. Data are presented as mean ± SD ($n = 6$ samples/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus saline RA; $^{\dagger}p < 0.05$, $^{\ddagger}p < 0.01$ versus Avastin RA; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ versus right untreated eye. RE: right eye; LE: left eye.

USA) in 1.0 mL ice-cold sterile normal saline. The homogenates were centrifuged at 10,000 rpm for 20 minutes at 4°C and filtered prior to assay. A portion of the filtrate (10 μL) was used for total cellular protein levels. For collection of blood samples, the rat pups were euthanized by decapitation. Mixed arterial-venous blood samples were collected in sterile Eppendorf tubes and placed on ice for 30 minutes, prior to centrifugation at 3000 rpm at 4°C.

2.3. Assay of VEGF, sVEGFR-1, and IGF-I. VEGF, sVEGFR-1, and IGF-I levels were determined in the serum and retinal and choroidal homogenates using commercially available rat

Table 1: Growth parameters.

	Room air (RA)		50% O_2		IH (50% O_2/12%O_2 cycling)	
	Saline	Avastin	Saline	Avastin	Saline	Avastin
P23						
% change in body weight	745.7 ± 1.3	$449.6 \pm 16.9^{\S\S}$	$308.7 \pm 16.4^{**}$	$524.0 \pm 28.8^{\S\S}$	$644.9 \pm 16.6^{**}$	$525.4 \pm 22.2^{\S\S}$
% change in length	101.1 ± 1.1	$81.8 \pm 3.1^{\S\S}$	$77.1 \pm 2.8^{**}$	$91.1 \pm 2.9^{\ddagger\S\S}$	$94.5 \pm 2.4^{**}$	$92.6 \pm 3.7^{\dagger}$
Brain/body weight ratio	0.027 ± 0.0006	0.0092 ± 0.091	$0.053 \pm 0.003^{**}$	$0.04 \pm 0.002^{\S\S}$	0.023 ± 0.0008	$0.04 \pm 0.0014^{\S\S}$
Lung/body weight ratio	0.0097 ± 0.0008	0.015 ± 0.002	$0.021 \pm 0.001^{**}$	0.016 ± 0.002	0.009 ± 0.0003	$0.02 \pm 0.0014^{\ddagger}$
P45						
% change in body weight	2886.6 ± 111.2	$2328.4 \pm 76.7^{\S\S}$	$2373.1 \pm 109.5^{**}$	$2677.7 \pm 109.2^{\dagger}$	2613.2 ± 84.6	2583.2 ± 81.2
% change in length	247.9 ± 4.3	$187.8 \pm 4.7^{\S\S}$	$193.3 \pm 3.5^{**}$	$205.9 \pm 3.7^{\dagger\S}$	$206.8 \pm 4.2^{**}$	193.7 ± 4.3
Brain/body weight ratio	0.041 ± 0.002	$0.011 \pm 0.003^{\S\S}$	$0.01 \pm 0.0003^{**}$	0.01 ± 0.0003	$0.009 \pm 0.0003^{**}$	0.0093 ± 0.004
Lung/body weight ratio	0.091 ± 0.005	$0.008 \pm 0.0006^{\S\S}$	$0.0062 \pm 0.0002^{**}$	$0.0065 \pm 0.0003^{\dagger}$	$0.006 \pm 0.0007^{**}$	$0.005 \pm 0.0005^{\ddagger}$

Data are mean ± SD ($n = 18$ per group; $^{**}p < 0.01$ versus saline RA; $^{\dagger}p < 0.05$ and $^{\ddagger}p < 0.001$ versus Avastin RA; $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ versus saline). IH: intermittent hypoxia.

Table 2: Serum levels of VEGF, sVEGFR-1, and IGF-I.

Growth factors	Room air (RA)		50% O_2		IH (50% O_2/12% O_2 cycling)	
	Saline	Avastin	Saline	Avastin	Saline	Avastin
P23						
VEGF (pg/mL)	79.9 ± 17.6	82.9 ± 25.3	$32.7 \pm 27.2^{**}$	$19.7 \pm 2.3^{\ddagger}$	$8.9 \pm 10.8^{**}$	$1.01 \pm 2.3^{\ddagger}$
sVEGFR-1 (pg/mL)	716.8 ± 130.0	665.7 ± 285.9	$594.6 \pm 144.0^{*}$	$412.7 \pm 51.7^{\ddagger\S}$	$387.6 \pm 99.7^{**}$	$350.3 \pm 75.0^{\ddagger}$
IGF-I (pg/mL)	4197.5 ± 33.0	4222.7 ± 99.3	4017.4 ± 152.2	4137 ± 95.2	4222.9 ± 86.7	$3151.0 \pm 51.8^{\ddagger\S\S}$
P45						
VEGF (pg/mL)	73.3 ± 36.1	$33.0 \pm 25.2^{\S}$	$30.8 \pm 2.4^{**}$	17.3 ± 14.6	$39.5 \pm 20.1^{**}$	$98.5 \pm 110.8^{\ddagger\S\S}$
sVEGFR-1 (pg/mL)	671.8 ± 194.4	623.6 ± 166.4	$518.6 \pm 154.6^{*}$	519.9 ± 106.4	$304.3 \pm 50.5^{**}$	$380.5 \pm 124.4^{\ddagger}$
IGF-I (pg/mL)	4044.3 ± 112.1	4018.6 ± 60.8	3985.9 ± 159.3	4036.0 ± 89.1	$3214.0 \pm 277.3^{**}$	$3144.3 \pm 32.7^{\ddagger}$

Data are mean ± SD; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus saline RA; $^{\ddagger}p < 0.01$ versus Avastin RA; $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ versus saline ($n = 10$/group). IH: intermittent hypoxia.

sandwich immunoassay kits (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Data from the homogenates were standardized using total cellular protein levels as previously described [29, 31–34]. Validation studies comparing serum and plasma VEGF levels show no differences [35]. In addition, recovery studies done by the manufacturer, of human VEGF spiked to three different levels throughout the range of the assay in various matrices showed 102% recovery for serum (range: 92–115%, $n = 5$); 97% recovery for EDTA plasma (range: 82–113%, $n = 5$); 93% recovery for heparin plasma (range: 82–102%, $n = 5$); and 100% recovery for citrate plasma (range: 88–113%, $n = 5$), providing further evidence that either plasma or serum can be used to determine VEGF levels with equivalent validity.

2.4. Total Cellular Protein Assay. On the day of the assay, retinal homogenates were assayed for total protein levels using the dye-binding Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

2.5. ADPase Staining. ADPase staining of the retinas and computer imaging were carried out as previously described [29, 31–34].

2.6. Retinal H&E Staining. Eyes were enucleated, rinsed in phosphate-buffered saline (PBS), fixed in Hartmann's fixative, and sent to New York University Experimental Pathology Histology Core Laboratory, NY, NY, USA, for processing and staining using standard histological techniques. Images were captured at 40x magnification using an Olympus BX53 microscope, DP72 digital camera, and CellSens imaging software (Olympus, Center Valley, PA, USA), attached to a Dell Precision T3500 computer (Dell, Round Rock, TX, USA).

2.7. Vascular Density Quantification. Digital images of the ADPase-stained retinal flatmounts take at 10x magnification were analyzed using WimRetina retinal vessel quantification image analysis software (Wimasis, Munich, Germany), as shown in Figure 1(a). The four quadrants of three retinas from each group ($n = 12$ measurements per group) were analyzed in a masked manner for vascular density (%, calculated by dividing the number of pixels of the vessels by the total number of pixels of the region of interest), total vascular area, number of branching points (where two or more segments converge), number of segments (number of individual vessel segments), and mean segment length.

2.8. Statistical Analysis. Data were analyzed using analyses of variance (ANOVA) for normally distributed data comparing differences among the oxygen groups (RA versus 50% O_2 versus IH) within the saline- and Avastin-treated groups

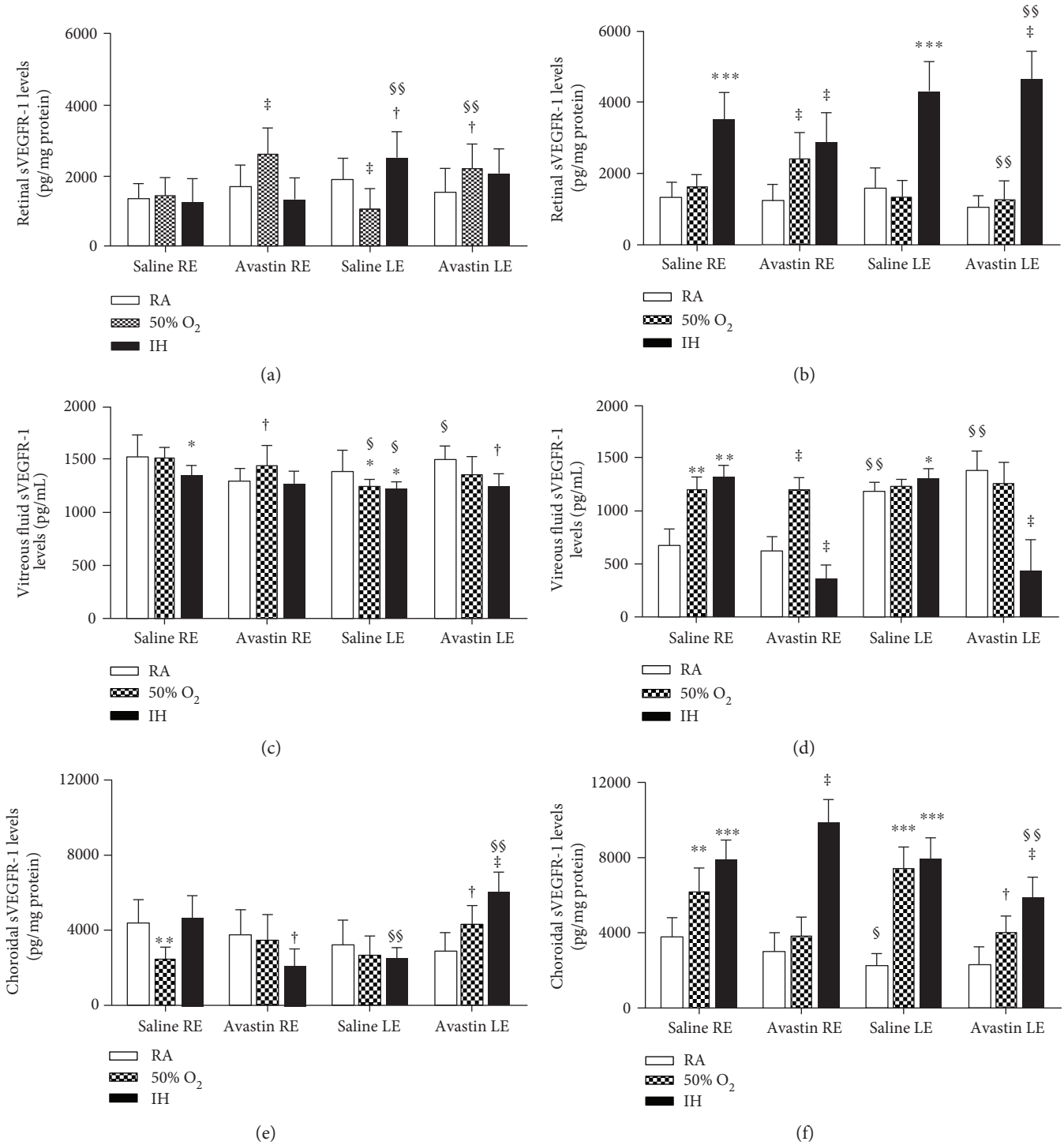

Figure 2: Effects of Avastin on retinal (a, b), vitreous fluid (c, d), and choroidal (e, f) sVEGFR-1 levels in adolescent 23-day-old (a, c, and e) and pubertal 45-day-old (b, d, and f) rats exposed to room air (RA), hyperoxia (50% O_2), and intermittent hypoxia (IH). Groups are as described in Figure 1. Data are presented as mean ± SD ($n = 6$ samples/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus saline RA; $^{\dagger}p < 0.05$, $^{\ddagger}p < 0.01$ versus Avastin RA; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, versus right untreated eye. RE: right eye; LE: left eye.

following Bartlett's test for equality of variances. For non-normally distributed data, the Kruskal-Wallis test was used. Post hoc analysis was performed using the Tukey, Bonferoni, and Student-Newman-Keuls tests for significance. Unpaired *t*-tests were conducted to compare saline versus Avastin within each oxygen environment following Levene's test for equality of variances for normally distributed data. Mann–Whitney *U* test was used for nonnormally distributed data. Significance was set at $p < 0.05$, and data are reported as mean ± SD. All analyses were two-tailed and performed using SPSS version 20.0 (SPSS Inc., Chicago, IL).

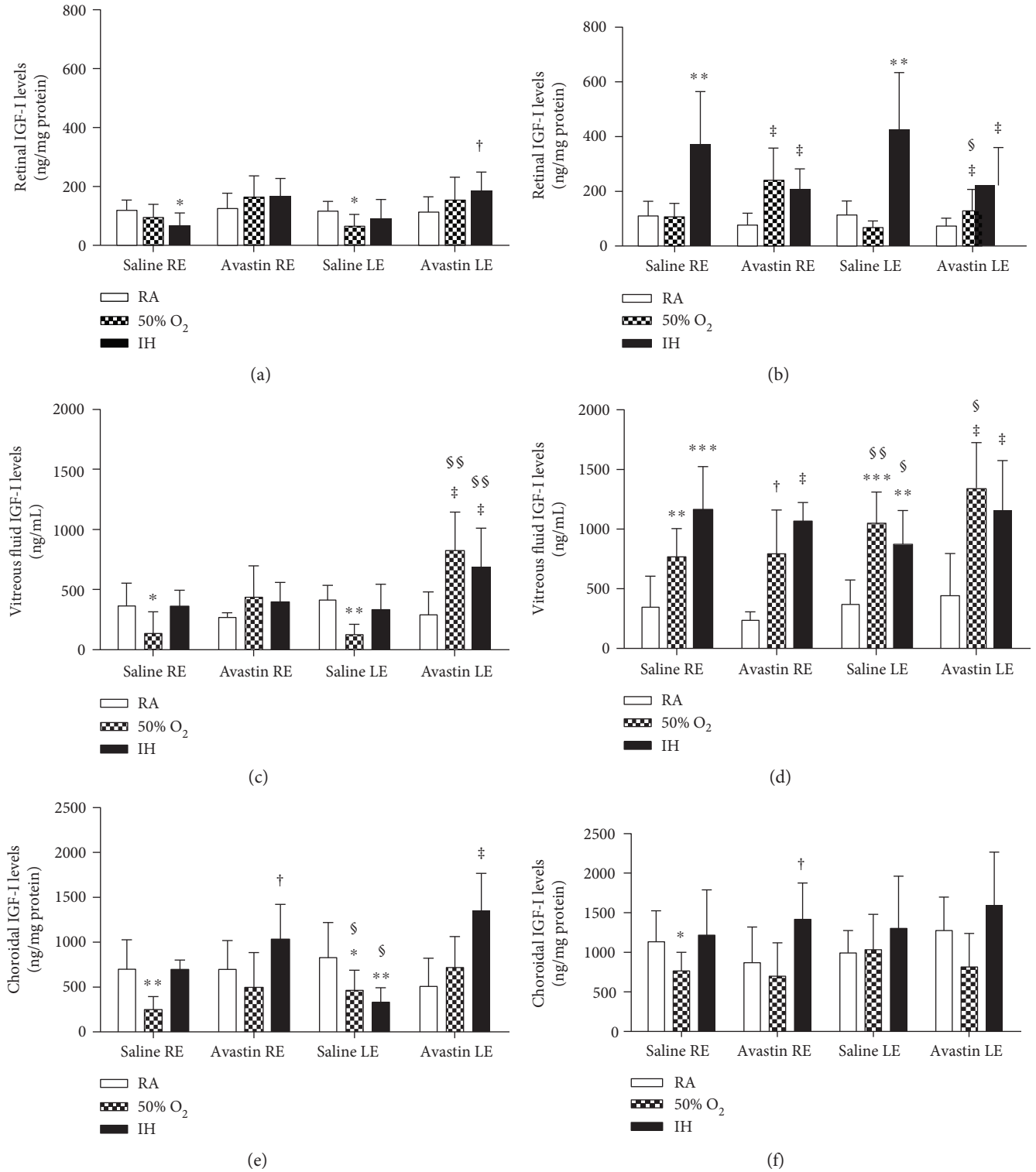

Figure 3: Effects of Avastin on retinal (a, b), vitreous fluid (c, d), and choroidal (e, f) IGF-1 levels in adolescent 23-day-old (a, c, and e) and pubertal 45-day-old (b, d, and f) rats exposed to room air (RA), hyperoxia (50% O_2), and intermittent hypoxia (IH). Groups are as described in Figure 1. Data are presented as mean ± SD ($n = 6$ samples/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus saline RA; $^{\dagger}p < 0.05$, $^{\ddagger}p < 0.01$ versus Avastin RA; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ versus right untreated eye. RE: right eye; LE: left eye.

3. Results

3.1. Avastin Decreases Somatic Growth in RA. Growth parameters are listed in Table 1. In RA, percentage change in somatic growth was significantly reduced with Avastin treatment at P23 and P45. Similar reductions in brain/body weight and lung/body weight ratios occurred at P45. Hyperoxia itself reduced somatic growth at P23 with minimal

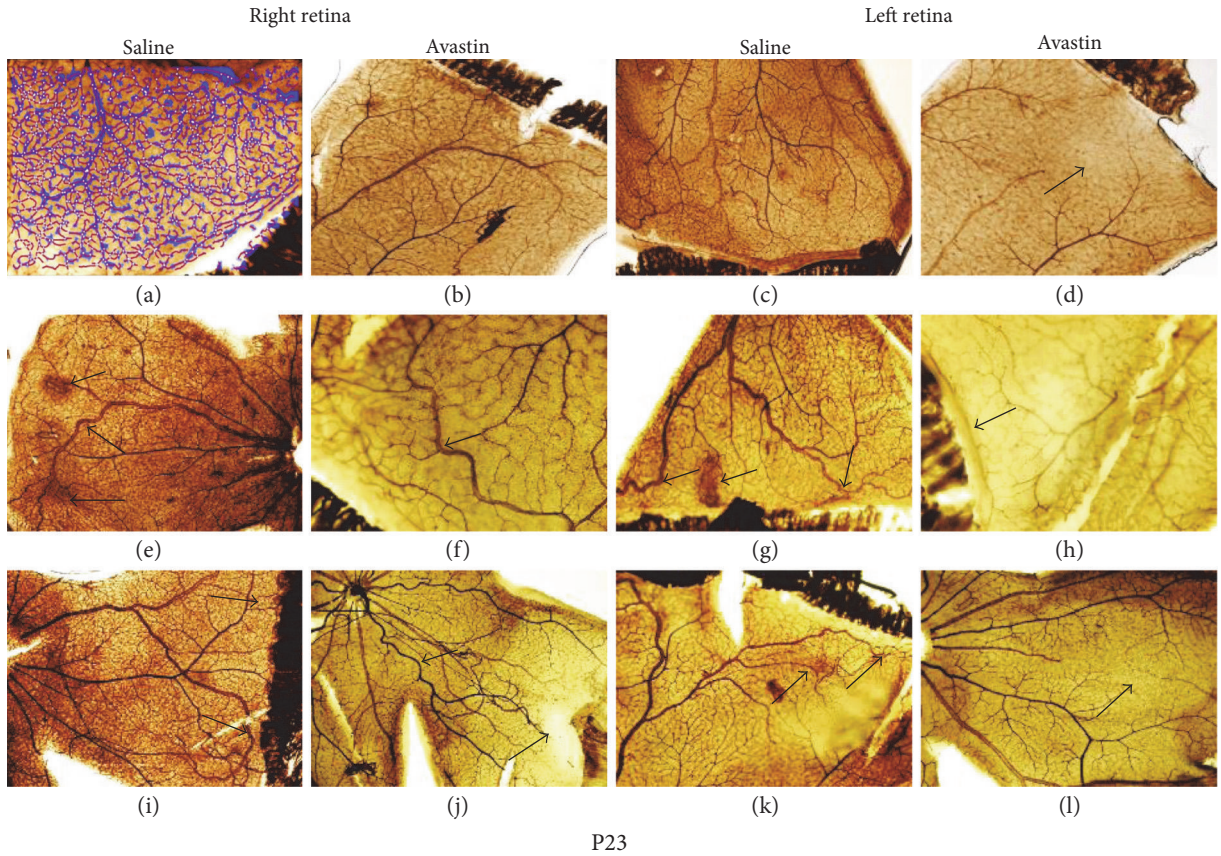

Figure 4: Retinal flatmounts showing ADPase-stained retinas from 23-day-old rat exposed to RA (a–d), 50% O2 (e–h), and IH (i–l). (a), (e), and (i) are right untreated retinas from saline-treated groups; (b), (f), and (j) are right untreated retinas from Avastin-treated groups; (c), (g), and (k) are saline-treated left retinas; and (d), (h), and (l) are Avastin-treated left retinas. (a) is a representative image of the WimRetina analysis for quantitation of vascular density parameters. Images are 10x magnification. Scale bar, 100 μm.

catch-up growth at P45. Avastin treatment in hyperoxia increased somatic growth although weight accretion did not quite achieve control levels. There were no significant effects of Avastin on brain/body or lung/body weight ratios in response to treatment in hyperoxia. IH caused similar reductions in somatic growth with minimal adjustments in response to Avastin treatment. Treatment with Avastin in IH increased brain/body and lung/body weight ratios at P23.

3.2. Avastin Increases VEGF in IH. To establish whether Avastin has effects on systemic VEGF, we examined the levels in serum at P23 and P45 (Table 2). At P23, Avastin had no effect on serum VEGF although the levels were significantly suppressed with hyperoxia and IH. At P45, the levels of VEGF in the untreated animals exposed to hyperoxia and IH remained lower than those in the RA animals. Animals treated with Avastin in RA had lower VEGF levels compared to those treated with saline, while treatment in IH caused a robust increase at P45. To determine whether Avastin affects the untreated eyes, we examined VEGF levels in the retina, VF, and choroid (Figure 2). In the retina, Avastin caused similar elevations in VEGF levels in the right eye and in the left eyes exposed to hyperoxia and IH at P23 (Figure 2(a)). At P45, retinal VEGF levels remained substantially higher in the left and right eyes of all groups exposed to IH despite treatment (Figure 2(b)). At P23, there was a significant increase in VF VEGF levels in the right untreated eyes compared to RA (Figure 2(c)). At P45, Avastin increased VF VEGF levels in the left and right eyes exposed to IH (Figure 2(d)). In the choroid, Avastin increased VEGF levels in the right eyes exposed to IH and in the left eyes exposed to hyperoxia and IH (Figure 2(e)). At P45, choroidal VEGF remained elevated in the saline-treated right and left eyes exposed to hyperoxia and IH compared to RA controls, and in the Avatin-treated right eye exposed to IH (Figure 2(f)).

3.3. Avastin Decreases sVEGR-1 in IH. sVEGFR-1 is an endogenous inhibitor of VEGF action. To establish whether Avastin influences systemic sVEGFR-1, we examined the levels in serum at P23 and P45 (Table 2). Avastin had no effect on serum sVEGFR-1 levels in any oxygen environment, although the levels were suppressed with hyperoxia and IH at P23 and sustained until P45. At P23, Avastin increased retinal sVEGFR-1 levels in the untreated right and treated left eyes exposed to hyperoxia (Figure 3(a)). At P45, all IH exposed eyes had higher sVEGFR-1 levels regardless of treatment (Figure 3(b)). At P23, Avastin suppressed VF sVEGFR-1 levels in the untreated right eye in RA and in the treated left eye in IH (Figure 3(c)). At P45, Avastin suppressed sVEGFR-1 levels in the VF of both untreated and treated eyes exposed

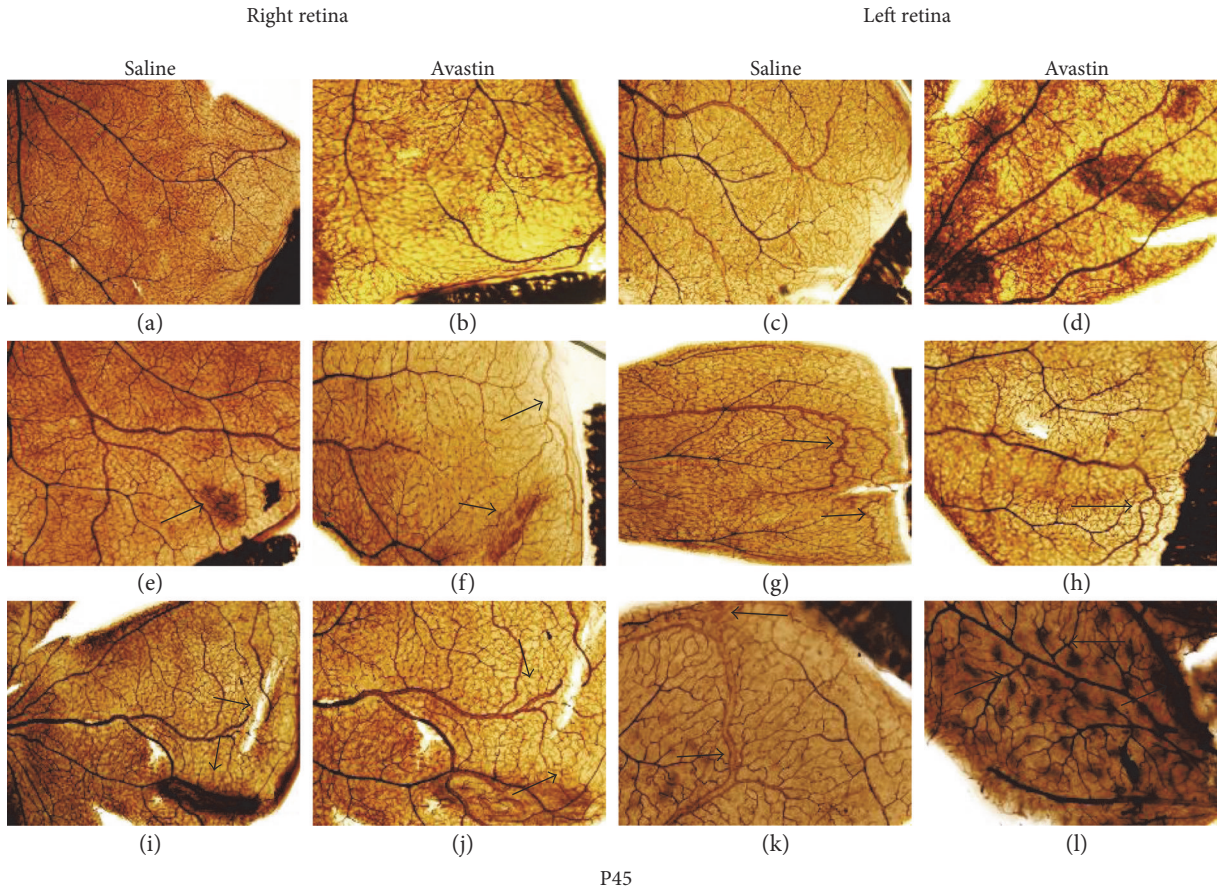

Figure 5: Retinal flatmounts showing ADPase-stained retinas from 45-day-old rats. Groups are as described in Figure 4. Images are 10x magnification. Scale bar, 100 μm.

to IH (Figure 3(d)). In the choroid, a different response was noted at P23. Avastin treatment suppressed sVEGFR-1 levels in the untreated right eyes exposed to IH but increased it in the treated eyes exposed to hyperoxia and IH (Figure 3(e)). At P45, choroidal sVEGFR-1 levels were elevated in the eyes exposed to hyperoxia and IH. Avastin treatment appeared to decrease the levels in the right untreated eyes exposed to hyperoxia and in the left-treated eyes exposed to hyperoxia and IH (Figure 3(f)).

3.4. Avastin Increases Retinal and Choroidal IGF-I in IH. IGF-I is a permissive factor for VEGF action. To establish whether Avastin influences systemic IGF-I, we examined the levels in serum at P23 and P45 (Table 2). Avastin suppressed serum IGF-I levels in IH at P23. However, at P45, both saline and Avastin-treated groups in IH had lower serum IGF-I levels. At P23, Avastin caused significant increases in retinal IGF-I levels particularly when administered in IH (Figure 4(a)). At P45, IH increased retinal IGF-I levels in the saline-treated eyes, while hyperoxia increased it in the Avastin-treated eyes (Figure 4(b)). At P23, Avastin increased VF IGF-I levels in the hyperoxia- and IH-exposed treated left eyes (Figure 4(c)). At P45, hyperoxia and IH caused elevations in VF IGF-I levels in all eyes (Figure 4(d)). Choroidal IGF-I levels were 3-4-fold higher than that of the retina. Avastin increased choroidal IGF-I levels when administered in IH at P23 (Figure 4(e)). Choroidal IGF-I levels remained elevated at P45 in all eyes exposed to IH (Figure 4(f)).

3.5. Avastin Causes Long-Term Retinal Neovascularization and Hemorrhage. At P23, Avastin-treated left eyes in RA (Figure 1(d)), hyperoxia (Figure 1(h)), and IH (Figure 1(l)) had decreased branching elements and a more limited capillary plexus compared to saline control left eyes particularly those exposed to hyperoxia. There was a difference in retinal vasculature seen in the fellow eye as well, with the fellow right eye consistently showing greater amounts of neovascularization and anastomoses in RA (Figure 1(b)), vessel dilatation in hyperoxia (Figure 1(f)), and disorganized tortuous vessels, with decreased branching at the periphery in IH (Figure 1(j)). The untreated right and left eyes showed characteristics consistent with OIR following exposure to hyperoxia (Figures 1(e) and 1(g)) and IH (Figures 1(i) and 1(k)). At P45, retinal vessels of Avastin-treated left eyes were still prominent, but greater amounts of intraretinal hemorrhage were seen in RA (Figure 5(d)) and a greater degree in IH (Figure 5(l)). There was also a suppressive effect on the untreated fellow right eyes with decreased capillary networks in RA (Figure 5(b)) and in hyperoxia (Figure 5(f)), but in IH, there was evidence of

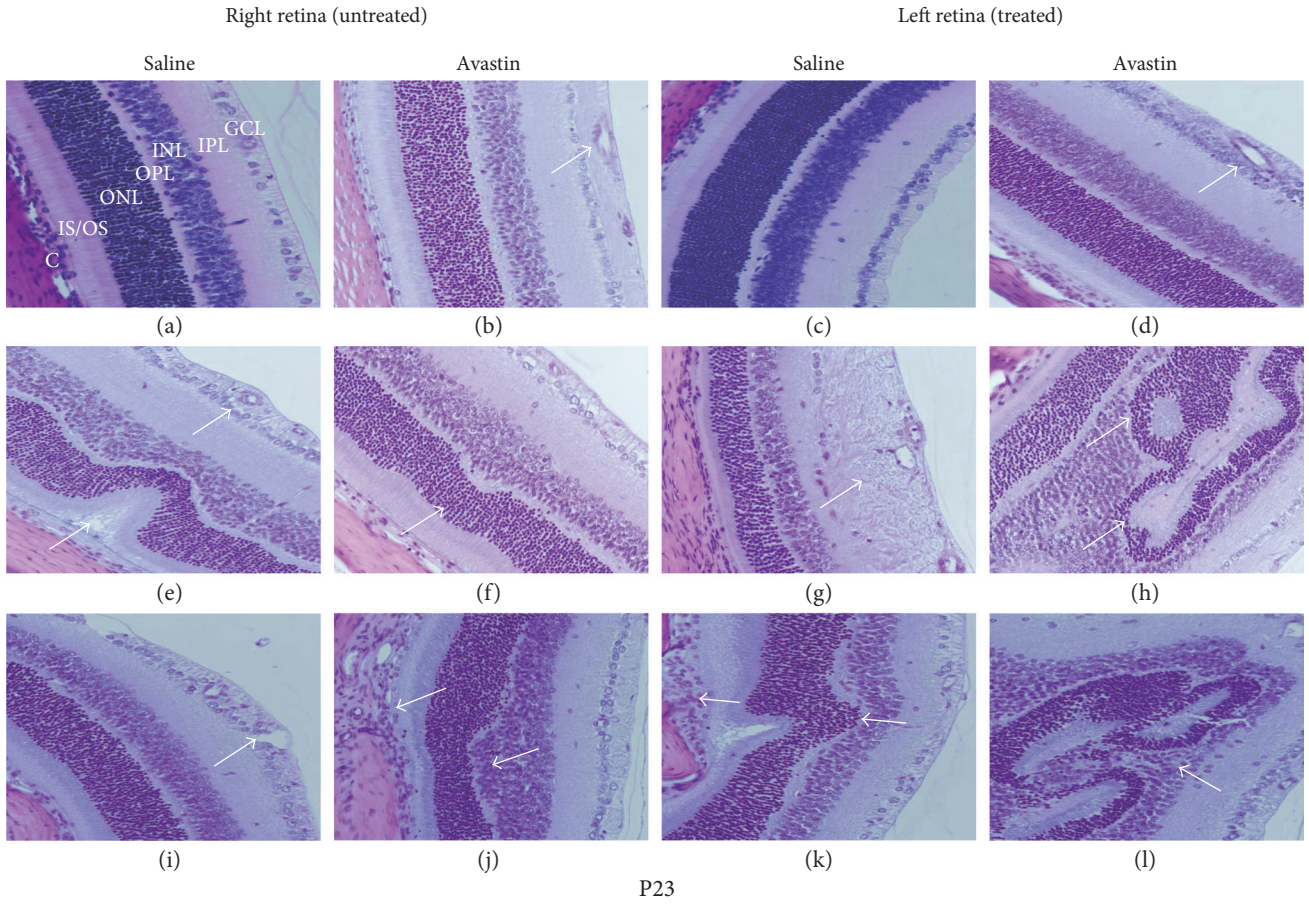

FIGURE 6: H&E stain of retinal layers from 23-day-old rat exposed to RA (a–d), 50% O_2 (e–h), and IH (i–l). (a), (e), and (i) are right untreated retinas from saline-treated groups; (b), (f), and (j) are right untreated retinas from Avastin-treated groups; (c), (g), and (k) are saline-treated left retinas; and (d), (h), and (l) are Avastin-treated left retinas. Images are 40x magnification. Scale bar, 20 μm.

dilated abundant vascular networks (Figure 5(j)). In the saline-treated eyes, characteristics consistent with OIR persisted at P45 in the hyperoxia (Figures 5(e) and 5(g)) and IH (Figures 5(i) and 5(k)) groups.

3.6. Avastin Causes Severe Retinal Abnormalities. Figures 6 and 7 show the H&E-stained retinal layers from P23 and P45 rats, respectively. The layers are labeled in Figure 6(a): NFL (nerve fiber layer), GCL (ganglion cell layer), IPL (inner plexform layer), INL (inner nuclear layer), OPL (outer plexiform layer), ONL (outer nuclear layer), IS/OS (photoreceptor inner segment/outer segment laye), and C (choroid)). A–D represent the RA groups, E–H are the corresponding hyperoxia (50% O_2) groups, and I–L are the corresponding IH (50% O_2/12% O_2) groups. All images were acquired at the central retina where most of the damage was located. At P23, Avastin treatment in RA caused NFL loss and increased blood vessel size (arrow) in the left retina (Figure 6(d)). Similar effects were noted in the untreated right retina (Figure 6(b), arrows). Avastin treatment in hyperoxia caused severe abnormalities in the IPL and INL (Figure 6(h), arrows). Saline treatment in the left eye caused significant NFL swelling and GCL degeneration (Figure 6(g), arrow). In the untreated right eye, there were mild retinal folds in the Avastin group (Figure 6(f), arrow), moderate retinal folds with photoreceptor loss, and increased blood vessel caliber and wall thickness (Figure 6(e), arrows). Severe abnormalities in the IPL and INL persisted with Avastin treatment in IH (Figure 6(l), arrow), while in the untreated eye, there was mild retinal folds and choroidal neovascularization (Figure 6(j), arrows). Saline treatment in IH caused retinal folds, photoreceptor loss, and choroidal neovascularization (Figure 6(k), arrow), while in the untreated right retina, there was mild NFL swelling and blood vessel dilatation (Figure 6(i), arrow). At P45, Avastin treatment in RA caused retinal folds (arrow), photoreceptor degeneration, and moderate loss of the NFL and GCL (arrows) in the left retina (Figure 7(d)). In the untreated right retina, Avastin caused mild retinal folds (Figure 7(b), arrows). Avastin treatment in hyperoxia caused mild retinal folds and loss or narrowing of the outer plexiform layer (Figure 7(h), arrows). Treatment with Avastin in IH caused retinal folds, moderate INL loss, and degeneration of NFL and GCL (Figure 7(l), arrows). Saline treatment in IH caused marked choroidal dilation (Figure 7(k), arrow). In the untreated right retina, Avastin caused mild retinal folds, loss of OPL, and GC atrophy (Figure 7(j), arrows).

3.7. Avastin Effects On Vascular Density. Quantification of vascular density for the right contralateral eyes (RE) and

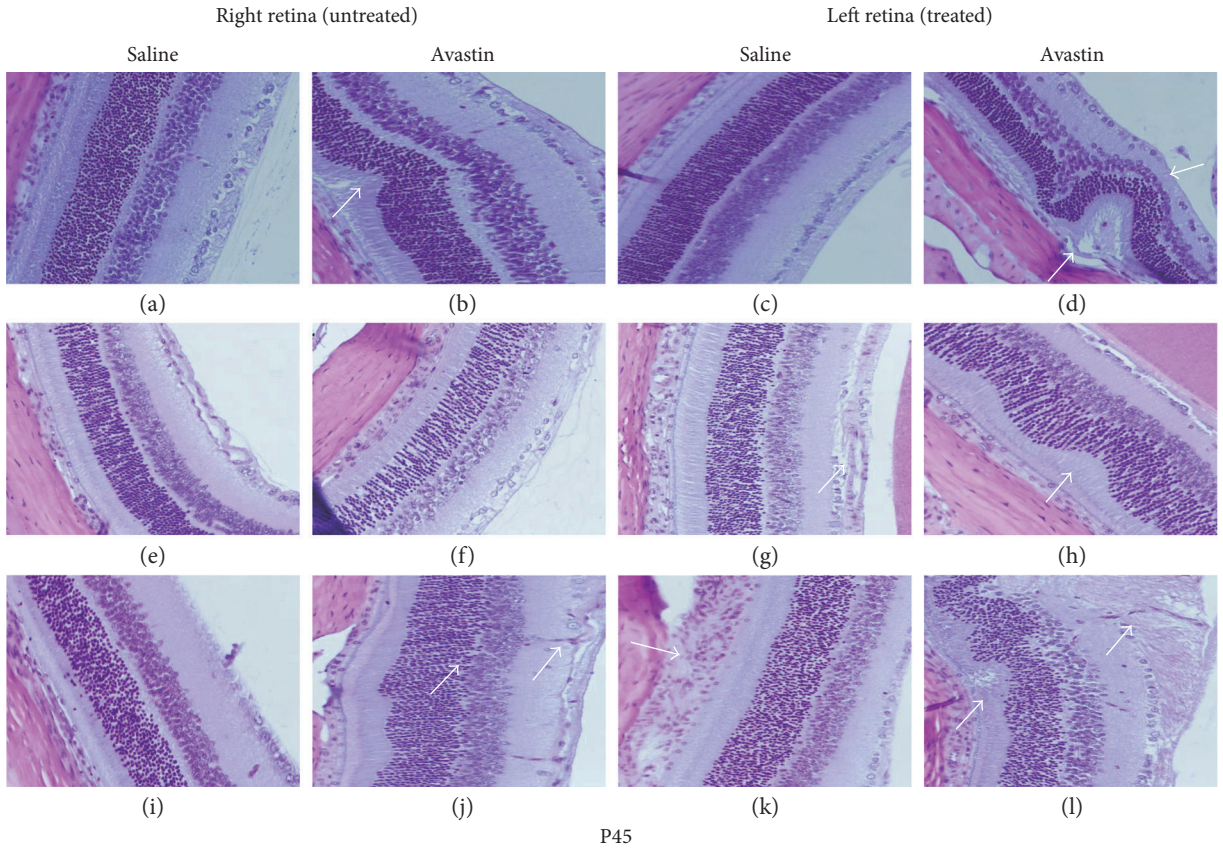

Figure 7: H&E stain of retinal layers from 45-day-old rats. Groups are as described in Figure 6. Images are 40x magnification. Scale bar, 20 μm.

Table 3: Quantitation of retinal vasculature (right eyes).

	Room air (RA)		50% O_2		IH (50% O_2/12% O_2 cycling)	
	Saline	Avastin	Saline	Avastin	Saline	Avastin
P23						
Vascular density (%)	35.8 ± 3.6	$34.1 \pm 6.7^{\S\S}$	$33.4 \pm 2.1^{**}$	31.4 ± 3.3	$33.5 \pm 4.7^{**}$	25.6 ± 4.5
Total vessel network	23134 ± 7786	18391 ± 4537	$15330 \pm 1803^{**}$	$15020 \pm 2031^{\dagger}$	$16141 \pm 3644^{**}$	$12867 \pm 2292^{\ddagger\S\S}$
Branching points	522.5 ± 41.1	415.5 ± 49.4	$316.5 \pm 45.4^{**}$	$261.8 \pm 59.4^{\ddagger\S\S}$	$343.0 \pm 33.8^{**}$	$235.3 \pm 63.1^{\ddagger\S\S}$
Number of segments	904.0 ± 103.2	$732.6 \pm 127.2^{\S\S}$	$580.8 \pm 98.1^{**}$	$493.8 \pm 99.7^{\ddagger\S\S}$	$619.5 \pm 117.5^{**}$	$449.3 \pm 109.8^{\ddagger\S\S}$
Mean segment length	26.0 ± 2.8	$29.0 \pm 1.4^{\S\S}$	26.8 ± 1.7	$30.8 \pm 3.4^{\S\S}$	27.3 ± 5.3	29.0 ± 2.3
P45						
Vascular density (%)	31.3 ± 4.5	33.1 ± 1.8	$36.0 \pm 3.1^{*}$	34.5 ± 2.3	$36.9 \pm 4.4^{**}$	$30.9 \pm 4.2^{\S\S}$
Total vessel network	15764 ± 2492	16849 ± 3147	$18180 \pm 1929^{*}$	18792 ± 2616	$19177 \pm 2281^{**}$	$15483 \pm 2488^{\S\S}$
Branching points	320.7 ± 83.5	287.8 ± 58.6	$401.3 \pm 56.0^{*}$	$241.3 \pm 65.2^{\S\S}$	$451.8 \pm 63.4^{**}$	$290.4 \pm 68.9^{\S\S}$
Number of segments	599.3 ± 127.4	628.4 ± 127.6	$718.3 \pm 73.8^{*}$	711.2 ± 105.0	$784.0 \pm 140.3^{**}$	$530.0 \pm 123.8^{\S\S}$
Mean segment length	26.3 ± 1.2	$31.0 \pm 4.7^{\S\S}$	25.3 ± 1.5	$26.7 \pm 4.2^{\dagger}$	25.5 ± 3.4	$29.7 \pm 2.7^{\S\S}$

Data are mean $\pm$ SD; $^{*}p < 0.05$, $^{**}p < 0.01$ versus saline RA; $^{\dagger}p < 0.05$, $^{\ddagger}p < 0.01$ versus Avastin RA; $^{\S\S}p < 0.01$ versus saline ($n = 3$ retinas/group; 12 measurements per group). IH: intermittent hypoxia.

the left-treated eyes (LE) is presented in Tables 3 and 4, respectively. Data showed that exposure to 50% O_2 and IH significantly reduced vascular density and branching points, and the effects worsened with Avastin treatment. The effects of Avastin treatment in IH persisted until P45.

4. Discussion

Whereas laser photocoagulation is the "gold standard" for the clinical treatment of ROP, there are instances when its use is associated with poor anatomic and visual outcomes.

Table 4: Quantitation of retinal vasculature (left eyes).

	Room Air (RA)		50% O_2		IH (50% O_2/12% O_2 Cycling)	
	Saline	Avastin	Saline	Avastin	Saline	Avastin
P23						
Vascular density (%)	34.3 ± 3.2	32.5 ± 1.5	32.7 ± 5.1	30.4 ± 6.4	36.2 ± 4.3	31.4 ± 3.4§§
Total vascular area	14928 ± 1332	17045.0 ± 1981§§	15544± 4433	14654 ± 4274†	17963 ± 2611**	14339± 2668‡§§
Number of branching points	278.0 ± 13.4	347.3 ± 76.0§§	289.2 ± 103.8	267.8 ± 97.6	407.7 ± 113.1**	255.0 ± 71.2†§§
Number of segments	515.7 ± 23.3	622.3 ± 127.6§§	533.2 ± 177.8	512.4 ± 78.1	721.5 ± 178.5**	487.8 ± 132.3†§§
Mean segment length	29.0 ± 1.4	27.7 ± 3.1	30.2 ± 4.5	29.8 ± 4.2	25.5 ± 2.7*	30.0 ± 3.2§§
P45						
Vascular density (%)	34.5 ± 3.4	29.8 ± 4.5	37.9 ± 2.0	31.6 ± 3.3§	38.1 ± 8.1	24.1 ± 5.8‡§§
Total vessel network	17233 ± 2158	16076 ± 2604	22101 ± 5106*	17434 ± 3190§	19773 ± 4041	12715 ± 2772†§§
Branching points	364 ± 76.3	277.0 ± 58.6§§	522.8 ± 124.8**	347.3 ± 55.8§§	518.0 ± 112.1**	198.3 ± 91.0†§§
Number of segments	653.0 ± 119.0	524.0 ± 118.7§§	896.0 ± 187.9**	622.5 ± 85.1§§	876.8 ± 222.2*	382.8 ± 137.9†§§
Mean segment length	26.8 ± 2.4	27.0 ± 2.1	24.5 ± 0.6	28.3 ± 3.3‡§§	24.5 ± 7	34.5 ± 4.2‡§§

Data are mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$ versus saline RA; $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.01$ versus Avastin RA; $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ versus saline ($n = 3$ retinas/group; 12 measurements per group). IH: intermittent hypoxia.

The use of pharmacotherapy is contemplated in these high-risk cases and is gaining popularity. Although safe and effective in adults with minimal systemic adverse outcomes, there is a concern that intravitreal exposure may have a far different safety profile in small neonates [15]. Although adverse events associated with Avastin in ROP are rare, they are not negligible and include retinal detachment, vitreous and retinal hemorrhage, choroidal rupture, and spreading via the bloodstream into the fellow eye [16–21, 36–39]. These adverse events suggest that the immature retina may be vulnerable to VEGF blockade which may cause breakdown of the blood-ocular barrier [40]. More importantly, if Avastin interferes with the immature blood-ocular barrier, it is possible that it may cross into the systemic circulation and have inhibitory effects on VEGF in the developing neonate's vital organs, such as the brain [21] and lungs [41]. There are no studies examining the effects of Avastin treatment in the setting of neonatal IH. This is crucial since preterm infants at risk for severe ROP experience several hundred episodes of IH over the first few weeks of life [3]. For this reason, we used a well-established model of OIR which closely resembles neonatal IH experienced by ELGANs [29–34]. An important finding that concurs with others is that Avastin leaks into the fellow untreated eye and the systemic circulation, providing evidence that the contralateral eye should never be used as a pure control eye if manipulations are performed in the study eye. Avastin has long-term adverse effects on the retina resulting in hemorrhage, neovascularization, and photoreceptor abnormalities, particularly when administered in IH. Given that Avastin is a permanent VEGF inhibitor and that VEGF is a vascular permeability factor, it is counter-intuitive that its use is associated with hemorrhage as reported in many human and animal studies including the present study. In addition, the manufacturer's package insert warns against the use of Avastin in patients with serious hemorrhage. Gastrointestinal bleeding, central nervous system (CNS) hemorrhage, and vaginal bleeding occurred up to 5-fold more frequently in patients receiving systemic Avastin. Serious or fatal pulmonary hemorrhage occurred in 31% of patients treated with Avastin (Avastin package insert). Our recent findings of pulmonary hemorrhage in rats treated with intravitreal Avastin concur with the package insert [41]. The robust rebound elevations in VEGF, including in the untreated fellow eye, may provide at least one mechanism. Thus, it is reasonable that preterm infants with chronic lung disease experiencing many episodes of IH will have elevated levels of VEGF via $HIF_{1\alpha}$ upregulation. VEGF, being a vascular permeability factor, will cause vascular leakiness and hemorrhage. This has been repeatedly shown in our rat model of OIR [29, 31–34]. Administration of intravitreal Avastin in the setting of IH will result in adverse ocular and systemic effects, due to drug leakage from damage vessels.

Serum VEGF levels are key indicators of the potential of intravitreal Avastin to cause systemic effects. Previous studies found decreased serum VEGF levels in rats that received murine VEGF-A antibody [16], as well as in human infants who received 0.25 or 0.5 mg intravitreal Avastin [42]. These studies suggest the ability of VEGF inhibitors to escape the vitreous and enter into the general circulation to cause considerable alterations in serum VEGF. No systemic sequelae were noted in these studies; however, both had relatively short study periods. In the present study, serum VEGF levels showed two new long-term patterns not previously seen. In rats raised in room air without OIR, intravitreal Avastin caused long-term anti-VEGF effects systemically as serum VEGF levels were significantly lower at P45. This resulted in a substantial difference in weight accretion which is most likely due to altered blood vascular and/or organ growth. This finding is troubling since VEGF is important for the development of the vital organs. In stark contrast to the rats raised in RA, the animals treated with intravitreal Avastin in IH showed a robust rebound elevation in systemic VEGF levels at P45, with no reciprocal body weight accretion. The accumulation of VEGF concurrent with IH-induced vascular impairment may lead to vessel permeability and hemorrhage.

The increase in serum VEGF may represent a long-term compensatory systemic upregulation after the initial suppression by Avastin. It was interesting to note that Avastin had no substantial effect on serum sVEGFR-1, the endogenous inhibitor of VEGF which acts as a VEGF trap, or IGF-I which is a permissive factor for VEGF. sVEGFR-1 is associated with normalized angiogenesis [43]. However, Avastin treatment in IH caused a persistent decrease in serum sVEGFR-1 at P45. Similar latent systemic effects following intravitreal Avastin was demonstrated by Jalali et al. [19] who reported hepatic dysfunction in a 3-month old infant. Together, these findings confirm that Avastin enters general circulation and stays for weeks, which may result in permanent systemic adverse effects [42]. Therefore, treatment of preterm infants with already compromised blood-retinal barrier and retinal microvascular impairment will allow for even more drug to enter the systemic circulation, thus causing worse morbidities.

Long-term human infant studies of intravitreal Avastin are lacking. Martinez-Castellanos et al. [8] had the longest prospective case study of 5 years and actually used an adult dose of 1.25 mg Avastin per injection with encouraging results. All eyes in that study showed excellent anatomical success as well as visual outcomes; however, the study only included 13 patients and did not have a control group. In our study, the long-term effects of Avastin were most evident in the ocular compartment as evidenced by the retinal flatmounts and H&E stains. It should be noted that Avastin was administered on day 14, and samples were collected at day 23. Therefore, the increased retinal VEGF levels one week later may represent rebound effects. At P45, all IH exposed animals had higher retinal and choroidal VEGF levels, including the Avastin-treated groups. These high levels correlated with characteristics consistent with ROP (Figure 5) and are consonant with previous findings of a "late reactivation" phenomenon of ROP after intravitreal Avastin [17]. There are also case reports of choroidal ischemia with exudative retinal detachment, retinal break with macular hole, perivascular exudation and arterial narrowing with optic atrophy, and RPE/choroidal rupture [17–19]. A bilateral effect of unilateral Avastin has been previously reported [20]. Our data provide support to those previous findings, particularly when Avastin was administered in IH, which is most likely due to increased vessel permeability. The retinal folds and photoreceptor damage noted in the present study was also consistent with the previous reports [23].

One phenomenon that is often overlooked is the role of VEGF in the choroid and RPE. The use of VEGF inhibitors (particularly in the premature neonate) will alter choroidal blood flow [44] and result in apoptosis of the photoreceptors [45, 46]. Indeed, Avastin has been shown to cause inactivation of RPE cells, profound retinal dysfunction, and rapid and progressive dysfunction of cone receptors as determined by ERG [46]. The images of photoreceptor abnormalities (particularly in the intermittent hypoxia groups) as demonstrated in our H&E stains, as well as those of others [23], confirm the devastating effects of Avastin on the photoreceptors. There is evidence that intravitreal Avastin injection may be beneficial in ROP, especially in cases of posterior or severe disease. However, a strong case can be made regarding the potential adverse effects of leakage into the systemic circulation and possibly delayed, long-term adverse ocular and systemic effects, confirming that the drug is not restricted to the intended site. This fact alone should be a cause for concern and warrants the parsimonious use of anti-VEGF therapies in the setting of neonatal IH. As with all medical therapies, one must seriously consider the risk-benefit ratio when deciding on using intravitreal Avastin in the preterm neonate with an already compromised blood retina barrier and who experiences frequent IH episodes. We agree with Morin et al. [21] that more long-term follow-up studies are warranted, particularly when anti-VEGF therapies are used in the preterm infant.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Review Article

Use of Anti-VEGF Agents in Glaucoma Surgery

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A number of antivascular endothelial growth factor agents are currently available to treat various ocular conditions. These agents have similar, but distinct, biologic qualities and have been explored in the management of neovascular glaucoma and in glaucoma surgery. Several different delivery methods are described, and because these medications are routinely given as intraocular injections, some benefits over traditional antifibrotic medications when used in glaucoma surgery are noted. These agents effectively induce regression of anterior segment neovascularization and facilitate initial surgical management of neovascular glaucoma, but the long-term outcome of this condition remains dependent on definitive management of the underlying process. Use in trabeculectomy or tube shunt procedures for other types of glaucoma has shown promise in modulating bleb morphology but has not yet been found to be as effective as traditional antifibrotic agents. There are reports of persistently raised intraocular pressure after repeated use of the anti-VEGF agents, possibly related to frequency of injection. These medications have wide application in the field of surgical glaucoma, but a definitive role has yet to be defined.

1. Introduction

The potential of antivascular endothelial growth factor (anti-VEGF) agents to modify the disease course of neovascular glaucoma (NVG) was recognized shortly after their use in the treatment of age-related macular degeneration was reported. These medications were noted to induce rapid regression of the anterior segment neovascularization that characterizes NVG. This response has changed the way that newly diagnosed NVG is managed, although the effect of these agents on long-term clinical outcomes is less clear. Due to the role of VEGF in fibrosis, the anti-VEGF agents have been widely used not only in NVG but also to modify the wound healing response in traditional glaucoma surgery. Experiments have been performed considering the effect on bleb morphology, trabecular function, and retinal ganglion cell survival. We review the current use of these agents in various surgical glaucoma scenarios, as well as the direct effects of their injection on intraocular pressure (IOP).

2. Anti-VEGF Agents

The effect of VEGF on wound healing is related to its role in both vascularization and fibrosis of tissues involved. Each of the VEGF isoforms is active in normal and pathologic vascular endothelial growth and permeability. These growth factors have been found to affect fibrosis and collagen deposition in normal wound healing [1]. Due to this dual mechanism of action, the ability to block this family of molecules has the potential to impact diseases where there is pathologic overexpression of VEGF or when it is desirable to modulate the normal healing response, as in glaucoma filtering surgery.

Each of the anti-VEGF agents that are currently used to treat ocular conditions has been applied specifically in glaucoma management and surgery. Pegaptanib (Macugen, Pfizer, New York), bevacizumab (Avastin, Genentech Inc., San Francisco, CA), ranibizumab (Lucentis, Genetech Inc., San Francisco, CA), and aflibercept (Eylea, Regeneron,

New York) vary in their affinity for the subtypes of the VEGF molecule.

Pegaptanib is an aptamer that selectively prevents the $VEGF_{165}$ isomer of VEGF-A from binding to its receptors. Bevacizumab is a full-sized monoclonal antibody that binds all isomers of VEGF-A. Ranibizumab is the antigen-binding fragment (Fab) of a similar antibody that has a slightly higher affinity for all isomers of VEGF-A. Aflibercept is a recombinant fusion protein that also binds all isomers of VEGF-A and also binds VEGF-B and the related placental growth factor (PlGF) [2].

Each of these agents has been described as efficacious in the initial management of NVG [3–6] and has been used as an experimental surgical adjunct in traditional glaucoma filtering surgeries [1, 7–9]. There is extensive literature addressing the differential clinical response of each medication in the management of retinal diseases, but there are not currently comparative studies in either the management of NVG or as agents for use in glaucoma surgery. For the purposes of this review, the different agents will be highlighted as they have been reported, but it should be noted that there is no current evidence to suggest one is more efficacious than another in managing NVG or filtering glaucoma surgery. It is also anticipated that biosimilar drugs to each of these agents will become available. The most widely reported one, Razumab (Intas Pharmaceuticals, Ahmedabad, India), is a biosimilar to ranibizumab and is approved for use in India. Limited data is currently available on its role in NVG or glaucoma filtering surgery.

3. Delivery Methods Addressing NVG

For the management of NVG, the initial delivery of the anti-VEGF agents is generally in a standard intravitreal injection, although other methods have been described. Waisbourd et al. described some success inducing regression of neovascularization following four times daily topical administration of bevacizumab [10]. Several authors have reported rapid regression of anterior segment neovascularization following injection of bevacizumab into the anterior chamber [11–13]. A complicating factor to the standard intravitreal injection is that patients who present with a new diagnosis of NVG often have very elevated IOP, and the additional intravitreal volume exacerbates this situation. Patients with elevated IOP are frequently managed medically in clinic until the IOP reaches a more acceptable level. Alternatively, an anterior chamber paracentesis can be performed at the time of the intravitreal injection. In this setting, there may also be a role for these alternative methods of delivery.

4. Delivery Methods in Filtering Surgery

Use of antifibrotic agents such as 5-fluorouracil (5-FU) and mitomycin C (MMC) during traditional glaucoma surgery has been confined primarily to either saturated sponges at the time of surgery or subconjunctival injection during or after surgery. These routes of administration are used due to their immediate and local effect and, more importantly, to limit their toxicity to intraocular structures. The anti-VEGF agents are considered safe for intraocular injection, and coincidentally, investigators have used injections into the vitreous and anterior segment, as well as the traditional modes of topical and subconjunctival administration.

Nomoto et al. demonstrated in a rabbit model that intravitreal administration resulted in a higher concentration of bevacizumab within the eye compared to a subconjunctival administration; however, this has limited application to glaucoma surgery as in this case the site of desired action is extraocular [14]. Moisseiev et al. observed intravitreal concentrations of bevacizumab in patients undergoing vitrectomy and showed that, in patients without prior vitrectomy, the half-life was approximately 5 days [15]. They also showed that, in one patient who had undergone a prior vitrectomy, the calculated half-life was greatly reduced to less than 1 day.

The application of these findings to both the treatment of NVG and the management of glaucoma filtering surgery is not yet completely understood. In general, when a rapid effect is needed to control anterior segment neovascularization, intravitreal or intracameral injection of an anti-VEGF agent will be effective. Given the shorter half-life concentrations observed in postvitrectomy eyes, faster recurrence of anterior segment neovascularization might be expected in the absence of more definitive therapy such as panretinal photocoagulation (PRP) when anti-VEGF agents are injected into either the anterior chamber or a postvitrectomy posterior chamber. It is possible that a similar effect will become pertinent in the use of anti-VEGF agents in glaucoma filtering surgery. Direct application by sponges or injection will result in much higher local concentrations initially; however, a more prolonged low-level effect could be observed following injection into the vitreous. The clinical relevance of these effects is currently unknown.

5. Indications and Outcomes in Glaucoma Surgery for NVG

Neovascular glaucoma occurs when an ischemic process induces secondary angle closure due to fibrovascular proliferation on the iris and into the anterior chamber angle. The most common causes include diabetic retinopathy, retinal vein occlusions, and ocular ischemic syndrome. Historically, outcomes have been poor, as glaucoma filtering surgeries have a high rate of failure and recurrence of the condition is common [16]. Even with adequate control of the underlying condition, visual outcomes have been poor. Control of elevated IOP often requires multiple interventions including medications, tube shunt surgery, and cyclodestructive procedures.

With the advent of the anti-VEGF agents, the success in controlling active neovascularization has been greatly enhanced. Several authors have reported positive short- and long-term results of various injection regimens in NVG with the various anti-VEGF medications [6, 11–13, 17]. With adequate control of the neovascularization, glaucoma surgery has become possible, although it still depends on control of the underlying condition. The long-term success of trabeculectomy in NVG has not been definitively shown to be

improved by injecting bevacizumab as compared to MMC alone, although changes in bleb vascularity have been reported [18, 19]. Several authors have also reported similar results with glaucoma tube shunt implantation in NVG. Although there are beneficial effects including less anterior segment bleeding, the long-term outcomes with regard to visual acuity and IOP control are more dependent on definitive control of the underlying condition than on any specific perioperative anti-VEGF injections [20–22].

Laser therapy is often employed in the management of NVG. Panretinal photocoagulation (PRP) may be performed to address the underlying retinal disease and to reduce retinal oxygen demand and release of VEGF prior to surgical intervention for NVG. In a retrospective, consecutive case-control study, Ehlers et al. compared combined PRP and intravitreal bevacizumab to PRP monotherapy [23]. The authors reported that combination treatment resulted in more rapid decrease in IOP and increased frequency and faster regression of neovascularization. Cyclophotocoagulation (CPC) diode laser is often reserved for eyes with poor visual potential or when other surgical options have failed. CPC laser is effective, quick, and useful for patients who are unable to undergo incisional surgery. Gosh et al. evaluated combined CPC and intravitreal bevacizumab in eyes with NVG and reported rapid regression of neovascularization, IOP control, and symptomatic relief [24].

6. Indications and Outcomes in Glaucoma Surgery Aside from NVG

The role of antifibrotic agents in traditional glaucoma filtering surgery is well known. With the use of 5-FU and MMC, trabeculectomy in particular became more successful at reaching target IOP [25]. With the increase in efficacy, however, there has also been an increase in the incidence of bleb-related complications. Consequently, there is great interest in using a more focused approach to wound healing modulation. VEGF has several roles in wound healing, and the previously mentioned differential affinity of the different anti-VEGF agents may someday be used to exploit this. $VEGF_{165}$ and $VEGF_{121}$ more directly affect angiogenesis while the isomer $VEGF_{189}$ has more of an impact on fibrosis [8].

Multiple studies have evaluated the use of bevacizumab or ranibizumab as an alternative or adjunct to MMC at the time of trabeculectomy [26–30]. As mentioned above, routes of administration include topical, subconjunctival, intracameral, and intravitreal [31]. Many of the reports have shown a difference in bleb morphology in eyes treated with anti-VEGF agents. Early postoperative results have shown less vascular and more diffuse blebs compared to MMC; however, this effect appears to fade and longer-term outcomes show more vascularity and higher IOP when they were used as single agents compared with MMC [26, 29, 32].

There are also reports of subconjunctival injection of bevacizumab for use in rescuing failing filtering blebs [33, 34]. The use of 5-FU in particular is well described for this, and Freiberg et al. showed a decrease in the number of 5-FU injections required when used in conjunction with bevacizumab [33]. In a similar pattern of results to those evaluating stand-alone use in trabeculectomy, bleb vascularity was reportedly improved following these treatments; however, they did not demonstrate an improvement in long-term IOP. Postoperative use of anti-VEGF agents may have benefit in bleb rescue, but studies have not demonstrated a significant effect on IOP to date.

In glaucoma tube shunt surgery, the use of antifibrotic agents is less well defined than in trabeculectomy. Several large studies have addressed the use of MMC during tube shunt implantation and have failed to show a long-term effect on IOP [35, 36]. There are, however, newer reports of a limited role using MMC and 5-FU in tube shunt surgery [37]. Many studies have evaluated the use of anti-VEGF agents as adjuncts in tube shunt surgery for NVG, but only a few small studies have examined their use for the same procedures in other forms of glaucoma [38, 39]. Rojo-Arnao et al., using a postoperative series of bevacizumab injections, showed a decreased hypertensive phase without a long-term impact on IOP [38]. Desai et al. used intravitreal ranibizumab at the time of tube implantation surgery and found a trend toward improved outcome [39].

There have been some reports of rapid conjunctival dehiscence or necrosis with the subconjunctival injection of bevacizumab or ranibizumab. Georgalas et al. reported a case of conjunctival necrosis that occurred in a patient with a long-standing trabeculectomy shortly after a ranibizumab injection for AMD [40]. A prospective trial by Sengupta et al. comparing bevacizumab with either sponges or injection to MMC sponges used at the time of filtering surgery documented a case of conjunctival necrosis in the bevacizumab injection group [41]. Finally, Miraftabi and Nilforushan reported two cases of severe conjunctival dehiscence following placement of a glaucoma drainage implant with intraoperative subconjunctival injection of bevacizumab [42]. While most studies document a lesser effect of the anti-VEGF agents compared to MMC or 5-FU, these agents are not without risk and complications may still occur in a subset of patients.

7. Side Effects of Anti-VEGF Agents

Many adverse effects of anti-VEGF agents have been reported, including vitreous hemorrhage, lens injury, retinal detachment, central retinal artery occlusion, morphologic changes in corneal fibroblasts, and endophthalmitis. Elevated IOP, transient or sustained, is often a concern for patients with either ocular hypertension or pre-existing glaucoma [43].

In the initial treatment of NVG, additional intraocular volume from an injection frequently leads to worsened IOP elevation, necessitating anterior chamber paracentesis or aggressive medical management. This acute IOP elevation from anti-VEGF injection occurs in all eyes, but with a normally functioning outflow pathway, the majority of patients undergoing injections for conditions not resulting in NVG will return to a normal IOP in a matter of minutes [44]. Aside from this acute rise in IOP, persistent elevation in IOP does occur in a small subset of patients. Jalil et al. first reported a case in a patient with pre-existing ocular hypertension and

receiving bevacizumab, but subsequent cases have been described in patients receiving each of the various agents and frequently with no prior history of ocular hypertension or glaucoma [45–48]. The reason for this phenomenon is unclear. There are reports of possible direct trabecular damage from contaminants, as well as some evidence suggesting that a low-lying inflammatory reaction leads to the effect [49, 50]. Another recent report suggested that the risk of requiring glaucoma surgery is elevated with accumulating numbers of injections, becoming more marked when patients require more than seven annual injections [51]. Regardless of the exact mechanism, closer observation of IOP is recommended as the frequency of injection increases.

8. Conclusion

The anti-VEGF agents have rapidly changed the management of many retinal conditions, including AMD, diabetic retinopathy, and vein occlusions. Their impact on surgical glaucoma is less clear. They have made the largest impact in the initial management of NVG. A single injection is often sufficient to induce regression of active neovascularization, which then facilitates initial surgical management and simplifies control of the underlying condition. Unfortunately, the long-term outcome of NVG remains poor with few studies showing a benefit to visual acuity or IOP with the adjunctive use of anti-VEGF medications. In traditional glaucoma surgeries, the use of anti-VEGF therapy has also met with limited success. Changes in bleb morphology after trabeculectomy or tube show the potential of these agents to influence the healing process; however, the long-term effects on IOP have yet to be realized.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Clinical Study

Effect of Preoperative Intravitreal Bevacizumab on the Surgical Outcome of Neovascular Glaucoma at Different Stages

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Purpose. To evaluate the effect of preoperative intravitreal bevacizumab (IVB) injection on the surgical outcome of Ahmed glaucoma valve (AGV) implantation according to the angle status in neovascular glaucoma (NVG) eyes. *Materials and Methods*. This retrospective study included 70 NVG patients who underwent AGV implantation and were followed up for at least 12 months. An IVB injection before AGV implantation was administered to 45 eyes (IVB group), while it was not administered to 25 eyes (control group). Subgroup analyses were done at different stages in terms of the extent of peripheral anterior synechiae (PAS). *Results*. Mean follow-up period after AGV implantation was 27 ± 15 months. The IVB group showed higher prevalence of the eyes with less than 50% of PAS than that of the control group (78% versus 44%). The overall success rate 1 year postoperatively was 80% and 64% for the IVB and control groups, respectively ($P = 0.142$). When PAS extent was less than 50%, preoperative IVB had a marginally positive effect on surgical outcome (HR = 0.39, $P = 0.064$, per 1-time IVB injection). *Conclusions*. Preoperative IVB may enhance the success rate of AGV implantation in NVG eyes, before PAS has extensively formed. Further prospective randomized studies controlling the extent of PAS are warranted.

1. Introduction

Neovascular glaucoma (NVG) is still a medical and surgical challenge for ophthalmologists. The main causes of NVG are ischemic retinal conditions, such as proliferative diabetic retinopathy (PDR), central retinal vein occlusion (CRVO), and ocular ischemic syndrome (OIS) [1]. New vessels (NV) that are formed at the iris and anterior chamber angle and contracture of the fibrovascular membrane at the angle result in progressive angle closure and intraocular pressure (IOP) elevation [2]. The principle reason of IOP elevation is secondary angle closure due to peripheral anterior synechiae (PAS) [2].

Several angiogenic factors are involved in the neovascularization of the anterior segment in NVG [1]. Among them, the role of vascular endothelial growth factor (VEGF) type A has been well characterized in the pathogenesis of NVG [3, 4]. Since a significantly increased level of VEGF was detected in the aqueous humor in the eyes of patients with NVG [4], treatment that particularly targets this angiogenic factor has emerged. Bevacizumab (Avastin; Genentech Inc., South San Francisco, CA), which is a monoclonal antibody that was approved for treatment of metastatic colon cancer in 2004 by the US Food and Drug Administration, has also been used to treat NVG. Many reports have been published about the effect of intravitreal bevacizumab (IVB) injection on NVG [5–14]. Adjuvant IVB injection may lead to regression of NV in the iris and angle, thus, reducing the incidence of hyphema and potentially enhancing the surgical outcome of Ahmed glaucoma valve (AGV) implantation in NVG [10, 12]. However, its role in treating NVG is currently limited. Further, IVB showed no significant effect on the long-term surgical outcome in some publications [5, 9, 10, 12].

The effect of IVB on medical treatment of NVG at different stages was previously reported in 2008 [15]. This study reported that while IVB might stabilize iris NV and control IOP in patients with early-stage open-angle NVG, it could not control IOP in advanced angle-closure NVG. One of the limitations of this previous study, as noted by the authors, was the absence of a control group [15].

In the present study, we compared the surgical outcomes of the eyes of patients with NVG, in terms of IOP between those who received a preoperative IVB injection with AGV implantation (IVB group) and those who underwent AGV implantation alone (control group). Additionally, we performed subgroup analysis based on the anterior chamber angle status (i.e., the extent of PAS) to assess the adjuvant effect of preoperative IVB on the outcome of AGV implantation at different stages of NVG.

2. Materials and Methods

2.1. Subjects. The medical records of patients who underwent AGV implantation for the treatment of NVG from Nov. 2010 to Dec. 2015, by a single surgeon (KRS) at Asan Medical Center, were retrospectively reviewed. NVG was diagnosed by neovascularization of the iris and/or iridocorneal angle, with an IOP of more than 21 mmHg by Goldmann applanation tonometry (GAT) [12]. Among them, patients with a follow-up period of less than 1 postoperative year, younger than 18 years, and who had undergone previous glaucoma surgery, including a cyclodestructive procedure, were excluded. Further, patients with no preoperative information about detailed angle status (assessed using gonioscopy) were excluded. Subgroups were categorized according to anterior chamber angle status, with a cut-off point of 50% PAS (less than 50% versus more than 50%), as assessed by gonioscopy using a Sussman lens. This study was approved by the institutional review board of Asan Medical Center, Seoul, Korea, and conformed the tenets of the Declaration of Helsinki.

2.2. Treatment Plan of Neovascular Glaucoma. After diagnosis of NVG, IVB injection was done as an outpatient procedure with topical anesthesia. After draping with 5% povidone iodine solution, 0.05 cc of bevacizumab (25 mg/cc) was injected intravitreally via the pars plana, approximately 3.5 mm from the limbus, with a 30-gauge needle. After injection, visual acuity (VA) was tested to verify the perfusion of the optic nerve. If required, anterior chamber paracentesis was done to prevent excessive IOP elevation. Topical prophylactic antibiotics (moxifloxacin or gatifloxacin) were prescribed 4 times per day, for 3–5 days after IVB injection. Repeated injections of IVB were performed at the discretion of the glaucoma specialist, if required.

Panretinal photocoagulation (PRP) was done before AGV implantation for the majority of patients included in this study, except where there was severe media opacity in the eye, such as corneal edema, dense cataract, hyphema, and/or vitreous hemorrhage. PRP was administered to 38 (84%) of the 45 eyes in the IVB group and to 18 (72%) of 25 eyes in the control group ($P = 0.212$ by chi-square test).

AGV implantation was performed by a single glaucoma specialist (KRS) using a standardized surgical technique. The Ahmed-FP7 (New World Medical Inc., CA) model was used for all eyes. Initially, a subconjunctival lidocaine injection was administered, either supratemporally or supranasally. A corneal traction suture was then performed, before a fornix-based conjunctival pocket was made (either supratemporally or supranasally). Although the supratemporal site was preferred, the sites were chosen according to the surgeon's discretion, depending on factors, such as conjunctival scarring, presence of peripheral anterior synechiae, and depth of the anterior chamber, which affected the entry of the tube into the anterior chamber. To prevent excessive postoperative fibrosis, vessels were not cauterized during conjunctival dissection and scleral flap formation. After exposure of the scleral bed, measuring about 5×7 mm, a limbal-based partial-thickness scleral flap was prepared using a Beaver blade. The AGV was primed with balanced saline solution to confirm patency and a polypropylene (5-0 Prolene) thread was incorporated into the tube lumen. The tube was then ligated near the tube-plate junction with an absorbable polyglactin (8-0 Vicryl) suture, and the 5-0 Prolene thread was removed. The plate was placed on the sclera about 8–10 mm behind the limbus and was secured to the sclera with 9-0 nylon. The tube was trimmed to an appropriate length and inserted into the anterior chamber through a 23-gauge needle tract beneath the scleral flap. The tube was then fixed on the sclera with a 9-0 nylon suture, and the scleral flap was closed with two 9-0 nylon sutures. Finally, the conjunctiva was reapproximated to the limbus with an 8-0 Vicryl. No subconjunctival injection of antibiotics or dexamethasone was given. Corticosteroid ointment and a pressure patch were applied at the end of the surgery. Postoperatively, topical antibiotics, steroid, and atropine were administered 2–4 times per day for 4 weeks, 4–8 times per day for 4 weeks, and 2 times per day for 2 weeks, respectively.

2.3. Data Collection. The following preoperative baseline data were collected: age, gender, the cause of NVG (e.g., PDR, CRVO, and OIS), IOP, best-corrected VA (BCVA, which was measured using the Log minimal angle of resolution [LogMAR]), presence of NV at angle, the extent of PAS, and follow-up period after AGV implantation. The main outcome measures were IOP and BCVA at final visits. Surgical success was defined as an IOP between 6 and 21 mmHg, without loss of light perception (LP), and with or without the use of antiglaucoma medication [12]. Surgical failure was defined as an IOP of more than 21 mmHg or less than 6 mmHg at two consecutive follow-up visits, the loss of LP, or a need for additional glaucoma interventions [12]. A significant change in the VA was defined as a change of two or more Snellen line VA, or a change in category (e.g., count fingers to hand motions) after surgery [10]. For statistical analysis, we used a LogMAR value of 2.6 to represent vision of counting fingers and used extrapolated LogMAR values of 2.7, 2.8, and 2.9 to represent hand motion, light perception, and no light perception, respectively [16]. One was randomly chose if both eyes were eligible.

2.4. Statistical Analysis. Data were presented as mean ± standard deviation for continuous variables or as numbers with percentages for categorical variables. Comparisons between the groups were done using an unpaired *t*-test, Mann–Whitney *U* test, chi-square test, and Fisher's exact test, as appropriate. A Cox proportional hazard analysis was done to assess risk factors for surgical failure. For subgroups that were categorized according to the extent of PAS, comparisons of

Table 1: Baseline demographics and ocular characteristics of participants (total of 70 eyes).

	Total ($n = 70$ eyes)	IVB group ($n = 45$ eyes)	Control ($n = 25$ eyes)	P value (IVB versus control)
Gender (male/female, number of patients)	56/14	38/7	18/7	0.212
Age (years)	58 ± 13	59 ± 10	57 ± 18	0.556
Causes of NVG, number (%)				
PDR	47 (67)	33 (73)	14 (56)	
CRVO	8 (11)	5 (11)	3 (12)	0.110
OIS	12 (17)	7 (16)	5 (20)	
Baseline IOP (mmHg)	40.5 ± 9.2	40.3 ± 9.7	41.0 ± 8.4	0.759
Baseline BCVA (LogMAR)	2.01 ± 1.11	1.96 ± 1.07	2.12 ± 1.20	0.556
Presence of NVA, number (%)	66 (94)	42 (93)	24 (96)	1.000
PAS extent, number (%)				
Less than 50%	46 (66)	35 (78)	11 (44)	**0.004**
More than 50%	24 (34)	10 (22)	14 (56)	
Preoperative PRP, number (%)	56 (80)	38 (84)	18 (72)	0.212
Postoperative follow-up (months) [range]	27 ± 15 [12–68]	26 ± 16 [12–68]	27 ± 14 [12–67]	0.721

IVB = intravitreal bevacizumab; NVG = neovascular glaucoma; PDR = proliferative diabetic retinopathy; CRVO = central retinal vein occlusion; OIS = ocular ischemic syndrome; IOP = intraocular pressure; BCVA = best-corrected visual acuity; MAR = minimal angle of resolution; NVA = neovascularization of angle; PAS = peripheral anterior synechiae; PRP = panretinal photocoagulation. Continuous variables are represented as mean ± standard deviation.

surgical outcomes between the IVB group and the control group were done separately. A Cox proportional hazard analysis was also performed for each subgroup. For Cox proportional hazard analysis, independent variables with $P < 0.10$ in univariate analysis were selected and entered for multivariate analysis. All statistical analyses were performed using the SPSS version 18.0 (SPSS, Chicago, IL, USA) statistical package.

3. Results

A total of 70 patients met the inclusion criteria and included in the final analysis, of which a preoperative IVB injection was administered to 45 eyes (IVB group) and was not administered to 25 eyes (control group). For the IVB group, an average of 1.56 (maximum value of 4) preoperative IVB injections was administered to each eye. All patients were Korean. The mean age was 58 ± 13 years, and mean follow-up period after AGV implantation was 27 ± 15 months. The main causes of the NVG were PDR for 47 cases (67%), CRVO for 8 cases (11%), and OIS for 12 cases (17%). Table 1 summarizes baseline characteristics for both groups. There was no significant difference in age, gender, cause of NVG, baseline IOP, BCVA, and follow-up period between the two groups. However, there was significant difference in the extent of PAS between the two groups. The proportion of the eyes where the extent of PAS of more than 50% was greater in the control group (22% versus 56%; $P = 0.004$ by chi-square test).

Table 2 summarizes changes in the IOP and BCVA after AGV implantation in both groups. No significant differences were found between the two groups, in terms of the IOP measured at 1 year postoperatively and at final visit, BCVA at the final visit, or changes in the BCVA. Subgroup analysis according to PAS extent also showed no significant differences between the two groups.

The success rates after 1 year and at the final visit are summarized in Table 3 and Figure 1. For all the eyes that were analyzed, the success rate at the final visit was 78% for the IVB group and 56% for the control group, which was marginally significant ($P = 0.057$ by chi-square test). In the subgroup where the extent of PAS was less than 50%, the success rate 1 year postoperatively was 89% in the IVB group and 64% in the control group ($P = 0.057$). In the subgroup where the extent of PAS was more than 50%, the success rates 1 year postoperatively and at final visit were similar between the two groups. The differences between the IVB group and the control group tend to be larger in the subgroup where the extent of PAS was less than 50%, which suggests that IVB plays a greater role during early stages of NVG (Figure 1).

The risk factors for surgical failure were assessed by a Cox proportional hazard analysis for all the eyes (Table 4). The univariate model indicated that PRP, PAS extent, and IVB had possible associations ($P < 0.10$). When these variables were entered into a multivariate model with backward elimination approach, only PRP and the extent of PAS showed marginal significance (hazard ratio [HR] = 0.40 and 2.25, $P = 0.072$ and 0.075, resp.). For the subgroup where the extent of PAS was less than 50%, the IVB injection showed a protective effect of surgical failure, with a HR of 0.39 and borderline significance ($P = 0.064$, per 1-time IVB injection) (Table 5). For the eyes where the extent of PAS was more than 50%, the IVB group (compared to the control group) had no significant association with surgical failure ($P = 0.775$).

4. Discussion

Traditional management of NVG includes antiglaucoma medications, PRP, glaucoma filtering surgery, and/or drainage device implantation [10]. Despite these modalities,

Table 2: Changes of intraocular pressure and best-corrected visual acuity after Ahmed glaucoma valve implantation in the 2 groups.

Total	IVB group ($n = 45$ eyes)	Control ($n = 25$ eyes)	P value
Baseline IOP (mmHg)	40.3 ± 9.7	41.0 ± 8.4	0.759
PO 1-year IOP (mmHg)	15.3 ± 3.7	15.6 ± 2.8	0.660
Final IOP (mmHg)	15.5 ± 4.2	16.6 ± 4.4	0.290
Final BCVA (LogMAR)	1.62 ± 1.19	1.92 ± 1.29	0.334
BCVA increased	16 (36)	8 (32)	
BCVA unchanged	20 (44)	8 (32)	0.320
BCVA decreased	9 (20)	9 (336)	
PAS less than 50%	IVB group ($n = 35$ eyes)	Control ($n = 11$ eyes)	P value
Baseline IOP (mmHg)	40.7 ± 9.6	38.6 ± 8.1	0.497
PO 1-year IOP (mmHg)	15.5 ± 3.5	15.6 ± 2.2	0.958
Final IOP (mmHg)	15.7 ± 4.1	15.6 ± 2.2	0.880
Final BCVA (LogMAR)	1.51 ± 1.17	0.91 ± 1.14	0.140
BCVA increased	14 (40)	5 (46)	
BCVA unchanged	14 (40)	4 (36)	1.000
BCVA decreased	7 (20)	2 (18)	
PAS more than 50%	IVB group ($n = 10$ eyes)	Control ($n = 14$ eyes)	P value
Baseline IOP (mmHg)	38.7 ± 10.3	42.9 ± 8.4	0.281
PO 1-year IOP (mmHg)	14.5 ± 4.4	15.7 ± 3.3	0.447
Final IOP (mmHg)	14.5 ± 4.4	17.4 ± 5.6	0.182
Final BCVA (LogMAR)	2.00 ± 1.25	2.71 ± 0.73	0.128
BCVA increased	2 (20)	3 (21)	
BCVA unchanged	6 (60)	4 (29)	0.273
BCVA decreased	2 (20)	7 (50)	

IVB = intravitreal bevacizumab; IOP = intraocular pressure; PO = postoperative; BCVA = best-corrected visual acuity; MAR = minimal angle of resolution; PAS = peripheral anterior synechiae. Continuous variables are represented as mean ± standard deviation and categorical variables as number (%).

Table 3: Success rates (%) after Ahmed glaucoma valve implantation in the 2 groups for total participants and subgroups according to extent of peripheral anterior synechiae.

Total ($n = 70$ eyes)	IVB group	Control	P value
PO 1 year	80 (36/45)	64 (16/25)	0.142
Final visits	78 (35/45)	56 (14/25)	0.057
PAS less than 50% ($n = 46$ eyes)	IVB group	Control	P value
PO 1 year	89 (31/35)	64 (7/11)	0.057
Final visits	86 (30/35)	64 (7/11)	0.107
PAS more than 50% ($n = 24$ eyes)	IVB group	Control	P value
PO 1 year	50 (5/10)	64 (9/14)	0.678
Final visits	50 (5/10)	50 (7/14)	1.000

IVB = intravitreal bevacizumab; PO = postoperative; PAS = peripheral anterior synechiae.

NVG is still difficult to manage and sometimes results in devastating visual loss. Therefore, early diagnosis of NVG and proper treatment to minimize visual loss and control IOP are essential. The main mechanism of NVG is retinal ischemia [5]. PRP ablates the ischemic retina to decrease tissue oxygen demand, thereby reducing VEGF release and the formation of NV [17]. When delivered before IOP elevation, PRP can reduce the incidence of NVG in ischemic ocular conditions [17]. Therefore, PRP is the essential treatment for NVG. However, it is often difficult to administer laser treatment to the eyes of patients with NVG that have a presence of cloudy media due to corneal edema that results from elevated IOP, hyphema, and/or vitreous hemorrhage. In our study, PRP was given to a total of 80% of the eyes before AGV implantation. More eyes in the IVB group underwent PRP than those in the control group (84% versus 72%), but there was no statistically significant difference ($P = 0.212$).

Recently, anti-VEGF agents have emerged as adjuvant therapy for retinal ischemia, since the level of VEGF is increased in the aqueous or vitreous of the eyes of patients with NVG [4, 18]. Angiogenic factors, including VEGF, promote the creation of fibrovascular membranes, which lead to an increase in IOP. Boyd et al. [19] reported that since the level of VEGF in aqueous had a temporal correlation with the course of NV formation in eyes of patients with ischemic CRVO, anti-VEGF treatment during the early stages of NGV might be therapeutically beneficial. Bevacizumab, an anti-VEGF monoclonal antibody, has been widely used as off-label to treat neovascular age-related macular degeneration, diabetic macular edema, and CRVO [5]. The half-time of a single dose (0.05 cc) of the IVB injection is about eight to nine days in the human eyes [20, 21].

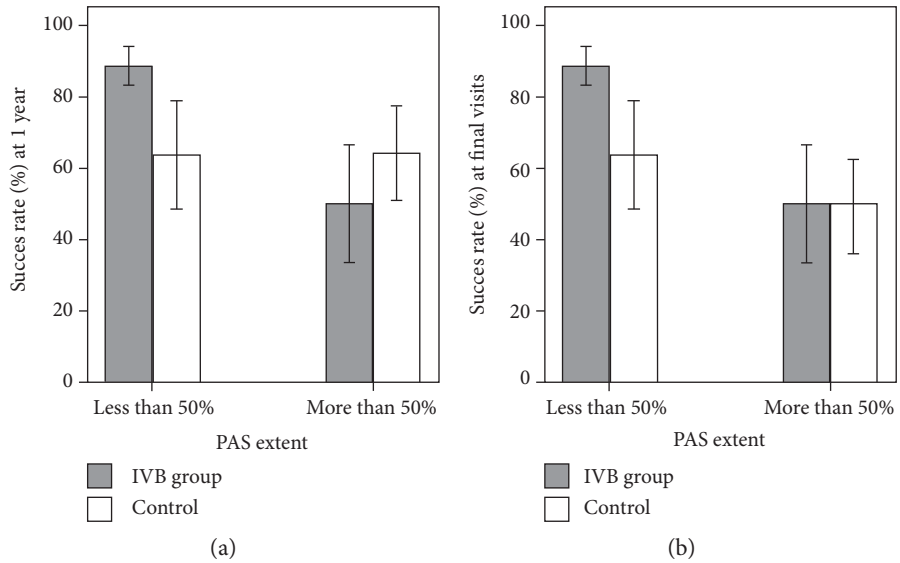

Figure 1: Graphs showing the success rates (with standard error) of glaucoma surgery, (a) at postoperative year 1 and (b) at final visits, between the preoperative intravitreal bevacizumab (IVB) group and the control group, according to the extent of peripheral anterior synechiae (PAS).

Table 4: Result of cox proportional hazard analysis for assessing risk factors of surgical failure (total of 70 eyes).

Variables	Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Preoperative PRP (versus not done)	**0.38 (0.14–1.02)**	**0.056**	0.40 (0.15–1.09)	0.072
Presence of NVA (versus absence)	0.91 (0.20–4.20)	0.907		
PAS more than 50% (versus less than 50%)	**2.33 (0.96–5.62)**	**0.061**	2.25 (0.92–5.47)	0.075
IVB group (versus control)	**0.47 (0.20–1.11)**	**0.085**		
Number of IVB injection (per 1-time injection)	**0.60 (0.35–1.03)**	**0.062**		
Baseline IOP (per 1 mmHg)	1.03 (0.99–1.08)	0.144		
Baseline BCVA (per 1 LogMAR)	1.23 (0.78–1.93)	0.368		

HR = hazard ratio; CI = confidence interval; PRP = panretinal photocoagulation; NVA = neovascularization of angle; PAS = peripheral anterior synechiae; IVB = intravitreal bevacizumab; IOP = intraocular pressure; BCVA = best-corrected visual acuity; MAR = minimal angle of resolution. Variables with $P < 0.10$ in univariate analysis (PRP, PAS extent, IVB, and number of IVB injection) were entered into multivariate analysis. Multivariate model using a backward elimination approach based on likelihood ratio; variables were entered in the model if $P < 0.05$ and removed if $P > 0.10$ in the saturated multivariate model.

Therefore, the effect of IVB may be transient and repeated injections are sometimes needed [15]. In our study, as many as 4 times, IVB injections were performed in the eyes of the IVB group (mean 1.56 times).

There have been many studies reporting the effect of bevacizumab in the eyes of patients with NVG [5–15, 17]. Wakabayashi et al. [15] retrospectively reviewed 41 patients with different stages of NVG, including iris NV without elevated IOP, open-angle NVG, and angle-closure NVG. In that study, all patients received IVB as an initial treatment, which showed no effect on controlling IOP and stabilizing NV, except for patients with angle-closure NVG [15]. Another study by Sahyoun et al. [12], which compared the surgical outcomes of NVG patients who did and did not receive preoperative IVB, reported that although preoperative IVB was not associated with a better surgical success, IOP control, or VA, it significantly decreased postoperative hyphema and was associated with a requirement for fewer antiglaucoma medications. In 2015, Arcieri et al. [10] reported a prospective, randomized clinical trial on the efficacy of concomitant and postoperative IVB injection with AGV implantation, compared with AGV implantation alone. They concluded that although there was no difference in the survival success rates between the two groups, the IVB group required fewer antiglaucoma medications and showed more frequent regression of iris NV [10]. Another retrospective, comparative study revealed that IVB had no long-term effects on patients with NVG and had a limited, temporizing role in the treatment of NVG [5].

A substantial portion of the eyes of patients with NVG did not respond to medical therapy with antiglaucoma medication and ultimately required surgical treatment. The success rate of conventional filtering surgery is relatively low for patients with NVG [12]. A drainage implant, such

Table 5: Result of cox proportional hazard analysis for assessing risk factors of surgical failure at different stages of peripheral anterior synechiae.

Variables	PAS less than 50% ($n = 46$ eyes)				PAS more than 50% ($n = 24$ eyes)	
	Univariate		Multivariate		Univariate	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Preoperative PRP (versus not done)	0.34 (0.08–1.37)	0.130			0.51 (0.12–2.13)	0.354
Presence of NVA (versus absence)	0.39 (0.08–1.90)	0.242			NA*	
IVB group (versus control)	**0.29 (0.08–1.10)**	**0.069**			1.19 (0.37–3.81)	0.775
Number of IVB injection (per 1-time injection)	**0.39 (0.15–1.06)**	**0.064**	0.39 (0.15–1.06)	0.064	0.91 (0.51–1.61)	0.743
Baseline IOP (mmHg)	1.05 (0.98–1.13)	0.186			1.02 (0.95–1.09)	0.624
Baseline BCVA (LogMAR)	1.05 (0.89–1.88)	0.881			1.25 (0.56–2.78)	0.582

*All 24 eyes had NVA. HR = hazard ratio; CI = confidence interval; NVA = neovascularization of angle; PAS = peripheral anterior synechiae; IVB = intravitreal bevacizumab; IOP = intraocular pressure; BCVA = best-corrected visual acuity; MAR = minimal angle of resolution; NA = nonapplicable. Variables with $P < 0.10$ in univariate analysis were entered into multivariate analysis. Multivariate model using a backward elimination approach based on likelihood ratio; variables were entered in the model if $P < 0.05$ and removed if $P > 0.10$ in the saturated multivariate model.

as an AGV, is of particular use in these conditions and is less likely to fail in comparison to trabeculectomy [5]. To the best of our knowledge, a comparative study on the efficacy of IVB in patients with NVG at different stages of the disease has not been previously published. Therefore, in the current study, we retrospectively reviewed medical records of patients, who consecutively underwent AGV implantation for the treatment of NVG, and enrolled those with detailed angle description, which was assessed by a glaucoma specialist using gonioscopy. Our study comprised a relatively large number of eyes of patients with NVG (70 eyes in total) and a long follow-up period, with an average of 27 months. Due to the relatively short half-life of intraocular bevacizumab, repeated preoperative injections of IVB were performed as needed, with an average of 1.70 times for the eyes where the extent of PAS were less than 50% and 1.51 times for those where PAS was more than 50% ($P = 0.481$). Although not statistically significant, IVB showed more adjuvant positive effect on surgical success rates at postoperative 1 year and final visits when PAS had not formed extensively, as illustrated in Figure 1.

We also assessed risk factors for surgical failure using a Cox proportional hazard analysis. In univariate analysis, PRP, PAS, and IVB were putative factors for all the eyes, while the PRP and PAS were the related factors in a multivariate model with borderline significances. Nakano et al. [8] retrospectively reviewed 181 eyes of patients with NVG and found that angle closure had the greatest effect on an NVG-IOP prognosis, with a HR of 3.059, which is in line with our results. Olmos et al. [5] who reviewed 163 eyes of patients with NVG with a mean follow-up of 12 months reported that PRP was the most important prognostic factor, with its long-lasting antiangiogenic effect. In our study, IVB showed some protective effect against surgical failure in the group where the extent of PAS was lower. One IVB injection had the effect of reducing the risk of surgical failure by 61% (HR = 0.39, $P = 0.064$). Therefore, IVB showed a positive effect on the surgical outcome of NVG, particularly during the early stages of NVG with an open angle.

Our study has some limitations. First, in the process of enrolling participants, the possibility of selection bias should not be ruled out. From the beginning of the study, we focused on the effect of IVB on different stages of NVG, so detailed information of angle status before AGV implantation was required. Additionally, retrospective chart review did not reveal patients with NVG who were excluded from IVB before AGV implantation for any reason, similar to a previous study [5]. Further, angle status after IVB injection and/or AGV implantation was not assessed due to lack of information on chart. Therefore, the direct assessment of the effect on regression of NV was not done. Another limitation is that the amount of laser ablation might be different among subjects, although most patients received PRP as wide as possible except the area of major vessel arcade. Quantitative assessment of PRP (sessions, total of spots) was therefore challenging, and instead, we only checked if the patient received PRP or not. Next, the extent of PAS was different between the 2 groups, showing 78% of the eyes had PAS less than 50% in the IVB group, but only 44% of the eyes had PAS less than 50% in the control group. In other words, a higher proportion of the eyes with PAS less than 50% underwent a preoperative IVB injection than those with PAS more than 50%. So, one might think that the eyes with severe PAS could be tendentiously not treated with IVB and presented a worse HR for surgical outcome. It is difficult to give a clear answer because there was no predetermined indication about IVB injection for the eyes with NVG, and we could not find the reason why IVB injection was not performed in the control group retrospectively. Although there was no statistical significance, the control group showed slightly higher baseline IOP, showed worse baseline BCVA, had more eyes with OIS which was known to have a bad prognosis, and showed lower rate of PRP than the IVB group. In other words, participants with relatively severe stages of NVG and/or media opacity might be more likely to be distributed in the control group. This could lead to a confounding effect in the success rate and Cox proportional hazard analysis to assess the risk factors of surgical failure.

In summary, there was no difference in terms of the final IOP and BCVA between the IVB group and the control group. However, IVB injection before AGV implantation showed the possibility of enhancing the surgical success rate when PAS is still not yet extensively formed. Further prospective, well-designed, and randomized controlled studies assessing the effect of IVB will be warranted.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Neovascular Age-Related Macular Degeneration in the Very Old (≥90 Years): Epidemiology, Adherence to Treatment, and Comparison of Efficacy

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Purpose. To investigate neovascular age-related macular degeneration (AMD) in patients aged ≥90 years from several perspectives for a comprehensive overview: prevalence, presenting characteristics, treatment adherence, reasons for discontinuation, and efficacy of antivascular endothelial growth factor (VEGF) treatment comparing Ranibizumab and Aflibercept. *Methods*. In this retrospective chart review, we determined the prevalence and presenting characteristics by reviewing all data for patients referred to our department with treatment-naïve neovascular AMD. By looking at historical cohorts, we determined adherence to treatment, reasons for discontinuation, and treatment outcomes after loading dose, 12 months, and 24 months. *Results*. Patients aged ≥90 years constituted 7% of the patients. Treatment was discontinued in 51%, primarily because of death and treatment burden. Mean change in best-corrected visual acuity was 3.2, 1.5, and −2.2 ETDRS letters at 4, 12, and 24 months, respectively. Aflibercept was superior to Ranibizumab in visual and anatomic outcomes. After two years of treatment, patients losing ≥15 ETDRS letters made up 19% in the Aflibercept group and 26% in the Ranibizumab group. *Conclusions*. We propose that the very old patients with neovascular AMD may constitute a distinctive group warranting special attention and possibilities for individualized therapy. Possible differences between anti-VEGF agents need further investigations.

1. Introduction

Age-related macular degeneration (AMD) is the most common reason for irreversible vision loss in the developed world [1]. Neovascular AMD represents a late stage of the disease, where vascular endothelial growth factor (VEGF) mediated development of choroidal neovascularizations (CNV) [2]. From only being able to postpone the inevitable scarring of the macula and loss of central vision, we can now improve or at least stabilize visual acuity and visual function in the majority of patients by the widespread introduction of anti-VEGF treatment [3–5].

The pivotal phase 3 trials MARINA and ANCHOR studies demonstrated that monthly Ranizbiumab (Lucentis, Novartis International AG, Basel, Switzerland) improved mean visual acuity for all subtypes of neovascular AMD [6, 7]. Its efficacy was later compared to that of Aflibercept (Eylea, Bayer AG, Leverkusen, Germany) in the noninferiority VIEW trails, where Aflibercept treatment was able to reach similar outcomes [8]. Aflibercept is similar to Ranibizumab in its pharmacodynamic properties but differs in its pharmacokinetics due to a VEGF-trap design that enables treatment with potentially 8 weeks' intervals. Many clinical and observational studies have confirmed the efficacy of Ranibizumab and Aflibercept and explored the possibility individualizing therapy depending on the patient's characteristics [9, 10]. One interesting aspect is how anti-VEGF efficacy is affected by *age*. Several studies find that the change in best-corrected visual acuity (BCVA) is negatively correlated with age [11–22]. Subgroup analyses of the two years outcomes in the MARINA study

found that unlike the younger patients which experienced a significant improvement of BCVA, patients ≥85 experienced no change in BCVA [13]. Similar results were reported for the first-year outcomes of the ANCHOR study [14]. Considering that anti-VEGF treatment experiences from daily clinical practice can be somewhat less promising than that seen in clinical trials [3], no change in BCVA may be hard to achieve in this patient group.

Ageing is an inevitable biological process that significantly affects the cells and organs of the body. Macula undergoes a range of changes [23]. The number of retinal pigment epithelium (RPE) cells decreases while the number of photoreceptors remains relatively stable [24]. Thus, each RPE cell must support more photoreceptors, which increases the metabolic and phagocytic stress on the RPE cells by which they respond by increasing in size and becoming multinucleated [25]. These aged and stressed RPE cells develop a higher VEGF response [26]. Taken together, increasing age leads to increased fragility of the macula [23], which we speculate may be a part of the reason for why the oldest individuals experience worse treatment response.

The average life expectancy is 80 years in developed countries such as Denmark, but diving into the statistics reveals that although 60+ years old individuals constitute 25%—every fourth—of the population, only <1% reaches 90+ years of age [27]. These very old individuals (defined as having aged ≥90 years) represent a small group that may significantly differ from the rest in terms of macular biology. However, another important aspect is also their opinion on the need for treatment and the burden of treatment in a monthly/bimonthly treatment regime. In cancer research, it is a well-documented phenomenon that some elderly refuse treatment because the perceived gain life expectancy does not outweigh the loss in quality of life [28]. Therefore, there may be several aspects among the very old patients with neovascular AMD with potential to influence the treatment response.

In this study, we investigated neovascular AMD in patients aged ≥90 years from several perspectives to give a comprehensive overview. First, we looked at their overall prevalence in a large tertiary retinal center and their presenting characteristics. Then, we looked at their adherence to anti-VEGF treatment and reasons for discontinuation. Finally, we evaluated results from anti-VEGF treatment and compared efficacy of Ranibizumab and Aflibercept using historical cohorts where each was the primary choice of treatment.

2. Materials and Methods

2.1. Study Design and Patient Eligibility. This study is a retrospective review of patients attending the Department of Ophthalmology at Zealand University Hospital, Roskilde, Denmark. All aspects of this study were conducted in accordance with the ethical principles stated in the Declaration of Helsinki. According to the national law and local hospital research guidelines, no institutional review board approval is required for retrospective observational clinical studies that review routine clinical practice.

We first determined the epidemiological aspects of patients with neovascular AMD aged ≥90 years: the prevalence and their presenting characteristics. For this part of the study, we reviewed all data for patients referred to our department in the year 2014. Eligible for analyses were patients with treatment-naïve neovascular AMD regardless of the status of their lesion (i.e., whether or not it was deemed treatable) or enrollment to anti-VEGF treatment. Diseases that share features with neovascular AMD (e.g., polypoidal choroidal vasculopathy) were not included.

Then, we looked at adherence to treatment, reasons for discontinuation, and treatment outcomes for a 2-year period comparing Aflibercept and Ranibizumab. For this part of the study, we included treatment results from all our patients aged ≥90 years diagnosed with treatment-naïve neovascular AMD from 2009 to 2012 (where Ranibizumab was the primary choice of treatment) and from 2014 to 2015 (where Aflibercept was the primary choice of treatment). In 2013 where Aflibercept was introduced in our clinic, we were concerned about the ongoing discussions about Aflibercept increasing the risk of cerebrovascular events [29] and allocated patients with selected comorbidities to Ranibizumab treatment. Hence, we did not include patients from 2013 to avoid selection bias.

2.2. Access to Retinal Care. In Denmark, primary sector units (practicing ophthalmologists and general practitioners) and hospital departments refer all patients suspected of neovascular AMD to ophthalmology departments since this is the only access to free-of-charge anti-VEGF treatment. Within 1-2 workdays after receiving an electronic referral, the patient is invited and booked for detailed retinal diagnosis. From the time of booking, the patient is in most cases seen within two weeks, depending on the patient's availability and the availability of the department. Patients are offered free-of-charge transportation between their home and the hospital department, including patients that may need wheelchair or special support.

2.3. Retinal Diagnosis. All patients referred for retinal diagnosis undergo a comprehensive ophthalmic examination including dilated fundus examination, measurement of best-corrected visual acuity (BCVA) in each eye using the Early Treatment of Diabetic Retinopathy Study (ETDRS) chart [30], retinal imaging using Heidelberg HRA-Spectralis Spectral Domain optical coherence tomography (OCT) (Heidelberg Engineering, Heidelberg, Germany), and retinal angiography using fluorescein and indocyanine green. All BCVAs were measured by personnel specifically trained in ETDRS measurements. We used CC-100 charts (Topcon Corp., Tokyo, Japan). All patients started from the top of the chart and read each letter of each horizontal line and progressed downwards until reaching a row where a minimum of three letters on a line could not be read. Each eye was tested individually and was scored according to correctly identified number of letters. Patients read the chart at 4 meters (adding 45 letters to the final score), but if the patient was unable to read the letters, then the chart was read at 1 meter (adding 15 letters to the final score).

Treatment was initiated in eyes with neovascular AMD as defined in the Clinical Age-Related Maculopathy Staging System (grade 5) [31] and in the presence of active CNV and no predominance of fibrosis and/or atrophy. In other words, this included active subfoveal CNV as evaluated by the presence of leakage in fluorescence angiography with the presence of one or more characteristics such as hemorrhage, exudates, serous detachments, and intraretinal edema. If the BCVA was ≥20 ETDRS letters or the patient had cardiovascular events within ≤3 months, treatment was commenced on a case-by-case basis with special emphasis on the state of the patient's contralateral eye.

2.4. Treatment and Follow-Up. All eyes received three consecutive monthly intravitreal injections with either Aflibercept (0.05 mL) or Ranibizumab (0.05 mL). Choice of Aflibercept or Ranibizumab was based on our local guidelines which were Ranibizumab prior to widescale Aflibercept introduction in 2013 and Aflibercept as first line of treatment starting from 2014 based on national guidelines for the treatment of neovascular AMD. Injections were given by physicians or specially trained injection nurses [32]. After the third injection, the patients were reevaluated in follow-ups using dilated fundus examination, measurement of best-corrected visual acuity (BCVA), and OCT scans to determine whether or not the macula was dry so that additional injections may be warranted. The reevaluation was after 4 weeks for Ranibizumab and 8 weeks for Aflibercept. We followed a pro re nata (PRN) treatment regime for both Aflibercept and Ranibizumab. Retreatment criteria, based on local guidelines and recommendations from the Danish Ophthalmological Society, were the same during the study period (2009 to 2017) [33]: the presence of subretinal or intraretinal fluid on OCT or retinal hemorrhage either new or persisting. In case of loss of visual acuity with no subretinal or intraretinal fluid, we repeated retinal angiography with fluorescein and indocyanine green to evaluate the presence of active CNV, which was another retreatment criterion. In eyes with development of untreatable retinal tubuli or fibrotic scar, or BCVA < 20 ETDRS letters with a dry macula, treatment was stopped. If additional injections were needed, the patient was booked for two to three anti-VEGF injections and reevaluated. Injections after the loading dose phase were given with 4 weeks intervals for Ranibizumab and 8 weeks for Aflibercept. If no additional injections were needed due to a dry macula, we booked the patient for reevaluation. After consecutive reevaluations with a dry macula, the disease was considered provisionally inactive and the patient was referred to a local primary sector ophthalmologist for future controls.

2.5. Data Analysis and Statistics. The prevalence of patients with neovascular AMD with an age ≥ 90 was calculated including a confidence interval for the prevalence estimate with a continuity correction [34]. We reviewed the identified patients' clinical characteristics using OCTs, angiographies, and BCVA. BCVA was obtained from treatment databases.

Categorical variables are presented using numbers and percentages and compared using the χ^2-test and Fisher's exact test in case any subcategory had $n < 5$. Continuous variables were checked for normal distribution using the Kolmogorov-Smirnov test. Where normal distribution was present, data was presented using mean and standard deviation (SD) and tested using parametric tests. Age did not fit normal distribution and was right tailed, so this parameter was presented in median and interquartile range (IQR) and tested using Mann–Whitney *U* test.

Using data from eligible patients enrolled between 2009–2012 and 2014-2015, we determined which patients discontinued treatment and noted the reason. Based on different reasons for discontinuation, we explored adherence to treatment over time using a time-to-event curve. We defined the start point as date of treatment start and followed the patient until either event or censoring. Time-to-event curves were made specifically for each reason for discontinuation by censoring the other reasons.

Visual and anatomical treatment outcomes were measured as change in BCVA and average central retinal thickness (CRT) after approximately 4 months (first reevaluation after treatment commencement), 12 months, and 24 months. The CRT was defined as the average thickness of an area with a diameter of 1 mm around the fovea including any subretinal hyperreflective material. Due to different reasons for treatment discontinuation, we analyzed data using the last observation carried forward (LOCF) method to account for missing data for all patients that had at least one reevaluation after treatment commencement. LOCF was not made for 24 months follow-up for patients started in treatment in 2015 since most of these patients did not have their follow-up at time of analysis (March 2017). We assumed that using the LOCF on these patients would bias the results towards better outcomes in the Aflibercept groups because 12 months outcomes in general are better than 24 months outcomes.

When a patient's both eyes were eligible for our analyses, we only included data from one eye (the first eye diagnosed with neovascular AMD or the right eye in case both eyes were diagnosed simultaneously) to avoid statistical problems with assumptions of independent sampling. In the analyses, we first looked at whether the change in BCVA and average CRT was significant using a two-tailed one sample *t*-test with a test value of 0. Changes in BCVA and average CRT were then compared between patients receiving Aflibercept with patients receiving Ranibizumab using a two-tailed independent samples t-test. Effect size was calculated using Cohen's *d*. Cohen defined the following interpretation as a rule of thumb: 0.2 small, 0.5 moderate, and 0.8 large [35].

Statistical analyses were made in SPSS version 23 (IBM, Armonk, NY, USA). Figures were made using Prism 7 (GraphPad Software, La Jolla, CA, USA). *P* values below 0.05 were interpreted as sign of statistical significance.

3. Results

3.1. Epidemiology: Prevalence and Presenting Characteristics. A total of 282 patients with neovascular AMD were referred to our clinic for retinal diagnosis during the year 2014. Twenty of these patients were ≥90 years, corresponding to

Table 1: Reasons for discontinuation of anti-VEGF treatment using either Aflibercept or Ranibizumab among patients with neovascular age-related macular degeneration aged ≥90 years.

	Aflibercept ($n = 54$)	Ranibizumab ($n = 62$)	P value
Death during follow-up, n (%)	7 (13)	9 (15)	0.809
Burdened by treatment/opted out of treatment, n (%)	6 (11)	10 (16)	0.434
Inactive CNV, dry macula, and referral of patient to the primary sector, n (%)	5 (9)	3 (5)	0.470
Treatment stopped due to development of a fibrotic/untreatable lesion, n (%)	6 (11)	11 (18)	0.314

P values were calculated using the χ^2-test for all categories, but *inactive CNV, dry macula, and referral of patient to the primary sector* was calculated using Fisher's exact test due to groups with <5.

a prevalence of 7.1% (CI 95%: 4.5 to 10.9%). Age ranged from 90 to 99 years with median 92 years and IQR 90 to 95 years. Eleven (55%) were females and nine (45%) males.

All patients presented with new neovascular AMD in one eye only, and all patients presented with a macular condition in the contralateral eye: early AMD in seven patients (35%), geographic atrophy in five patients (25%), old fibrotic AMD in five patients (25%), macular hole in one patient (5%), vitreomacular traction in one patient (5%), and subretinal drusen in one patient (5%).

Mean BCVA was 48 ETDRS letters (SD: 15 ETDRS letters). Average CRT was mean 454 μm (SD: 125 μm). Mean lesion size was 3563 μm (SD: 1081 μm) measured using the greatest linear dimension. Eleven eyes (55%) had predominantly classic lesion, seven eyes (35%) had predominantly occult lesion, and two eyes (10%) had RAP. In 15 eyes (75%), lesions were hemorrhagic in their appearance. All 20 patients were deemed eligible for treatment.

3.2. Treatment Adherence and Reasons for Discontinuation. For this part of the study, we included treatment results from all our patients aged ≥90 years diagnosed with treatment-naïve neovascular AMD from 2009 to 2012 (where Ranibizumab was the primary choice of treatment) and from 2014 to 2015 (where Aflibercept was the primary choice of treatment) and which during the follow-up period of 2 years was not switched from one anti-VEGF to another.

We identified a total of 116 patients treated with either Aflibercept ($n = 54$) or Ranibizumab ($n = 62$). During the 2 years follow-up, 59 patients (51%) discontinued treatment. Reasons for discontinuation are presented in Table 1 and did not differ significantly depending on choice of anti-VEGF.

Using time-to-event analyses, we explored the impact of each of these factors on the treatment adherence (Figure 1). Death was an issue throughout the follow-up period. Patients who did not wish to continue treatment due to the burden of treatment made this choice within the first year. Discontinuation due to treatment results differed: discontinuation due to fibrotic/untreatable lesions was more likely to happen after the loading dose and within the first year, whereas discontinuation due to inactive CNV/dry macula happened after the first year.

3.3. Visual and Anatomical Outcomes. Of the 116 patients treated with either Aflibercept or Ranibizumab, 106 (91%) remained in treatment for follow-up after the loading dose phase. These patients were included for a comparison of Aflibercept and Ranibizumab efficacy in terms of visual and anatomical outcomes. Patient characteristics are summarized in Table 2 and were similar in terms of demographics, lesion characteristics, and BCVA.

Overall, anti-VEGF therapy improved the BCVA at 4 months (after the loading dose phase) (mean change 3.2 (SD: 15.5) ETDRS letters, $P = 0.036$; one sample t-test) and stabilized at 12 and 24 months (mean change 1.5 (SD: 16.5) ETDRS letters, $P = 0.342$; mean change −2.2 (SD: 20.1) ETDRS letters, $P = 0.288$; one sample t-test, resp., for 12 and 24 months). The average CRT decreased significantly and remained decreased at all follow-ups (mean change −130 (SD: 143) μm, $P < 0.001$; mean change −117 (SD: 150) μm, $P < 0.001$; mean change −115 (SD: 158) μm, $P < 0.001$).

The mean number of treatments during 2 years was 5.7 (SD: 3.0). For Aflibercept and Ranibizumab, the mean number of treatments was 5.6 (SD: 2.9) and 5.8 (SD: 3.1), respectively. These numbers were influenced by 51% of the patients discontinuing treatment during the follow-up. Looking only at patients that continued treatment during the 2 years, the overall mean number of treatments increased to 7.2 (SD: 2.9). For Aflibercept and Ranibizumab, these numbers increased to a mean of 6.7 (SD: 3.0) and 7.9 (SD: 2.9), respectively.

Aflibercept treatment significantly improved BCVA at 4 months at mean 5.5 ETDRS letters ($P = 0.014$; one sample t-test) (Table 3). Although there was a small improvement at the later follow-ups at 12 and 24 months, its size was small and did not reach statistical significance (Table 3). The Δ average CRT decreased significantly at 4 months ($P < 0.001$; one sample t-test) and remained significantly decreased at 12 ($P < 0.001$; one sample t-test) and 24 months ($P < 0.001$; one sample t-test) (Table 3).

Ranibizumab treatment was not associated with a significant improvement or worsening of BCVA at 4 months or 12 months but lead to a significant decrease of 5.8 ETDRS letters after 24 months ($P = 0.028$; one sample t-test) (Table 4). The Δ average CRT decreased significantly at 4 months ($P < 0.001$; one sample t-test) and remained significantly decreased at 12 ($P < 0.001$; one sample t-test) and 24 months ($P < 0.001$; one sample t-test) (Table 4).

Overall, Aflibercept treatment was superior to Ranibizumab treatment in ΔBCVA at all time points. However, the mean differences between the groups were small initially and did not reach statistical significance until after 24 months: after 4 months (4.4 (CI 95%: −1.6 to 10.3) ETDRS letters, $P = 0.149$), after 12 months (4.5 (CI 95%: −1.9 to 10.8)

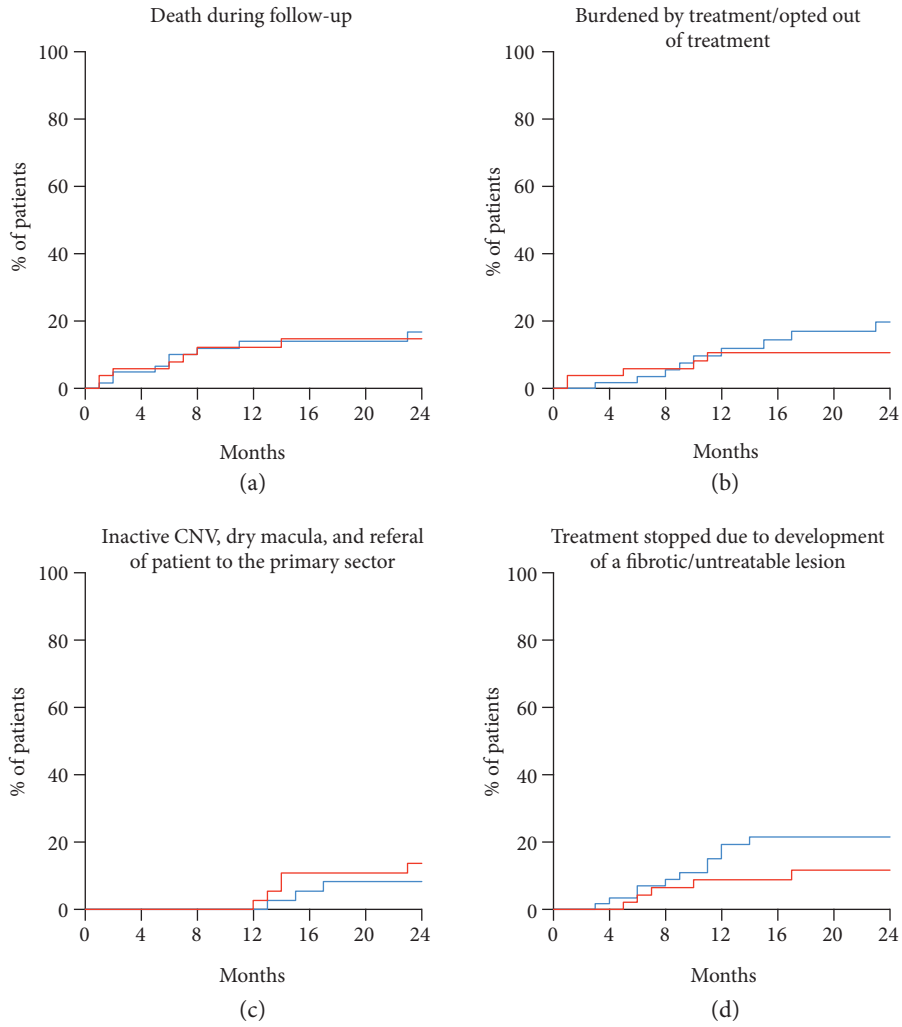

Figure 1: Treatment adherence in relation to different reasons for discontinuation shown in time-to-event curves. Comparing Aflibercept (red) with Ranibizumab (blue) did not show any statistically significant differences. (a) Death was an issue throughout the entire follow-up period. (b) Patients feeling burdened by the treatment opted out mostly within the first year. (c) Treatment discontinuation due to inactive CNV/dry macula was only seen after the first year. (d) Treatment discontinuation due to a fibrotic/untreatable lesion was mostly within the first year.

Table 2: Baseline factors of patients with neovascular age-related macular degeneration aged ≥90 years enrolled in Aflibercept or Ranibizumab treatment, which remained in treatment for at least the follow-up after the loading dose phase.

	Aflibercept ($n = 49$)	Ranibizumab ($n = 57$)	P value
Age, years, median (IQR)	91 (90 to 93)	91 (90 to 92)	0.400
Gender, n (%)			0.370
Female	35 (71)	45 (79)	
Male	14 (29)	12 (21)	
BCVA, ETDRS letters, mean (SD)	50 (14)	48 (18)	0.510
Average CRT, mean (SD)	433 (130)	445 (114)	0.660
Lesion type, n (%)[†]			0.309
Predominantly classic	16 (35)	18 (34)	
Predominantly occult	25 (54)	34 (64)	
Retinal angiomatous proliferation	5 (11)	1 (2)	

IQR: interquartile range; SD: standard deviation; BCVA: best-corrected visual acuity; CRT: central retinal thickness.[†]No data on lesion type for three patients in the Aflibercept group and four patients in the Ranibizumab group due to allergies to the contrast agents, lack of cooperation, or inaccessible data. P values were calculated using the Mann–Whitney U test for age, χ^2-test for gender, independent samples t-test for BCVA and average CRT, and Fisher's exact test for lesion type due to groups with <5.

Table 3: Two-year results on Aflibercept treatment for neovascular age-related macular degeneration in patients aged ≥90 years.

	Aflibercept ($n = 49$)	
	Mean (95% CI)	P value
ΔBCVA, ETDRS letters		
4 months	5.5 (1.1 to 9.9)	0.014
12 months	3.9 (−0.9 to 8.8)	0.106
24 months	3.4 (−3.4 to 10.2)	0.320
Δ average CRT, μm		
4 months	−148 (−185 to −111)	<0.001
12 months	−142 (−181 to −104)	<0.001
24 months	−141 (−185 to −97)	<0.001

BCVA: best-corrected visual acuity; CRT: central retinal thickness; CI: confidence interval. P values were calculated using the one sample t-test with test value = 0.

Table 4: Two-year results on Ranibizumab treatment for neovascular age-related macular degeneration in patients aged ≥90 years.

	Ranibizumab ($n = 57$)	
	Mean (95% CI)	P value
ΔBCVA, ETDRS letters		
4 months	1.2 (−3.0 to 5.3)	0.570
12 months	−0.5 (−4.8 to 3.7)	0.800
24 months	−5.8 (−10.9 to −0.6)	0.028
Δ average CRT, μm		
4 months	−114 (−155 to −73)	<0.001
12 months	−95 (−138 to −52)	<0.001
24 months	−98 (−143 to −52)	<0.001

BCVA: best-corrected visual acuity; CRT: central retinal thickness; CI: confidence interval. P values were calculated using the one sample t-test with test value = 0.

ETDRS letters, $P = 0.164$), and after 24 months (9.2 (0.8 to 17.5) ETDRS letters $P = 0.031$) (Figure 2). This corresponded to Cohen's d values of 0.3, 0.3, and 0.5, for 4, 12, and 24 months, respectively, indicating an initially small effect size that grows to a moderate effect size at 24 months. We determined the rate of patients with loss of ≥15 ETDRS letters in each group, which also showed that the differences between the groups have a tendency of growing over time (Figure 3). After 24 months, 19% in the Aflibercept group had lost ≥15 ETDRS letters, whereas this number was 26% in the Ranibizumab group.

The decrease in Δ average CRT was compared between Aflibercept and Ranibizumab. Here, we saw a small but nonsignificant more decrease among those treated with Aflibercept group at all time points (34 μm, 47 μm, and 43 μm, resp., for 4, 12, and 24, months) (Figure 4). These changes corresponded to Cohen's d values of 0.2, 0.3, and 0.3 for 4, 12, and 24 months, respectively, indicating a small effect size.

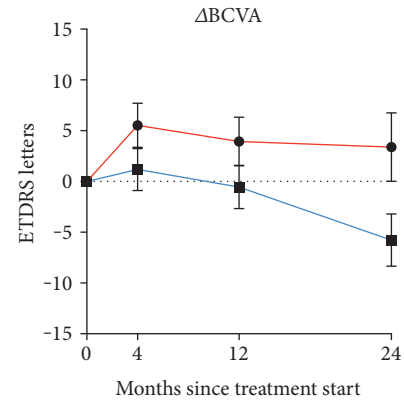

Figure 2: Change in best-corrected visual acuity of eyes with neovascular age-related macular degeneration in patients aged ≥90 years treated with either Aflibercept (red) or Ranibizumab (blue). Dots and whiskers indicate mean and standard error.

4. Discussion

Patients aged ≥90 years constitute 7% of patients with neovascular AMD—approximately one out of every 14. These patients have a high rate of treatment discontinuation, where death and burden of treatment play a considerable role. We also found that in these patients, Aflibercept therapy was superior to Ranibizumab, although the effect sizes were only small to moderate. Difference between groups in the CRT was small and not statistically significant.

Different aspects of anti-VEGF treatment may explain our results. Klettner et al. investigated the efficacy of Aflibercept, Ranibizumab, and Bevacizumab in an experimental setting using a RPE/choroid organ culture, where VEGF in the supernatant was measured throughout 7 days and using different concentration of anti-VEGFs [36]. First, Aflibercept required the lowest concentration for short-term VEGF inhibition when compared to Ranibizumab and Bevacizumab [36]. Second, one regular dose of Aflibercept inhibited VEGF completely until the 7th day, whereas VEGF could be detected after 72 hours with Ranibizumab treatment and after 12 hours with Bevacizumab treatment [36]. Brinkmann et al. compared the uptake of Aflibecept, Ranibizumab, and Bevacizumab in vitro using ARPE-19 cell cultures, where Aflibercept had significantly faster uptake when compared with Ranibizumab and Bevacizumab [37]. These findings on cellular level may not necessarily have a clinical significant impact on a broader level as seen in the VIEW studies [8]. However, considering that RPE with age becomes more fragile and develops a more potent VEGF response [23–26], patients aged ≥90 years may constitute a group where the aging process of RPE is at its utmost and where the pharmacokinetic differences between Aflibercept and Ranibizumab give rise to clinically measureable differences. However, the differences between Aflibercept and Ranibizumab on CRT had a small effect size in this study. It will be interesting to see how anti-VEGF drugs with more potent pharmacokinetic properties now in phase 3 trials, such as Abicipar/AGN-150998 (Allergan, Irvine, CA, USA) and Brolucizumab/RTH-258 (Novartis), will work on patients aged ≥90 years.

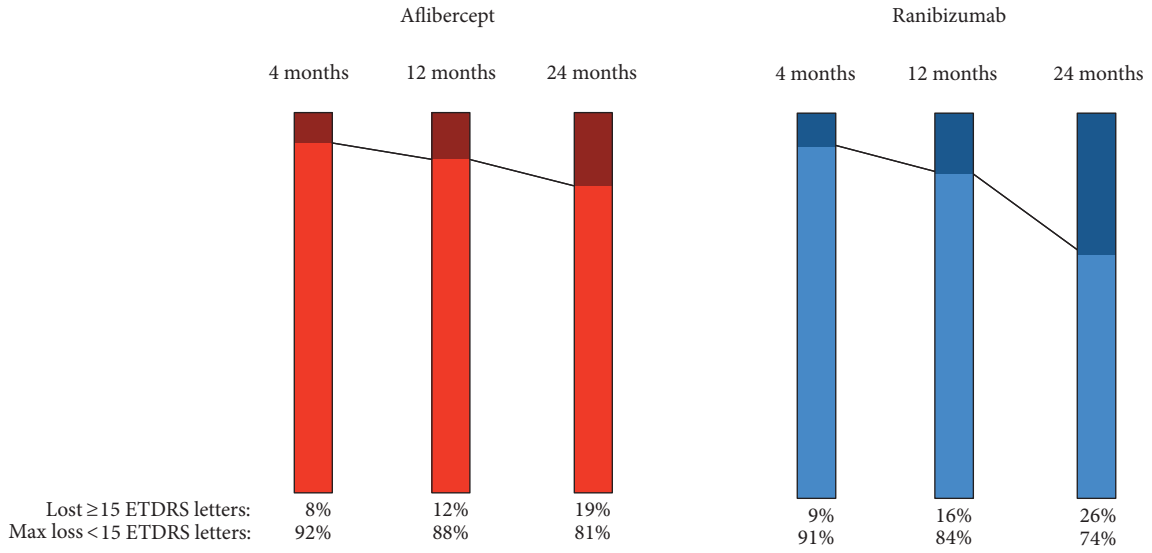

Figure 3: Rate of patients with neovascular age-related macular degeneration aged ≥90 years that experience a loss of ≥15 ETDRS letters in best-corrected visual acuity during treatment with either Aflibercept (red) or Ranibizumab (blue).

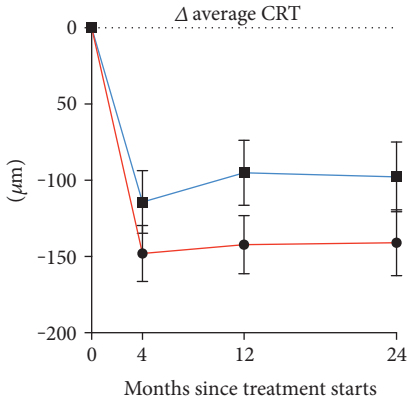

Figure 4: Change in average central retinal thickness of eyes with neovascular age-related macular degeneration in patients aged ≥90 years treated with either Aflibercept (red) or Ranibizumab (blue). Dots and whiskers indicate mean and standard error.

Another explanation to our findings is that Aflibercept allows eight weeks treatment intervals, which gives more flexibility from a clinical point-of-view. It is our experience that flexibility is much needed in the very old for a number of practical reasons, such as localized infections around the eye, systemic infections, fall trauma, or other conditions that may shift the priorities and the focus of the patient. For such cases, the eight weeks coverage of Aflibercept is less likely to undertreat the patients compared with the four weeks coverage of Ranibizumab. Indeed, when looking at the number of treatments, we only found that on average, ~1 additional treatment was given with Ranibizumab, underscoring our speculations of undertreatment of the Ranibizumab group.

We used a PRN regime, which some studies suggest may be inferior to the treat and extend (T&E) regimen in terms of visual outcomes [38]. In a systematic review and a network meta-analysis, Danyliv et al. compared the two regimens and found that although T&E was associated with better outcomes compared to PRN, the effect size was quite small and clinically irrelevant: ~2 more ETDRS letters at 12 months and 2-3 more ETDRS letters at 24 months [38]. This small effect size comes at a considerable cost: the T&E regimen is associated with significantly more injections [38]. However, considering that the pharmacokinetic properties of anti-VEGF drugs may play a larger role in patients aged ≥90 years, which are in risk of undertreatment, there may also be a potential for a larger gain using a T&E regime. Future studies may shed light on these aspects.

A considerable number of our patients discontinued treatment due to death or inacceptable burden of treatment. These aspects reflect the specific difficulties when dealing with patients aged ≥90 years [28]. In our study, we did not find any significant difference between the Aflibercept and Ranibizumab in the treatment discontinuation. We initially speculated that a greater number of injections might lead to a difference, but the number of injections between the groups only differed slightly. Details about burden of treatment warrant further investigation as we might be able to overcome any specific aspects proving to be an obstacle for helping our patients; for example, it would be interesting to know whether the perceived burden is due to specific factors such as the injections or the frequent visits.

Although our health system is based on a free-of-charge concept and even offers free-of-charge transportation to hospitals, actually utilizing that system may not be as straightforward in this group of very old as in others. Some of these patients informed that they did not seek help after several months, which is particularly problematic since timely treatment can be important factor for neovascular AMD [39, 40]. It is hard to speculate whether these challenges reflect a generation issue in not wanting to burden others, cognitive decline that are seen in among the very old, or a concept of accepting that vision declines with age.

Interesting exploratory studies may provide better insight into the future.

Limitations should be noted when interpreting our results. This was retrospective observational study where historic cohorts were compared. Although we tried to minimize selection bias by not including participants from 2013, where Ranibizumab was used on patients with cardiovascular comorbidities; a better study design would include a randomized allocation of patients to Aflibercept and Ranibizumab. We used LOCF handle missing data due to treatment discontinuation, and in that regard, it should be noted that a considerable number of our patients discontinued treatment for different reasons. However, we do not suspect that this results in skewed data between Aflibercept and Ranibizumab since the groups did not differ in treatment discontinuation. Finally, it should be noted that the mean difference between Aflibercept and Ranibizumab was at ~2 ETDRS lines at most, and as a rule of thumb, a clinically significant difference is 3 ETDRS lines [41].

5. Conclusion

Patients aged ≥90 years constitute a small but important proportion of those referred for treatment of neovascular AMD. These very old patients have a high rate of treatment discontinuation, where death and burden of treatment play a considerable role. Although both Aflibercept and Ranibizumab decreased the average CRT, Aflibercept seemed superior to Ranibizumab in terms of change in BCVA after the loading dose and after 12 and 24 months in patients aged ≥90 years. Important reasons may be pharmacokinetic differences between the two drugs or the relative more flexibility of treatment every 8 weeks versus 4 weeks with Aflibercept versus Ranibizumab; however, studies with prospective and randomized design are needed for more conclusive results. We propose that the very old patients constitute a distinctive group that may warrant special attention.

Conflicts of Interest

Author Torben Lykke Sørensen declares that there is no conflict of interest regarding the publication of this paper. Author Yousif Subhi has previously received travel grants from Novartis and Bayer.

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Research Article

Asymmetric Outcomes of Type 1 Retinopathy of Prematurity after Bilateral Intravitreal Ranibizumab Treatment

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Purpose. To present cases with retinopathy of prematurity (ROP), who were treated with intravitreal injection of ranibizumab (IVR) and had unpredictable asymmetric outcomes. *Methods.* A retrospective review was performed in infants with type 1 ROP and had bilateral IVR (0.25 mg/0.025 mL) as initial treatment. Patients were classified into the asymmetric outcome group and the symmetric outcome group. *Results.* Eighty-four patients (168 eyes) were included. There were 18 eyes of 9 patients (10.7%) in the asymmetric outcome group and 150 eyes of 75 patients (89.3%) in the symmetric outcome group. In the symmetric outcome group, 86 eyes (57.3%) had ROP regression, 60 eyes (40%) had reactivation requiring laser treatment, and 4 eyes (2.7%) progressed to retinal detachment requiring vitrectomy. In the asymmetric outcome group, one of the eyes of the 9 patients had ROP regression with/without reactivation after IVR, while the contralateral eyes had negative response, including remarkable posterior fibrosis, partial or total retinal detachment, and vitreous hemorrhage. There was statistically significant difference between the birth weight of the two groups. *Conclusion.* Contralateral eyes with ROP can take a different clinical course after ranibizumab treatment. High rate of reactivation after IVR is another concern that ophthalmologists should pay attention to.

1. Introduction

Retinopathy of prematurity (ROP) is a retinal vasoproliferative disorder. It continues to be a significant cause of childhood blindness. Laser photocoagulation is the current standard treatment for ROP [1]. Although cryotherapy and laser treatment can cure ROP disease in most cases, they may cause complications such as peripheral visual field defect and myopic shift. Since the role of vascular endothelial growth factor (VEGF) in the pathophysiology of ROP has been well studied, the use of anti-VEGF agents is emerging as a treatment for ROP [2–4]. The only prospective, controlled, randomized, multicenter trial about anti-VEGF treatment for ROP—Bevacizumab Eliminates the Angiogenic Threat of Retinopathy of Prematurity (BEAT-ROP) study—showed that bevacizumab was effective in treating ROP and was more effective than laser treatment in zone 1 ROP cases [5]. On the other hand, reports about reactivation and retinal detachment after injection were not rare [6–8]. What is more, many questions remain unanswered, including the optimal dose and timing of injection, systemic safety, and long-term complications.

In this report, we describe nine cases of type 1 ROP that had asymmetric outcomes after intravitreal injection of ranibizumab (IVR, Lucentis®) treatment.

2. Materials and Methods

This was a retrospective study which was conducted in the referral ROP screening center in Xin Hua Hospital, affiliated to Shanghai Jiao Tong University School of Medicine. The medical records of patients who were diagnosed with type 1 ROP and had bilateral IVR as initial treatment from January 2012 to December 2014 were reviewed. Patients were

classified into asymmetric outcome group (different additional treatments or asymmetric anatomic outcomes in two eyes) and symmetric outcome group. Patients with a follow-up of less than six months were excluded. Each patient's parents or legal guardians were required to sign a consent form before any examination or treatment. This study was approved by the Ethics Committee of Xin Hua Hospital.

Infants were screened if they were born at gestational age of less than 32 weeks or/and their birth weight was less than 2000 g or if they had an unstable clinical course as determined by the infant's neonatologist [9]. Patients' age, gender, family, and birth history, as well as systemic and other ocular anomalies, were noted. Patients were screened by binocular indirect ophthalmoscopy and RetCam (Clarity Medical Systems, Pleasanton, California, USA) fundus photography. Ultrasound examination was given to patients whose fundus was invisible due to corneal opacity or leucocoria. IVR was provided to patients with type 1 ROP. Infants treated with IVR were examined a day after the procedure and weekly thereafter until full vascularization of the retina was observed. If they did not respond positively to the treatment, conventional laser treatment and/or surgery was performed. No second injection of IVR was given to patients. IVR (0.25 mg/0.025 mL), laser treatment, lensectomy, and vitrectomy were performed by the same surgeon (PQZ). Systemic conditions of infants were checked every month after injection by neonatologists.

We performed statistical analysis with the program IBM SPSS 22 (SPSS Inc., Chicago, IL). Continuous variables were summarized as mean and standard deviation (SD) because data were normally distributed. An independent t-test was used to compare continuous data between the group with asymmetric outcome and the group with symmetric outcome. P value <0.05 was considered statistically significant.

3. Results

During the study period, 168 eyes of 84 infants were diagnosed with type 1 ROP and received bilateral IVR as initial treatment. Among them, 18 eyes of 9 patients (10.7%) had asymmetric outcomes in contralateral eyes after IVR (Table 1). The remaining 150 eyes of 75 patients (89.3%) had symmetric outcomes. In the symmetric outcome group, 32 eyes (21.3%) had aggressive posterior retinopathy of prematurity (APROP), 30 eyes (20%) were classified as zone I stage 3+, 16 eyes (10.7%) were classified as zone II stage 3+, 20 eyes (13.3%) were classified as zone I stage 2+, and 52 eyes (34.7%) were classified as zone II stage 2+. In the asymmetric outcome group, the gestational age ranged from 27 to 32 weeks, with a mean of 29.6 ± 1.8 weeks; the birth weight ranged from 980 to 1690 g, with a mean of 1222.2 ± 216.6 g; and the IVR injection time ranged from 34 to 42 weeks postmenstrual age (PMA), with a mean of 37.0 ± 2.4 weeks (Table 2). There was a statistically significant difference between the BW of the asymmetric outcome group and symmetric outcome group. There were no statistically significant differences between the mean GA, PMA, and postnatal age (PNA) at IVR.

In the symmetric outcome group, 86 eyes (57.3%) had ROP regression after IVR, 60 eyes (40%) had reactivation requiring additional laser treatment, and 4 eyes (2.7%) of 2 patients which progressed to retinal detachment required lens-sparing vitrectomy. All eyes had flat retinas at the last visit. The time between reactivation and the initial IVR was 16~108 days, with an average of 56.8 ± 17.1 days. The mean PMA at reactivation was 43.4 ± 3.4 weeks.

In the asymmetric outcome group, one of the eyes of the 9 patients had ROP regression with later reactivation in 8 of them after IVR requiring secondary laser treatment, while the contralateral eyes had negative responses requiring additional surgical treatment, including remarkable posterior fibrosis, partial or total retinal detachment, and vitreous hemorrhage (VH) (Table 1). Retinas were attached in 16 eyes (88.9%) at the last visit. The follow-up period of all eyes ranged from 8 to 30 months, with a mean of 14.4 ± 8.9 months. No noticeable systemic complications related to IVR were observed.

3.1. Infant 2. Infant 2 was born at 29 weeks of gestation with a birth weight of 1.2 kg. At 37 weeks PMA, both eyes were diagnosed as stage 3+ ROP in zone I with mild preretinal hemorrhages. The infant received bilateral IVR at 37^{+4} weeks PMA. The right eye regressed first and recurrence occurred in zone II, which was diagnosed as stage 2 ROP without plus at 43 weeks PMA and required laser treatment. Unpredictably, the left eye was diagnosed as stage 5 ROP with marked posterior fibrosis and VH at 39 weeks PMA (10 days post-IVR). The disease rapidly progressed to the shallow anterior chamber and finally received a lensectomy and vitrectomy at 57 weeks PMA. The retina was not reattached (Figure 1).

3.2. Infant 7. Infant 7 was born at 31 weeks of gestation with a birth weight of 1.38 kg. The infant was transferred to our clinic at 41^{+6} weeks PMA with stage 3+ ROP in zone I and received IVR in both eyes at 42 weeks PMA. Regression of ROP was first noted in both eyes, and then severe vitrial and preretinal hemorrhages were noted at 43^{+2} weeks PMA (nine days post-IVR) in the right eye. Hemorrhages continued to progress after laser treatment; and at 47 weeks PMA (38 days post-IVR), hemorrhages covered the macula and the eye, which eventually required LSV treatment. In the left eye, we observed persistent zone II avascularity that required laser treatment (Figure 2).

4. Discussion

We described nine cases of ROP that had asymmetric outcomes after bilateral IVR as initial treatment. Similar ROP cases having asymmetric outcomes after intravitreal injection of bevacizumab (IVB) have been reported [8, 10]. However, no such cases have been reported after IVR.

It is difficult to explain why although ranibizumab was administered for both eyes on the same time, but asymmetric outcomes were observed in some patients. The only factor that influenced the outcome in our series was BW. The mean BW of the asymmetric outcome group (1222.2 ± 216.6 g) was smaller than that of the symmetric outcome group (1412.2 ± 335.6 g, $P = 0.001$). We hypothesize that subtle differences in the levels of moieties in the vitreous

Table 1: Patient characteristics of infants treated had different outcomes in two eyes.

Cases/gender/eye	GA (weeks)	Birth weight (g)	Zone	Stage	Plus	PMA, weeks at IVR	First appearance of different outcome post-IVR (days)	Clinical course	PMA, weeks at laser treatment	Surgery	Final retinal reattachment	Follow-up (months)
1/M/OD	31	1350	I	3	Yes	37	22	Regression first; then recurrence as zone II stage 2 at 42 weeks PMA	43	—	y	28
1/M/OS	31	1350	I	3	Yes	37	22	Marked posterior fibrosis; then S4B at 42 weeks PMA; S5 at 43 weeks PMA	43	LSV at 45 weeks PMA	Partial reattachment	28
2/M/OD	29	1200	I	3	Yes	37	10	Regression first; then recurrence as zone II stage 2 at 43 weeks PMA	43	—	y	30
2/M/OS	29	1200	I	3	Yes	37	10	S5 with marked posterior fibrosis and VH	—	Lensectomy and vitrectomy at 57 weeks PMA	n	30
3/F/OD	30	1100	I	3	Yes	35	27	Regression first; then recurrence as zone II stage 2 at 40 weeks PMA	41	—	y	10
3/F/OS	30	1100	I	3	Yes	35	27	Marked posterior preretinal hemorrhage at 38 weeks PMA; then S4B at 56 weeks PMA	41	LSV at 58 weeks PMA	y	10
4/M/OD	28	1000	I	3	Yes	36	18	Regression first; then recurrence as zone II stage 2 at 47 weeks PMA	47	—	y	29
4/M/OS	28	1000	I	3	Yes	36	18	Marked posterior fibrosis and VH then S5 at 47 weeks PMA	47	Lensectomy at 65 weeks PMA, vitrectomy at 104 weeks PMA	n	29
5/M/OD	27	980	I	3	Yes	36	116	Regression of ROP but persistent zone II avascularity	43	—	y	18
5/M/OS	27	980	I	3	Yes	36	116	Regression of ROP but persistent zone II avascularity; then	43	LSV at 53 weeks PMA	y	18

Table 1: Continued.

Cases/gender/eye	GA (weeks)	Birth weight (g)	Zone	Stage	Plus	PMA, weeks at IVR	First appearance of different outcome post-IVR (days)	Clinical course	PMA, weeks at laser treatment	Surgery	Final retinal reattachment	Follow-up (months)
6/F/OD	27	1100	I	3	Yes	34	63	S4A at 52 weeks PMA Regression first; then recurrence as zone II stage 1 at 43 weeks PMA	43	—	y	9
6/F/OS	27	1100	I	3	Yes	34	63	Regression first; then recurrence as zone II stage 2 and severe VH at 43 weeks PMA	43	—	y	9
7/M/OD	31	1380	I	3	Yes	42	9	Regression first; then VH appeared at 43 weeks PMA and continued to progress	43	LSV at 47 weeks PMA	y	8
7/M/OS	31	1380	I	3	Yes	42	9	Regression of ROP but persistent zone II avascularity	43	—	y	8
8/F/OD	31	1200	APROP		Yes	36	22	Regression of ROP	—	—	y	8
8/F/OS	31	1200	APROP		Yes	36	22	Plus regressed but VH progressed to fibrosis at 40 weeks PMA and TRD at 42 weeks PMA	—	LSV at 52 weeks PMA	y	8
9/F/OD	32	1690	II	3	Yes	40	4	Plus sign regressed but peripheral fibrosis progressed and then stage 4A at 42 weeks PMA	41	LSV at 42 weeks PMA	y	11
9/F/OS	32	1690	II	3	Yes	40	4	Regression of ROP but persistent zone II avascularity	41	—	y	11

APROP: aggressive posterior retinopathy of prematurity; F: female; GA: gestational age; LSV: lens-sparing vitrectomy; M: male; OD: right eye; OS: left eye; PMA: postmenstrual age; TRD: tractional retinal detachment; VH: vitreous hemorrhage.

Table 2: Characteristics compared with infants between asymmetric and symmetric outcome.

	Asymmetric outcome group	Symmetric outcome group	P value (independent t-test)
Number of patients (eyes)	9 (18)	75 (150)	
Gestational age at birth (weeks)	29.6 ± 1.8	29.4 ± 2.1	0.556
Birth weight (g)	1222.2 ± 216.6	1412.2 ± 335.6	0.001
PNA at IVR (days)	52.1 ± 13.2	45.5 ± 13.8	0.946
PMA at IVR (weeks)	37.0 ± 2.4	35.9 ± 2.3	0.707

PMA: postmenstrual age; PNA: postnatal age; IVR: intravitreal injection of ranibizumab.

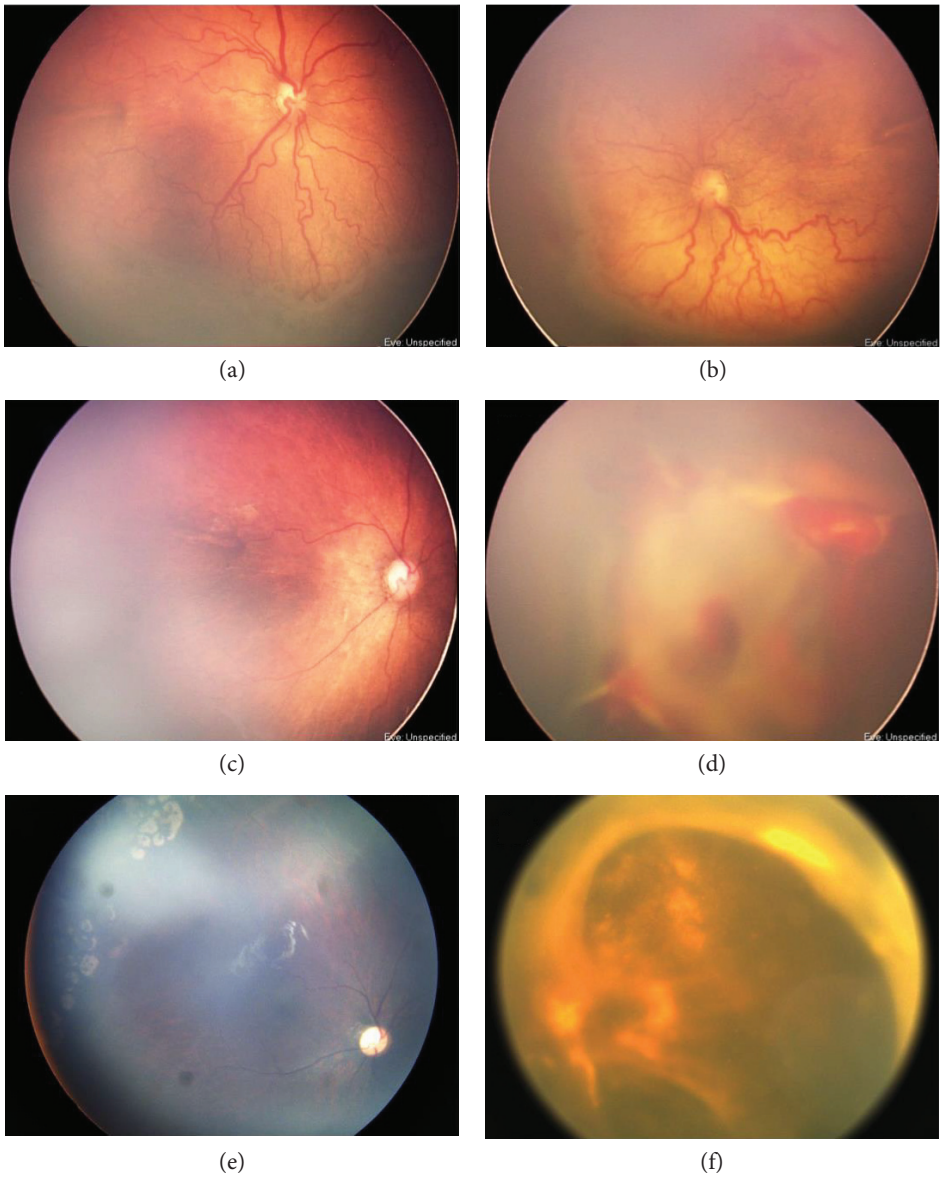

Figure 1: Before treatment, both eyes were diagnosed as stage 3+ ROP in zone I ((a) and (b)). Ten days after IVR, the right eye revealed regression (c), but the left eye revealed stage 5 ROP with marked posterior fibrosis and vitreous hemorrhages (VH) (d). The right eye received laser treatment at 43 weeks PMA, and the retina was flat at the last follow-up (e). The left eye received a lensectomy and vitrectomy at 57 weeks PMA, and the retina was not reattached at the last follow-up (f).

hemorrhage, such as VEGF, erythropoietin, and insulin-like growth factor-1, [11], may have caused contralateral eyes to follow an asynchronous disease course. Thus, the injection time and dose suitable for one eye was not suitable for the other eye. In infant 2, for instance, RetCam fundus photography revealed that both eyes had zone I, stage 3+ ROP before IVR treatment with the same degree of ridged membrane formation and preretinal hemorrhage; however, at 10 days

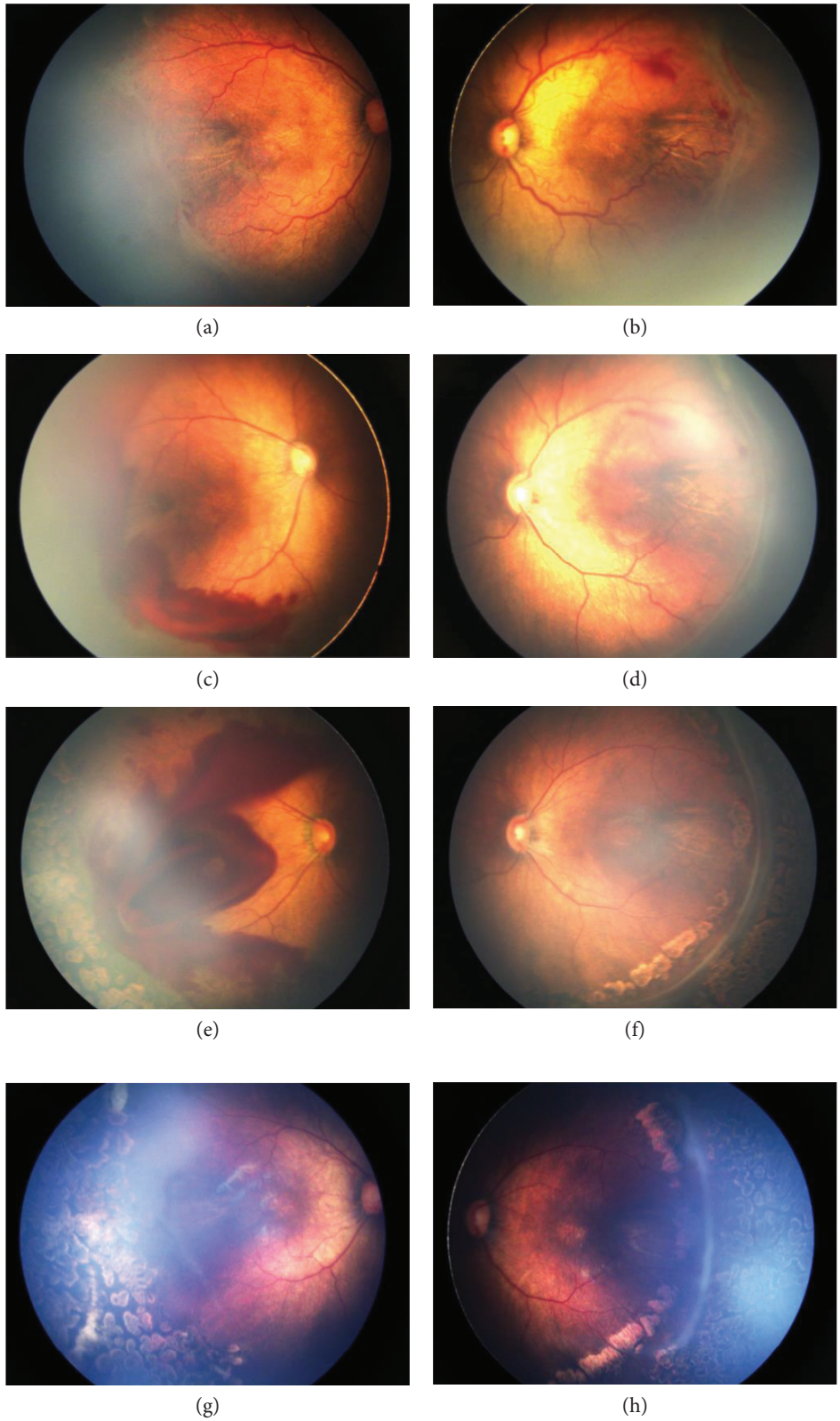

Figure 2: Before treatment, both eyes were diagnosed as stage 3+ ROP in zone I ((a) and (b)). Nine days post-IVR, regression of ROP and plus disease was noted in both eyes, but there were vitreous and preretinal hemorrhages only in the right eye ((c) and (d)). Thirty-eight days post-IVR, hemorrhages in the right eye continued to progress even after laser treatment (e) and covered the macula; and the eye eventually required LSV treatment. One month after LSV treatment, the retina of the right eye was flat with peripheral laser spots, and there was no sign of hemorrhages. The left eye received laser treatment due to persistent zone II avascularity at 43 weeks PMA (f), which was resolved by the last follow-up (h).

post-IVR, the left eye revealed a marked extraretinal fibrovascular proliferation (EFP) and VH, while the retina of the right eye was flat with regressed plus disease.

The reactivation rate of ROP after IVR was relatively high. The reported rate of reactivation after IVB was 0~4.3% [5, 12]. Compared with ranibizumab, bevacizumab has a longer half-life [13, 14], it reduces serum VEGF levels more significantly, and systemic VEGF suppression lasts longer [15, 16]. Although this may make ranibizumab a better option for premature patients, it may also translate into a higher chance of reactivation [6]. The reported rate of recurrence with conventional laser therapy was about 26% [5, 17]. Unlike laser treatment destroying the retina, retinal vessels continue to develop after IVR. This can theoretically decrease the supplementary laser spots needed after reactivation and the subsequent destruction of peripheral visual fields, which might offer potential vision benefits [17]. Moreover, the interval from treatment to reactivation of anti-VEGF treatment was longer than that of laser therapy [5]. Approximately ninety percent of infants demonstrated reactivation after IVB within a 10-week window from approximately 45 to 55 weeks of adjusted age [18]. The mean reactivation PMA after IVR in this study was 43.4 ± 3.4 weeks, which was earlier than IVB. Thus, the follow-up examinations after anti-VEGF treatment should last longer than laser treatment [18].

The timing of the administration of anti-VEGF therapy is of utmost importance [19]. The mean injection time in the asymmetric outcome group was later than the treatment of zone I ROP in the BEAT-ROP study (34 ± 1 weeks PMA) [5]. All of our patients received IVR within phase II of ROP [5, 20], and they all had plus disease before the initial treatment. It is possible that an older PMA is a risk factor for complications after IVR treatment.

Anti-VEGF agents might exacerbate preexisting fibrosis and retinal detachment due to traction [21]. In patients with proliferative diabetic retinopathy, a decline in VEGF levels with active neovascularization due to anti-VEGF treatment may inhibit angiogenesis and promote fibrosis driven by connective tissue growth factor [22]. In patients with ROP, there are several reports of vitreoretinal traction band formation and retinal detachment following anti-VEGF therapy [7, 8, 21, 23]. In our cases, the deteriorated eyes of infants 1, 3, 4, 5, and 8 that eventually progressed to stage 4 or 5 all had EFP and vitreous or/and preretinal hemorrhages before IVR. EFP combined with vitreous or preretinal hemorrhages may be an indication of poor prognosis of anti-VEGF treatment for ROP.

Vitreous or preretinal hemorrhages are other major ocular complications associated with IVR. Vitreous or preretinal hemorrhages were observed in 8% of the eyes after IVB, and all were absorbed after a few weeks [24]. Infant 6 in our series had preretinal hemorrhages in her left eye 63 days post-IVR due to the recurrence of ROP. Preretinal hemorrhages were eventually resolved. Infant 7 had preretinal hemorrhages in his right eye nine days post-IVR, and hemorrhages aggravated and expanded covering the macula. Thus, both recurrences of ROP and IVR itself can cause vitreous or preretinal hemorrhages. Any vitreal-retinal tractive force or vascular contractive force exerted on neovascularization could lead to bleeding.

The limitations of this study include its retrospective nature, the small-size cohort, and the varied follow-up time in a number of patients. We still have questions to be answered, regarding the optimal dosing, timing, indications, and prognostic factors of IVR treatment. Further studies are urgently needed to provide evidence-based answers to these questions.

In conclusion, our study demonstrated that contralateral eyes with ROP can take a significantly different clinical course after IVR, which is very rare in patients treated with laser [5, 25]. The high rate of reactivation is another concern that ophthalmologists should pay attention to. The use of anti-VEGF agents causes the outcome of treatment of ROP to be unpredictable with no consensus on the safety, indications, suitable timing, and doses. Weekly or even tighter follow-up schedule is required to detect vitreoretinal traction band formation and retinal detachment in time.

Disclosure

Qiujing Huang and Qi Zhang are the co-first authors. The sponsors and funding organization had no role in the design or conduct of this research. An earlier version of this work was presented as a poster at the 7th Chinese Congress of Research in Vision and Ophthalmology (2015).

Conflicts of Interest

No conflicting relationship exists for any author.

Acknowledgments

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Clinical Study

Comparison of Progression Rate of Retinal Pigment Epithelium Loss in Patients with Neovascular Age-Related Macular Degeneration Treated with Ranibizumab and Aflibercept

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Purpose. Retinal pigment epithelium (RPE) loss in neovascular age-related macular degeneration (nAMD) seem to have a linear progression but might be influenced by the treatment. The purpose of the study is the comparison of RPE loss over three years in patients treated with intravitreal ranibizumab to patients who were switched to aflibercept. *Methods.* A retrospective analysis with 96 eyes switched to aflibercept was conducted. The progression rate of RPE loss was evaluated in patients who showed atrophy one year prior to switch ($n = 17$) or on switch date ($n = 19$). The RPE loss was evaluated by spectral domain optical coherence tomography (SD-OCT). Further, 22 eyes from patients treated with ranibizumab were compared. *Results.* The median yearly progression of RPE loss after square root transformation showed no significant difference in the year prior to switch compared to the year after switch ($p = 0.854$). In patients who received only ranibizumab, the median yearly progression of RPE loss was 0.15 mm/y, for aflibercept patients, 0.13 mm/y. This difference was not statistically significant ($p = 0.172$). *Conclusions.* There seems to be a linear progression rate of RPE loss in patients treated with ranibizumab as well as in patients with aflibercept. No significant increase of progression rate was found after switch to aflibercept.

1. Introduction

Age-related macular degeneration (AMD) is a common cause for legal blindness in the elderly population reaching a global prevalence—according a recent meta-analysis of 129,664 individuals aged 45–85 years—of 8.69% [1].

Central vision is commonly impaired in AMD patients due to choroidal neovascularization (CNV) or due to fibrosis and/or loss of retinal pigment epithelium (RPE). CNV and associated edema can be controlled today by chronic treatment with inhibitors of the vascular endothelial growth factor (VEGF); however, fibrosis or loss of the RPE—once they occur—appears irreversible. Since fibrosis and RPE loss are associated with photoreceptor loss, central vision is impaired. RPE loss is common in nonneovascular AMD, but can also be associated with neovascular AMD (nAMD), either as sequelae of CNV or as concomitant nonneovascular AMD. Changes in the cell environment as a result of oxidative stress have been proposed to further damage RPE cells, which are already dysfunctional due to the underlying disease [2, 3].

Inhibition of VEGF is the current first-line therapy for nAMD and beneficial for most patients. However, VEGF is also a relevant physiologic factor within the retina and the RPE and its full suppression might impair the RPE and the underlying choriocapillaris [2, 4]. In the large Comparison of Age-Related Macular Degeneration Treatments Trials (CATT), more RPE atrophy appearance was found in patients treated on a monthly basis than those with pro re nata regiment with fewer treatments [5].

In addition to treatment frequency, different VEGF-binding affinities as well as other properties of currently used

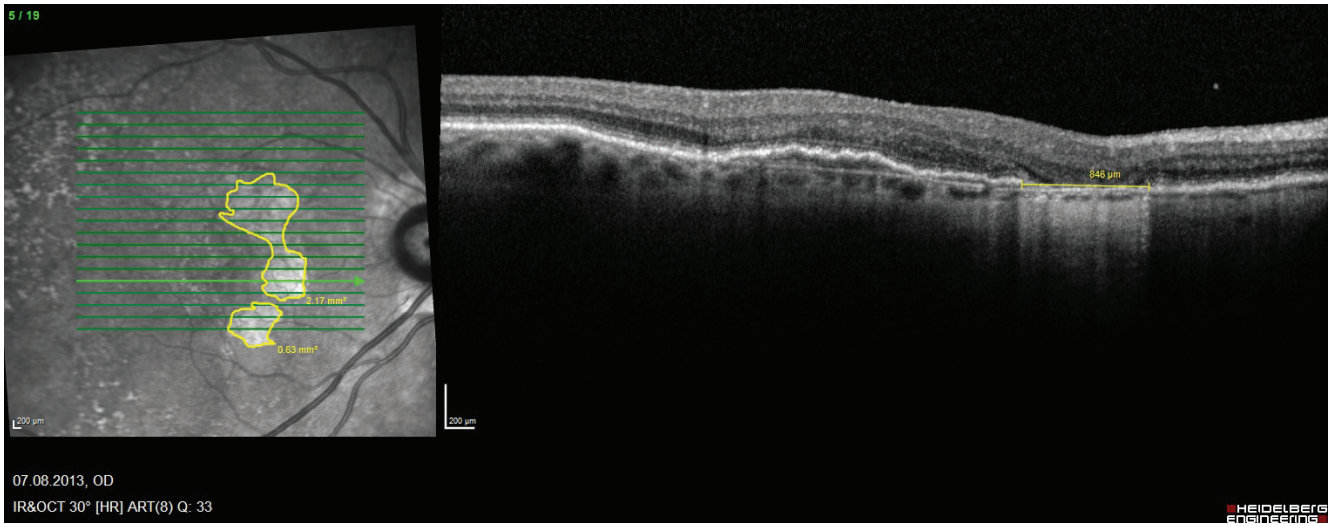

Figure 1: Representative case with RPE loss of a patient receiving intravitreal ranibizumab.

VEGF inhibitors might play a role on their effect on RPE and choroid. In effect, differences—especially on the choroid—have been described for ranibizumab and aflibercept in animal models and in clinical studies [4, 6].

In the past, fundus photography and fluorescein angiography (FA) have been the standard for evaluating RPE loss; however, there are key disadvantages in using these imaging modalities. Currently, fundus autofluorescence and SD-OCT have become the most widely used imaging technologies for evaluating loss of the RPE. Recently, it has been shown that RPE loss progression rates in nonneovascular AMD—after square root transformation—have a linear progression using different imagining modalities [7]. Despite the current advances in RPE imaging and potentially first upcoming treatment options to prevent RPE loss, there is no formal consensus terminology of RPE loss in AMD and its precise definition. One aspect however—most experts agree upon—is the total loss of RPE in areas with hypertransmission seen in SD-OCT.

In this context, a retrospective analysis on the progression rate of RPE loss over time in patients with nAMD being switched from ranibizumab or bevacizumab to aflibercept was conducted and compared to nAMD patients of similar age being treated with ranibizumab only.

2. Material and Methods

The retrospective study was conducted at the Department of Ophthalmology at the City Hospital Triemli in Zurich and included 118 eyes with nAMD. The study was conducted according to the Declaration of Helsinki and was approved by the local ethics committee in Zurich. Written informed consent was collected from all study subjects, prior to investigation-related procedures.

The study was conducted based on the following patient selection. A total of 96 eyes initially treated with ranibizumab or bevacizumab were switched to aflibercept due to insufficient response defined as persistent intraretinal or subretinal fluid despite at least three intravitreal injections within four months prior to the switch (consecutive cases between November 2012 and March 2013). Patients aged from 68 to 95 years were included in the study. For the statistical analysis, RPE loss lesions were included under the following conditions: RPE loss diameter was more than 300 μm; atrophy lesions without signs of suspected CNV in SD-OCT including PED or fibrosis, entitled as CNV independent RPE loss; and RPE loss area fully within the $20^\circ \times 15^\circ$ scan frame at one year before switch or on switch date.

All patients were imaged at all visits using the Heidelberg Spectralis system (Heidelberg Engineering, Heidelberg, Germany) in follow-up mode. A standard set of 19 B-scans (512 A-scans; $20^\circ \times 15^\circ$) was used at all times. The total area of hypertransmission on SD-OCT within the $20^\circ \times 15^\circ$ and the maximum diameter of RPE loss on the fovea crossing OCT scans was measured by two graders (JW and MW).

Measurements were taken one year prior to switch, at the time of switch, and one, two, and in some patients as well three years after switch. The identical OCT setting was used for the follow-up scans. For independence of growth rate from initial lesion size a square root transformation was performed [8]. All eyes were treated, except for the loading dose at switch, using a treat-and-extend strategy as described recently [9].

In addition, the progression rate of RPE loss (hypertransmission on SD-OCT) was retrospectively examined in 22 patients with nAMD, who were treated only with ranibizumab over 3 years. Patients who developed RPE atrophy according to the same criteria as above within 4 to 40 months after initiation of ranibizumab therapy were included.

The yearly progression rates of RPE loss were statistically compared.

2.1. Image Analysis. The scanning laser image analysis program of the Heidelberg Eye Explorer (Version 1.9.10.0) was used to measure RPE loss. Total loss of RPE was predefined in the OCT B-scans by the pattern of increased choroidal hyperreflectivity due to hypertransmission and absence

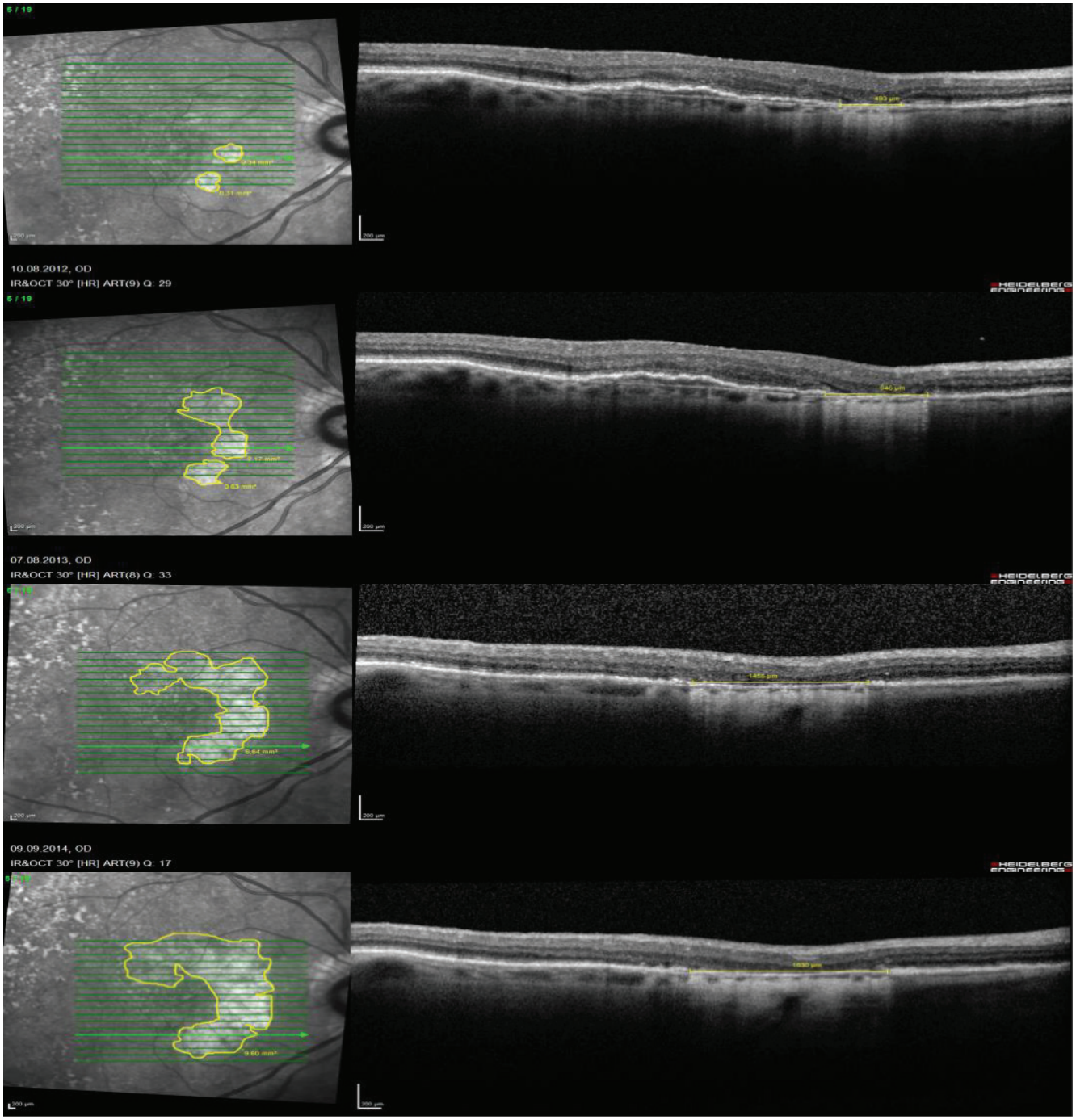

Figure 2: Yearly progression of RPE loss in the right eye of a patient with nAMD and intravitreal ranibizumab therapy.

of the photoreceptor inner segment/outer segment junction (Figure 1). The total area was measured with the draw region tool—based on the OCT B-scans—in the infrared image. In addition, the maximum diameter of the absolute RPE loss was measured on the fovea crossing B-scan. The yearly progression rates—based on the square root transformation—were calculated (Figure 2).

2.2. Statistical Analysis. All analyses were conducted using SPSS Version 20. Figures were created in Graphpad Prism Version 5. For quantitative variables, mean, standard deviation (SD), median, 1st quartile (Q1), 3rd quartile (Q3), minimum (Min), and maximum (Max) are given.

All measurements of the area of RPE loss were square root transformed to account for different baseline values [8]. For comparison of RPE loss one year prior to and one year after switch to aflibercept, differences of square root transformed values were compared with an exact Wilcoxon signed rank test. For comparison of RPE loss between the treatment groups, curve estimation regression

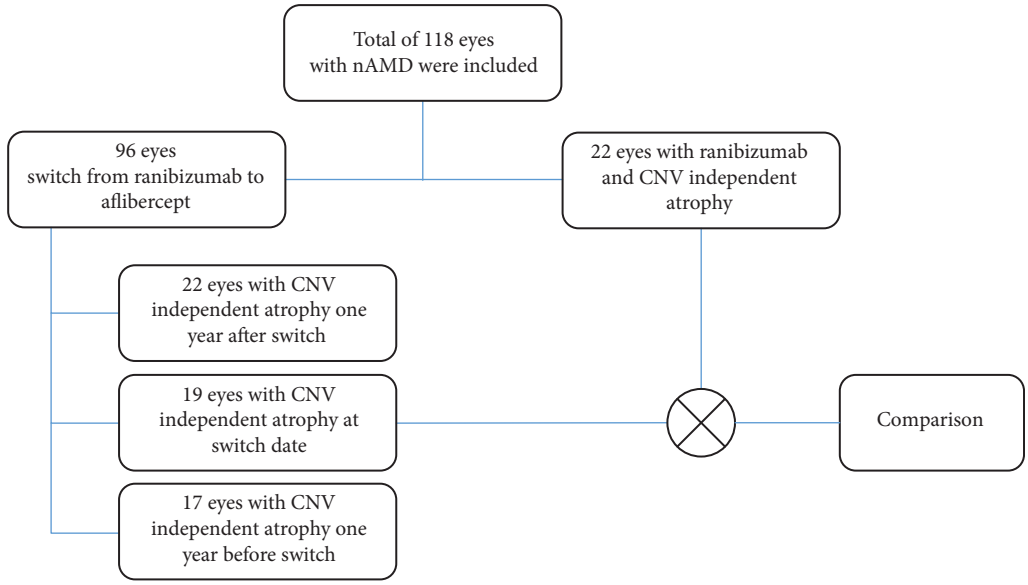

Figure 3: Overview of the included eyes and the different patient groups.

Table 1: Baseline characteristics of enrolled therapy switch patients.

Patients	RPE loss (mm^2)					Number of injections (N)		
	One year before switch	On switch date	One year after switch	Two years after switch	Three years after switch	Total	Prior to switch 24 months	After switch 24 months
P1	0.18	0.29	0.53	0.60	1.15	*56*	13	22
P2	0.23	0.27	0.35	0.42		*53*	22	21
P3	0.18	0.33	0.41	0.65	0.84	*44*	20	24
P4	1.44	2.88	3.25	3.85	3.94	*28*	19	4
P5	1.05	1.51	1.81	2.23		*47*	22	18
P6	0.35	0.54	0.69	0.75		*30*	16	17
P7	2.95	3.34	4.19	5.37		*64*	20	18
P8	2.82	3.06	3.40	3.80	3.79	*53*	12	17
P9	4.26	4.29	4.40	4.68		*26*	13	13
P10	1.02	1.07	1.80	2.01		*39*	16	23
P11	0.26	0.32	0.34	0.48	0.54	*40*	21	16
P12	0.97	1.13	1.37			*61*	22	12
P13	2.95	3.57	4.25	4.72		*50*	20	18
P14	1.93	2.48	2.94	3.34		*44*	21	11
P15	2.75	3.06	3.20			*36*	14	6
P16	3.38	3.52	5.17			*46*	23	7
P17	0.06	0.15	0.35			*28*	20	7
P18	0	0.55	0.66	1.06		*9*	5	4
P19	0	0.71	1.50	1.74	2.13	*36*	19	17

statistics was performed and the slopes of the square root transformed values were compared with an exact Mann-Whitney U test.

In addition, the injection rates of both treatment groups were calculated and compared with an exact Mann-Whitney U test or an exact Wilcoxon signed rank test for inter- and intragroup comparisons, respectively.

3. Results

3.1. Clinical Demographics. The study analyzed retrospectively 96 eyes of patients who were switched to aflibercept from ranibizumab or bevacizumab. The CNV independent lesions of RPE loss were measured. On each patient, the atrophy measurements were performed in only one eye.

Table 2: Baseline characteristics of enrolled ranibizumab patients.

Patients	RPE loss (mm^2)					Number of injections (N)
	First year	Second year	Third year	Fourth year	Total	Since RPE loss, 36 months
P1	0.29	0.31	0.59	0.90	*29*	18
P2	0.31	0.34	0.36	0.38	*47*	20
P3	0.06	0.17	0.21	0.35	*20*	6
P4	0.67	2.78	4.54	5.45	*6*	3
P5	0.08	0.54	1.62	2.84	*21*	16
P6	0.15	0.29	0.44	0.50	*31*	21
P7	0.45	0.48	0.85	1.33	*7*	2
P8	0.65	2.49	3.58	4.11	*26*	18
P9	0.40	0.75	1.17	1.85	*37*	26
P10	0.12	0.55	1.23	2.58	*35*	18
P11	0.39	0.55	0.70	0.88	*30*	23
P12	0.36	0.40	0.42	0.50	*31*	22
P13	0.08	0.65	2.80	6.64	*44*	22
P14	0.32	0.72	1.02	1.52	*27*	19
P15	0.26	0.36	0.41	0.42	*49*	19
P16	0.72	0.98	1.33	1.71	*27*	23
P17	0.33	0.58	0.70	0.98	*25*	20
P18	0.22	0.49	1.38	2.53	*22*	12
P19	0.56	0.68	0.86	1.35	*25*	21
P20	0.35	2.14	5.10	8.51	*18*	15
P21	0.15	0.16	0.19	0.24	*27*	18
P22	0.98	1.33	1.61	1.96	*39*	31

All 96 eyes were retrospectively evaluated for hypertransmission on SD-OCT one year prior to switch regarding the above-mentioned inclusion criteria for RPE loss regions. A CNV independent RPE loss was present in 17 patients one year before switch. In 19 patients, a RPE loss could be measured on switch date. In 9 of these 19 patients, a follow-up exam of two years was possible; in 6 of these patients, a follow-up exam three years after switch was conducted.

The median age of the patients within the therapy switch group that was statistically analyzed was 85.16 years (SD ± 11.31). Ten of the treated eyes were from female patients.

Most of 96 patients were treated only with ranibizumab before switch. Regarding the 19 patients who were included into the statistical analysis for comparison between two treatment groups, two patients were treated with ranibizumab and bevacizumab before switch.

A total of 17 patients who showed RPE loss one year prior to switch could be included for an intraindividual comparison of RPE loss prior to and after switch to aflibercept. The median number of injections for these 17 patients 24 months before switch was 20 and 17 in a follow-up time of 24 months after switch. The injection rate over 24 months was in median 2 injections less per patient after switch; the difference was not statistically significant ($p = 0.132$).

For a comparison between the two treatments, 22 patients with only ranibizumab therapy were compared to 19 patients after switch to aflibercept (Figure 3).

Table 3: Enlargement rate (mm) ($n = 17$).

Time	Mean	SD	Median	Q1	Q3	Min	Max
Prior to switch	0.12	0.11	0.11	0.05	0.16	0.01	0.50
After switch	0.14	0.10	0.11	0.07	0.20	0.02	0.40

The median age of the ranibizumab patients was 87.36 years (SD ± 0.71). The mean injection rate of the ranibizumab eyes was 0.54 injections per month and 0.71 injections per month for the 19 aflibercept eyes. This difference was statistically significant ($p = 0.011$).

Details on the anti-VEGF treatments are shown in Tables 1 and 2.

The analysis included patients who showed nAMD with classic or occult CNV (analog to CNV lesion types 1 and 2) [10]. The examined RPE loss regions showed different patterns comparable to the aspects known from geographic atrophy in nonneovascular AMD (focal, diffuse, patchy, and banded) [11].

The intraclass correlation coefficient (ICC) of the two independent graders amounted to 0.98 (ICC 2.1).

3.2. Comparison of RPE Loss prior to and after Switch to Aflibercept. The median progression rate of RPE loss was after square root transformation with 0.11 mm identical in the year prior to and after switch to aflibercept ($p = 0.854$). Descriptive statistics for enlargement rate before and after switch to aflibercept is shown in Table 3.

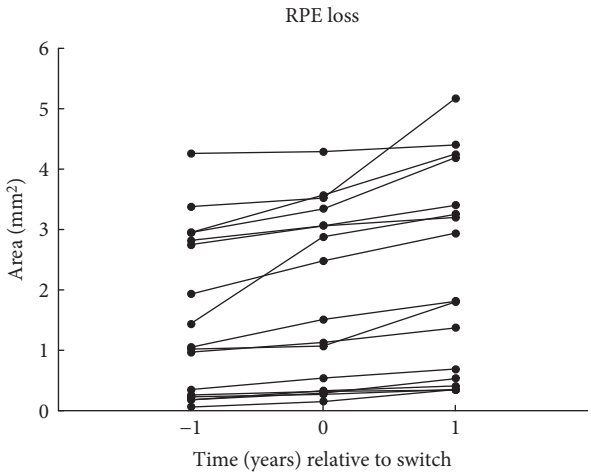

Figure 4: Course of RPE loss ($n = 17$).

The course of atrophy area over time before and after therapy switch is represented by Figure 4.

3.3. Comparison of Course of RPE Loss between Aflibercept and Ranibizumab Patients. The patients of comparable age treated with aflibercept or ranibizumab had no significant differences in the yearly RPE loss (after square root transformation). Table 4 shows the RPE baseline area of RPE loss for each drug.

Also within each group no significant change in the yearly rate of RPE loss could be found. The overall median yearly progression was 0.13 mm for aflibercept patients and 0.15 mm for ranibizumab patients (Table 5). This difference was not statistically significant ($p = 0.172$). The significant difference in the area of RPE loss at baseline was accounted for by using the square root transformation. Subsequently, the slope of the square root transformed area was calculated. R^2 was between 0.858 and 1.000 with a mean of 0.959. Thus, all courses of RPE loss could be described as a linear regression (Figure 5).

Further details on each group (number of injections, size of RPE loss at baseline, and the RPE loss for each year and each drug) are shown in Tables 1 and 2.

4. Discussion

The presented study seems to show a linear progression of CNV independent RPE loss in patients with nAMD under anti-VEGF treatment over at least three years of treatment.

Our mean progression rate before switch is 0.30 mm^2/year and 0.39 mm^2/year after switch, which is similar to the average rate of progression in the work of Bhisitkul et al. [12]. However, we did not include CNV lesions in the atrophy measurements. Until now, it is not known if we can compare atrophy lesions in nAMD and nonneovascular AMD. Based on the limited data in the literature, we assume that atrophy progression could have similar characteristics, especially regarding the CNV independent RPE loss in nAMD.

The primary aim for our study was to investigate, whether there is an early detectable, significant difference in the growth of atrophy independent from the CNV region after anti-VEGF therapy switch. No significant acceleration or deceleration of RPE loss was found when patients were switched from ranibizumab or bevacizumab to aflibercept. The number of aflibercept injections in the year after switch was 1.6 injections lower than in the year prior to switch using ranibizumab. Fewer injections with aflibercept might have despite longer anti-VEGF effects in the eye [13] led to the same rate of RPE loss as a more frequently applied drug with shorter activity in the eye. However, in both groups being treated on one drug for several years with decreasing numbers of injections per year (Tables 1 and 2), RPE loss remained constant and no deceleration could be found.

This study might indicate that progression of CNV independent RPE loss in nAMD has a linear progression over time independent of the duration of treatment and the anti-VEGF drug used.

It has however to be taken into consideration that the selected population was initially insufficiently responding to anti-VEGF therapy with ranibizumab and bevacizumab. This selected patient population required quite extensive treatment prior to switch (mean of 18.5 injections/24 months) and was still frequently treated after switch to aflibercept (mean of 14.9 injections/24 months). This is on the one hand reassuring since—despite heavy treatment—no acceleration of RPE loss could be found over several years; however, these eyes might have high levels of endogenous produced VEGF potentially protective from RPE damage.

Our findings for yearly growth rates of atrophy regions are in line with previous studies for nAMD and for slow-growing atrophy in nonneovascular AMD [3, 11]. This might relate to a reduced number of anti-VEGF injections used in a treat-and-extend setting compared to monthly treatment. Based on clinical experience, intravitreal anti-VEGF injections can induce a reduction in choroidal thickness [14], which has been confirmed by in vivo studies in primates. Understandably the reduction in choroidal flow, a reduced fenestration in the choriocapillaris and a reduced choriocapillaris density—as shown in primates—could lead to impairment of the RPE [4]. We have shown priorly in our study population that intravitreal anti-VEGF therapy leads to a significant reduction in choroidal thickness and a switch to three monthly injections of aflibercept from prior intensive anti-VEGF therapy induces a further reduction in choroidal thickness [6]. Whether the choroidal thickness plays a significant role in the development of atrophy in nAMD is currently unclear. But there are study results for nonneovascular AMD that indicate a choroidal thinning in the eyes with geographic atrophy compared to normal eyes of similar age [15].

The treat-and-extend treatment strategy used in the study has an OCT-based proactive component; however, it is not a continuous VEGF suppression. Between injections, this treatment allows some recovery of VEGF levels, which might be sufficient to prevent accelerated RPE loss. Furthermore—even though intriguing—it has so far not been shown in a clinic that any reduction in choroidal thickness is necessarily associated with a RPE impairment.

Even though our study does not allow drawing absolute conclusion due to the absence of a today impossible control

Table 4: Baseline area of RPE loss.

Group	Mean	SD	Median	Q1	Q3	Min	Max
Aflibercept ($n = 19$)	1.74	1.42	1.13	0.33	3.06	0.15	4.29
Ranibizumab ($n = 22$)	0.36	0.24	0.33	0.15	0.48	0.06	0.98

Table 5: Relative increase of RPE loss.

Group	Mean	SD	Median	Q1	Q3	Min	Max
Aflibercept ($n = 19$)	0.14	0.09	0.13	0.07	0.19	0.04	0.40
Ranibizumab ($n = 22$)	0.25	0.22	0.15	0.11	0.40	0.02	0.78

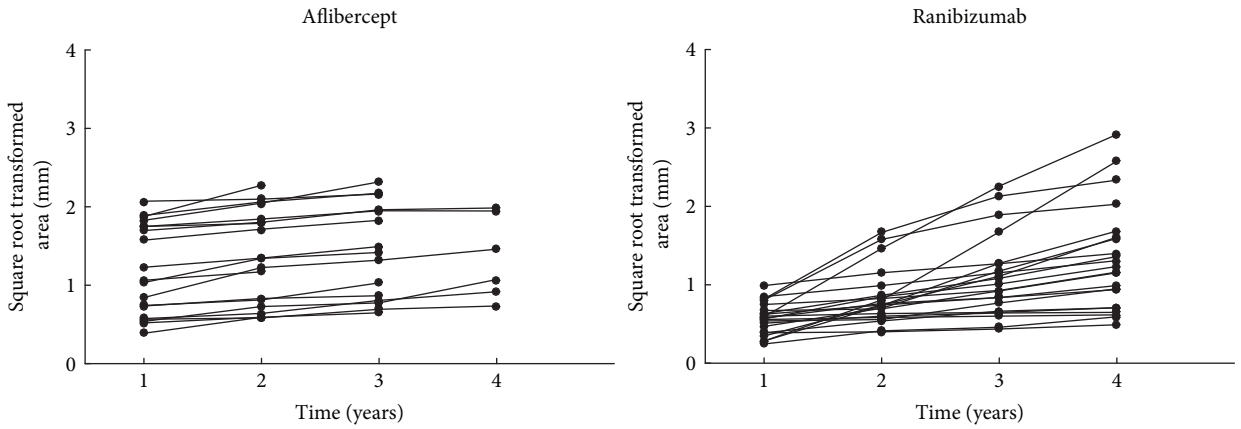

Figure 5: Course of RPE loss for patients receiving aflibercept ($n = 19$) and ranibizumab ($n = 22$).

group (untreated nAMD), we could not detect a very different rate of RPE loss progression between anti-VEGF drugs with different affinities to VEGF as shown within the switching group and the group treated with ranibizumab only.

Limitations of the presented study are its retrospective nature, the limited number of patients showing any RPE loss one year after switch to aflibercept, and the relatively small sample size in general. Further, the limitation of RPE loss analysis based on SD-OCT with 19 B-scans and infrared imaging has to be mentioned.

Reassuring are the consistent RPE loss rates in long-term follow-up of several years in an identical follow-up imaging mode with minimal loss of patients in follow-up. Currently, there is no consistent terminology for RPE loss. Much of the terminology dates to the era of fundus photography and fluorescein angiography. We opted for OCT technology since image acquisition is easy and provides good quality even without mydriasis. In contrast, autofluorescence imaging shows much worse quality in undilated eyes and is quite uncomfortable for patients. To date there is no consensus on terminology using OCT technology for RPE imaging; however, hypertransmission associated with loss of the overlaying IS/OS band appears to be a consistent indicator for absolute RPE loss. This excludes however possibly impaired RPE or a reduced RPE density. It has been shown that different imaging modalities measure RPE loss differently; the progression rates in different modalities have however been quite consistent [3, 8].

It has to be mentioned that there could be an influence of different baseline RPE losses for the comparison of the two groups. As seen in Figure 5, some patients of the solely ranibizumab group had a stronger RPE loss increase. However, in this group also, the injection rates were higher as in comparison to the aflibercept patients after switch. The results of CATT showed more often development of atrophy in patients treated on a monthly basis than with pro re nata regiment with fewer treatments [5]. Maybe the increased RPE loss in this group can be a sign for the presumed argument that a higher number of injections lead to a faster RPE loss.

5. Conclusion

In conclusion, our data did not show an increase of CNV independent atrophy progression rate in nAMD after switch from bevacizumab or ranibizumab to aflibercept in comparison of follow-up exams from one year before until up to three years after switch. Further, a comparable RPE loss progression could be seen in a control group of patients, who received only ranibizumab over a three-year period.

Disclosure

Both funding organizations had no role in the design or conduct of this research.

Competing Interests

The authors Magdalena A. Wirth and Juliana Wons declare the freedom of any potential conflicting interests, certifying that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interests; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript. The "Stiftung wissenschaftliche Forschung, Fonds Ophthalmologie, City Hospital Triemli" received research grants from Novartis Schweiz AG and Bayer Schweiz AG and payments for invited talks or advisory board participations for Matthias D. Becker and Stephan Michels from Allergan, Novartis, Alimera, Bayer, Roche, Pfenex, and Clanotech.

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