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RETINAL VASCULAR DEVELOPMENT WITH 0.312 MG INTRAVITREAL BEVACIZUMAB TO TREAT SEVERE POSTERIOR RETINOPATHY OF PREMATURITY

A Longitudinal Fluorescein Angiographic Study

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Purpose: To report the outcome of intravitreal 0.312 mg bevacizumab (IVB) monotherapy in acute retinopathy of prematurity (ROP) and to describe the vascular development over time.

Methods: Seventeen prematurely born infants were treated with IVB (0.312 mg in 0.025 mL per eye) because of acute ROP in posterior Zone II or Zone I, including aggressive posterior ROP. Infants were examined by fluorescein angiography (FA) using RetCam II or III (Clarity Medical Systems Inc) before IVB (n = 21 eyes), within 6 weeks (n = 23 eyes), 8 to 13 weeks (n = 22 eyes), and up to 45 months (n = 10 eyes).

Results: Acute ROP regressed in 19 out of 27 analyzed eyes (70%), including 100% and 80% of posterior Zone II and Zone I eyes, respectively, but only 25% of aggressive posterior ROP eyes. Early recurrences (11%, all aggressive posterior ROP) and late reactivations (18%) were observed within 1 week and at 9 to 12 weeks, respectively. All eyes showed leakage at the junction of the vascularized zone and capillary malformation on FA before treatment. Vessel branching abnormalities and circumferential vessel formation were typical FA features after treatment. Vascular outgrowth after one IVB became complete in 87.5% of eyes for which FA was available up to at least 9 weeks after IVB.

Conclusion: A single dose of 0.312 mg bevacizumab was efficient to induce regression of ROP in posterior Zone II and most of Zone I cases, but not in aggressive posterior ROP. FA describes vascular abnormalities, the importance of which warrants further investigation.

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Children born at a gestational age (GA) ≤32 weeks and/or a birth weight (BW) <1,500 g have an increased risk for developing acute retinopathy of prematurity (ROP), one of the few preventable causes of childhood blindness.[1] The disease shows a biphasic course and reflects the incompletely formed retinal vasculature at birth.[2] Major players in the pathomechanism include increased vascular endothelial growth factor (VEGF) expression, altered insulin-like growth factor-1 (IFG-1) expression and others.[3–5]

An International classification system has been developed to characterize the clinical phenotype and to define treatment guidelines, which are based on three major characteristics of ROP (stages, zones, and plus disease).[6–8] Plus disease is present when the large retinal vessels are tortuous and dilated in at least two quadrants at the posterior pole.[7] In addition, information concerning rubeosis iridis, dilated persistent tunica vasculosa lentis, retinal or vitreal hemorrhage, and rigid pupils are of importance. Treatment requiring ROP (Type 1 ROP) is defined as 1) Zone I with any stage and plus disease or 2) Zone I and Stage 3 without plus disease, or 3) Zone II Stage 2 or 3 with plus disease. Aggressive posterior ROP (APROP) is particularly

severe, develops early and rapidly at the posterior pole and always requires treatment.[7]

Classic treatment options include laser photocoagulation or cryotherapy. Both treatments aim at neutralizing the hypoxia-induced VEGF gradient through ablation of the peripheral retina. In recent years, anti-VEGF injections (bevacizumab, ranibizumab, and pegaptanib) have gained much attention in the treatment of neovascular disorders and have also been applied to infants with treatment requiring ROP, either as first line therapy or in combination with laser.[9] The underlying hypothesis for using anti-VEGF medication rather than laser is the attempt to cede VEGF-induced neovascularization, preserve the avascular periphery, and have it vascularized once pathologic vascularization has faded. However, the impact of intravitreal anti-VEGF treatment on physiologic vascularization and organ development is currently unknown.[10] It was shown in primates that after intravitreal injection of 1.25 mg bevacizumab (the adult dose), the anti-VEGF antibody was detectable in the circulation for several weeks.[11] Similarly, the anti-VEGF drug was detectable for at least two weeks in the serum of premature infants who received between 0.5 mg and 1 mg bevacizumab in one or both eyes.[12,13]

Since both treatment regimens have potential side effects, it is important to identify those ROP phenotypes for which anti-VEGF medication might be superior to classic laser photocoagulation and vice versa. The first major prospective, controlled, and randomized study to compare the use of bevacizumab versus classic laser photocoagulation was performed in the bevacizumab eliminates the angiogenic threat of retinopathy of prematurity study in ROP Stage 3+ in Zone I or posterior Zone II.[14] The dose of bevacizumab was 0.625 mg per eye, which ultimately corresponds to the same dose administered in adults in one eye. It was shown that Zone I disease profited most from the anti-VEGF treatment. Based on this report, the majority of studies published to date used a similar dose.[9]

The aim of this study was to characterize the efficacy of bevacizumab at 0.312 mg per eye in infants with treatment requiring ROP in Zone I and posterior Zone II including APROP, and to describe the effects of the treatment on the developing retinal vasculature by FA.

Material and Methods

Study Description

This is a multi-centre, retrospective study conducted at the Departments of Ophthalmology and Neonatology, Justus-Liebig-University Giessen, and the Children's Hospitals in Siegen and Fulda. The study followed the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of the Justus-Liebig-University of Giessen (JLU 17/15). Informed consent was obtained from the parents for all examinations, including FA and treatment after detailed explanation of the different treatment options, that is, laser photocoagulation or IVB.

All infants born prematurely at the NICUs in Giessen, Siegen, and Fulda were screened routinely for signs of ROP according to the German guidelines, that is, all infants below 1,500 g BW or GA $\leq$32 weeks, and also older and heavier babies, if oxygen supplementation was given for more than 3 days. Screening was done using a Shuttle, RetCam II or III (Clarity Medical Systems Inc, Pleasanton, CA) to obtain digital fundus images at the NICUs by a trained ophthalmologist (Giessen) or paediatrician (Siegen) or infants were referred to the Hospital in Giessen because the local ophthalmologist had detected possibly treatment-requiring disease with indirect ophthalmoscopy (Fulda). The final treatment decision was always based also on indirect binocular ophthalmoscopy.

In cases of severe ROP estimated to have a non-optimal outcome with laser photocoagulation by an experienced pediatric ophthalmologist (B.L.), infants were treated with IVB injection and enrolled in the present study. This included infants with ROP Stage 3+ in posterior Zone II or Zone I, or with APROP. Posterior Zone II was defined by a circle centred on the optic disc and a diameter 3 times the distance optic disc − fovea in accordance with the classification used in the bevacizumab eliminates the angiogenic threat of retinopathy of prematurity study.[14]

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Fluorescein Angiography (FA)

FA was done using the RetCam II or III (Clarity Medical Systems Inc). At the department of Ophthalmology in Giessen, FA examinations are normally scheduled once before treatment, and within the first 6 weeks, between 8 weeks and 13 weeks and beyond 20 weeks after treatment. Unfortunately, due to unrelated neonatal complications or technical issues, not all planned FAs could be performed or were of sufficiently high quality for subsequent analysis. On the other hand, in some cases, FA was performed more frequently if considered necessary to follow disease progression. Generally, all FAs were done to increase the clinical information before any decision for treatment, including assessment of leakage in the anterior segment and the retinal vasculature.

FA was performed in the operating room of the eye department or at the NICU of Giessen using a bolus of 10% fluorescein solution (Alcon Pharma GmbH, 79108 Freiburg, Germany) intravenously administered at a dose of 0.1 mL/kg body weight, followed by an isotonic saline flush. Sedation was provided by experienced neonatologists. When a treatment requiring case was identified at the NICUs in Siegen and Fulda, FA was performed under GA in Giessen directly before IVB. Bilateral lid specula were used to allow going back and forth between eyes for bilateral imaging in one session.

ROP Classification and Documentation

ROP was classified on the retinal images in accordance with original international classification of retinopathy of prematurity criteria.[6] When classifying ROP, the nasal and temporal extensions were assessed separately. Assignment to zone was based on the most central extension of retinopathy.

Criteria for early retreatment (= treatment failure) were persistent or progressive activity of vascular changes (progressive plus disease, fibrovascular proliferation, and vascular changes at the junction or posterior to the junction). Reactivation of ROP (late recurrence) was diagnosed when fibrovascular proliferation reappeared 10 weeks to 12 weeks after the initial IVB, after a period of no or low grade vascular activity signs (see **Table S1, Supplemental Digital Content**, http://links.lww.com/IAE/A490).

Bevacizumab Injection and Rescue Treatment

Intravitreal injection of bevazicumab (IVB) was done under general anaesthesia at the Department of Ophthalmology in Giessen, as described also in the technical requirements of the German Ophthalmological Society. In medically unstable cases, injection was done in the NICUs on bedside under strict asepsis. A dose of 0.312 mg in 0.025 mL bevacizumab prepared by the Hospital Pharmacy (Avastin; Genentech Inc, South San Fracisco, CA) per eye was injected 1 mm to 1.5 mm behind the limbus with a 30 G needle after local disinfection of skin and conjunctiva with 0.04% Polyhexanid (Lavanid 2; Serag-Wiessner AG, 95119 Naila, Germany) or 5% Polidon-iode (Braunol; Braun Melsungen, Melsungen, Germany) and after maximal mydriasis obtained with 2.5% phenylephrine (Minims; Bausch+Lomb, Kingston upon Thames, United Kingdom), tropicamide (Mydriatikum Stulln; Pharma Stulln, Stulln, Germany), and custom-made 0.1% atropine eye drops as needed. Postoperatively, 1 mg dexamethasone, 3,500 I.E. neomycin, and 6,000 I.E. polymyxin-B per 1 mL (Isopto-max eye drops; Alcon) were applied 4 times per day for 1 week and atropine 0.1% eye drops twice daily for 3 days.

In case of early treatment failure, a second IVB was performed, alone or together with laser therapy. Late reactivations were treated with laser therapy. Transpupillary laser ablation therapy was performed with a diode laser (Ocu-Light SL, 810 nm; Iridex Corp, Mountain View, CA) and a 20D or 28D lens (Volk Optical Inc, Mentor, OH) in a near confluent technique. Cryotherapy was applied to areas of previous laser treatment but yet continued vascular proliferation into the area of laser scars. In case of significant peripheral traction, 23 gauge lens sparing vitrectomy was performed to release this traction. Definitions of reactivation, treatment failure, treatment success, and indications for cryotherapy or vitrectomy are listed in **Supplemental Digital Content** (see **Table S1**, http://links.lww.com/IAE/A490).

Analysis of Vascular Outgrowth

We measured the outgrowth using a method that determines the ratio between the distance of the centre of the disc to the border of the vascularized zone (D→B) and the centre of the disc to the fovea distance (D→F). With full vascularization out to the ora serrata, the ratio is 4 for the nasal part and 5 for the temporal part of the retina.

Results

Study Population

Seventeen preterm infants with ROP Stage 3+ in posterior Zone II or in Zone I, or with APROP, born between August 2008 and August 2014, were included in the study (Table 1). ROP Stage 3+ in posterior Zone II was diagnosed in 5 infants (mean GA 24 2/7 weeks ± 4 days, mean BW 653 ± 90 g), ROP Stage 2+ or 3+ in

Table 1. Disease Classification, Clinical Parameter, and Treatment Details of the Enrolled Infants

Case	Stage ROP	ROP Zone ICROP	GA (weeks)	BW (g)	Age at Treatment (PMA in weeks)				Age and Reason of Death
					IVB	Laser	Cryo	Vitrec	
1	3+	pz II	25 2/7	560	36 3/7				
2	3+	pz II	24 5/7	770	34 3/7				
3	3+	pz II	23 3/7	540	35 5/7*	35 5/7†			
4	3+	pz II	24 2/7	730	34 5/7				
5	3+	pz II	24 1/7	665	34 1/7				
6	3+	z I	24 2/7	380	34 1/7				
7	3+	z I	24 2/7	480	35 6/7				
						47 2/7			
						54 1/7			
8	3+	z I	26 5/7	490	36 1/7				
9	3+	z I	23 3/7	710	34 4/7				
10‡	3+	z I	25 4/7	980	34 0/7				40 6/7, BPD
11‡	3+	z I	22 0/7	420	33 6/7				40 2/7, BPD
12	3+	z I	26 2/7	920	34 1/7				
13	APROP		24 5/7	690	35 6/7				
					36 4/7*				
						37 4/7			
14	APROP		21 5/7	460	34 0/7				
15‡	APROP		23 3/7	696	32 0/7				35 0/7, NEC
16	APROP		24 3/7	430	34 3/7				
						45 4/7			
								76 6/7	
							77 3/7		
							81 5/7	81 5/7	
								83 1/7	
17	APROP		23 4/7	630	33 1/7				
					34 6/7*	34 6/7*			
					36 6/7*				
						42 6/7†			

*Right eye only.
†Left eye only.
‡Children expired of unrelated reason.
BPD, bronchopulmonary dysplasia; GA, gestational age; IVB, intravitreal bevacizumab; NEC, necrotizing enterocolitis; PMA, postmentrual age; Vitrec, vitrectomy; ICROP, international classification of retinopathy of prematurity.

Zone I in 7 cases (mean GA 24 4/7 weeks ± 10 days, mean BW of 625 ± 227 g), and APROP in 5 infants (mean GA 23 4/7 weeks ± 7 days, mean BW 581 ± 113 g), when rapid progression within days was observed (Figure 1).

Interestingly, in all 3 groups the mean postnatal age at first treatment was similar, that is, about 10 weeks (Table 2). Once treatment requiring ROP was diagnosed, treatment was performed within 2 days at maximum according to German guidelines. Three children died from bronchopulmonary dysplasia (n = 2) or necrotizing enterocolitis (n = 1) (Table 1). Therefore, only 27 of the 33 treated eyes could be included in the analysis of treatment outcome (Table 2). Overall, 19/27 eyes (70%) were treated successfully with a single IVB of 0.312 mg. When only eyes with posterior Zone II disease were assessed, treatment was successful in 100% (n = 9) of cases, and still in 80% (n = 8) of eyes with Zone I disease. In contrast, only 25% (n = 2) of eyes with APROP responded to treatment. **Supplemental Digital Contents** (see **Figures S1–3**, http://links.lww.com/IAE/A487, http://links.lww.com/IAE/A488, http://links.lww.com/IAE/A489) show, for each group, one example of treatment outcome as seen with FA. Three eyes (11% of all eyes) had no response to the initial IVB and received retreatment within the first week. Five eyes (18%) had late reactivation of the disease about 11 weeks after the initial IVB. All three eyes with early treatment failure and 3/5 eyes with late reactivation had been classified as APROP.

FA Features

FA was performed before treatment, within the first 6 weeks posttreatment (short-term, 36–40 weeks postmenstrual age), between 8 weeks and 13 weeks posttreatment (mid-term, 42–48 weeks postmenstrual age), and beyond 20 weeks posttreatment (long-term) to

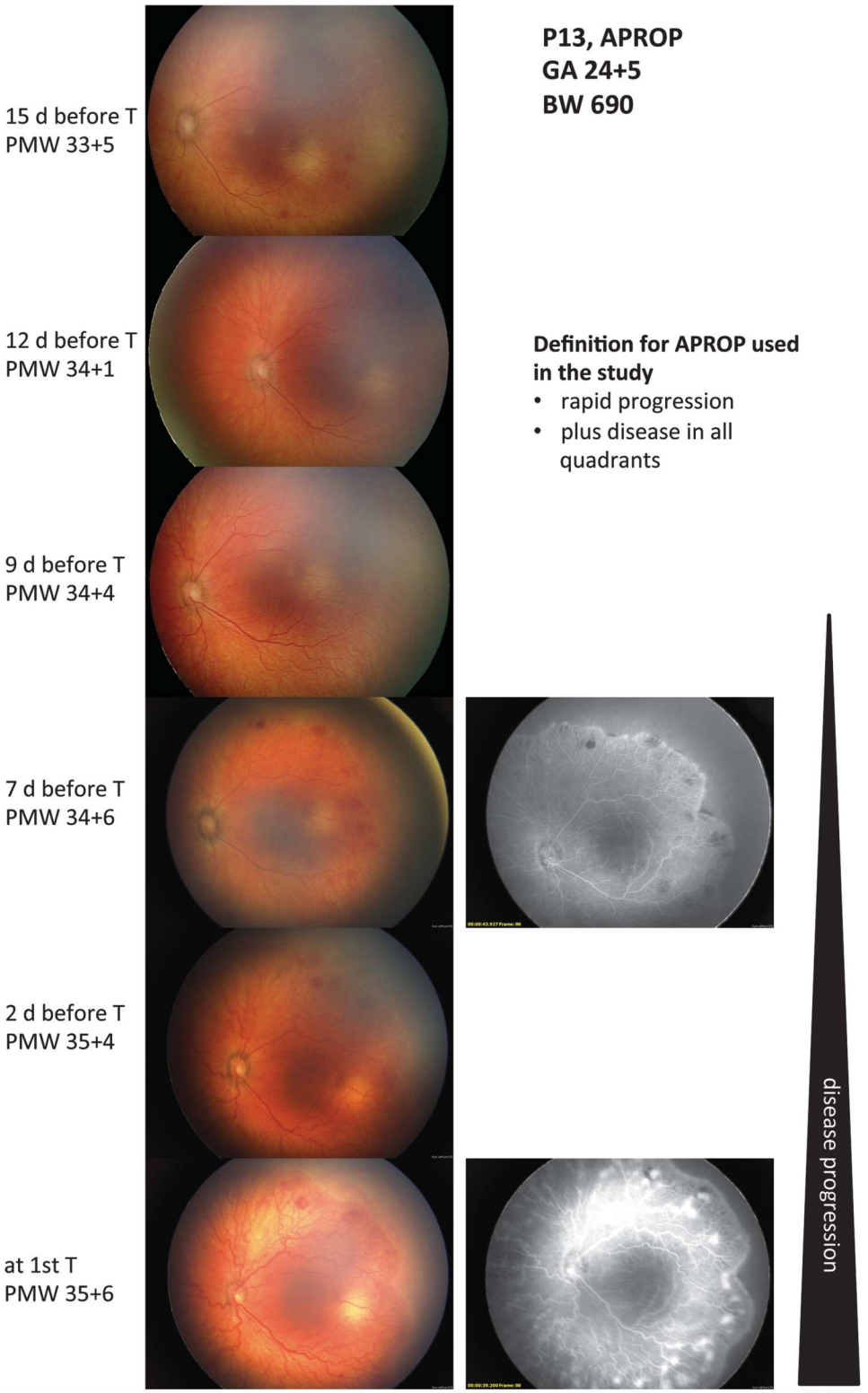


Fig. 1. Documentation of rapid progression of vascular changes in Patient 13 with aggressive posterior retinopathy of prematurity (APROP). This patient was seen 6 times within 15 days before treatment. At each examination date, fundus photography was performed, and twice fluorescein angiography (7 days before treatment and at the day of treatment). Initially, there were no significant changes. From 7 days before treatment onwards, rapid progression of vascular abnormalities (i.e., tortuosity of the large vessels, dye leakage at the junction of the vascular–avascular zone and at different sites within the vascularized area, bleeding, beginning flat intraretinal neovascularization) were observed classified as characteristic of APROP, and therefore prompt treatment was indicated.

Table 2. Demographics and Treatment Characteristics of Participants

Score	Total	Posterior Zone II	Zone I	APROP
Mean BW (g)	620 ± 327	653 ± 90	625 ± 227	581 ± 113
Mean GA (weeks)	24 2/7 ± 9 days	24 3/7 ± 4 days	24 5/7 ± 11 days	23 4/7 ± 7 days
Time to 1, T (weeks)	10 2/7 ± 9 days	10 5/7 ± 6 days	10 0/7 ± 10 days	10 2/7 ± 9 days
Total eyes treated	33	9	14	10
No. eyes included in analysis of treatment	27	9	10	8
Treatment success	19 (70%)	9 (100%)	8 (80%)	2 (25%)
Retreatment necessary	8 (30%)	0 (0%)	2 (20%)	6 (75%)
Early treatment failure	3 (11%)	0 (0%)	0 (0%)	3 (37.5%)
Time to retreatment	10 ± 3 days	—	—	10 ± 3 days
Late reactivation	5 (18%)	0 (0%)	2 (20%)	3 (37.5%)
Time to retreatment	11 0/7 ± 4 days	—	11 3/7 ± 0 days	10 6/7 ± 6 days

BW, birth weigh; GA, gestational age; T, treatment.

follow-up disease activity and vascular growth (Table 3). In this analysis, only FAs after the first anti-VEGF treatment were analyzed for consistency of results. Vascular abnormalities observed in FA were classified according to descriptions suggested by Lepore et al[15,16] (definitions of features summarized in Table 4). A representative image displaying each feature is shown in Figure 2. The prevalence of the abnormalities at the different time points of examination is displayed in Table 5.

At the Vascular–Avascular Junction

FA features seen at the vascular–avascular junction included leakage at the active site (Feature 1), abnormal vascular branching (Feature 2) at the large arteriolar level (2a), small arteriolar level (2b), or precapillary level (2c), circumferential vessel formation (i.e., "naked" arteriovenous shunts, Feature 3), hyperfluorescent lesions (i.e., cotton wool like structures or vascular tufts) (Feature 4), and capillary malformations such as capillary tufts (commonly referred to as "popcorn"), focal dilations, and rosary bead-like lesions (Feature 5) (see Figure 2, A–G and Table 4). All eyes (100%, n = 20) examined displayed Features 1 and 5 before treatment, and most of them displayed Feature 4 (90%, n = 18). Concerning the different ROP forms, all eyes examined with posterior Zone II disease (100%, n = 4) displayed, in addition to Features 1 and 5, also Features 2b and 4. This was not the case for Zone I disease and APROP, where only 87% (n = 7) displayed Feature 4 and 50% (n = 4) and 62% (n = 5) Feature 2b, respectively.

At the short-term examination, Feature 2a was always present in Zone I disease (100%, n = 10), but absent in posterior Zone II or APROP. In the latter two groups, Feature 2b was present in 86% (n = 6) of posterior Zone II ROP, and in 50% (n = 3) of APROP. The remaining features were present at low frequency.

At the mid-term examination, Feature 2a was again seen in all Zone I disease eyes (100%, n = 10), whereas only 25% (n = 2) of posterior Zone II and none of the APROP eyes were positive. Feature 3 was observed in almost three quarters of eyes (73%, n = 16), without any correlation to the form of ROP (75%, n = 6; 80%, n = 8; and 50%, n = 2, for posterior Zone II; Zone I; and APROP, respectively).

Long-term examinations revealed the presence of Feature 3 in all eyes examined (100%, n = 10). In addition, albeit at low numbers (n = 2), all eyes (100%) with posterior Zone II showed Feature 2b. Feature 2a was seen in two thirds of cases (n = 4) of Zone I disease.

Inside Vascularized Zone

FA features seen inside the vascularized zone included areas of hypofluorescence (Feature 6) and periarteriolar loss of capillary beds (Feature 7) (Figure 2, H and I and Table 4).

Before treatment, Feature 7 was present in 70% (n = 14) of all eyes, and Feature 6 in 25% (n = 5) (Table 5). Feature 6 was present in 50% (n = 4) of Zone I disease eyes, and only sporadically in the other 2 phenotypes. In contrast, the prevalence of Feature 7 increased from posterior Zone II disease (50%, n = 2) through Zone I disease (62%, n = 5) to APROP (87%, n = 7).

From Week 6 posttreatment onwards, Feature 6 was no longer observed, except in 2 eyes (20%) with Zone I disease at the mid-term evaluation. Similarly, Feature 7 was no longer observed in Zone I disease. In contrast, it was observed in 71% (n = 5) of eyes with posterior Zone II disease and 83% (n = 5) with APROP at the short-term evaluation, and in 75% (n = 6) and 50% (n = 2) at the mid-term

Table 3. Longitudinal Fluorescein Angiography Data for All Participants With Vascular Abnormalities Described as Features (see Table 4 for Definition)

Case	Age at T	FA Pre-T	FA Post-T (Weeks Posttreatment)			FA Description (Features Described in Table 4) OU, Unless Otherwise Stated
			Short-term (<6 weeks)	Mid-term (8–13 weeks)	Long-term (>20 weeks)	
1	36 3/7	+				1,2b,4,5,10
			3			2b,7,10
				9		2a,7,9
3	35 5/7	−				
			6			3,7 (OD)
				11		3,7
4	34 5/7	−				
			3			2b,9
				12		1,3
					21	2b,3
5	34 1/7	+				1,2b,4,5,7
			4			2b,7,10
				11		1,2b,3,7
6	34 1/7	−				
			4			2a,3,10
				10		2a,3,10
					25	2a,3
					187	2a,3
7	35 6/7	+				1,4,5,6,7,8
			4			2a,8
				11		1,2a,5,8
						Laser treatment OU at 12 and 19 weeks postinjection, stabilization
8	36 1/7	+				1,4,5,7 (OS)
			3			2a,10
				12		2a,3,6,9
					27	2a,3
9	33 4/7	+				1,2a,5 (OS)
			5			1,2a,c
				11		2a,3
11*	33 6/7	+				1,2b,4,5
			Died	Died		
12	34 1/7	+				1,2a,b,c,4,5,6,7,10
			2			2a,c,10
				13		1,2a,c,3
					20	1,2b,3
13	35 6/7	+				*1,2b,4,5,7*
						Injection 5 days after IVB OD, Laser 1 weeks later OU, stabilization
14	34	+				1,5 (OD)
			3			2b,3,7,10
				12		3,9,11e
					24	3
15*	32	+				1,2b,3,4,5,6,7,8 (OD)
			Died	Died		
16	34 3/7	+				1,4,5,7,8
			4			7,10
				8		7,10
						Recurrence 11 weeks post-IVB, subsequently Laser, Cryo and Surgery, OD finally stable condition, OS complicated surgery with unfavorable outcome

(*continued on next page*)

Table 3. (*Continued*)

Case	Age at T	FA Pre-T	FA Post-T (Weeks Posttreatment) Short-term (<6 weeks)	Mid-term (8–13 weeks)	Long-term (>20 weeks)	FA Description (Features Described in Table 4) OU, Unless Otherwise Stated
17	33 1/7	+	1			1,2b,4,5,7 1,2b,4,5 (OD) 7,9 (OU) *Injection and Laser 1 weeks after IVB OD, second IVB 2 weeks later OD, Laser 9 weeks later OS, finally stable condition*

FA, fluorescein angiography; OD, rigth eye; OS, left eye; OU, both eyes; T, treatment.

evaluation, respectively. Feature 7 was not seen at the long-term follow-up.

Macula

FA features analyzed in the macula included absence of the foveal avascular zone (Feature 8), hypoperfusion (Feature 9), and hyperfluorescence due to leakage (Feature 10) (Figure 2, J–L and Table 4). The prevalence of these features before treatment was generally limited, with 25% (n = 5), 5% (n = 1), and 30% (n = 6) for Features 8, 9, and 10, respectively. The prevalence of Feature 8 decreased over time, whereas it increased for Feature 9 from the short-term to the mid-term examination. Feature 10 was seen in 61% (n = 14) of all eyes at the short-term examination, equally distributed among the phenotypes, but was only seen in 9% (n = 2) at the mid-term screening, where it was only present in eyes with APROP (50% of those). None of the features was observed at the long-term examination.

Table 4. Fluorescein Angiography Features Used in This Study to Describe Vascular Abnormalities

At the junction vascular–avascular retina	
1	Dye leakage at the site of active ROP
2	Abnormal vascular branching At the large arteriolar level Small arteriolar level Precapillary level
3	Circumferential vessel ("naked" arteriovenous shunt)
4	Hyperfluorescent lesion (cotton wool like, vascular tuft = popcorn posterior to the ridge)
5	Capillary tuft formation, focal dilatation of capillaries, rosary bead like lesions inside the vessels
Inside vascularized zone	
6	Areas of hypofluorescence
7	Periarteriolar loss of capillary bed
Macula	
8	Absence of FAZ
9	Hypoperfusion
10	Hyperfluorescence due to leakage

Vascular Outgrowth Into Periphery

For 16 eyes that received IVB successfully as monotherapy, vascular outgrowth into the periphery was documented up to 9 weeks at minimum (range 9–187 weeks) (Figure 3). Calculating the ratio between the distances of the centre of the disc to the border of vascularized zone (D→B) and the centre of the disc to the fovea (D→F) in a 30 weeks old term born child without ROP and unrelated to this study (Figure 3, A and B), we show that the ratio is 4 for the nasal and 5 for the temporal retina in case of full retinal vascularization. This is also schematically visualized in Figure 3C. For the 16 eyes analyzed in this substudy, the mean ratio for the temporal vascularized zone was 4.61 ± 0.27 for all but 2 eyes from one patient (Patient 5), and the mean ratio for the nasal zone (without Patient 5) was 3.73 ± 0.2 (Figure 3D). Patient 5 showed an asymmetric vascularization, as in both eyes the temporal part was not completely vascularized, but the nasal part was (Figure 3D, highlighted in gray). The degree of vascularization did not change significantly from 9 weeks to 11 weeks posttreatment (temporal ratio 4.3 ± 0.3, nasal ratio 3.8 ± 0.16) to 20 weeks to 27 weeks posttreatment (temporal ratio 4.6 ± 0.25, nasal ratio 3.6 ± 0.17), and 187 weeks posttreatment (Patient 6, Figure 3D).

Discussion

In this study, we describe the effect of intravitreal 0.312 mg/0.025 mL bevacizumab monotherapy IVB in 27 eyes with posterior Zone II or Zone I, Stage 3+ ROP, or APROP. We also report on the FA features observed before and after treatment using definitions set in place by Lepore et al,[15,16] and categorized by us.

Treatment success rates for posterior Zone II (100%) and Zone I (80%) disease were within the success rates reported in the literature.[13,14] However, in most of the previous studies, 0.625 mg per eye were

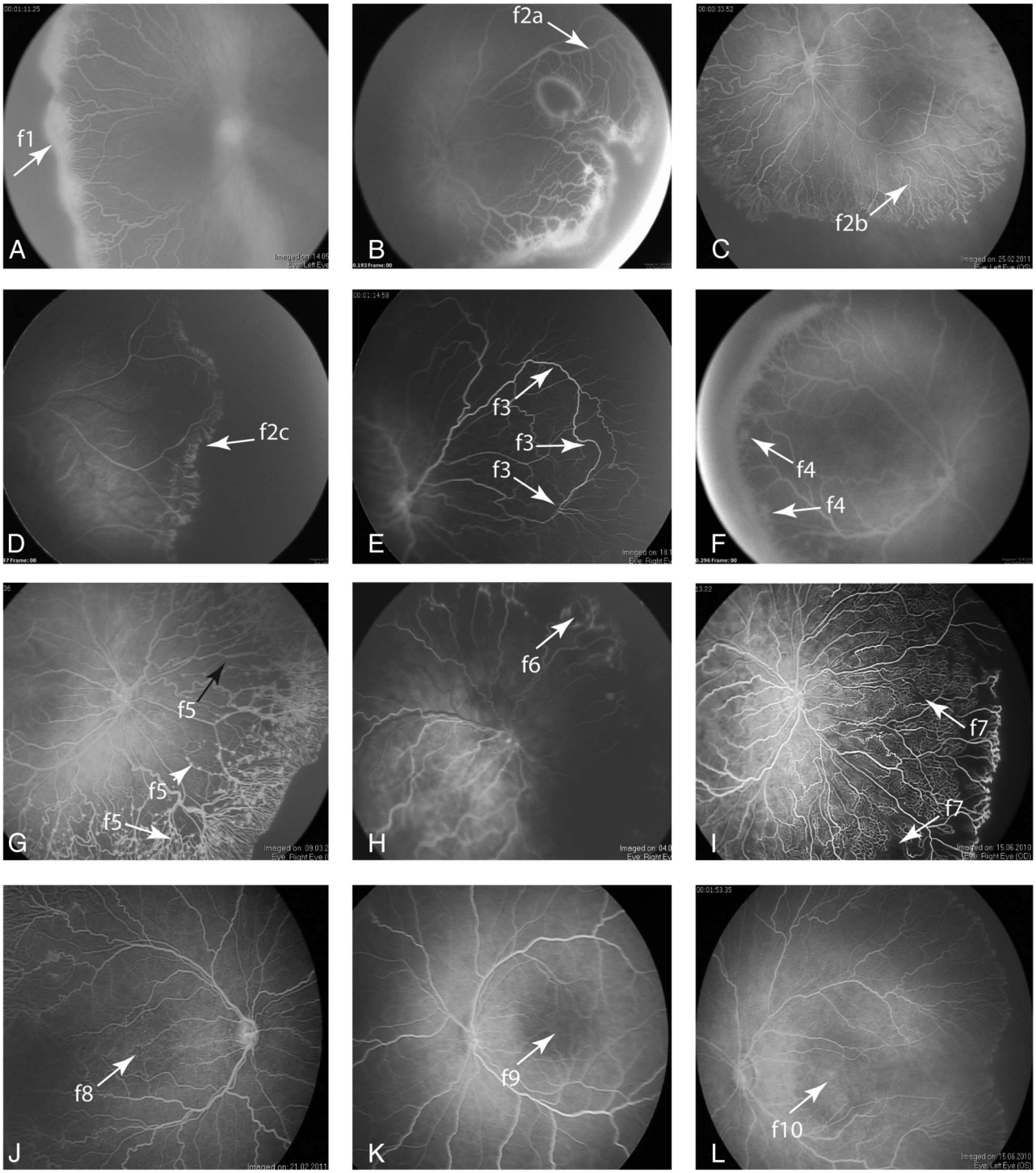


Fig. 2. Representative FA images displaying the features used in this study to describe vascular abnormalities in acute retinopathy of prematurity (ROP) leading to treatment and observed as they changed after a single intravitreal injection of 0.312 mg bevacizumab. Features are marked with a white arrow unless otherwise stated. **A.** Leakage of fluorescein from neovascularization at the vascular–avascular junction in Stage 3+ ROP (f1). **B.** Abnormal vascular branching at the large arteriolar level (f2a). **C.** Abnormal vascular branching at the small arteriolar level (f2b). **D.** Abnormal vascular branching at the precapillary level (f2c). **E.** Circumferential vessel (naked arteriovenous shunt) (f3). **F.** Hyperfluorescent lesions in form of cotton wool-like structures (f4). **G.** Capillary tuft formation (white arrow), focal dilatation of capillaries (black arrow), and rosary bead-like lesions (white arrowhead) inside the vessels (f5). **H.** Areas of hypofluorescence due to retinal of choroidal filling errors (f6). **I.** Periarteriolar loss of capillary bed (f7). **J.** Absence of foveal avascular zone (f8). **K.** Hypoperfusion of the macula (f9). **L.** Hyperfluorescence due to leakage in the macula (f10).

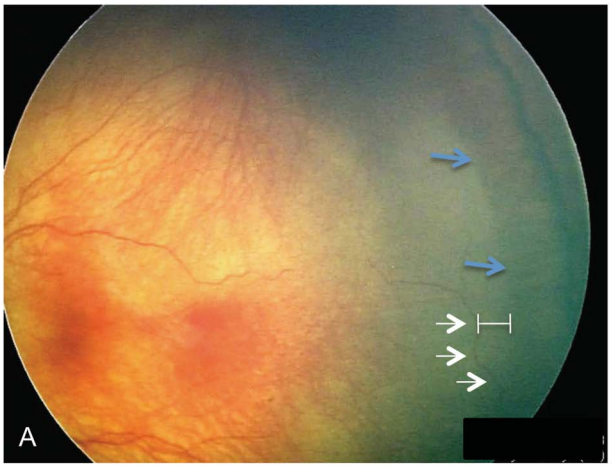
Table 5. Prevalence of Vascular Abnormalities Defined as Features Seen in FA According to Table 3

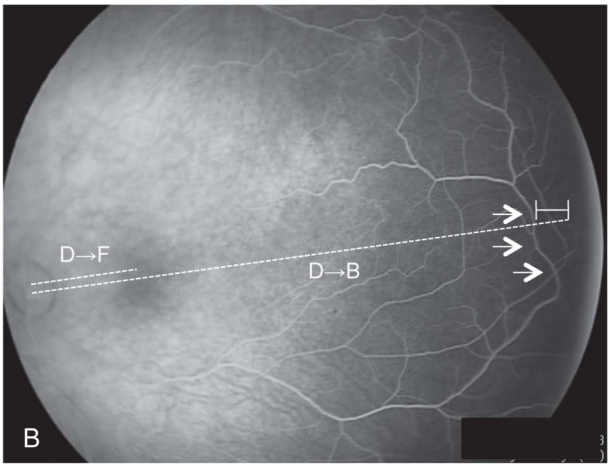
	Time Point	1	2			3	4	5	6	7	8	9	10
			a	b	c								
Total	Pre-T, n = 20	**20 (100)**	3 (15)	13 (65)	2 (10)	3 (15)	18 (90)	**20 (100)**	5 (25)	14 (70)	5 (25)	1 (5)	6 (30)
	<6 weeks, n = 23 (%)	3 (13)	10 (43)	9 (39)	4 (17)	5 (22)	0 (0)	1 (4)	0 (0)	10 (43)	2 (9)	4 (17)	14 (61)
	7–13 weeks, n = 22 (%)	8 (36)	12 (54)	2 (9)	2 (9)	16 (73)	0 (0)	2 (9)	2 (9)	8 (36)	2 (9)	6 (27)	2 (9)
	>20 weeks, n = 10 (%)	2 (20)	4 (40)	4 (40)	0 (0)	**10 (100)**	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Post-Zone II	Pre-T, n = 4 (%)	**4 (100)**	0 (0)	**4 (100)**	0 (0)	0 (0)	**4 (100)**	**4 (100)**	0 (0)	2 (50)	0 (0)	0 (0)	2 (50)
	<6 weeks, n = 7 (%)	0 (0)	0 (0)	6 (86)	0 (0)	1 (14)	0 (0)	0 (0)	0 (0)	5 (71)	0 (0)	2 (29)	4 (57)
	7–13 weeks, n = 8 (%)	4 (50)	2 (25)	2 (25)	0 (0)	6 (75)	0 (0)	0 (0)	0 (0)	6 (75)	0 (0)	2 (25)	0 (0)
	>20 weeks, n = 2 (%)	0 (0)	0 (0)	**2 (100)**	0 (0)	**2 (100)**	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Zone I	Pre-T, n = 8 (%)	**8 (100)**	3 (37)	4 (50)	2 (25)	2 (25)	7 (87)	**8 (100)**	4 (50)	5 (62)	2 (25)	0 (0)	4 (50)
	<6 weeks, n = 10 (%)	2 (20)	**10 (100)**	0 (0)	4 (40)	2 (20)	0 (0)	0 (0)	0 (0)	0 (0)	2 (20)	0 (0)	6 (60)
	7–13 weeks, n = 10 (%)	4 (40)	**10 (100)**	0 (0)	2 (20)	8 (80)	0 (0)	2 (20)	2 (20)	0 (0)	2 (20)	2 (20)	0 (0)
	>20 weeks, n = 6 (%)	2 (33)	4 (66)	2 (33)	0 (0)	**6 (100)**	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
APROP	Pre-T, n = 8 (%)	**8 (100)**	0 (0)	5 (62)	0 (0)	1 (12)	7 (87)	**8 (100)**	1 (12)	7 (87)	3 (37)	1 (12)	0 (0)
	<6 weeks, n = 6 (%)	1 (17)	0 (0)	3 (50)	0 (0)	2 (33)	0 (0)	1 (17)	0 (0)	5 (83)	0 (0)	2 (33)	4 (66)
	7–13 weeks, n = 4 (%)	0 (0)	0 (0)	0 (0)	0 (0)	2 (50)	0 (0)	0 (0)	0 (0)	2 (50)	0 (0)	2 (50)	2 (50)
	>20 weeks, n = 2 (%)	0 (0)	0 (0)	0 (0)	0 (0)	**2 (100)**	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

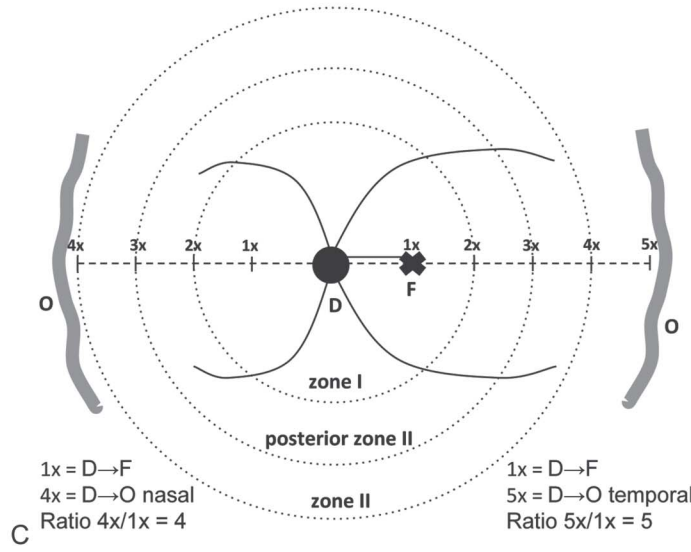
Highlighted in bold are those values that are common for all individuals in this cohort.
T, treatment.

applied, which corresponds to half of the adult dose per eye. Since the anti-VEGF molecules exit the eye and act on other organs, dosage of bevacizumab is particularly important in premature infants who are extremely fragile and display rapid growth of all organs. Surprisingly, only few studies used lower bevacizumab doses to verify efficacy in the treatment of acute ROP. Harder and colleagues reported on the outcome of 29 patients (57 eyes) with Zone I or Zone II plus disease, and showed that all except one eye were successfully treated with 0.375 mg bevacizumab.[17] A group from Japan even used only 0.25 mg bevacizumab in 6 eyes with Zone II and 2 eyes with Zone I disease and observed late recurrence only in Zone I disease.[18] These data, together with the results from our study suggest that a single dose of 0.312 mg bevacizumab is sufficient to reliably treat active ROP in posterior Zone II plus disease and very likely in Zone I disease, and hence could be considered preferable over 0.625 mg, since the lower the anti-VEGF levels injected the less likely unwarranted systemic side effects should occur. In line with this, large and multicentre studies currently aim at identifying the lowest effective dosage of bevacizumab (NCT02390531) or, alternatively, ranibizumab (NCT02375971, NCT02134457) in ROP.

The diagnosis of APROP is challenging and agreement of its diagnosis is imperfect even among experts.[19] Furthermore, outcome rates depend on whether bevacizumab was given as monotherapy or combination therapy with laser or cryotherapy.[20–28] In one case of APROP, simultaneous laser treatment combined with high dose of 0.75 mg IVB was effective.[20] Another small case series described successful treatment of APROP with administration of high dose 0.75 mg bevacizumab alone (n = 2) or in combination with laser (n = 2).[26] In a single-case description, 0.66 mg bevacizumab as first line therapy was initially sufficient to induce regression of neovascularization, but early recurrence occurred at 2 weeks needing laser treatment to completely halt the disease.[25] Law et al reported on 12 eyes with APROP treated with high dose 0.75 mg bevacizumab followed by laser or vitrectomy treatment within 72 hours. Still, 3 of the 12 eyes (25%) progressed to tractional retinal detachment.[21] In contrast, Dani et al[22] reported on 4 patients with APROP receiving either 0.625 mg bevacizumab alone (n = 2) or in combination with laser coagulation (n = 2) and observed a 100% success rate with no recurrences. Likewise, Mintz-Hittner and Kuffel[28] reported one case of APROP and successful treatment outcome after a single 0.625 mg bevacizumab injection. In a more recent chart review, 14 eyes with APROP were all successfully treated with 0.625 mg







Pat	wks after IVB	OD temp Ratio	OD nasal Ratio	OS temp Ratio	OS nasal Ratio
1	9	4.84	3.74	4.54	4.12
3	11	4.01	3.75	nd	nd
4	21	4.65	3.61	4.64	3.77
5	11	3.34	3.47	3.28	3.29
6	187	4.48	3.86	4.46	4.01
8	27	4.87	3.69	4.7	3.65
9	11	4.81	3.73	nd	nd
12	20	4.42	3.8	4.23	3.43
14	24	5.1	3.82	4.79	3.28
Mean temp:		4.61 +/- 0.27 (without P5)			
Mean nasal:		3.73 +/- 0.2 (without P5)			

D

Fig. 3. Principle and results of vascular outgrowth analysis (**A**) fundus image of a 30 weeks old term born girl examined under general anaesthesia because of incontinentia pigmenti. This girl did not have any form of ROP, was not born preterm, and is not part of the study presented in this manuscript. The fundus photo and the fluorescein angiography image in **B** are presented herein, because the temporal border of the vasculature and the ora serrata are clearly visible (blue arrows) and illustrate our approach to quantify vascular outgrowth. The white line indicates the area between an arcade forming large vessel (white arrows) and the ora serrata. **B.** Fluorescein angiography image of the girl described in **A** shows the optic disc and the vasculature with the arcade identified before (white arrows). The white line corresponds to the white line in **A** and indicates the distance from this vessel to the ora serrata. The short dashed line marks the distance from the centre of the disc to the fovea (D→F), the long dashed line marks the distance from the centre of the optic disc to the border of the vascularized zone (D→B). **C.** Schematic of the size of the retina from ora serrata to ora serrata in the horizontal meridian. With the distance from the centre of the optic disk to the foveal centre as reference, the distance from the centre of the optic disk to the ora serrata is about 5 times larger (×5) for the retina temporal to the optic disc, and about 4 times larger (×4) for the retina nasal to the optic disc. These anatomic relationships are also the basis of the classification in zones as set in place in the international classification of retinopathy of prematurity guidelines.[6] D, optic disc; F, foveal centre; O, ora serrata. **D.** Results of vascular outgrowth in 16 eyes with retinopathy of prematurity after monotherapy with 0.312 mg intravitreal bevacizumab. The table lists the relative distances to the temporal and nasal border of vascularization at the time of the last follow-up with FA. Patient 5 is highlighted in gray because of a significantly smaller temporal vascularized zone compared with all other patients. This patient was not included into the calculation of the mean value of the relative distances shown in the last two lines of the table.

bevacizumab alone.[23] A retrospective study in Sweden observed an even better or at least similar outcome of APROP disease in four eyes with a single IVB of 0.4 mg bevacizumab compared with combination therapy of 0.625 mg with laser or cryotherapy.[24]

A large study from Turkey observed very good outcome rates of 92% after a single IVB of 0.625 mg in 31 eyes diagnosed with APROP, and 1 or 2 repeated injections regressed the disease in all remaining cases.[27] This is in contrast to the results presented here, where 75% of APROP cases needed laser photocoagulation for stopping the disease, or even cryotherapy and vitrectomy. Repeated bevacizumab injections for the management of APROP did not appear to be

sufficient in our study (Table 1). The reason for the discrepancy of treatment efficacy compared with other studies might be related to differences in BW, GA, and our diagnosis of APROP. With a mean age of 28.2 weeks and BW of 1,005 g, the Turkish infants were about 4 weeks older and 400 g heavier than the patients enrolled in our study. Since age at treatment was similar at about 33 weeks to 35 weeks, the time frame from birth to treatment was about 6 weeks in the Turkish study and 10 weeks in the present study. The phenotype of APROP might be comparable in both cases but the maturation of the vasculature or growth factor concentrations might vary.

Vascular outgrowth into the periphery is an important feature, which can be well documented with FA, and the presence of a peripheral avascular zone of less than 2 disc diameters may be considered normal in healthy infants up to 11 years.[29] A study in 20 eyes with ROP treated with IVB as monotherapy showed that in more than 50% of the eyes (n = 11 eyes), peripheral avascular areas of more than 2 disc diameters were present up to 4 years after treatment, even though the outcome of the IVB was considered to be satisfactory.[30] Very recently, large peripheral avascular zones were also observed in children with congenital glaucoma or cataract up to 7.5 years of age, indicating that complete vascularization to the ora may not be the rule.[31] Since we could not identify the ora serrata on the FA images, we were not able to use this reference point. We therefore developed a method based on the distances of morphological structures (OD, fovea, peripheral border of vascularization). In case of full vascularization, the calculated ratios of 5 for the temporal and 4 for the nasal retina are essentially similar to what has been the basis for the definition of zones in ROP in 1985.[6] If we consider all eyes with a vascularized zone of more than the ratio 4 temporally (i.e., temporal arcade or Zone III) and 3 nasally to be fully vascularized, only 12.5% of eyes (n = 2) included in this substudy would be classified as incompletely vascularized by this definition. Vascularization into the periphery after one IVB appears to occur rapidly, since the level of vascularization at 9 weeks to 11 weeks posttreatment did not differ compared with later examination times in our cohort.

In our study, the time of onset of late recurrence was about 11 weeks after the first treatment. In previous reports, varying mean time intervals have been observed, for example 14 weeks and 19 weeks for Zone I and posterior Zone II disease, respectively, in the BEAT-ROP study,[14] and 14 weeks in Stage 3+ disease.[32] However, the range of onset varied significantly between 4 weeks and 25 weeks, resulting in the fact that even at postmenstrual age beyond 50 weeks, late recurrences were reported.[14] The observation that features typical for acute ROP can be reactivated after a prolonged period of time, regardless of the dose of anti-VEGF drug administered, indicates that the underlying mechanisms leading to the onset of an active ROP continue to be active and may only be temporarily blocked. Long-term follow-up therefore appears mandatory.

In our patient cohort, the course of the different FA features throughout the treatment period was not uniform, as some features decreased in frequency after treatment, thus indicating regression of the disease, whereas others increased with time, the consequences of which are currently not well understood.

Not surprisingly, all or almost all eyes examined with FA showed signs of active ROP at the vascular–avascular junction before treatment, such as leakage (Feature 1), hyperfluorescent lesions (Feature 4), and capillary malformations (Feature 5). Especially the occurrence of dye leakage at the junction in all patients, which is the most important sign for severe ROP and need for surgery is in line with the literature,[33] as is the presence of Features 4 and 5.[15] In all our patients, all three features decreased after treatment, indicating calming of disease activity at the junction followed by a more controlled outgrowth of vessels into the periphery.

Abnormal vascular branching (Feature 2) had different patterns in the 3 forms of ROP treated in our study. In posterior Zone II disease and APROP, the abnormal branching events occurred rather at the small arteriolar level (Feature 2b), whereas this was not the case for Zone I disease, where these branching events occurred almost exclusively at the large arteriolar level (Feature 2a) (Table 5). Interestingly, this was true regardless of whether FA was analyzed before or after treatment. What is even more interesting is the frequency of the appearance: in posterior Zone II disease, Feature 2b was present before treatment, decreased after treatment and reappeared at the long-term examination. In contrast, in Zone I disease, Feature 2a was present at low frequency before treatment, increased to 100% at the short-term and mid-term examination, and again decreased over time. Only in APROP, the frequency decreased after treatment and disappeared completely. Since Lepore et al[15] also observed persistence of abnormal branching after IVB treatment, these reactions of the vasculature to anti-VEGF treatment may be physiologic but materialize in different ways, potentially depending on the immaturity of the vessels at the time of treatment. Interestingly, the reaction of the vasculature to laser

treatment seems to be different, as abnormal branching events were reduced after treatment.[15]

Circumferential vessels or so-called "naked" arteriovenous shunts (Feature 3) appeared in 75% of cases at the mid-term examination, and in 100% of cases at the long-term examination, but were present only at low frequency before treatment. Lepore et al[15] observed a high frequency of this feature before and after treatment and described it as a long-lasting characteristic of IVB. Since we found these vessels in all groups, including all eyes successfully treated with single IVB injection, they do not seem to be associated with an increased risk for late recurrence, as suggested by others.[15,33,34] It is even possible that these shunts are a typical characteristic of continuing retinal vascular development, outgrowth into the periphery, and remodelling.

The highest incidence of capillary bed loss (Feature 7), which is different from areas of hypofluorescence due to retinal or choroidal filling errors (Feature 6), was observed before treatment in eyes with APROP (7 out of 8 eyes, 87%), an observation that is in line with prior studies that identified this feature as characteristic for APROP.[35] Interestingly, the prevalence of this feature decreased over time, an observation not in line with data presented by Lepore et al[15] for the IVB treated group. In their study, the rate only decreased in the laser treated group. Since we and others showed that oxygen levels have an influence on the size of the periarteriolar capillary free zone in different animal models of ROP, the question of whether this feature may represent a marker for an altered oxygen supply at birth or within the first weeks of life warrants more investigations.[36,37]

Macular abnormalities, frequently observed before treatment (100%) and after IVB (75%) in the Lepore study were seen less frequently by us in the eyes before treatment and only sporadically after IVB.[15] The reasons for these differences remain speculative but would indicate differences in study populations.

A major limitation of our study is the small sample size, which precludes a statistically relevant analysis, specifically in the APROP group, and that IVB monotherapy was not compared with laser monotherapy. Since this was a retrospective study, data analysis is based on routine clinical documentation and the definition of study arms was not possible. In line with this, no control group of imaging data from healthy infants was included. Variability of dosing is always an issue with bevacizumab, which needs to be prepared from a stock solution at the local pharmacy. In addition, we were not able to obtain FA data from every child for all time points pretreatment or posttreatment, which further decreases the number of cases available.

Nonetheless, the total number of 75 FA imaging data sets examined in this study enabled us to reveal interesting new clinical data in a sufficient number of cases. Specifically, we provide longitudinal imaging data even before treatment that help define eyes as either posterior ROP or APROP. However, this classification may still be somewhat arbitrary and not uniformly accepted or applied by others. Classification is nonetheless crucial as our data show the worst prognosis with IVB 0.312 mg in APROP eyes. Hence, our results may not be directly comparable with previous reports. Since in our study, the APROP phenotype, as one may expect, had the highest need for early and late retreatment, uniform classification among investigators is highly desirable, also in view of finding the optimal treatment regimen for APROP. Sufficient sample sizes can only be addressed in large and multicentre studies with ample photodocumentation.

To summarize, we show here that the use of lower doses of bevacizumab, that is, a quarter of the adult dose very likely shows similar results of disease silencing in posterior Zone II and Zone I disease compared with the use of half the adult dose. At times when systemic consequences of anti-VEGF medication for the developing organism of the infants are still unknown, this information is significant for the research community. In contrast, 0.312 mg bevacizumab did not seem to be the treatment of choice for APROP in our hands. Experience with higher dose anti-VEGF reported by others is also not conclusive, partially related to the loose definition of APROP. Fluorescein angiography is helpful in defining the junction of the vascularized zone and the vascular outgrowth, identifying avascular areas within the vascularized zone, the degree of leakage, and further vessel alterations such as shunt formation, the importance of which remains unknown with regard to any possible late sequelae.

Key words: acute retinopathy of prematurity, aggressive posterior retinopathy of prematurity, fluorescein angiography, bevacizumab, vascular development.

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