Systemic Counterregulatory Response of Placental Growth Factor Levels to Intravitreal Aflibercept Therapy

Claus Zehetner,[1] Nikolaos E. Bechrakis,[1] Martin Stattin,[1] Rudolf Kirchmair,[2] Hanno Ulmer,[3] Martina T. Kralinger,[1] and Gerhard F. Kieselbach[1]

[1]Department of Ophthalmology, Medical University Innsbruck, Innsbruck, Austria
[2]Department of Internal Medicine, Medical University Innsbruck, Innsbruck, Austria
[3]Department of Medical Statistics, Informatics and Health Economics, Medical University Innsbruck, Innsbruck, Austria

Correspondence: Claus Zehetner, Medical University Innsbruck, Department of Ophthalmology, Anichstr. 35, 6020 Innsbruck, Austria; claus.zehetner@i-med.ac.at.

Submitted: February 15, 2015
Accepted: April 5, 2015

Citation: Zehetner C, Bechrakis NE, Stattin M, et al. Systemic counterregulatory response of placental growth factor levels to intravitreal aflibercept therapy. *Invest Ophthalmol Vis Sci*. 2015;56:3279–3286. DOI:10.1167/iovs.15-16686

Purpose. Placental growth factor (PlGF) has been implicated as a contributor to resistance against anti-VEGF therapy. The purpose of the present study was to analyze the systemic levels of PlGF, VEGF-A, and VEGF-B in patients with neovascular age-related macular degeneration (AMD) after treatment with aflibercept, ranibizumab, or bevacizumab.

Methods. Totals of 19 patients were treated with intravitreal aflibercept, 19 with ranibizumab, and 18 with bevacizumab. The cytokine levels were measured by ELISA just before the injection, and 7 days and 1 month thereafter. Age- and sex-matched participants ($n = 22$) served as controls.

Results. The median PlGF plasma concentration at baseline was <12.0 pg/mL in the control group as well as in all three anti-VEGF treatment cohorts. After intravitreal aflibercept injection, a significant upregulation of systemic PlGF could be observed in all treated patients (38.0 [31.0–44.0] pg/mL after 1 week [$P < 0.001$] and 16.0 [0.0–19.0] pg/mL [$P = 0.005$] after 4 weeks). No significant effects on plasma PlGF concentrations could be detected in those treated with ranibizumab and bevacizumab. The systemic VEGF-A levels were significantly reduced 1 and 4 weeks after intravitreal aflibercept ($P < 0.001$, $P < 0.001$) and bevacizumab ($P < 0.001$, $P < 0.01$) injections. No significant effects on plasma cytokine concentrations could be observed in the ranibizumab cohort. No significant effects on systemic VEGF-B could be observed in any of the treatment groups.

Conclusions. In this study, we report a significant systemic upregulation of the proangiogenic cytokine PlGF after intravitreal administration of aflibercept. This might represent a counterregulatory response to antiangiogenic therapy.

Keywords: aflibercept, ranibizumab, bevacizumab, age-related macular degeneration (AMD), VEGF-A, VEGF-B, placental growth factor (PlGF)

Age-related macular degeneration (AMD) is the leading cause of severe and irreversible vision loss in aging western populations. Abnormal angiogenesis is the pathophysiologic hallmark of neovascular AMD.[1]

Multiple proangiogenic factors are consistently upregulated during neovascularization, and the process is, in part, mediated by transcriptional regulation via the hypoxia-inducible factor (HIF-1) cascade. The activation of HIF-1 induces an upregulation of several vasoactive gene products, including placental growth factor (PlGF), VEGF-A, and VEGF-B. This triggers a shift of the balance between pro- and antiangiogenic factors in favor of neoangiogenesis.[2,3] The discovery that the pathognomonic development of choroidal neovascularization (CNV) is mediated by the VEGF family of ligands gave rise to the development of antiangiogenic therapies for the treatment of neovascular AMD. The inhibition of VEGF-A currently is the most efficient therapy in arresting choroidal neoangiogenesis and vascular permeability.

Three anti-VEGF substances are in use currently for the treatment of patients with neovascular AMD in clinical routine. Ranibizumab (Lucentis; Novartis Pharma AG, Basel, Switzerland and Genentech, Inc., South San Francisco, CA, USA), a recombinant, humanized, antibody Fab (48 kD), and bevacizumab (Avastin; Genentech, Inc.), a recombinant human monoclonal IgG1 antibody (149 kD), both of which selectively inhibit human VEGF-A.[4,5] Aflibercept (Eylea; Bayer, Basel, Switzerland and Regeneron Pharmaceutical, Inc., Tarrytown, NY, USA) is the newer addition to the armamentarium of antiangiogenic therapeutics used in ophthalmology. Eylea is the intraocular formulation of ziv-aflibercept, a drug used in oncology (Zaltrap; Regeneron Pharmaceutical, Inc.), that has been specifically purified and buffered to minimize the risk of eye toxicity when injected intravitreally. It is a 110 kD, soluble decoy receptor that contains extracellular VEGF receptor sequences (VEGFR-1/Flt-1 and VEGFR-2) fused to an IgG backbone. Aflibercept not only blocks multiple isoforms of VEGF-A, such as bevacizumab and ranibizumab, but also the proangiogenic VEGFR1 ligands PlGF and VEGF-B.[6]

The rationale behind the development of aflibercept was to provide a more potent and prolonged anti-VEGF effect. Data on equilibrium dissociation constants (KD) demonstrated that aflibercept binds VEGF-A with an approximately 100-fold higher binding affinity than ranibizumab or bevacizumab, thereby neutralizing VEGF-A with greater potency.[7] It has been

shown that aflibercept requires less frequent dosing compared to ranibizumab, but regarding clinical outcomes in the treatment of neovascular AMD, the exponentially higher affinity of aflibercept for the target cytokine, and the potential benefit from blocking the two other proangiogenic cytokines PLGF and VEGF-B does not translate into better visual acuity or more effective reduction of retinal thickness.[8]

Chronic suppression with serial intravitreal injections of VEGF antagonists usually is required for maintaining disease control, and neither of the available medications causes a complete regression of the choroidal neovascular membrane. In line with this, not all patients respond to treatment, with some developing into nonresponders. There is a need for identification of factors that confer antiangiogenic resistance on the neovascular membrane and a greater understanding of counter-regulatory mechanisms induced by VEGF suppression.

The factors that contribute to drug resistance and persistent activity of neovascular AMD are not clearly defined. It recently has been shown that intravitreal aflibercept and bevacizumab significantly reduce plasma VEGF-A levels.[9–11] Currently, data on the systemic pharmacodynamic effects of intravitreal antiangiogenic therapy on the proangiogenic cytokine PlGF in patients with neovascular AMD are lacking. This prompted us to perform a nonprespecified secondary ELISA analysis of our samples of patients randomized to treatment with intravitreal ranibizumab and aflibercept, and to consecutively recruit an additional bevacizumab treatment cohort to determine systemic effects on the target cytokines VEGF-B and PlGF.[12]

Placental growth factor has been implicated in resistance against anti-VEGF regimens. It is an amplifier of VEGF-driven angiogenesis, and promotes endothelial cell proliferation and vascular permeability.[13] Systemic upregulation of circulating levels of PlGF after antiangiogenic therapy with combined anti-VEGF/PLGF inhibitors has been reported by oncological studies on tumor neoangiogenesis.[14,15] Alterations of PlGF levels could potentially promote an escape from angiogenesis inhibition and may be responsible for the decreased therapeutic effect or persistence of the neovascular tissue in patients undergoing intravitreal antiangiogenic therapy.

The factors that contribute to drug resistance and persistent activity of the neovascular membrane are not clearly defined. The purpose of the present study was to analyze the plasma levels of PlGF, VEGF-A, and VEGF-B in patients with neovascular AMD treated with intravitreal anti-VEGF injections of aflibercept, ranibizumab, or bevacizumab. To the best of our knowledge, there are no published data from a prospective series determining differences in the systemic counterregulatory response to blockage of these key mediators of neoangiogenesis.

Methods

Subjects

This prospective trial was conducted in accordance with the Declaration of Helsinki and was performed after obtaining approval from the institutional review committee of the Medical University Innsbruck. Informed consent was obtained from every included participant. None of the patients received intravitreal injections for at least 6 months before inclusion. Patients with comorbidities that might involve vascular disease or neoangiogenesis were excluded. According to this criterion, patients with a history of cancer, or other vasoproliferative diseases, diabetes, uncontrolled hypertension, stroke, myocardial ischemia, and peripheral arterial disease were excluded from the study. Furthermore, patients on anti-inflammatory medications, such as steroids or nonsteroidal anti-inflammatory drugs (NSAID), were not included. To avoid factors that might affect pharmacokinetics of the anti-VEGF agents, no patients that had undergone vitrectomy or were on dialysis were included.

We performed a secondary analysis of the plasma samples obtained from 38 patients with neovascular AMD. The study subjects had been randomized by permuted block randomization to treatment with aflibercept or ranibizumab between June 2013 and January 2014. A total of 19 patients received intravitreal injections of aflibercept and 19 received ranibizumab. The primary objective of the initial prospective study was to analyze possible effects of these intravitreally administered anti-VEGF agents on VEGF-A via ELISA.[12] The aim of the present study, in which plasma samples were subjected to secondary analysis, was to investigate the effects of aflibercept on plasma VEGF-B and PlGF concentrations with two additional ELISA assays. For this study, an additional non-randomized cohort of patients treated with bevacizumab was recruited through a prospective consecutive case series to determine the effects of this drug on VEGF-B and PlGF.

No patients had any history of prior vitrectomy, photodynamic therapy, macular or peripheral laser photocoagulation, or intravitreal injections of steroids. In all patients, the IOP was within normal limits and none of the patients was concomitantly using glaucoma medication.

In the aflibercept as well as the ranibizumab cohort, 17 of 19 patients (89.5%) were treatment-naïve; two patients had been pretreated with intravitreal anti-VEGF in each cohort. All four had received pretreatment with ranibizumab, and the shortest interval between inclusion and last anti-VEGF pretreatment was 8 months. In the bevacizumab cohort, one of 18 patients had been pretreated with intravitreal bevacizumab 6 months before inclusion in the study; all others were naïve to anti-VEGF treatment (94.4%). Losses to follow-up were due to failure to attend medical appointments (no show) in all cases. The control group comprised 22 age- and sex-matched participants without any history of ocular and other systemic pathologies. Specifically, subjects with AMD, chorioretinal abnormalities, diabetes, hypertension, and vasoproliferative disorders were excluded from the control cohort.

Injection Technique

The intravitreal dose of aflibercept was 2 mg, equivalent to 50 μL, of ranibizumab 0.5 mg, equivalent to 50 μL, and of bevacizumab 1.25 mg, equivalent to 50 μL. All intravitreal injections were performed under sterile conditions in the operating room, including topical administration of povidone-iodine. The medication was administered by injection 3.5 to 4.0 mm posterior to the limbus using a 30-gauge needle. After removal of the needle, a sterile cotton tip applicator was used to prevent reflux. No antibiotics were given before or after injection.

Collecting Blood Samples

Blood samples were obtained 1 to 3 hours before intravitreal injection and 1 and 4 weeks after intravitreal injection. Blood samples were drawn from all patients by venous puncture with minimal stasis. For the VEGF assay, blood samples were collected in tubes containing EDTA. Centrifugation was performed at 1000*g* for 20 minutes immediately after sampling. Plasma was stored at 20°C until the assay, which was performed within 4 weeks after sampling.

ELISA Assay

Plasma concentrations of free PlGF, VEGF-A, and VEGF-B were determined by ELISA (Quantikine Human PlGF ELISA Kit,

Investigative Ophthalmology & Visual Science

TABLE. Demographic Data and Plasma Cytokine ELISA Measurements

			Baseline					1 wk			4 wk			
	Age	Sex, M/F	*n*	PlGF, pg/mL	VEGF-A, pg/mL	VEGF-B, pg/mL	*n*	PlGF, pg/mL	VEGF-A, pg/mL	VEGF-B, pg/mL	*n*	PlGF, pg/mL	VEGF-A, pg/mL	VEGF-B, pg/mL
Aflibercept	78.0 (67.0–82.0)	8/11	19	<12.0 (0.0–11.0)	43.0 (30.0–57.0)	118.0 (103.0–138.0)	19	38.0 (31.0–44.0)*	<9.0 (0.0–9.0)*	118.0 (105.0–145.0)	19	16.0 (0.0–19.0)*	17.0 (0.0–25.0)*	112.0 (98.0–143.0)
Ranibizumab	79.0 (72.0–85.0)	6/13	19	<12.0 (0.0–11.0)	59.0 (39.0–80.0)	113.0 (100.0–131.0)	18	<12.0 (0.0–11.8)	54.0 (42.3–74.0)	106.0 (97.8–135.3)	16	<12.0 (0.0–14.8)	58.5 (43.0–95.5)	122.0 (98.0–135.5)
Bevacizumab	71.0 (67.5–84.8)	9/9	18	<12.0 (0.0–14.3)	54.5 (37.5–113.3)	96.5 (69.8–156.8)	18	13.5 (0.0–15.0)	17.5 (10.3–27.5)*	100.5 (68.8–158.8)	17	<12.0 (0.0–15.8)	17.0 (10.5–31.5)†	100.0 (70.5–145.0)
Control	77.0 (71.8–79.3)	12/10	22	<12.0 (0.0–11.0)	61.0 (38.3–112.3)	111.0 (98.8–119.5)								

Values are medians (25th–75th percentiles). *P* values for comparing plasma cytokine levels with baseline measurements. *P* values of <0.05 were considered to indicate statistical significance, Friedman test.

* $P < 0.001$.

† $P < 0.01$.

#DPG00 and Quantikine VEGF ELISA Kit, #DVE00; R&D Systems Europe, Abingdon, UK; and VEGF-B ELISA Kit, #ABIN415602; Antibodies-Online, Inc., Atlanta, GA, USA) as described by the manufacturer. Briefly, for the Quantikine assays, 100 µL assay diluents were added to each well of 96-well polystyrene microplates, then 100 µL standard or samples (EDTA-plasma) were added to each well, mixed by gently tapping the plate frame for 1 minute, and incubated for 2 hours at room temperature. Thereafter, washing with wash buffer (400 mL) was performed three times, followed by addition of 200 mL polyclonal antibody conjugate to each well, incubation for 2 hours at room temperature, and washing again with wash buffer three times. Subsequently, 200 µL substrate solution were added to each well, incubated for 25 minutes at room temperature, and finally, 25 µL stop solution were added to each well. The concentration was determined by an ELISA reader at 450 nm. ELISA readings were reported in whole numbers without decimal places. For PlGF measurements, the minimum detectable dose (MDD) defined by the manufacturer was 12 pg/mL, 9 pg/mL for VEGF-A, and 8 pg/mL for VEGF-B.

Statistical Analyses

All statistical analyses were performed using SPSS 20.0 (IBM Corporation, Armonk, NY, USA) statistical software packages. Continuous data are given as median and interquartile range (IQR) in parentheses and qualitative data as percentages. To test for normal distribution, the Kolmogorov-Smirnov test was used. Not all cytokine measurements were normally distributed, and, in part, exact values were not given because the values were below the minimum detectable dose of the respective ELISA assay. Therefore, nonparametric testing was applied, and a conservative value of 11 pg/mL for PLGF and 8 pg/mL for VEGF-A was imputed for each value below the MDD to perform the calculations. None of the VEGF-B measurements was below the MDD. Kruskal-Wallis, Mann-Whitney *U* test, and χ^2 tests were used for comparisons between treatment groups. Within treatment group comparisons were performed with the Friedman and Wilcoxon signed-rank test. *P* values < 0.05 were considered to indicate statistical significance.

RESULTS

There were no differences between the aflibercept, ranibizumab, bevacizumab, and the control cohort with respect to age ($P = 0.741$) and sex distribution ($P = 0.486$). There were no significant differences between the pretreatment plasma levels of PlGF ($P = 0.162$), VEGF-A ($P = 0.102$), and VEGF-B ($P = 0.412$) of patients randomized to intravitreal aflibercept, ranibizumab, or bevacizumab and the control cohort. Details on demographics and cytokine levels are summarized in the Table.

PlGF Concentration

The median PlGF plasma concentration was <12.0 pg/mL in the control group as well as in all three anti-VEGF treatment cohorts at baseline. The systemic pretreatment PlGF levels were lower than the MDD in the majority of study subjects (78.2%).

In the ranibizumab group, no significant effects on systemic PlGF levels could be observed and the median PlGF levels remained <12.0 pg/mL at all sample time points tested.

At 1 week after intravitreal aflibercept injection, a significant feedback upregulation of systemic PlGF could be observed with a median (IQR) PLGF measurement of 38.0 (31.0–44.0) pg/mL. The increase of PlGF compared to

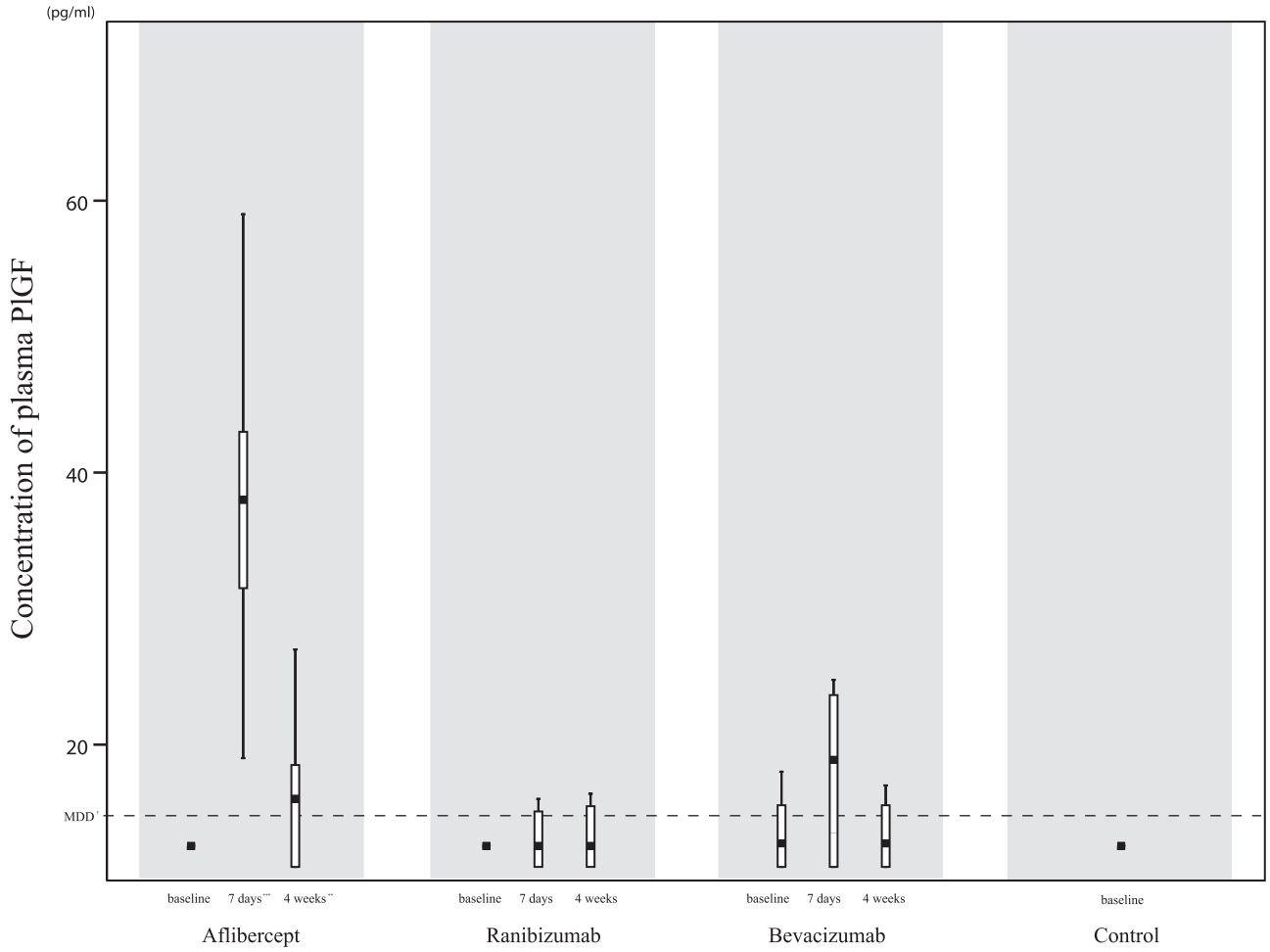

Figure 1. Plasma levels of PlGF before and after intravitreal injection of anti-VEGF therapeutics in patients with neovascular AMD. In those treated with aflibercept, PlGF levels significantly increased after 7 days, and this increase persisted throughout 4 weeks. No significant effects were seen in the ranibizumab and bevacizumab cohort. Statistically significant differences (***$P < 0.001$, **$P < 0.01$). †The MDD of PlGF defined by the manufacturer was 12 pg/mL. All measurement outliers are below the MDD.

pretreatment occurred in all patients treated with aflibercept (19 of 19 patients, 100%; $P < 0.001$). After 4 weeks, the PlGF levels remained significantly elevated compared to pretreatment values with a median PlGF of 16.0 (0.0–19.0) pg/mL ($P = 0.005$).

In those treated with intravitreal bevacizumab, a statistically nonsignificant increase of plasma PlGF was measured with an increase to a median of 13.5 (0.0–15.0) pg/mL after 7 days ($P = 0.397$) and <12.0 (0.0–15.8) pg/mL after 4 weeks ($P = 0.837$; Fig. 1).

VEGF-A Concentration

The median plasma VEGF-A concentration of controls was 61.0 (38.3–112.3) pg/mL. The pretreatment plasma concentrations of VEGF-A were 43.0 (30.0–57.0) pg/mL in the aflibercept cohort, 59.0 (39.0–80.0) pg/mL in the ranibizumab cohort, and 54.5 (37.5–113.3) pg/mL in the bevacizumab cohort.

After intravitreal injection of aflibercept plasma, VEGF-A measurements were significantly reduced to values below the MDD in 17 of 19 patients (89.5%), resulting in a median and an IQR of <9.0 pg/mL 7 days relative to baseline ($P < 0.001$). The reduction persisted throughout the 4 weeks of the sampling period with values below the MDD in 5 of 19 patients (26.3%) and a median VEGF-A concentration of 17.0 (0.0–25.0; $P < 0.001$).

The VEGF-A values were significantly lower in those treated with aflibercept comparing the plasma measurements at 7 days and 1 month after intravitreal injection to the respective VEGF levels in the ranibizumab and control cohort (7 days, $P < 0.001$; 4 weeks, $P < 0.001$).

A significant reduction of systemic VEGF-A levels also was observed after intravitreal bevacizumab injections with a median VEGF-A of 17.5 (10.3–27.5) pg/mL after 1 week ($P < 0.001$) with 4 of 18 (22.2%) measurements below the MDD. Compared to baseline, the 4-week measurements of VEGF-A remained significantly reduced to 17.0 (10.5–31.5) pg/mL and 4 of 17 (23.5%) samples were below the MDD ($P < 0.001$) in the bevacizumab group.

No significant effects on systemic VEGF-A could be observed in those treated with intravitreal ranibizumab; the VEGF-A levels in this group did not change significantly from the baseline with 59.0 (39.0–80.0) to 54.0 (42.3–74.0) pg/mL at 7 days ($P = 0.776$) and 58.5 (43.0–95.5) pg/mL at 4 weeks of follow-up ($P = 0.670$). The plasma measurements at 7 days and 1 month after intravitreal ranibizumab injection did not differ significantly compared to control ($P = 0.638$, $P = 0.827$; Fig. 2).

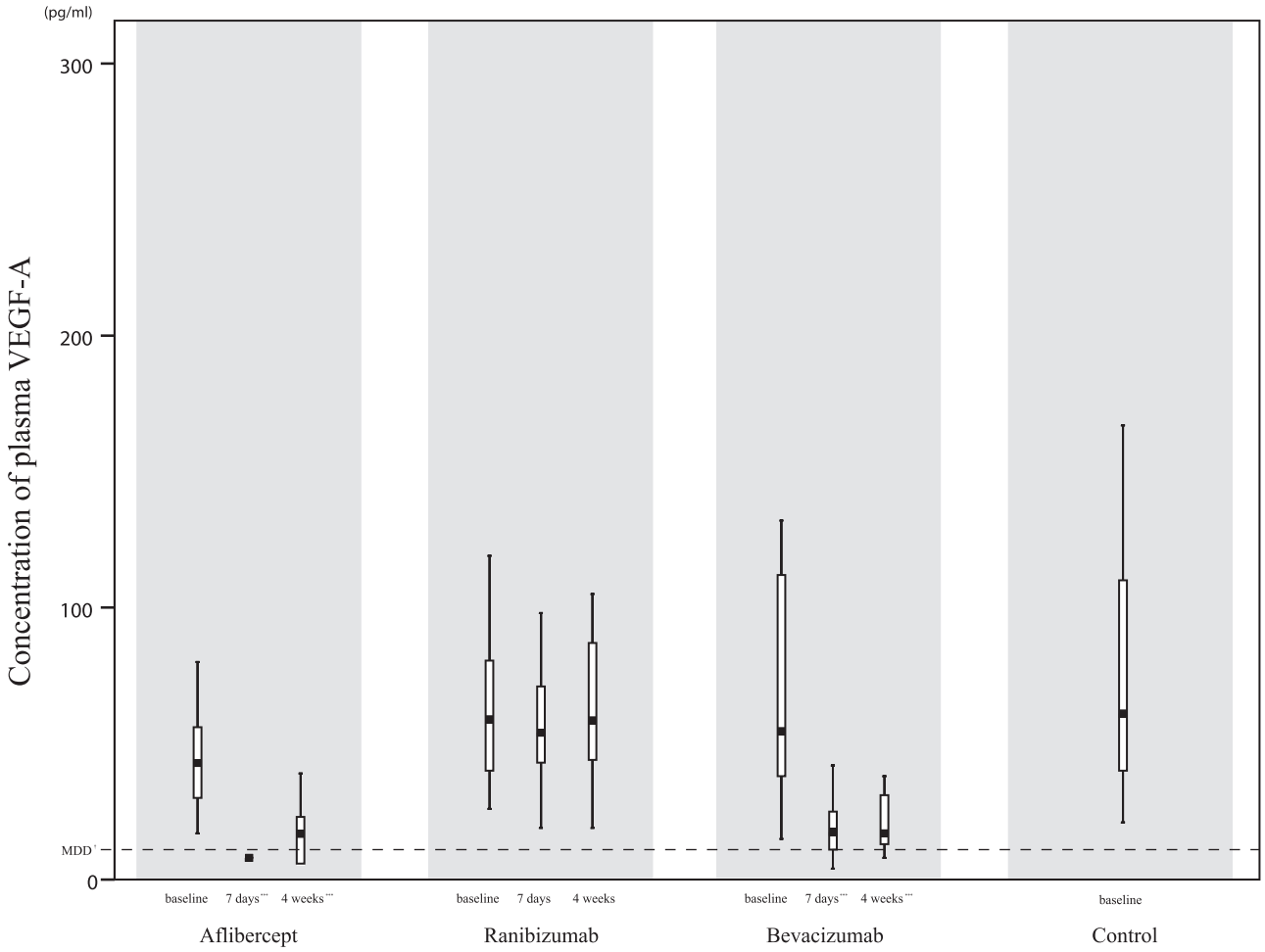

Figure 2. Plasma levels of VEGF-A before and after intravitreal injection of anti-VEGF therapeutics in patients with neovascular AMD. In those treated with aflibercept and bevacizumab, systemic VEGF-A levels were significantly reduced after 7 days, and this reduction persisted throughout 4 weeks. No significant effects were seen in the ranibizumab cohort. Statistically significant differences (***$P < 0.001$). †The MDD of VEGF-A defined by the manufacturer was 9 pg/mL. All measurement outliers were below the MDD.

VEGF-B Concentration

The median plasma VEGF-B concentration of controls was 111.0 (98.8–119.5) pg/mL. The pretreatment plasma concentration of VEGF-B was 118.0 (103.0–138.0) pg/mL in the aflibercept cohort, 113.0 (100.0–131.0) pg/mL in the ranibizumab cohort, and 96.5 (69.8–156.8) pg/mL in the bevacizumab cohort. No significant effects on systemic VEGF-B could be observed after intravitreal anti-VEGF injections in any of the three treatment groups. None of the ELISA measurements was outside of the quantification limits of the assay (Fig. 3).

Discussion

The process of neovascularization is regulated by the balance of pro- and antiangiogenic cytokines, and a shift of this balance in favor of angiogenesis promotes the pathologic development and persistence of the neovascular membrane in patients with AMD. The most important proangiogenic signaling circuit involves the VEGF family of cytokines.[16] Vascular endothelial growth factor-A, VEGF-B, and PlGF are all ligands of the two tyrosine kinase (TK) receptors VEGFR-1 and VEGFR-2. The proangiogenic activity of the VEGF cytokines is exerted through the dimerization of the receptors upon ligand binding and activation of the TKs.

Placental growth factor is a VEGF homolog that is expressed at low or undetectable levels under physiologic conditions.[17] It is upregulated in many diseases that involve pathological neoangiogenesis, and PlGF overexpression is required for the angiogenic effect of VEGF in the ischemic retina. Placental growth factor induces a substantial increase in vasculature, including the absolute number of vessels, vascular caliber, and increased vascular permeability.[18,19] Increased choroidal PlGF levels were measured in laser-induced CNV, and targeted blocking of PlGF with a monoclonal antibody dose-dependently inhibited the CNV formation.[20] Placental growth factor acts as a synergistic amplifier of VEGF-driven angiogenesis through different mechanisms.[13] By activating VEGFR-1, PlGF induces an intermolecular crosstalk between VEGFR-1 and VEGFR-2, which stimulates the activation of VEGFR-2 and enhances its response to VEGF. Placental growth factor and VEGF-A form a heterodimer that stimulates neoangiogenesis by inducing the formation of VEGFR-1/VEGFR-2 receptor dimers, which transphosphorylate each other in an intermolecular reaction. Placental growth factor also upregulates downstream target genes resulting in expression of VEGF, thereby further amplifying this synergism.[21]

The results of this study demonstrated that alterations in the systemic milieu of angiogenic cytokines occur after intravitreal application of VEGF inhibitors and that these alterations may

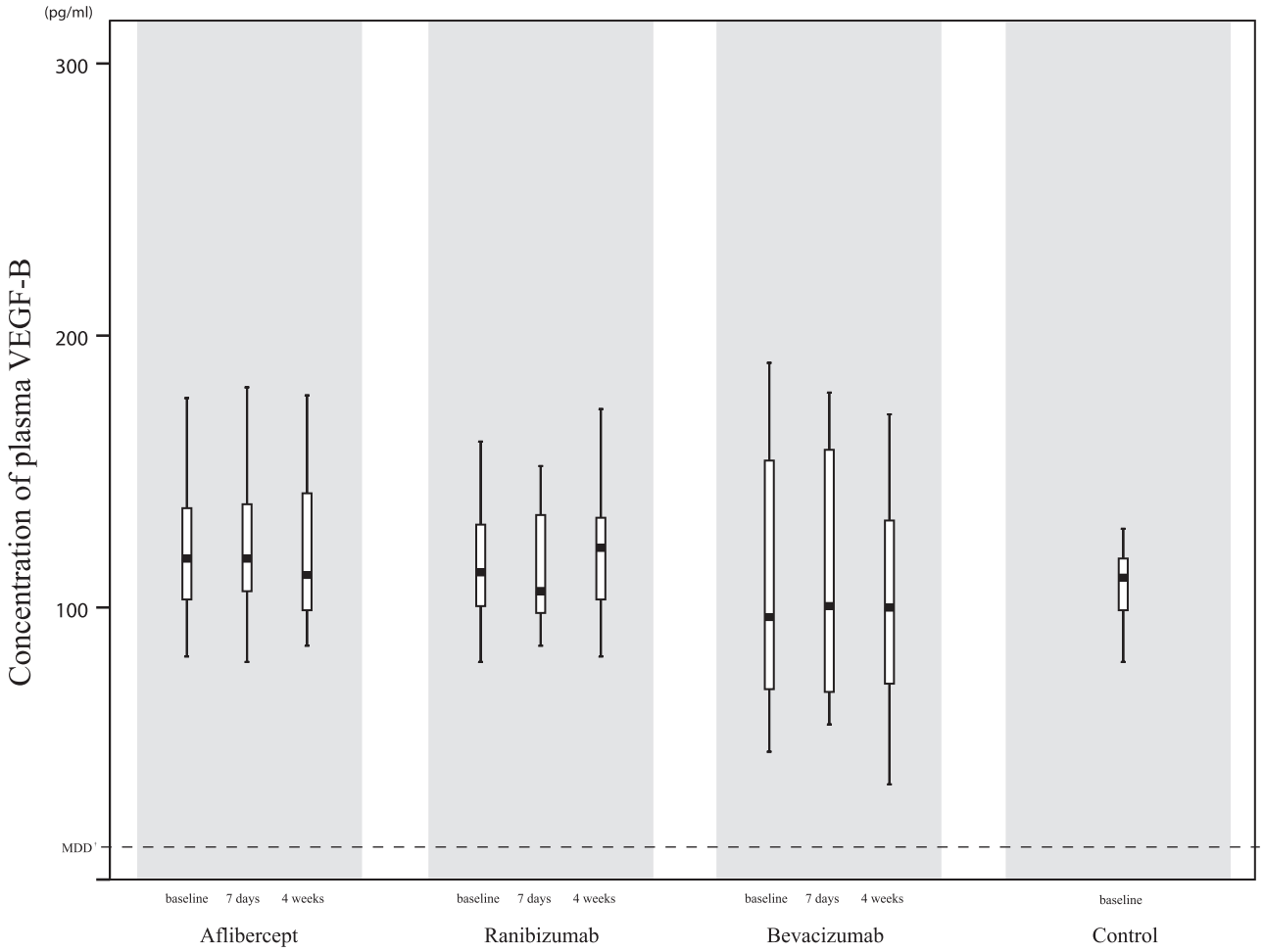

Figure 3. No significant effects on systemic VEGF-B could be observed after intravitreal anti-VEGF injections in any of the three treatment groups. None of the ELISA measurements was outside of the quantification limits of the assay. †The MDD of VEGF-A defined by the manufacturer was 8 pg/mL.

be clinically significant. Our data showed a significant reduction of VEGF-A in the plasma of patients 1 week after being treated with intravitreal aflibercept and bevacizumab, a reduction that persisted throughout the 1-month sampling period. The systemic inhibitory effect on VEGF-A was more pronounced in those patients receiving intravitreal aflibercept. These findings are consistent with recent reports of significantly suppressed systemic VEGF-A levels after intravitreal aflibercept and bevacizumab injections, and no clearly significant effects in those treated with ranibizumab.[9–11,22]

Specific pharmacodynamic properties of the drugs might help explain these differences in the systemic effects after intravitreal application. Data on binding affinities provide a rationale for the stronger reduction of plasma VEGF-A resulting from intravitreal aflibercept as it has an exponentially stronger binding affinity than ranibizumab or bevacizumab, thereby neutralizing VEGF-A with greater potency.[7] Besides differences in affinity, distinct molecular characteristics of the intravitreally used angiogenesis inhibitors might have a role. Aflibercept and bevacizumab share a similar molecular component, as they both comprise an immunoglobulin G Fc fragment. The Fc region equips these molecules with the ability to interact with the neonatal Fc receptor (FcRn). The FcRn modulates the transport of molecules that contain an Fc region across endothelial boundaries, like the blood–retina barrier, and it has been established that the long systemic half-life of IgGs compared to other proteins, such as ranibizumab, are due to their recycling and rescue from catabolic elimination by the FcRn via direct engagement of the Fc fragment.[23] Thus, the FcRn might not only mediate intraocular pharmacokinetics of therapeutic monoclonal antibodies, but also possible off-target effects in the systemic circulation by actively transporting the antiangiogenic agents across the blood–retina barrier.[24]

Aflibercept is a soluble decoy receptor generated by the fusion of human VEGF receptor domains. The aflibercept molecule comprises the second Ig domain of human VEGFR1 and the third Ig domain of human VEGFR2 expressed as an inline fusion with the constant region (Fc) of human IgG1. Like ranibizumab and bevacizumab, the two other anti-VEGF agents investigated in this study, aflibercept binds all isoforms of VEGF-A, but in contrast to these antibodies, aflibercept was developed also to bind VEGF-B and PlGF, the related proangiogenic VEGFR1 ligands. The systemic effects of intravitreal aflibercept on these two cytokines have not been studied yet. Therefore, we investigated these cytokine levels after exposure to aflibercept.

No significant effects on VEGF-B levels could be observed after intravitreal treatment with any of the three drugs. Though VEGF-B has been considered traditionally a prototypical angiogenic factor, current knowledge about its physiological functions as well as about its role in the VEGF receptor cascade are limited. The results from studies investigating the involvement of VEGF-B in neovascularization or possible

effects resulting from direct antagonization of this cytokine are conflicting and still subject to debate.[25–28]

Analyzing the systemic measurements of PlGF after intravitreal aflibercept injection, we found a significant systemic upregulation of this proangiogenic cytokine after 1 week, persisting throughout the 4-week sampling point. This could represent a host counter-regulatory response to antiangiogenic therapy with intravitreal aflibercept. In the bevacizumab cohort, a slight, though statistically nonsignificant increase after intravitreal application was observed. Ranibizumab induced no detectable effects on systemic PlGF.

Nonresponse to VEGF inhibition in the treatment of neovascular AMD or disease persistence, as reflected by activity and leakage of the neovascular membrane, might be attributed to several interacting mechanisms. Among them are participation of different pathways, pruning of the neovessel pericytes and compensatory actions of other growth factors that contribute to escape and survival of the neovascular membrane in the presence of VEGF inhibition. The selective therapeutic interference with factors of the proangiogenic signaling circuit for the treatment of pathologic neovascularization is likely to result in compensatory increases of other factors involved in this process. This may induce converse regulatory effects that might weaken the therapeutic efficacy of antiangiogenic drugs.

Escape pathways mediating resistance to VEGF-targeted therapy are well described in oncological studies. Counter-regulatory angiogenic pathways after VEGF blockade that involve upregulation of cytokines, such as PlGF, fibroblast growth factor (FGF), erythropoietin, or platelet-derived growth factor (PDGF), have been described; whether VEGF signaling inhibitors that slow angioneogenesis also can promote escape of neovascularization and disease progression still is under debate.[14,29]

Placental growth factor has been implicated in resistance to anti-VEGF regimens. A similar systemic upregulation of circulating levels of PlGF that we detected in patients treated with intravitreal aflibercept has been observed in several tumor studies induced by antiangiogenic therapy with combined anti-VEGF/PLGF inhibitors as well as with selective VEGF inhibitors.[15,30] Plasma PlGF levels have been shown to increase more than 10-fold in patients with colorectal cancer after treatment with inhibitors of VEGF-A signaling. A comparable systemic effect was observed in patients with glioblastoma or renal cell cancer.[31–33] Oncological treatment with the fusion protein sFLT0, which is a soluble decoy receptor similar to aflibercept and is composed of the VEGF/PlGF binding domain of VEGFR-1/Flt-1, resulted in an acute increase in the circulating levels of PlGF. This is in agreement with the results reported in the present study of a cohort of patients treated with intravitreal aflibercept. In animal models, dual anti–VEGF-A/PlGF inhibition induced increased secretion of systemic PlGF in tumor-bearing and nontumor-bearing mice indicating a host response.[15] In the tumor-bearing mice, a more invasive cell phenotype developed with the tumors becoming more invasive within the tumor microenvironment and resulting in an increase in distant metastases.[34]

A limitation of the study protocol is that it has been designed as a secondary ELISA assay analysis investigating the plasma concentrations of PlGF and VEGF-B. The primary study found a highly significant reduction of systemic VEGF-A concentrations in patients randomized to intravitreal aflibercept.[12] Placental growth factor and VEGF-B also are trapped by aflibercept, but no published data on the effects of this drug on the other two target cytokines are available. This study was intended to fill this specific lacuna. Besides aflibercept, bevacizumab has been shown to significantly reduce systemic VEGF-A. To identify possible counter-regulatory effects resulting from intravitreal bevacizumab, an additional prospective consecutive series of patients treated with bevacizumab was recruited for this study. These patients were nonrandomized. The strengths of the study are its prospective study design with homogenous pretreatment characteristics in all three cohorts compared to control and investigation of the systemic effects of all three of the currently used anti-VEGF drugs for AMD on the target proangiogenic cytokines, namely PlGF, VEGF-A, and VEGF-B.

Placental growth factor could be an important mediator in the set of puzzling factors affecting the equilibrium between pro- and antiangiogenic cytokines in the process of choroidal neovascularization. The study results suggest that a systemic upregulation of PlGF might be induced by intravitreal antiangiogenic therapy and will not be prevented by a dual VEGF-A/PlGF antagonist. This could be of translational relevance for the clinical outcomes in treatment of neovascular AMD and it would be worth investigating whether this systemic proangiogenetic shift has an impact on the cytokine milieu in CNV. The upregulation and increased secretion of PlGF into circulation upon intravitreal administration of aflibercept could potentially exert a countervailing trophic effect on neovascular tissue, and, thus, reduce the therapeutic effect of VEGF inhibition.[14,15] The treatment of neovascular ocular diseases has been revolutionized by VEGF antagonization, but there still is a substantial need for improvement and further insight into the regulatory response mechanisms induced by targeted VEGF blockage is required. Identification of factors that confer antiangiogenic drug resistance would enable development of the next generation of drugs for more effective treatment of neovascular AMD.

Acknowledgments

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The authors alone are responsible for the content and writing of the paper.

Disclosure: **C. Zehetner**, None; **N.E. Bechrakis**, None; **M. Stattin**, None; **R. Kirchmair**, None; **H. Ulmer**, None; **M.T. Kralinger**, None; **G.F. Kieselbach**, None

References

1. Klaver CC, Wolfs RC, Vingerling JR, Hofman A, de Jong PT. Age-specific prevalence and causes of blindness and visual impairment in an older population: the Rotterdam Study. *Arch Ophthalmol*. 1998;116:653–658.
2. Ozaki H, Yu AY, Della N, et al. Hypoxia inducible factor-1alpha is increased in ischemic retina: temporal and spatial correlation with VEGF expression. *Invest Ophthalmol Vis Sci*. 1999; 40:182–189.
3. Campochiaro PA. Ocular neovascularization. *J Mol Med (Berl)*. 2013;91:311–321.
4. Martin DF, Maguire MG, Fine SL, et al. Ranibizumab and bevacizumab for treatment of neovascular age-related macular degeneration: two-year results. *Ophthalmology*. 2012;119: 1388–1398.
5. Ferrara N, Damico L, Shams N, Lowman H, Kim R. Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. *Retina*. 2006; 26:859–870.
6. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A*. 2002;99:11393–11398.
7. Papadopoulos N, Martin J, Ruan Q, et al. Binding and neutralization of vascular endothelial growth factor (VEGF)

and related ligands by VEGF Trap, ranibizumab and bevacizumab. *Angiogenesis*. 2012;15:171–185.

8. Heier JS, Brown DM, Chong V, et al. Intravitreal aflibercept (VEGF trap-eye) in wet age-related macular degeneration. *Ophthalmology*. 2012;119:2537–2548.
9. Wang X, Sawada T, Sawada O, Saishin Y, Liu P, Ohji M. Serum and plasma vascular endothelial growth factor concentrations before and after intravitreal injection of aflibercept or ranibizumab for age-related macular degeneration. *Am J Ophthalmol*. 2014;158:738–744.
10. Avery RL, Castellarin AA, Steinle NC, et al. Systemic pharmacokinetics following intravitreal injections of ranibizumab, bevacizumab or aflibercept in patients with neovascular AMD. *Br J Ophthalmol*. 2014;98:1636–1641.
11. Carneiro AM, Costa R, Falcao MS, et al. Vascular endothelial growth factor plasma levels before and after treatment of neovascular age-related macular degeneration with bevacizumab or ranibizumab. *Acta Ophthalmol*. 2012;90:e25–e30.
12. Zehetner C, Kralinger MT, Modi YS, et al. Systemic levels of vascular endothelial growth factor before and after intravitreal injection of aflibercept or ranibizumab in patients with age-related macular degeneration: a randomised, prospective trial. *Acta Ophthalmol*. 2014;93:e154–e159.
13. Autiero M, Waltenberger J, Communi D, et al. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med*. 2003;9:936–943.
14. Sennino B, McDonald DM. Controlling escape from angiogenesis inhibitors. *Nat Rev Cancer*. 2012;12:699–709.
15. Bagley RG, Ren Y, Weber W, et al. Placental growth factor upregulation is a host response to antiangiogenic therapy. *Clin Cancer Res*. 2011;17:976–988.
16. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9:669–676.
17. De Falco S. The discovery of placenta growth factor and its biological activity. *Exp Mol Med*. 2012;44:1–9.
18. Carmeliet P, Moons L, Luttun A, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med*. 2001;7:575–583.
19. Hollborn M, Tenckhoff S, Seifert M, et al. Human retinal epithelium produces and responds to placenta growth factor. *Graefe's Arch Clin Exp Ophthalmol*. 2006;244:732–741.
20. Van de Veire S, Stalmans I, Heindryckx F, et al. Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. *Cell*. 2010;141:178–190.
21. Tjwa M, Luttun A, Autiero M, Carmeliet P. VEGF and PlGF: two pleiotropic growth factors with distinct roles in development and homeostasis. *Cell Tissue Res*. 2003;314:5–14.
22. Zehetner C, Kirchmair R, Huber S, Kralinger MT, Kieselbach GF. Plasma levels of vascular endothelial growth factor before and after intravitreal injection of bevacizumab, ranibizumab and pegaptanib in patients with age-related macular degeneration, and in patients with diabetic macular oedema. *Br J Ophthalmol*. 2013;97:454–459.
23. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol*. 2007;7:715–725.
24. Powner MB, McKenzie JA, Christianson GJ, Roopenian DC, Fruttiger M. Expression of neonatal Fc receptor in the eye. *Invest Ophthalmol Vis Sci*. 2014;55:1607–1615.
25. Silvestre JS, Tamarat R, Ebrahimian TG, et al. Vascular endothelial growth factor-B promotes in vivo angiogenesis. *Circ Res*. 2003;93:114–123.
26. Li Y, Zhang F, Nagai N, et al. VEGF-B inhibits apoptosis via VEGFR-1-mediated suppression of the expression of BH3-only protein genes in mice and rats. *J Clin Invest*. 2008;118:913–923.
27. Zentilin L, Puligadda U, Lionetti V, et al. Cardiomyocyte VEGFR-1 activation by VEGF-B induces compensatory hypertrophy and preserves cardiac function after myocardial infarction. *FASEB J*. 2010;24:1467–1478.
28. Kanda M, Nomoto S, Nishikawa Y, et al. Correlations of the expression of vascular endothelial growth factor B and its isoforms in hepatocellular carcinoma with clinico-pathological parameters. *J Surg Oncol*. 2008;98:190–196.
29. Forooghian F, Kertes PJ, Eng KT, Agron E, Chew EY. Alterations in the intraocular cytokine milieu after intravitreal bevacizumab. *Invest Ophthalmol Vis Sci*. 2010;51:2388–2392.
30. Kopetz S, Hoff PM, Morris JS, et al. Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. *J Clin Oncol*. 2010;28:453–459.
31. Willett CG, Boucher Y, Duda DG, et al. Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. *J Clin Oncol*. 2005;23:8136–8139.
32. Rini BI, Michaelson MD, Rosenberg JE, et al. Antitumor activity and biomarker analysis of sunitinib in patients with bevacizumab-refractory metastatic renal cell carcinoma. *J Clin Oncol*. 2008;26:3743–3748.
33. Batchelor TT, Sorensen AG, di Tomaso E, et al. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell*. 2007;11:83–95.
34. Paez-Ribes M, Allen E, Hudock J, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell*. 2009;15:220–231.

Investigative Ophthalmology & Visual Science