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CHANGES IN SYSTEMIC LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR AFTER INTRAVITREAL INJECTION OF AFLIBERCEPT OR BROLUCIZUMAB FOR NEOVASCULAR AGE-RELATED MACULAR DEGENERATION

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Purpose: To analyze and compare the effects of intravitreal brolucizumab versus aflibercept on systemic vascular endothelial growth factor (VEGF)-A levels in patients with neovascular age-related macular degeneration.

Methods: In this prospective interventional case series study, brolucizumab (6.0 mg/50 μL) or aflibercept (2.0 mg/50 μL) was injected intravitreally in 30 patients each. Blood samples were drawn at baseline and 7 days and 28 days after the first injection. Systemic VEGF-A levels were measured using enzyme-linked immunosorbent assay. Thirty healthy individuals served as controls.

Results: The median baseline systemic VEGF-A levels in the brolucizumab, aflibercept, and control groups were 10.8 (8.0–13.2), 12.0 (8.0–18.5), and 10.0 (8.0–15.1) pg/mL, respectively ($P = 0.315$). In the brolucizumab group, VEGF-A levels significantly decreased to 8.0 (8.0–11.5) pg/mL on Day 7 ($P = 0.0254$) and to 8.0 (8.0–8.0) pg/mL on Day 28 ($P < 0.001$). In the aflibercept group, VEGF-A levels significantly decreased to 8.0 (8.0–8.0) pg/mL on Day 7 ($P < 0.001$) but returned to the baseline level, 12.5 (8.5–14.6) pg/mL, on Day 28 ($P = 0.120$). Vascular endothelial growth factor–A levels were significantly different between the treatment groups after 28 days ($P < 0.001$).

Conclusion: Intravitreal brolucizumab resulted in a sustained reduction of systemic VEGF-A levels until 28 days posttreatment, which raises concerns regarding its safety and long-term effects.

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Age-related macular degeneration (AMD) is a leading cause of vision loss in the aging Western population.[1,2] The most important proangiogenic signaling circuit in the process of neovascularization involves the vascular endothelial growth factor (VEGF).[3] With the introduction of intravitreal anti-VEGF therapies, the management of neovascular (n) AMD has been revolutionized.[4]

Brolucizumab (Beovu; Novartis International AG, Basel, Switzerland) is the latest anti-VEGF medication for the treatment of nAMD, approved by the Food and Drug Administration of the United States and European Medicine Agency. It is a humanized single-chain variable fragment antibody that inhibits all isoforms of VEGF-A binding to the VEGF receptors, VEGF1 and VEGF2.[5,6] By contrast, aflibercept (Eylea; Regeneron, Tarrytown, NY and VEGF-Trap Eye; Bayer AG, Leverkusen, Germany) is a 110 kD fusion protein, which acts as a soluble decoy receptor binding to VEGF-A, VEGF-B, and placental growth factor (PlGF).[7] The

low molecular mass (26 kDa) of brolucizumab allows for a 10 times higher molar concentration than aflibercept, providing the potential for sustained VEGF-A suppression.[8] The increased molar concentration has also been postulated to contribute to its increased durability.[9] Brolucizumab is the first anti-VEGF agent labeled for a dosing interval of 8 to 12 weeks after a loading dose of three injections for three consecutive months.[11,12] It is well known that intravitreally administered aflibercept can cross the blood–retina barrier causing off-target effects in the systemic circulation.[11,12] However, there are no data on the systemic effects of intravitreal brolucizumab administration.

Structural differences between aflibercept and brolucizumab may lead to different effects on systemic VEGF-A levels on intravitreal application. Since the approval of brolucizumab in 2019, safety concerns have been raised because of reports of intraocular inflammation and retinal vascular occlusion.[6,13–16] Therefore, potential systemic side effects of intravitreal injection (IVI) of brolucizumab require great attention.

Thus, the primary purpose of this prospective study was to analyze and compare the effects of intravitreal aflibercept versus brolucizumab injection on systemic VEGF-A levels for the treatment of nAMD. To the best of our knowledge, this is the first study to report on the effects of intravitreal brolucizumab therapy on systemic VEGF levels.

Materials and Methods

Subjects

This prospective interventional case series study recruited 60 consecutive patients diagnosed with nAMD who were treatment naïve to intravitreal anti-VEGF for at least 6 months. At inclusion, a retinal specialist diagnosed and classified nAMD by fundus examination and fluorescence angiography. The central macular thickness and macular neovascularization size were measured using optical coherence tomography angiography (OCT-A; Heidelberg Spectralis OCT, Heidelberg Engineering, Heidelberg, Germany).

Treatment naïve patients with nAMD indicated for anti-VEGF therapy were prospectively recruited for blood sample collection within the framework of a biobank for degenerative macular diseases at the Department of Ophthalmology, Medical University Innsbruck, Innsbruck, Austria. The indication for a certain intravitreal anti-VEGF agent was determined depending on the patients' preference. Once the treatment was determined, informed consent was obtained for the drawing of blood samples at specific time points.

Although 30 consecutive patients were treated with an IVI of brolucizumab (6.0 mg/50 μL), the other 30 patients received aflibercept (2.0 mg/50 μL). Blood samples were collected at treatment indication, as well as 7 days and 28 days after the first IVI. Thirty participants without any history of ocular and systemic pathologies served as the control group at baseline.

The exclusion criteria were bilateral disease in need of concurrent therapy, a history of vitrectomy or uveitis, systemic inflammatory comorbidities, treatment with antiinflammatory medications, diabetes mellitus, renal diseases, systemic vasoproliferative disorders, a history of cancer, or previous cancer treatment with anti-VEGF drugs.

The study was conducted as per the tenets of the Declaration of Helsinki. The establishment of a biobank and the performance of consecutive cytokine analyses were approved by the Institutional Review Committee of the Medical University of Innsbruck (Innsbruck, Austria—No 1261/2020 & 1049/2021). Written informed consent to participate was obtained from all patients after explaining to them the nature and possible consequences of the study.

Blood Sample Collection

For the enzyme-linked immunosorbent assay, blood samples were collected within 1 hour before and 7 days and 28 days after the first IVI. Blood samples were collected with minimal stasis into citrate–theophylline–adenosine–dipyridamole tubes, which have been shown to prevent platelet activation, thereby minimizing the release of cytokines, including VEGF.[17] After centrifugation at 3,000 rpm for 20 minutes, plasma was collected, aliquoted, and stored at −80°C within 2 hours of collection, until further analysis.

Analysis of Cytokines in Plasma Samples by Enzyme-Linked Immunosorbent Assay

Systemic levels of free VEGF-A were determined using enzyme-linked immunosorbent assay (Quantikine

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ELISA Kit, R&D Systems Europe, Abingdon, United Kingdom; DVE00 for VEGF-A) as per the manufacturers' protocol. All samples were analyzed together in duplicate. The minimum detectable dose of VEGF-A concentration was 8.0 pg/mL.

Statistical Analyses

All statistical analyses were performed using SPSS Statistics 25 (IBM, Armonk, NY). We used a two-sided test for sample size estimation. Previous data on VEGF-A levels in patients receiving aflibercept were used for this purpose. Using type I error of $\alpha = 0.05$ and type II error of 10% (meaning $1-\beta$, that is, the power of 90%) at a SD of 20 pg/mL, we estimated a sample size of 27 per treatment group to achieve a medium-to-large effect size (r = 0.6–0.8).

We conducted the Kolmogorov–Smirnov test to evaluate all variables for normal distribution. Continuous data are reported as mean with SD for normally distributed data or median with interquartile range for nonnormally distributed data. We used the analysis of variance for normally distributed data and the Kruskal–Wallis test for nonnormally continuous data to compare baseline characteristics between the treatment and the control groups. For comparison of the normally distributed age and body mass index (BMI), we used the two-sample *t*-test. We used the chi-square test to compare the sex distribution and the distribution of the affected eye between the treatment and control groups. A binary logistic regression and multinominal logistic regression adjusted to age, sex, and BMI were used to compare VEGF-A levels between the treatment and control groups. Within the treatment groups, comparisons of systemic VEGF-A concentrations were performed using the Friedman and Wilcoxon signed-rank tests. A *P* value of <0.05 was considered statistically significant in all analyses.

Results

All the enrolled patients were observed for 1 month. There were no ocular or systemic complications during the follow-up.

Patients in the treatment groups were older than the healthy controls ($P = 0.001$; the Kruskal–Wallis test) but did not differ significantly in sex distribution ($P = 0.870$) and BMI ($P = 0.051$). There were no significant differences between the treatment groups in clinical characteristics (Table 1).

Systemic Vascular Endothelial Growth Factor-A Levels

A regression analysis adjusted for age, sex, and BMI found no significant difference in the baseline systemic VEGF-A levels between the treatment and control groups ($P = 0.316$; multinominal logistic regression; Table 2 and Figure 1).

In the aflibercept group, the median (interquartile range) systemic VEGF-A levels showed a significant decrease from 12.0 (8.0–18.5) pg/mL to 8.0 (8.0–8.0) pg/mL ($P < 0.001$; Wilcoxon signed-rank test), on Day 7 after the initial IVI, which returned to the baseline level (12.5 [8.5–14.6] pg/mL) ($P = 0.120$) on Day 28. A decrease in systemic VEGF-A levels was observed in 96% (29 of 30) of the patients 7 days after the IVI.

In the brolucizumab group, systemic VEGF-A levels significantly decreased from 10.8 (8.0–13.2) pg/mL to 8.0 (8.0–11.5) pg/mL ($P = 0.0254$) on Day 7 after the IVI. The levels remained lower (8.0 [8.0–8.0] pg/mL) than baseline on Day 28 ($P < 0.001$). After the IVI, a decrease in systemic VEGF-A levels was observed in 76% (23 of 30) of the patients on Day 7 and 86% (26 of 30) of those on Day 28. In addition, we observed a decrease in systemic VEGF-A levels from Day 7 to Day 28 ($P = 0.0245$).

The brolucizumab group showed significantly lower systemic VEGF-A levels than the aflibercept group 28 days after the IVI ($P < 0.001$, Mann–Whitney *U* test).

Systemic Vascular Endothelial Growth Factor-B Levels

A regression analysis adjusted for age, sex, and BMI revealed no differences in the systemic pretreatment levels of VEGF-B between the aflibercept (58.2 [27.8–139.8 pg/mL]) and brolucizumab groups (68.0 [49.9–323.3] pg/mL) compared with the control group (33.7 [12.4–70.2] pg/mL, $P = 0.295$). There were no differences within and across the treatment groups 7 days and 28 days after IVI (Figure 2).

Systemic Placental Growth Factor Levels

In a regression analysis adjusted for age, sex, and BMI, the treatment groups had significantly higher systemic PlGF levels compared with that of the control group ($P = 0.023$, Table 2 and Figure 3). In the aflibercept group, the systemic PlGF levels increased from 8.5 (8.0–14.9) pg/mL at the baseline to 33.1 (21.9–40.6) pg/mL on Day 7 after IVI ($P < 0.001$) and remained decreased after 28 days (11.3 [8.0–18.5] pg/mL, $P = 0.002$).

Table 1. Demographics and Clinical Baseline Characteristics of Patients

	Aflibercept	Brolucizumab	*P*	Control	*P*
N	30	30		30	
Age (SD)	78 (8)	80 (7)	0.252	72 (8)	0.001*
Sex (M/F)	12/18	13/17	1.00	11/19	0.870
Eyes (OD/OS)	13/17	15/15	0.796	—	—
BMI (SD)	26 (4)	24 (4)	0.755	27 (4)	0.051
Pseudophakia	19 (63)	19 (63)	1.0	—	—
MNV lesion size (mm^2)	1.05 (0.52–1.81)	1.10 (0.67–1.72)	0.878	—	—
CMT (μm)	365 (126)	419 (133)	0.084	—	—

Values are presented as mean with SD, median with interquartile range, or distribution.
*Indicates statistical significance ($P < 0.05$).
BMI, body mass index; OD, oculus dexter; OS, oculus sinister; MNV, macular neovascularization; CMT, central macular thickness.

In the brolucizumab group, we observed a significant increase in the systemic PlGF levels from the baseline (9.6 [8.0–13.3] pg/mL) to Day 7 after IVI (13.3 [8.3–16.2] pg/mL, $P = 0.015$). The systemic PlGF levels returned to the baseline values after 28 days (9.8 [8.0–15.6] pg/mL, $P = 0.433$).

Compared with Day 7, the systemic PlGF levels were significantly lower on Day 28 in the aflibercept and brolucizumab groups ($P < 0.001$ and $P = 0.033$, respectively).

The brolucizumab group showed significantly lower systemic PlGF levels than the aflibercept group 7 days after IVI ($P < 0.001$).

Systemic Inflammatory Marker

Compared with baseline, no significant difference was seen in C-reactive protein levels in both the aflibercept and brolucizumab groups, 7 days ($P = 0.263$ and $P = 0.532$, respectively) and 28 days ($P = 0.252$ and $P = 0.879$, respectively) after the IVI (Table 2).

Discussion

This study provides novel information about the effect of brolucizumab on systemic VEGF-A levels and offers a comparison of potential off-target effects of intravitreal brolucizumab versus aflibercept in the treatment of patients with nAMD. We found a significant reduction of systemic VEGF-A after the treatment with intravitreal brolucizumab. This decrease was observed after 7 days, and a further reduction was measured after 28 days. Patients treated with intravitreal aflibercept also showed a decrease of

Table 2. Systemic Cytokine and CRP Levels

	Aflibercept	Brolucizumab	*P*	Control	*P*
VEGF-A (pg/mL) BL (IQR)	12.0 (8.0–18.5)	10.8 (8.0–13.2)	0.158	10.0 (8.0–15.1)	0.316
VEGF-A (pg/mL) 7 days (IQR)	8.0 (8.0–8.0)	8.0 (8.0–11.5)	0.066		—
P (BL vs. 7 days)	<0.001*	0.0254*			—
VEGF-A (pg/mL) 28 days (IQR)	12.5 (8.5–14.6)	8.0 (8.0–8.0)	<0.001*		—
P (BL vs. 28 days)	0.120	<0.001*			—
VEGF-B (pg/mL) BL (IQR)	58.2 (27.8–139.8)	68.0 (49.9–323.3)	0.228	33.7 (12.4–70.2)	0.295
VEGF-B (pg/mL) 7 days (IQR)	63.2 (32.7–166.1)	56.7 (47.1–260.5)	0.535		—
P (BL vs. 7 days)	0.349	0.440			—
VEGF-B (pg/mL) 28 days (IQR)	59.8 (39.8–99.8)	68.5 (52.4–212.4)	0.133		—
P (BL vs. 28 days)	0.230	0.990			—
PlGF (pg/mL) BL (IQR)	8.5 (8.0–14.9)	9.6 (8.0–13.3)	0.751	8.0 (8.0–8.0)	0.023
PlGF (pg/mL) 7 days (IQR)	33.1 (21.9–40.6)	13.3 (8.3–16.2)	<0.001*		—
P (BL vs. 7 days)	<0.001*	0.015*			—
PlGF (pg/mL) 28 days (IQR)	11.3 (8.0–18.5)	9.8 (8.0–15.6)	0.277		—
P (BL vs. 28 days)	0.002*	0.433			—
CRP (mg/dL) BL (IQR)	0.16 (0.09–0.25)	0.15 (0.07–0.26)	0.518	0.23 (0.10–0.31)	0.772
CRP (mg/dL) 7 days (IQR)	0.18 (0.2–0.33)	0.15 (0.07–0.24)	0.257		—
P (BL vs. 7 days)	0.263	0.532			—
CRP (mg/dL) 28 days (IQR)	0.20 (0.10–0.30)	0.15 (0.08–0.25)	0.303		—
P (BL vs. 28 days)	0.252	0.879			—

Values are presented as median (IQR).
*Indicates statistical significance ($P < 0.05$).
IQR, interquartile range; BL, baseline; CRP, C-reactive protein.

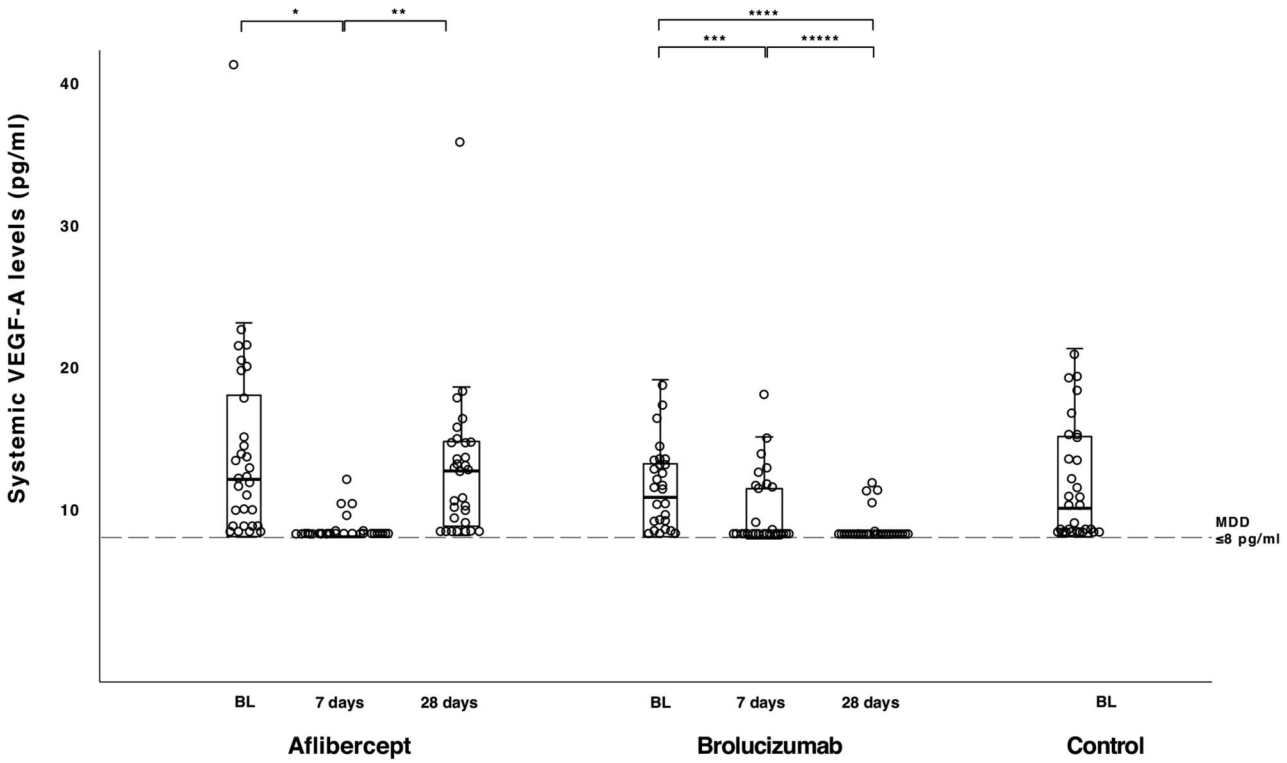

Fig. 1. Systemic VEGF-A levels before and after IVI of anti-VEGF. Compared with the baseline levels, systemic VEGF-A levels in patients with nAMD decreased significantly on Day 7 (*$P < 0.001$) in the aflibercept group and on Day 7 (***$P = 0.0254$) and Day 28 (****$P < 0.001$) in the brolucizumab group. The VEGF-A levels in the aflibercept group at 28 days were significantly higher and significantly lower than those at 7 days postinjection in the brolucizumab group (**$P < 0.001$ and *****$P = 0.0245$, respectively). nAMD, neovascular age-related macular degeneration; MDD, minimum detectable dose.

systemic VEGF-A after 7 days, but in contrast to the brolucizumab group, the reduction was less sustained and values returned to baseline after 28 days. Accordingly, the systemic VEGF-A levels were significantly lower in the brolucizumab group compared with those of the aflibercept group after 28 days. These findings provide data for systemic effects after intravitreal anti-VEGF therapy and may indicate a possibility for inadvertent off-target effects. None of the treatment groups showed any clinical signs of intraocular or systemic inflammation. Although aflibercept was also designed to bind VEGF-B and PlGF,[18] we could not observe any effects on the systemic VEGF-B in either the treatment or control group in this study. Intriguingly, the systemic upregulation of PlGF was more pronounced in the aflibercept group than in the brolucizumab group. This phenomenon of systemic counter regulation of circulating PlGF has already been reported in previous studies.[19–21]

Brolucizumab was designed for intraocular use alone; therefore, there is currently little knowledge about its systemic pharmacokinetics. Brolucizumab is a humanized single-chain antibody fragment that inhibits all isoforms of VEGF-A. With a molecular weight of 26 kDa, it is the smallest of all anti–VEGF-A antibodies used for intraocular injection (aflibercept −115 kDa and ranibizumab—48 kDa).[8,10] Owing to its small size, brolucizumab can be concentrated up to 120 mg/mL, allowing the administration of 6 mg in a single 0.5 mL IVI. On a molar basis, 6 mg of brolucizumab equals roughly 12 times the usual dose of aflibercept and about 22 times the ranibizumab dose.[10] The low molecular weight and high intravitreal concentration gradient between the vitreous and retina might provide effective retinal and choroidal penetration of brolucizumab. Higher molar doses of a drug are likely to be cleared from the eye over an extended period, thus prolonging the duration of its intravitreal and systemic action.[8,22] The estimated terminal elimination half-life of free systemic brolucizumab is 4 to 5 days, with about 0.5 ng/mL of free systemic brolucizumab measured 4 weeks after a single IVI.[23] By contrast, free systemic aflibercept could not be detected 2 weeks after a single IVI.[18,24] Consistent with these systemic pharmacokinetic reports, we found a sustained reduction in systemic VEGF-A levels after brolucizumab treatment, which returned to baseline levels 28 days after an aflibercept IVI. The molar weight of aflibercept is 4.4 times higher compared with the single-chain antibody fragment

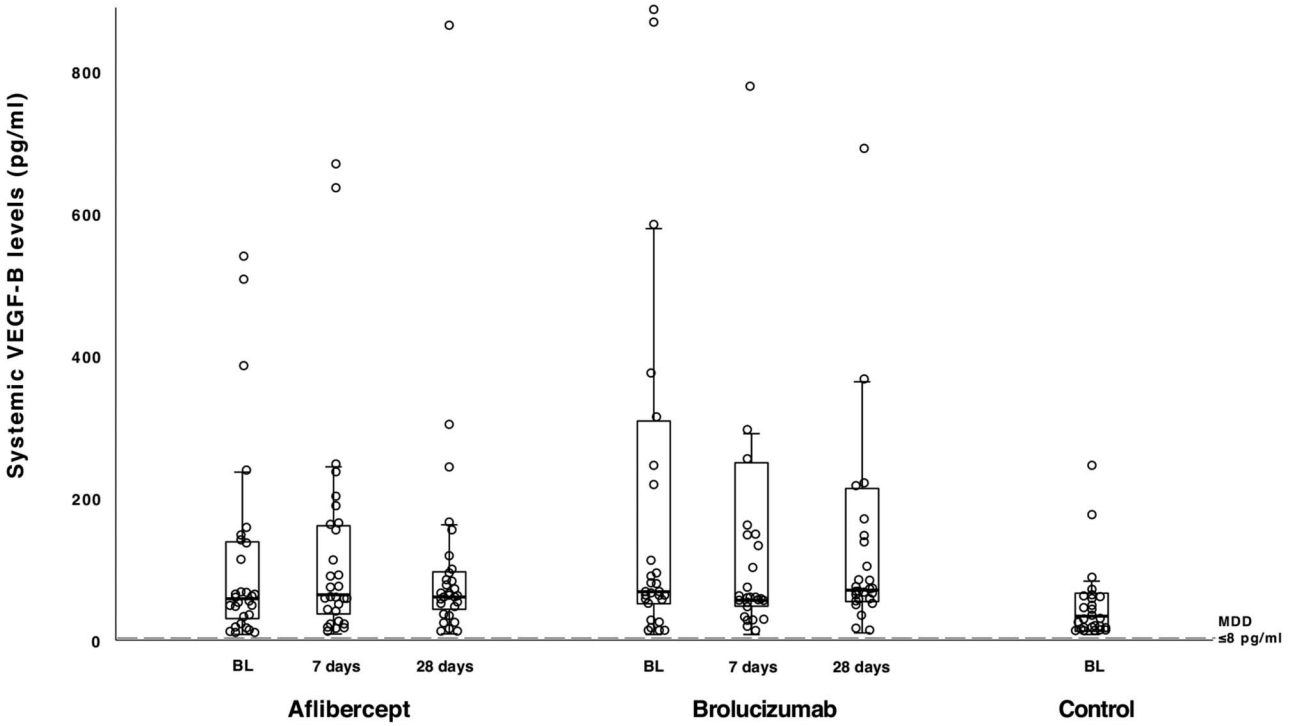

Fig. 2. Systemic VEGF-B levels before and after IVI of anti-VEGF. There was no significant difference in within and across group comparison regarding systemic VEGF-B levels in patients with nAMD treated with aflibercept or brolucizumab. There were three outliers >800 pg/mL in the aflibercept group, 11 outliers in the brolucizumab group, and four outliers in the control group that were not illustrated for a better presentation of the figure but are included in the median and interquartile range. nAMD, neovascular age-related macular degeneration; MDD, minimum detectable dose.

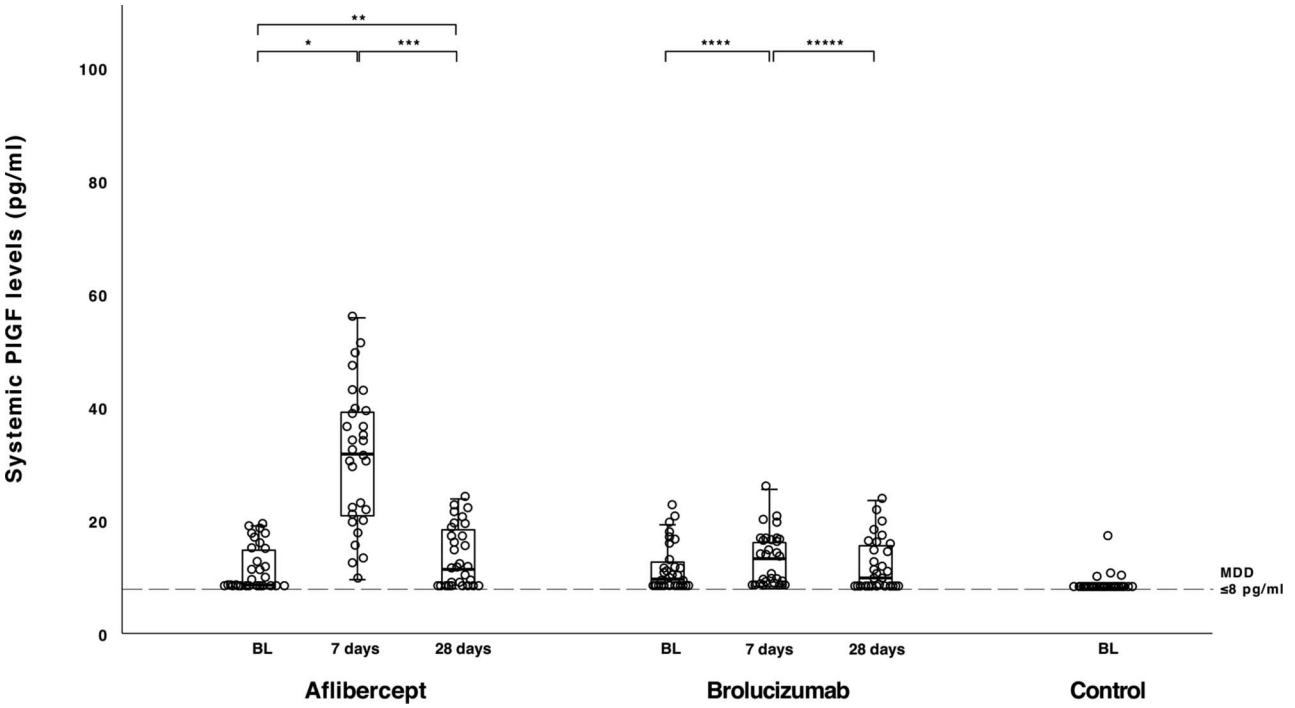

Fig. 3. Systemic PlGF levels before and after IVI of anti-VEGF. Compared with the baseline levels, systemic PlGF levels in patients with nAMD increased significantly on Day 7 (*$P < 0.001$) and Day 28 (**$P = 0.002$) in the aflibercept group and on Day 7 (****$P = 0.015$) in the brolucizumab group. The PlGF levels in the aflibercept group and the brolucizumab group were significantly lower at 28 days than at 7 days postinjection (***$P < 0.001$ and *****$P = 0.033$, respectively). nAMD, neovascular age-related macular degeneration; MDD, minimum detectable dose.

brolucizumab. Besides its active binding domains, aflibercept consists of the constant region (Fc) of human IgG1. It has been determined that the neonatal Fc receptor is responsible for the active transport of molecules containing an Fc domain across the blood–retinal barrier.[11,12,19,25–27]

The prolonged suppression of systemic VEGF-A levels after brolucizumab IVI increases the possibility of unexpected and unwanted systemic off-target effects. We should keep in mind that most patients with AMD are elderly with comorbidities. Vascular endothelial growth factor is a multifunctional cytokine involved in the regulation and function of healthy vessels and is closely linked to inflammatory mediators and response.[28,29] Besides its vascular protective function, it maintains the antiinflammatory properties of the vascular endothelium[30] and its blockade leads to a decrease in circulating lymphocytes.[31] Although there are no reported systemic adverse events from preliminary real-world studies or the Phase 3 HAWK and HARRIER studies, we should consider that premarketing studies of new drugs might not be reliable for detecting rare but important systemic adverse events.[32] Postmarketing investigations are hence crucial for the evaluation and characterization of a pharmaceutical's risk profile. Unfortunately, there are no data regarding the changes in VEGF-A levels in patients experiencing systemic side effects available currently. Although there are numerous reports describing an increase in nonocular hemorrhagic events, blood pressure elevation, myocardial infarction, and kidney disease after anti-VEGF therapy,[33–36] none of these reports include information regarding the systemic VEGF-A levels. A long-term prospective study stratified for patients at risk would be required to measure the systemic VEGF-A levels during these pathologic events and evaluate the differences in systemic adverse events between brolucizumab and other anti-VEGF agents.

Regardless of the lack of information on clinically meaningful systemic VEGF-A levels, the outcomes of this study might potentially be interesting for physicians using brolucizumab for treating patients with comorbidities.

A limitation of this study is that the healthy control group was recruited from sex-matched but not age-matched population. Nevertheless, the baseline systemic VEGF-A levels did not differ between the treatment and control groups. The analysis of systemic VEGF-A within each treatment group and across treatment groups are not affected by the given age distribution. All participants of this study were treated on-label. They received a loading dose of three consecutive IVI of either aflibercept or brolucizumab every four weeks. Thus, the study protocol impeded us from studying the effect of brolucizumab on systemic VEGF-A beyond 28 days after a single injection in treatment naïve patients. Future studies could investigate the response of systemic VEGF-A beyond 28 days after the completion of the loading dose. The strengths of the study include its prospective design and relatively large sample size per treatment group, allowing us to achieve a large effect size based on the power analysis.

In conclusion, we observed a sustained reduction of systemic VEGF-A levels in patients receiving intravitreal brolucizumab, with the greatest effect measured 28 days posttreatment. Patients treated with intravitreal aflibercept also showed a decrease of systemic VEGF-A, but in contrast to the brolucizumab group, the reduction was less sustained and values returned to baseline within 28 days. Although we did not observe any systemic adverse events, the prolonged effect of VEGF-A raises concerns regarding the safety and long-term effects of intravitreal brolucizumab.

Key words: aflibercept, age-related macular degeneration, brolucizumab, VEGF levels.

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