Intraocular Pharmacokinetics of Aflibercept and Vascular Endothelial Growth Factor-A

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Submitted: January 9, 2015
Accepted: May 24, 2015

Citation: Celik N, Scheuerle A, Auffarth GU, Kopitz J, Dithmar S. Intraocular pharmacokinetics of aflibercept and vascular endothelial growth factor-A. *Invest Ophthalmol Vis Sci*. 2015;56:5574–5578.
DOI:10.1167/iovs.15-16418

Purpose. To determine intraocular pharmacokinetics of aflibercept and VEGF-A in patients with neovascular age-related macular degeneration (nAMD) during a treatment period of 6 months.

Methods. Seven nonvitrectomized patients diagnosed with macular edema secondary to nAMD undergoing intravitreal injections (IVI) of aflibercept. Patients were treatment naïve at least for the last 2 months and received intravitreal injection of 2 mg aflibercept for the first time. Aqueous humor samples were obtained prior to each injection procedure during a 6-month period: three times monthly, then bimonthly. Over all 35 samples were analyzed with ELISA for unbound VEGF-A and a self-developed assay for unbound aflibercept.

Results. In all cases, wet AMD was inactive after IVI. Unbound aflibercept could be detected in all samples. Initial mean concentration of aflibercept was 305.4 ± 43.8 μg/mL and remained stable after the first injection with 0.8 ± 0.5 μg/mL. Initial mean level of unbound VEGF-A was 190.7 ± 26.9 pg/mL. A significant decrease of the concentration to 92.6 ± 10.2 pg/mL ($P < 0.05$, Wilcoxon rank sum test) after the first injection was observed. This level remained stable during further treatment.

Conclusions. Levels of unbound aflibercept and unbound VEGF-A remained stable after every month and every second month of IVI. The findings of these small case series support suggestions that treatment intervals with bimonthly IVI of aflibercept are sufficient due to a detectable remaining biologic active concentration of aflibercept.

Keywords: aflibercpt, VEGF, pharmacokinetics, macular edema

Treatment of neovascular age related macular degeneration (nAMD) with intravitreal injections (IVI) to inhibit VEGF-A has become the current therapeutic standard. For a long time, anti-VEGF drugs like pegabtanib (Macugen; Eyetech, New York, NY, USA), ranibizumab (Lucentis; Genentech, San Francisco, CA, USA), and bevacizumab (Avastin; Genentech) have been administered intensively in ophthalmologic practice. Recently, in November 2012 aflibercept (Eylea/VEGF Trap; Regeneron Pharmaceuticals, Tarrytown, NY, USA) was approved for the treatment of nAMD in Europe.[1] Aflibercept is a humanized recombinant fusion protein. It consists of fragments from the extracellular domains of human VEGF receptors 1 and 2 fused to the Fc portion of human IgG1.[2] Due to its higher binding affinity to VEGF-A compared with other anti-VEGF drugs, aflibercept is supposed to have a longer biological activity.[3] No data are available concerning the intraocular pharmacokinetics of aflibercept in humans, apart from a mathematic theoretical model.[3,4] Knowledge about the pharmacological activity could help to adjust the required treatment frequency properly. In this study, we measured the intraocular levels of aflibercept and VEGF-A in patients with nAMD during a 6-month follow-up.

Methods

Subjects

In a prospective, noncomparative, nonrandomized study, seven patients (Tables 1, 2) with a macular edema due to nAMD were enrolled. Informed written consent was obtained from all patients. The protocol was approved by the Ethics Committee of the University of Heidelberg and followed the tenets of the Declaration of Helsinki. Patients received intravitreal injections of aflibercept for the first time. Patients with any other intravitreal injections within 2 months prior to the study were excluded. Further exclusion criteria were other ocular diseases (i.e., glaucoma, uveitis, and all retinal diseases), a cataract surgery within the last 6 months or a previous vitrectomy.

Treatment and Sampling

One eye was treated in each patient according to the guidelines: initial 3 monthly injections, followed by bimonthly injections. During a 6-month follow-up including an upload phase and two further IVI five samples of aqueous humor were collected per patient. Approximately 0.1 mL of aqueous humor was obtained via limbal paracentesis with a 30-G needle on a

Table 1. Demographic Subject Data

Number of participants	7
Mean age	77.4 ± 6.7 (65–84) y
Sex	2 men, 5 women
Lens	4 phacic, 3 pseudophacic
Mean eye volume, calculated	6.7 ± 1.1 (5.5–8.5) mL

Summarized clinical characteristics of patients with exudative AMD. Value in parentheses is range.

1.0-mL tuberculin syringe prior to each injection procedure. Samples were immediately stored at −80°C. Patients were extensively monitored including best-corrected visual acuity (BVCA) and central macular thickness (CMT) with spectral-domain optical coherence tomography (SD-OCT; Heidelberg Engineering, Heidelberg, Germany) at each visit before intravitreal administration and 2 months after the last IVI. Best-corrected visual acuity was measured in Snellen and converted to logMAR units. Horizontal raster scans were performed to obtain the CMT, which was calculated automatically by the OCT software. If the automatically detected boundary lines did not match the membrane limitans interna and the Bruch's membrane, boundaries were corrected manually.

Aflibercept and VEGF-ELISA Assays

Concentrations of unbound aflibercept in aqueous humor were measured by a self-developed ELISA as no commercial ELISA specially designed for the detection of aflibercept is available. The principle of this assay is shown in Figure 1. Samples (100 µL) were transferred to 96-well plates precoated with anti-human IgG1 polyclonal antibody (anti-human IgG1 precoated 96-well strip plate; Cayman Chemical, Ann Arbor, MI, USA) and incubated for 1 hour at room temperature to bind aflibercept via its Fc domain to the solid phase of the wells. After three washes with 200 µL PBS/0.1% Tween 20, biotinylated VEGF-165 (Fluorokine Biotinylated Human VEGF, 40 ng in 100µL PBS/0.1%BSA; R&D Systems, Minneapolis, MN, USA) was added and incubated for 1 hour at room temperature, thereby forming a biotinylated VEGF-aflibercept complex via the Fc domain of aflibercept. Then the wells were washed three times with 200 µL PBS/0.1% Tween 20. Next, 100 µL alkaline peroxidase-coupled streptavidine (Extravidin-Peroxidase, diluted 1:2000 in PBS/0.1% BSA; Sigma-Aldrich, Dreieich, Germany) was added for 1 hour. After three washes with PBS/0.1% Tween 20, peroxidase activity, which corresponds to the amount of aflibercept was assayed by incubation with 100 µL peroxidase substrate solution (3,3′,5,5′ Tetramethylbenzidine [TMB)] liquid substrate system for ELISA; Sigma-Aldrich) for 10 minutes. After stopping the reaction with 100 µL 2N HCl absorbance was read in an ELISA reader at 450 nm. A typical calibration curve recorded with pure aflibercept in PBS is given in Figure 2. The linear range is 0 to 100 ng/mL. Samples were diluted with PBS to fall into the linear range and assayed in triplicate.

Table 2. Intraocular Levels of VEGF-A and Aflibercept

Unbound VEGF-A at baseline	190.7 ± 26.9 (136–218) pg/mL
Unbound VEGF-A after 1 mo	92.6 ± 10.2 (73–105) pg/mL
Unbound VEGF-A after bimonthly IVI	89.1 ± 12.2 (69–104) pg/mL
Estimated initial aflibercept concentration	305.4 ± 43.8 (235.3–362.3) µg/mL
Unbound aflibercept after 1 mo	0.8 ± 0.5 (0.3–1.5) µg/mL
Unbound aflibercept after bimonthly IVI	0.8 ± 0.2 (0.6–1.1) µg/mL

Immunoassay for intraocular aflibercept and VEGF-A: mean values ± SD (range).

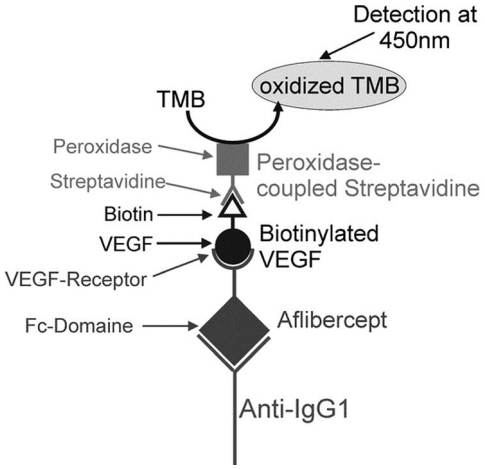

Figure 1. Principle of the ELISA for detection of aflibercept. Aflibercept is captured by anti-IgG coated to an ELISA plate. Biotinylated VEGF is added resulting in a biotinylated complex corresponding to the amount of aflibercept. The complex is quantitated by Alkaline Peroxidase-coupled Streptavidine. TMB is added to the wells and yields a blue color when oxidized in proportion to the amount of analyte. The absorbance is measured at 450 nm.

Unbound VEGF-A was determined applying a commercial solid-phase sandwich ELISA-Kit (Human VEGF Quantikine ELISA Kit; R&D Systems) according to the manufacturer's instructions.

Ocular Biometrics

The initial concentration of aflibercept was calculated considering the globe size. Ocular globe was estimated to be spherical and the ocular volume was calculated with the axial

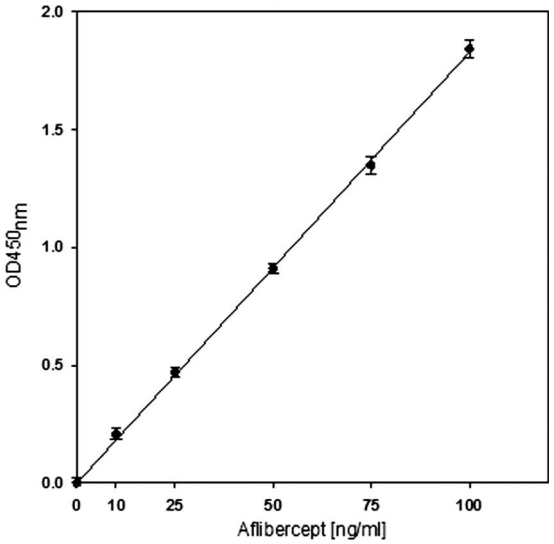

Figure 2. Typical ELISA standard curve of aflibercept. With measurements of serial dilutions of aflibercept the assay was calibrated. Each point represents the mean of three measurements (±SD). The detection range of the assay for humour aqueous humor analysis was 0 to 100 ng/mL.

length (AL) of the eye, which was measured by optic biometry (IOL Master; Carl Zeiss Meditec, Jena, Germany) for each study eye. The following formula for volume calculation was used:

$$\text{Volume[mL]} = 4/3 \times \pi(1/2 \times \text{AL[mm]})^3. \quad (1)$$

Statistical Analysis

Statistical analysis was performed using SPSS software (PASW Statistics 18; SPSS, Chicago, IL, USA). The ANOVA at different time points was calculated by the Friedman test followed by the Wilcoxon rank sum test. For the assessment of nonparametric association between two variables Spearman's rank correlation coefficient was performed.

Results

The unbound aflibercept and VEGF-A levels were measured in 35 aqueous humor samples of seven patients with nAMD. Mean age was 77.4 ± 6.7 years (age range, 65–84 years) with 5 female and 2 male patients. In a 6-month follow-up patients were treated with 3 monthly injections followed by 2 bimonthly injections. Samples were obtained prior to injection procedure in the same eye. Tables 1 and 2 give an overview about the clinical characteristics of the study population (Table 1) and about the measured values with the ELISA (Table 2). Injections and taps were well tolerated by the subjects without any adverse events. In all cases wet AMD was inactive after intravitreal treatments. After the first IVI the CMT decreased from 441 ± 78 μm (range, 279–510μm) to 316 ± 89 μm (225–472 μm; Fig. 3A). This change was statistically significant in the Wilcoxon test ($P = 0.018$). After the second IVI a further slight decrease of 262 ± 49 μm (204–351 μm) was measured in the CMT, which was not significant. The BCVA was 0.77 ± 0.41 logMAR (0.3–1.3 logMAR) at baseline. After the last IVI VA was improved to 0.64 ± 0.39 logMAR (0.2–1.3 logMAR), but without any statistical significance ($P < 0.05$; Fig. 3B). Mean ocular volume was 6.7 ± 1.1 mL (range, 5.5–8.5 mL) and was estimated from measured axial length. Considering the globe volume and the injected aflibercept concentration of 2 mg/mL the calculated initial mean concentration of aflibercept in the eye was 305.4 ± 43.8 μg/mL (range, 235.3–362.3 μg/mL). Mean concentration of unbound aflibercept, obtained 1 month after the first injection, was 0.8 ± 0.5 μg/mL (range, 0.3–1.5 μg/mL) and remained stable with small intra- and interindividual deviations (Fig. 4A). Initial mean level of VEGF-A was 190.7 ± 26.9 pg/mL (range, 136–218 pg/mL). Wilcoxon test showed a significant decrease ($P = 0.018$) after the first IVI to 92.6 ± 10.2 pg/mL (range, 73–105 pg/mL), which remained stable during the treatment period (Fig. 4B). A statistical correlation between VEGF-A and the CMT or BCVA could not be established.

Discussion

For the treatment of nAMD anti-VEGF medications have often to be injected monthly and over a long period of time. Less injections are desirable because of patients discomfort and injection-related risks like endophthalmitis. Aflibercept is supposed to have a stronger binding effect to VEGF than other anti-VEGF medications.[3,5] Concerning the pharmacokinetics of aflibercept only a few theoretical calculation models and experiments in animal eyes have been published.[3,4,6] Due to a different anatomy like smaller vitreous volumes pharmacokinetics in animal eyes are not transferable to human eyes. For this reason different half-life values in rabbits and monkeys were obtained for example for bevacizumab.[7,8] In the present study, we examined the pharmacokinetics of unbound aflibercept and VEGF-A in human eyes in serial aqueous humor probes with multiple IVI.

After the first IVI the concentration of VEGF-A decreased significantly. Interestingly, there were no significant differences between monthly and bimonthly treatment intervals in terms of the concentrations of unbound aflibercept and VEGF-A, respectively. We show the first insights of 35 serial concentration measurements during treatment with aflibercept in a 6-month follow-up. We investigated stable concentrations without exceptions and without big deviations after monthly and bimonthly IVI. A larger sample size could possibly show small deviations with only a small effect, but a relevant distinction is not expected. In further studies, a larger cohort with more statistical power could emphasize our findings. Nevertheless, our sample size was large enough to show a statistical significant decrease of the VEGF-concentration after the first IVI.

We had no evidence for clinical disease reactivity or nonresponse during different treatment intervals. Central macular thickness decreased significantly parallel to the concentration of VEGF-A after the first injection. Similarly to the concentrations of VEGF-A, levels of CMT did not show differences after monthly or bimonthly IVI. The results of BCVA almost remained stable during the study. Regarding our data the necessity of monthly IVI during the loading dose seems doubtful. Another study investigated a suppression time of approximately 8 weeks for VEGF during treatment with aflibercept.[9] In a mathematical model Stewart postulates an aflibercept-activity for even 10 to 12 weeks.[3] If this hypothesis is true, patients could benefit from less frequent injections. Nevertheless, the real effect of the upload dosage remains unclear. Possibly, the loading phase alters the disease state and potentially the VEGF production.

Unbound aflibercept with the capacity of binding VEGF-A remained during the loading phase as well as after bimonthly IVI at a similar level. However, free VEGF-A was also still detectable. This constellation might reflect an ongoing binding process, which was not completed at the time of sampling. Monthly and bimonthly IVI show similar levels of aflibercept without any accumulation. Monthly IVI do not induce an extended biologic active concentration of aflibercept. From the calculated initial concentration of 305.4 ± 43.8 μg/mL of aflibercept we found 0.8 ± 0.5 μg/mL unbound aflibercept after monthly and bimonthly IVI. We assume that the major part of the drug is bound to its targets like VEGF-A. Nevertheless, the rates of diffusion and active choroidal transport via the Fc receptor remain unknown.[2,10,11]

Small individual deviations were negligible in our study.

In previous experiments, it was shown that levels of mediators in aqueous humor correspond to the vitreous values.[12–16] In addition, we expect a steady state equilibrium between the two compartments within at least 1 month between IVI and probe collection. Aqueous humor is accessible to investigate pharmacokinetics in a small sample volume with the help of ELISA technique and offers the possibility to adapt treatment frequency of IVI. Commercial ELISA kits are available for various biological markers including VEGF-A. There is a discrepancy between our results on intraocular VEGF concentrations and the results of another group weeks.[9] We found higher concentrations of VEGF in the untreated eye and a remaining VEGF level during aflibercept treatment, whereas Fauser et al.[9] describe complete suppression of VEGF upon aflibercept. This might be explained by the use of different measurement methods. We used a conventional sandwich ELISA technique while Fauser et al.[9] applied the Luminex technology. The fluorescence-based method by

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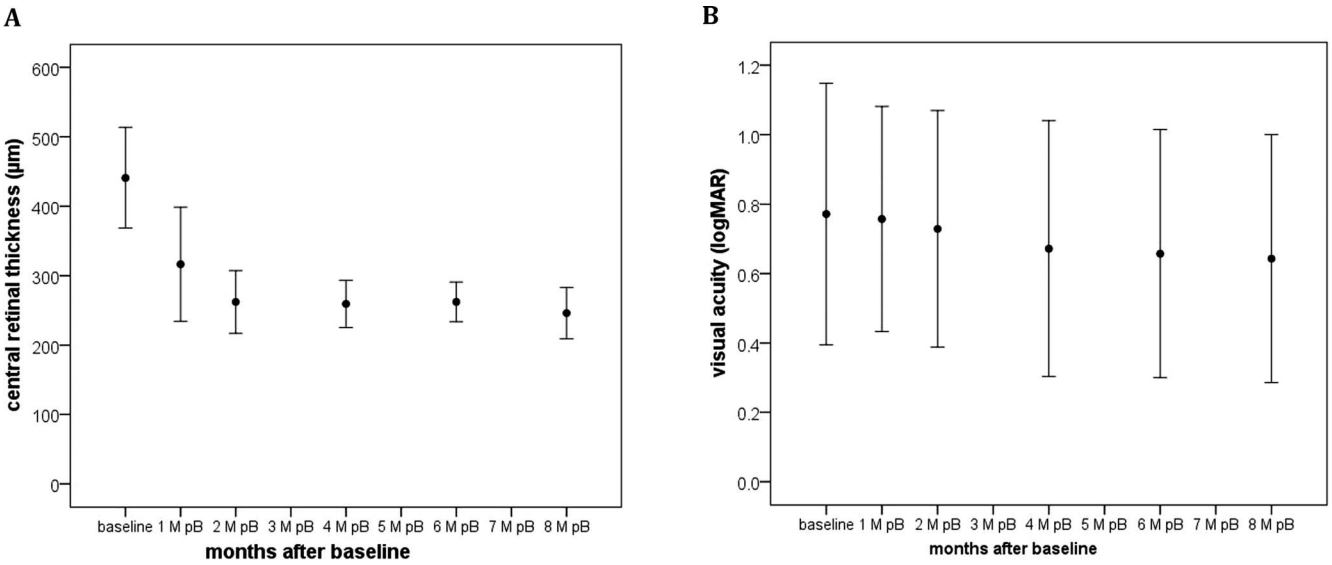

FIGURE 3. (A) Central retinal thickness (µm). Central macular thickness decreased significantly after the frist IVI and remained stable during further treatment. (B) Visual acuity (logMAR) in all patients during follow-up. Mean visual acuity (logMAR) remained stable during treatment with IVI of aflibercept. M, month; pB, post baseline.

Fauser et al.[9] is more sensitive (detection limit 2 pg/mL) than our conventional photometric method detection limit (9 pg/mL). However, our results are always far above the detection limit. It is conceivable that differences in the preparation of the samples for measurement may play a role. In particular specific calibrator diluents are used to stabilize and remove the analyte from nonspecific binding to other proteins. Thus, sample preparation in our assay may have been more efficient in increasing the availability of free analyte for detection. Accordingly, Carneiro et al.[17] found very similar results to ours in plasma samples by the ELISA technique.

With our self-developed ELISA, we showed that also aflibercept is measurable with the ELISA technique in aqueous humor samples. To minimize experimental irregularities all aqueous humor samples were stored at −80°C until analyzing altogether in the same experimental setting. As it was recently shown that bevacizumab did not lose its activity after one time freezing and thawing.[18] We assume that this is also true for aflibercept.

To have a comparable basic requirement, vitrectomized eyes were excluded from our study as it was already demonstrated that clearance of intravitreal drugs is more rapid.[19] Yanyali et al.[20] describe an inadequate clinical effect of bevacizumab in patients with vitrectomized eyes. This result is also expected for aflibercept and should be observed during clinical treatment with IVI although another study with rabbits could not show any different pharmacokinetics in vitrectomized eyes.[21]

In summary, IVI therapy can be monitored and adapted by measuring levels of biological markers and drugs like aflibercept in aqueous fluid. The initial injected high concentration of aflibercept declines to a very small level. Concentrations of free VEGF-A decline significantly after the first IVI, reflecting the binding affinity of aflibercept. There are no

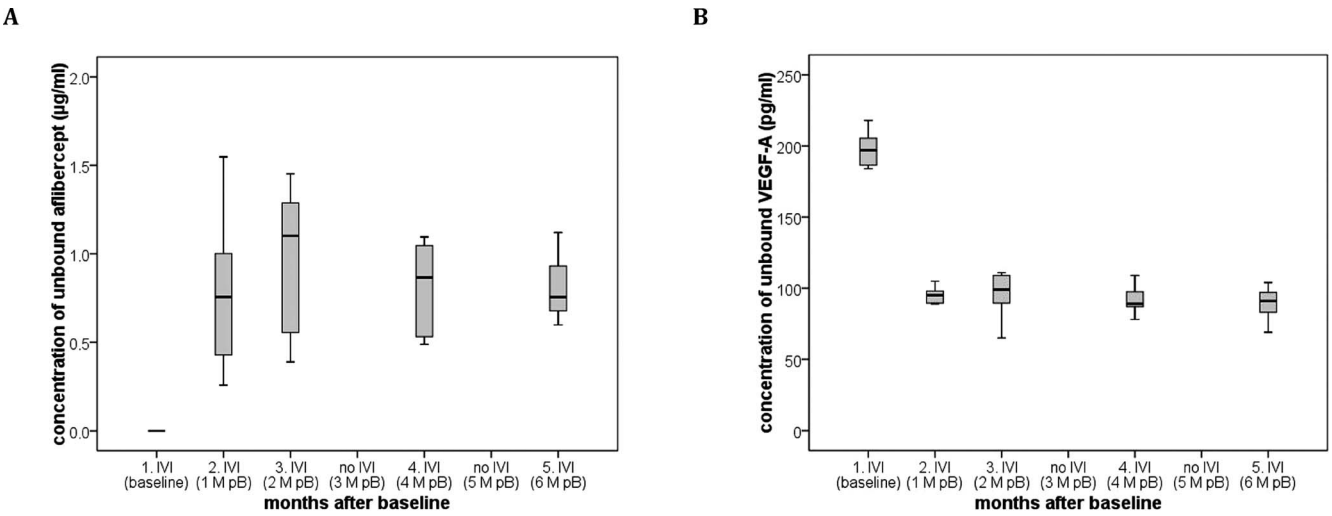

FIGURE 4. (A) Concentrations of unbound aflibercept (µg/mL) in aqueous humor of patients with neovascular AMD during treatment. Levels of unbound aflibercept remained stable during monthly and bimonthly IVI. (B) Concentrations of unbound VEGF-A (pg/mL) in aqueous humor of patients with neovascular AMD during treatment. Levels of VEGF-A decreased after the first injection and remained stable during the treatment period.

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significant distinctions between concentrations after monthly and bimonthly IVI for aflibercept and VEGF-A. Hence, an inhibition of VEGF-A is sufficient with bimonthly IVI of aflibercept. Further studies are necessary to recruit larger cohorts and to investigate the effective influence of the loading phase.

Acknowledgments

The authors thank M. Karl (laboratory technician) for excellent technical assistance.

Supported by Gertrud Kusen Foundation and German Ophthalmologic Society (2014 DOG poster prize; DOG, Munich, Germany; both NC).

Disclosure: **N. Celik**, None; **A. Scheuerle**, None; **G.U. Auffarth**, Abbott Medical Optics (F, C, R), Alcon Laboratories, Inc. (F, C, R), Alimera Sciences, Inc. (F), Bausch & Lomb Surgical (F, C, R), Carl Zeiss Meditec (F, R), Contamac (F), Glaukos Corporation (F), Heidelberg Engineering (F), HumanOptics (F, R), Novartis Pharmaceuticals Corporation (F, C, R), Oculentis (F, R), Physiol (F, R), Power Vision (F), Rayner Intraocular Lenses Ltd. (F, C, R), Technolas (F, C, R), Allergan (C, R), Bayer Healthcare Pharmaceuticals (R), Hoya (R), Kowa (R), OPHTEC (R); **J. Kopitz**, None; **S. Dithmar**, Bayer Healthcare Pharmaceuticals (F)

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