

The Influence of Intravitreal Ranibizumab on Inflammation-associated Cytokine Concentrations in Eyes With Diabetic Macular Edema

Shueh Wen Lim,¹ Esther Bandala-Sanchez,² Maria Kolic,¹ Sophie L. Rogers,¹ Annie K. McAuley,^{1,3} Lyndell L. Lim,¹ and Sanjeewa S. Wickremasinghe¹

¹Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, Victoria, Australia

²Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

³Department of Epidemiology and Preventative Medicine, Monash University, Australia

Correspondence: Sanjeewa S. Wickremasinghe, Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, Victoria, Australia; Sanj.Wickremasinghe@eyeandear.org.au.

Submitted: November 8, 2017

Accepted: September 9, 2018

Citation: Lim SW, Bandala-Sanchez E, Kolic M, et al. The influence of intravitreal ranibizumab on inflammation-associated cytokine concentrations in eyes with diabetic macular edema. *Invest Ophthalmol Vis Sci*. 2018;59:5382-5390. <https://doi.org/10.1167/iovs.17-23325>

PURPOSE. To evaluate the effect of intravitreal ranibizumab injections on aqueous concentrations of angiogenic or inflammatory cytokines in patients with diabetic macular edema (DME).

METHODS. Thirty eyes of 25 patients with center-involved DME were recruited to the study. All had a central macular thickness (CMT) of >300 μm and best-corrected visual acuity (BCVA) between 28 and 70 logMAR letters (Snellen equivalent 20/320–20/40). At baseline, all eyes had 0.1 mL of aqueous collected before ranibizumab treatment. At week 4, a second ranibizumab injection was administered and at week 8, aqueous sampling was repeated before a third ranibizumab injection. From week 12, all eyes were followed at 4-weekly intervals and the need for ranibizumab treatment was determined by BCVA and CMT measurements. Levels of 32 cytokines were assessed at baseline and at week 8 using a multiplex array assay.

RESULTS. Following two consecutive ranibizumab injections, there was a statistically significant reduction in VEGF ($P < 0.00001$), as well as IL-1 β ($P = 0.00006$), IL-7 ($P = 0.00002$), IL-8 ($P = 0.00023$), IL-10 ($P < 0.00001$), IL-12 ($P < 0.00001$), IL-17 ($P = 0.00024$), MCP-1 ($P = 0.00023$), and TNF- α ($P < 0.00001$). There was also an upregulation of soluble VEGF receptor-2 ($P = 0.00004$). A $P < 0.0015$ was considered significant in this study.

CONCLUSIONS. Ranibizumab treatment influences various inflammatory cytokine concentrations in addition to reducing aqueous VEGF concentrations in patients with DME. This may contribute to its therapeutic effect in patients with DME.

Keywords: anti-vascular endothelial growth factor, aqueous inflammatory cytokines, diabetic macular edema

Diabetic macular edema (DME) is a major cause of vision impairment in patients with diabetic retinopathy (DR). In DME, breakdown of the inner blood-retinal barrier leads to increased paracellular and transcellular permeability, which results in intraretinal and subretinal fluid accumulation and retinal dysfunction.¹ Although VEGF upregulation is important in increasing intraretinal vascular permeability, it has increasingly been shown that non-VEGF-dependent inflammatory pathways play a crucial role.^{2–8} There is mounting evidence that chronic subclinical inflammation contributes to the pathogenesis of DR.^{2,9} Early in the pathogenesis of DME, increased expression of inflammatory mediators, leukocyte adherence to retinal vascular endothelium and complement activation result in endothelial cell death, vascular leakage, and capillary closure.^{4,5}

Several cytokines, including those associated with inflammation, have been implicated in the pathogenesis of DME. VEGF is elevated in the serum as well as in the eyes of patients with DME and proliferative DR,^{10–16} and VEGF inhibition has been shown to increase vision and reduce macular thickness in many eyes with DME.^{17–29} Unfortunately the response is

variable and unpredictable with quick and significant improvements being observed in 50% of patients, whereas an intermediate response or no significant improvement is detected in others despite monthly injections.³⁰

Intravitreal anti-VEGF treatment is expected to reduce intraocular VEGF levels and reduce vascular leakage; however, the exact mechanisms and reasons for response to treatment remains unknown. There is currently conflicting evidence on the effect of anti-VEGF agents on intraocular cytokine concentrations.^{13,31–34} This study investigates the profiles of cytokines in the aqueous humor of patients with DME and investigates the effect of intravitreal ranibizumab injections on angiogenic and inflammatory cytokines.

METHODS

Patients

Patients with center-involved DME were recruited from the Royal Victorian Eye and Ear Hospital, Melbourne, Australia, between September 2015 and May 2016. Both eyes could be



recruited into the study if they both met all inclusion and exclusion criteria. Each separate study eye was allocated the next sequential study number. If the fellow eye of an already recruited study eye became eligible during the course of treatment, the second eye had the baseline visit at least 28 days after the last ranibizumab injection had been administered in the first eye, because the plasma half-life of ranibizumab is approximately 2 hours and ranibizumab has not been shown to decrease plasma VEGF beyond 7 days.^{35,36}

Key inclusion criteria were age ≥ 18 years, clinically significant macular edema as defined in the Early Treatment of Diabetic Retinopathy Study (ETDRS),³⁷ central macular thickness (CMT) 300 μm or greater as documented on optical coherence tomography (OCT), best-corrected visual acuity (BCVA) of 17 to 70 logMAR letters (20/400–20/40), and type 1 or 2 diabetes mellitus. Key exclusion criteria were loss of vision from ocular disease other than DR, intraocular surgery within 6 months before study entry, intravitreal anti-VEGF injection within 3 months or corticosteroids within 6 months before study entry, argon laser photocoagulation within 3 months before study entry, and previous systemic anti-VEGF treatment. (Please see the Appendix for full inclusion and exclusion criteria.)

At baseline and each visit thereafter, all patients underwent a complete ophthalmic examination that included BCVA by logarithm of the minimum angle of resolution (logMAR) chart, IOP measurements, slit lamp examination, and OCT. Fast macular thickness scans and 6 mm cross-hair scans were obtained using Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany). CMT was determined automatically and analyzed by OCT software. In all OCT maps, automated macular thickness detection was performed by the instrument's software analysis without manual operator adjustment.

This study was approved by the Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital, Melbourne, Australia (approval number 13/1123H) as part of the Diabetic macular edema: aqueous and Serum Cytokine profiling to determine the Efficacy of RaNibizumab (DISCERN) Study. Research adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained by a study investigator from all participants before enrollment in the study.

Visits and Treatment Schedule

All participants received an intravitreal ranibizumab (Novartis Pharma AG, Basel, Switzerland) 0.5 mg/0.05-mL injection 3.5 to 4.0 mm behind the corneal limbus at baseline, week 4 (± 7 days) and at week 8 (± 7 days). All injections were performed by a retinal specialist under sterile conditions, using a 30-gauge needle attached to a 1-mL tuberculin syringe.

From week 12 onward, patients were treated with "as required" dosing as per the RESTORE protocol up to 48 weeks.²¹ In summary, ranibizumab injections were continued until stable BCVA was reached (stable vision over two consecutive visits). Treatment could be suspended if either of the following criteria were met: if the BCVA was stable over two consecutive visits and the investigator felt that no further BCVA improvement could be achieved with continued intravitreal injections, or BCVA letter score of 84 (approximate Snellen equivalent 20/20) was observed over two consecutive visits. After suspension, injections could be resumed pro re nata if there was a decrease in BCVA due to DME progression, confirmed by clinical evaluation and/or OCT or other anatomic and clinical assessments, in the opinion of the investigator. Patients were then treated at monthly intervals until stable BCVA was reached again. Thus, re-initiation of intravitreal injections required at least two successive monthly treatments.

Aqueous Sampling

At baseline and month 2, undiluted aqueous humor samples (0.05–0.1 mL) were collected from participants before they received an intravitreal injection of ranibizumab for treatment of DME. All samples were collected via anterior chamber paracentesis at the corneal limbus under sterile conditions, using a 30-gauge needle with a tuberculin syringe. The specimens were immediately transferred to a sterile plastic tube and stored at -80°C until assayed.

Aqueous Processing and Analysis

The aqueous sample analysis was performed using a multiplex array assay, which is based on the Luminex xMAP technology. Levels of 32 different cytokines were measured, using multiplex detection kits from Bio-Rad (Hercules, CA, USA) and Millipore (Billerica, MA, USA). These included IL-1 β , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), platelet-derived growth factor subunit B and TNF- α , epidermal growth factor (EGF), erythropoietin (EPO), IFN- γ , basic FGF (FGFb), IFN- γ -induced protein 10 (IP-10), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , angiotensin-2 (Ang-2), regulated on activation, normal T-cell expressed and secreted (RANTES), VEGF soluble VEGF receptor-1 (sVEGFR-1), and soluble VEGF receptor 2 (sVEGFR-2).

The aqueous samples were thawed on ice, sonicated, and then spun at 9.3g for 10 minutes to remove any precipitates. Subsequently, samples were diluted 1:4 using the sample diluent buffer provided in the kit. Cytokine standards and aqueous humor samples were added to wells (50 μL /well) of a 96-well plate containing cytokine detection beads and incubated for 30 minutes. This was carried out at room temperature with the 96-well plate sealed and placed on an orbital shaker at 300 rpm. After incubation, the plate was washed with 100 μL of washing buffer three times, then detection antibody (25 μL) was added, and the plate was sealed and placed on an orbital shaker at 300 rpm for 30 minutes. The plate was subsequently washed again in washing buffer and 50 μL streptavidin phycoerythrin was added, with the plate sealed and placed on an orbital shaker at 500 rpm for another 10 minutes. The plate was then washed with 100 μL of washing buffer three times. The beads were resuspended in 125 μL assay buffer and shaken for 30 seconds at 1100 rpm. The analysis procedure was conducted as per the manufacturer's instructions. Standard curves were generated with the reference cytokine sample supplied in the kit using the Bio-Plex200 System (software version 5.1.1; Bio-Rad Laboratories). They were used to calculate the cytokine concentrations in aqueous humor samples. Aqueous humor samples were assayed in duplicate.

Statistical Analysis

All statistical analyses were undertaken using Stata IC 14.2 for Windows (StataCorp LP, College Station, TX, USA). Nonparametric methods were used to account for the truncation of cytokine measurement due to the minimum detectable level. Cytokine values are presented as median and interquartile ranges (IQRs). The Wilcoxon matched-pairs signed-ranks test was used to assess any significant changes between preinjection and 2-month postinjection values. Following regression of pre- and postinjection cytokine concentrations, possible outliers were identified above conservative thresholds for postregression leverage ($>3/10$), Cook's distance ($>4/30$),

TABLE 1. Baseline Clinical Characteristics of the Patients With DME

	Values
Characteristic	
Number of participants	25
Sex, male/female (%)	18/7 (72% male)
Mean age of patients, y, mean (\pm SD)	63.8 (9.6)
Glycated hemoglobin, %, mean (\pm SD)	7.51 (1.16)
Treatment	
Oral hypoglycaemic agent, <i>n</i> (%)	10 (40.0)
Insulin, <i>n</i> (%)	1 (4.0)
Oral hypoglycemic agent plus insulin, <i>n</i> (%)	13 (52.0)
Hypertension, <i>n</i> (%)	19 (76.0)
Dyslipidemia, <i>n</i> (%)	23 (92.0)
Duration of diabetes, y, <i>n</i> (%)	
<5	2 (8.0)
5–10	3 (12.0)
10–15	5 (20.0)
>15	15 (60.0)
Per-eye characteristics	
Number of study eyes	30
BCVA, logMAR, median (IQR)	61.5 (55–67)
CMT, μ m, mean (\pm SD)	484.5 (134.3)
Phakic lens status, <i>n</i> (%)	25 (83.3)
Posterior vitreous detachment, <i>n</i> (%)	0 (0.0)
Stage of DR, <i>n</i> (%)	
Mild NPDR	0 (0.0)
Moderate NPDR	15 (50.0)
Severe NPDR	11 (36.7)
Proliferative DR	4 (13.3)
History of focal photocoagulation, <i>n</i> (%)	10 (33.3)
History of panretinal photocoagulation, <i>n</i> (%)	7 (23.3)
History of intravitreal anti-VEGF, <i>n</i> (%)	9 (30.0)
History of intravitreal corticosteroids, <i>n</i> (%)	1 (3.3)

and/or standardized residuals (>3). A supplementary analysis repeated the signed-ranks test after removing any values that were flagged as possible outliers on any, or all, of these measures to confirm that results were not being driven by extremes.

For those cytokines that showed significant change from preinjection baseline, associations between concentrations of cytokines and VEGF and their change over the 2-month period were assessed with the Spearman rank correlation rho. Given that there are no agreed criteria for when to apply various multiple testing correction methods,^{38,39} multiple comparisons in this study need to be interpreted with caution. For this article, we consider findings with a *P* value less than 0.0015 as significant.

RESULTS

A total of 60 aqueous humor samples were obtained from 30 eyes of 25 patients. Baseline characteristics of patients are illustrated in Table 1. The mean (SD) patient age was 63.8 (9.6) years, with a median (IQR) BCVA of 61.5 letters (55–67), range 28–70 letters. Of the 30 study eyes, 26 (86.7%) had non-proliferative DR (NPDR), 4 (13.3%) had quiescent proliferative DR (PDR), all of which were regressed PDR after panretinal photocoagulation, and all 30 had clinically significant macula edema. Nine eyes (30%) had prior intravitreal anti-VEGF injection at a mean of 8 months before entry into the study, and one had previously received intravitreal corticosteroids 6 years before entry into the study. One eye (3.3%) underwent vitrectomy before the study. A statistically significant improvement in logMAR visual acuity letters before and after two

intravitreal ranibizumab injections of +7.4 letters was noted (95% confidence interval [CI] 4.9–9.9, paired *t*-test, *P* < 0.0001; range, –4 to +21 letters, SD +6.67 letters. Similarly, there was a significant reduction in CMT of –93.5 μ m (95% CI 47.6–139.5, paired *t*-test, *P* = 0.0003) following two injections.

Cytokine Levels in Aqueous Humor

Baseline cytokine concentration as well as cytokine changes after two doses of intravitreal ranibizumab were measured. Samples with measurable concentrations of cytokines and chemokines were included in the analysis. We were unable to detect IL-2, RANTES, EPO, and Ang-2 in any sample, at either baseline or at month 2, therefore they were excluded from further analysis.

Table 2 shows the median cytokine concentrations before and following intravitreal ranibizumab administration. Aqueous levels of nine angiogenic factors and cytokines decreased markedly over the 2-month period. In addition to VEGF (median change –190.71 [IQR –254.86, –154.01], *P* < 0.00001), aqueous levels of many inflammatory cytokines were significantly reduced. These included IL-1 β (–0.55 [IQR–0.77, 0.00], *P* = 0.00006), IL-7 (–5.93 [–9.39, –1.97], *P* = 0.00002), IL-8 (–4.71 [–13.37, –0.49], *P* = 0.00023), IL-10 (–6.30 [–9.98, –3.90], *P* < 0.00001), IL-12 (–15.99 [–22.43, –8.79], *P* < 0.00001), IL-17 (–1.15 [–4.28, 0.00], *P* = 0.00024), MCP-1 (–49.26 [–146.73, –5.70], *P* = 0.00023), TNF- α (–7.97 [–15.32, –3.17], *P* < 0.00001). There was also a statistically significant increase in sVEGFR-2 concentration after intravitreal ranibizumab injection (+93.34 [IQR +43.91, +171.94], *P* = 0.00004). Figure 1 illustrates the changes in aqueous cytokine concentrations post intravitreal ranibizumab. Concentrations of some cytokines did not show a statistically significant change following intravitreal ranibizumab injections. These included IL-1ra, IL-4, IL-13, IL-15, FGFb, IFN- γ , IP-10, MIP-1a, MIP-1b, sVEGFR-1, and EGF (Table 2).

Influence of Outliers

To address any potential effect of outliers influencing changes in the cytokine concentrations, we assessed the degree of leverage and influence for each cytokine. Figure 2 illustrates the median (IQR) change in the cytokines showing significantly increased or decreased concentrations from baseline to month 2 in the whole study population and after the exclusion of outliers. In all cases, the median change in cytokine concentrations was similar when the outliers were excluded compared with the changes observed with the use of all available data.

Influence of Baseline Characteristics

The influence of heterogeneity of baseline characteristics on the change in aqueous cytokine concentrations was also assessed. Thresholds of baseline clinical characteristics, such as BCVA (60–70 or >70 letters), CMT (300–450 or >450 μ m), and HbA1c (HbA1c <7.5% or > 7.5%), were arbitrarily chosen a priori to create relatively even groups to maintain power for comparison and give relatively clinically homogeneous subjects. These results are illustrated graphically as spaghetti plots of individual eyes' values by clinical subgroups of BCVA, CMT, and HbA1c for each of the cytokines that showed significant overall changes from baseline to month 2. In all, 10 cytokines that showed a significant change in cytokine concentration from baseline to month 2, the changes were similar in eyes irrespective of their baseline BCVA (Supplementary Fig. S1) or CMT (Supplementary Fig. S2). Similarly, the baseline HbA1c

TABLE 2. Changes in Aqueous Concentrations (pg/mL) of Inflammatory and Angiogenic Cytokines After Intravitreal Ranibizumab in DME

Variable	Preinjection Median (IQR)	Postinjection Median (IQR)	<i>P</i> [*]
IL-1 β	0.55 (0.00, 0.77)	0.00 (0.00, 0.00)	0.00006 †
IL-1ra	25.42 (0.00, 70.00)	16.83 (0.00, 59.06)	0.52309
IL-4	1.01 (0.82, 1.31)	0.91 (0.72, 1.10)	0.08818
IL-5	0.00 (0.00, 0.00)	0.00 (0.00, 0.38)	0.0215
IL-6	73.24 (9.73, 206.59)	17.78 (4.68, 77.04)	0.02422
IL-7	6.82 (3.87, 9.39)	0.00 (0.00, 0.00)	0.00002 †
IL-8	13.25 (7.79, 25.70)	7.72 (5.51, 10.23)	0.00023 †
IL-9	7.12 (3.12, 12.10)	3.44 (0.60, 6.82)	0.01025
IL-10	11.56 (9.24, 14.81)	4.97 (4.39, 5.57)	< 0.00001 †
IL-12	17.41 (12.67, 24.21)	1.78 (0.81, 3.04)	< 0.00001 †
IL-13	0.00 (0.00, 0.38)	0.00 (0.00, 0.00)	0.51738
IL-15	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.13384
IL-17	1.15 (0.00, 4.28)	0.00 (0.00, 0.00)	0.00024 †
Eotaxin	24.96 (20.25, 27.75)	19.80 (16.99, 22.45)	0.00279
FGFb	124.02 (108.00, 136.92)	114.47 (107.61, 135.37)	0.82169
G-CSF	13.45 (5.78, 27.77)	0.70 (0.00, 13.45)	0.00276
GM-CSF	1007.91 (733.85, 1162.91)	826.89 (671.47, 993.06)	0.02422
IFN- γ	10.87 (0.00, 19.90)	19.90 (14.05, 25.31)	0.14969
IP-10	299.38 (190.61, 448.73)	363.40 (231.39, 584.25)	0.09577
MCP-1	176.01 (126.36, 274.78)	135.48 (107.06, 153.13)	0.00023 †
MIP-1a	1.00 (0.66, 1.36)	0.90 (0.66, 1.28)	0.84462
MIP-1b	20.62 (8.70, 28.13)	16.76 (7.97, 29.12)	0.44471
PDGF	3.84 (0.90, 6.18)	6.18 (3.36, 8.94)	0.0213
TNF- α	9.46 (4.92, 15.32)	0.00 (0.00, 0.44)	< 0.00001 †
VEGF-A	232.76 (178.93, 294.61)	22.42 (18.54, 31.54)	< 0.00001 †
sVEGFR-1	115.78 (84.48, 140.63)	81.33 (59.15, 115.79)	0.06657
sVEGFR-2	342.43 (274.39, 377.65)	431.87 (377.65, 487.65)	0.00004 †
EGF	16.83 (0.00, 25.62)	10.48 (0.00, 25.62)	0.64909

Bold *P* values are significant at the 0.05 level.

* *P* value relates to Wilcoxon matched-pairs signed-rank test.

† *P* value significant after accounting for multiple comparison testing.

level (Supplementary Fig. S3) did not appear to influence the changes noted in the cytokine concentration.

Correlations of Inflammation-associated Cytokines With VEGF

At baseline, the aqueous concentrations of IL-7 ($\rho = 0.556$, $P = 0.0014$), IL-10 ($\rho = 0.851$, $P < 0.00001$), and IL-12 ($\rho = 0.927$, $P < 0.00001$) were correlated with VEGF concentration. Among all eyes recruited in the study, there appeared to be a single outlier with very high baseline VEGF concentration

TABLE 3. Correlation Between Changes in Aqueous Cytokines With Change in VEGF Following Two Ranibizumab Treatments

Aqueous Cytokine	Change in VEGF Concentration	
	Correlation, Spearman ρ	<i>P</i> [*]
IL-1 β	0.200	0.2895
IL-7	0.508	0.0041
IL-8	0.338	0.0674
IL-10	0.825	< 0.0001
IL-12	0.895	< 0.0001
IL-17	0.192	0.3098
MCP-1	0.196	0.2993
TNF- α	0.303	0.1039
sVEGFR-2	0.385	0.0772

Bold *P* values are significant at the 0.05 level.

* *P* value relates to Spearman's rho correlation coefficient.

(1059.15 pg/mL) and very significant reduction in VEGF after ranibizumab injections, to the lowest 25th percentile of study eyes (18.54 pg/mL). To assess the influence of this outlier, we also calculated the correlation of the cytokines with VEGF at baseline after excluding this eye from the analysis; only IL-10 ($\rho = 0.835$, $P < 0.0001$) and IL-12 ($\rho = 0.919$, $P < 0.0001$) remained significantly correlated with VEGF. Table 3 shows the correlation between changes in VEGF concentration from baseline to month 2 and changes in aqueous cytokine concentrations. Although there were reductions in IL-1 β , IL-7, IL-8, IL-10, IL-12, IL-17, MCP-1, and TNF- α levels at month 2, only changes in IL-10 ($P < 0.0001$) and IL-12 ($P < 0.0001$) were significantly correlated with the changes in VEGF. These correlations remained significant even after removing the outlier (IL-10 $\rho = 0.807$, $P < 0.0001$ and IL-12 $\rho = 0.883$, $P < 0.0001$). These associations are depicted in Figure 3.

DISCUSSION

This study examined the effects of intravitreal ranibizumab on inflammatory and angiogenic factors in the aqueous of patients with DME. After two monthly intravitreal ranibizumab injections, there was a significant reduction in concentrations of several inflammatory cytokines in addition to VEGF. These included IL-1 β , IL-7, IL-8, IL-10, IL-12, IL-17, MCP-1, and TNF- α .

Previous studies have shown that VEGF is important in increasing vascular permeability in DME.^{11,15,40} Although the mechanisms of DME development are still yet to be fully understood, there is evidence that chronic inflammation is a key mediator in its pathogenesis. Upregulation of inflammatory factors, including VEGF, IL-1 β , IL-6, IL-8, intercellular adhesion

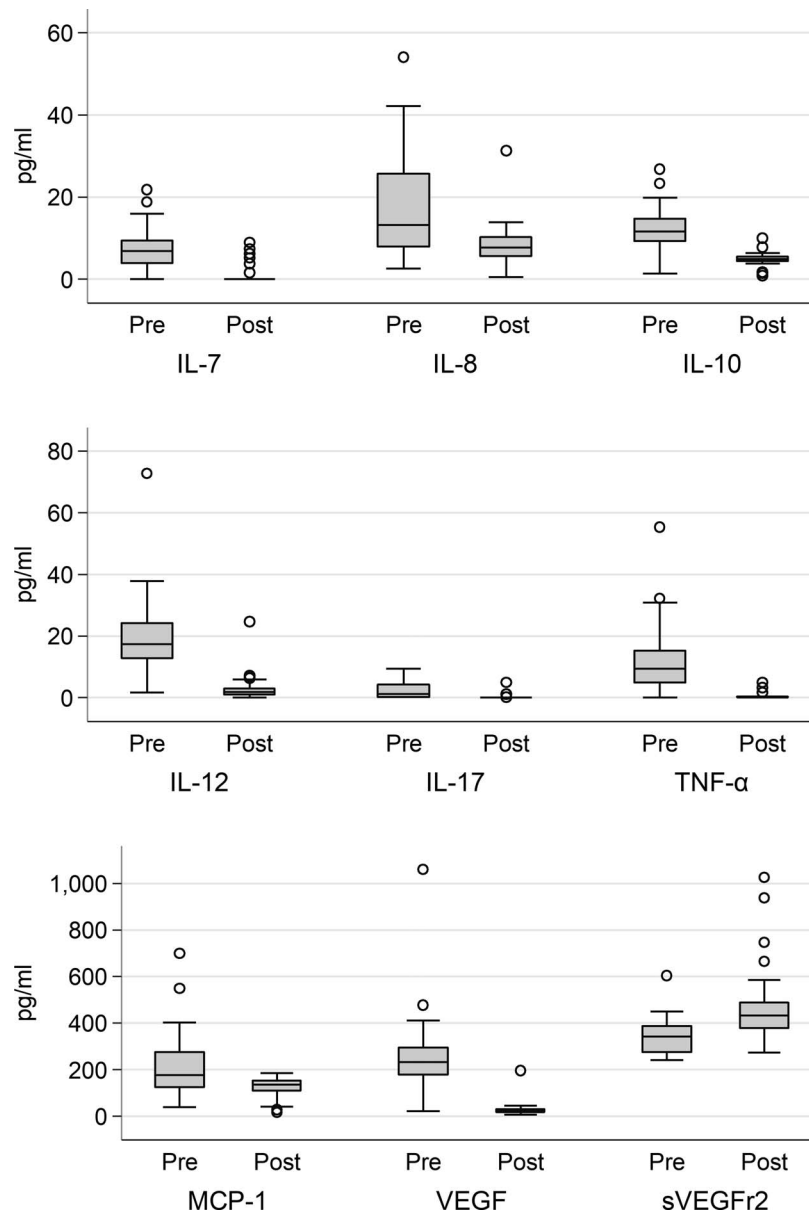


FIGURE 1. Median aqueous concentrations of inflammatory and angiogenic cytokines before and following two intravitreal ranibizumab injections in eyes with DME.

molecule-1, and MCP-1, have been shown in vitreous or aqueous humor of patients with DR.^{2,5,9,11,13,14} There is a significant amount of overlap in pathways of VEGF and cytokines, with previous studies demonstrating activation of VEGF by inflammatory mediators such as Toll-like receptor 3, IL-6, and MCP-1,⁴¹ upregulation of MCP-1 expression by VEGF-A,⁴² induction of VEGF expression by IL-6,^{43,44} and upregulation of sVEGFR-1 expression by IL-4 and GM-CSF.⁴⁵ Interactions between these key pathways led to endothelial cell damage, increased vascular permeability, and leakage.^{2,9}

Intraocular cytokine levels have been found to be elevated in patients with DME,^{10-12,15} some of which also demonstrated a positive correlation between some cytokines and macular volume and thickness as a surrogate of the severity of DME.^{12,46,47} To our knowledge, this is the first study to demonstrate a significant impact of intravitreal ranibizumab on various inflammatory cytokines. Consistent with previous studies,^{15,48,49} we found that ranibizumab lowered VEGF levels

in the aqueous of DME patients. However, we also found that ranibizumab reduced levels of several inflammatory cytokines. This is in marked contrast to other studies investigating the effect of bevacizumab on aqueous cytokine profiles in eyes with DME. These groups found that bevacizumab therapy only reduces VEGF concentrations, without significantly changing levels of inflammatory cytokine concentrations.^{13,15,34,49}

We also found that there was an increase in sVEGFR-2 levels after ranibizumab treatment. VEGF-A acts by binding to transmembrane VEGFR-1 and 2, but the latter seems to be the main receptor that mediates the bulk of the cellular responses to VEGF-A.⁵⁰ Soluble receptors generally bind to and inactivate their ligands, as they lack the intracellular domain needed for signal induction.⁵¹ The sVEGFR-2 has been shown to have anti-angiogenic activity.⁵² The upregulation of sVEGFR-2 may therefore contribute to the beneficial effect of anti-VEGF inhibition on DME reduction.

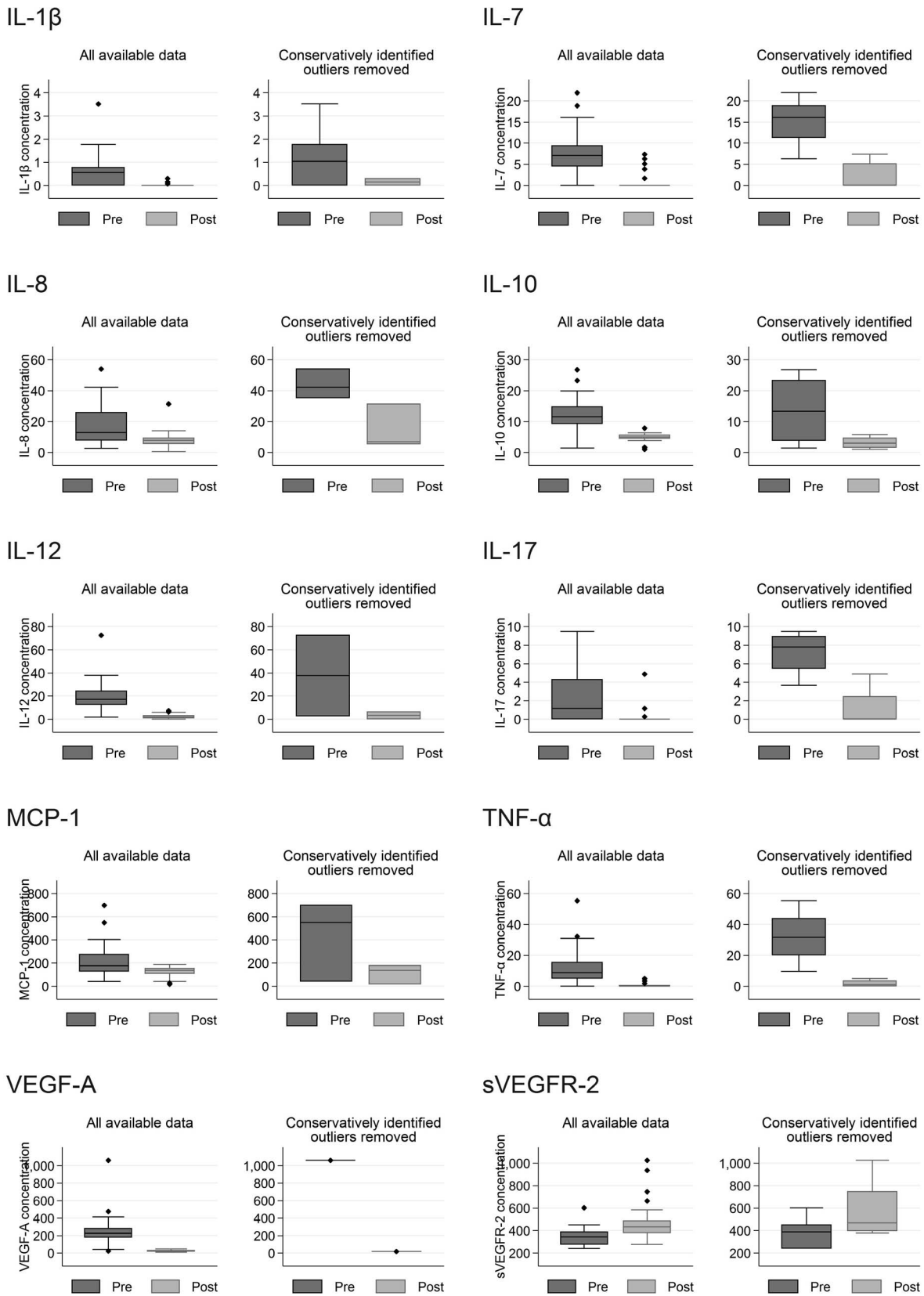


FIGURE 2. Median pre- and postinjection cytokine concentrations following the removal of all conservatively identified possible outliers.

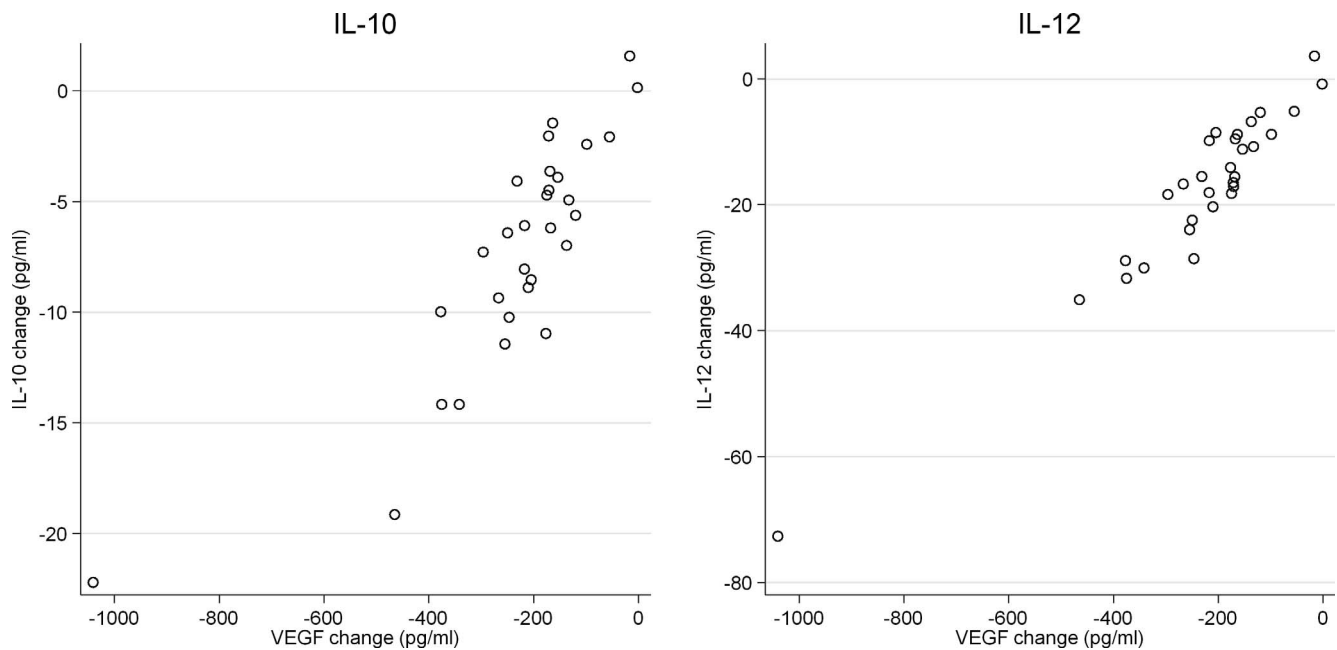


FIGURE 3. Correlation of selected aqueous cytokine concentrations with VEGF reduction following ranibizumab treatment. (*Left*) Complete data set. (*Right*) After exclusion of the outlier.

An additional finding from this study is that there was a strong correlation between baseline levels of aqueous VEGF and IL-10 and IL-12, and also between the change in aqueous VEGF concentration and that of IL-10 and IL-12. This suggests that the benefits that ranibizumab confers on vision and macular thickening in DME may be a result of this complex interplay between VEGF and inflammatory cytokines. IL-10 is not only a potent inhibitor of cytokine and chemokine production, but also proangiogenic. Some studies have shown that IL-10 has the ability to polarize macrophages to an M2 phenotype, which when stimulated, produces high levels of IL-10 and VEGF and low levels of proinflammatory cytokines, such as IL-6 and TNF- α .⁵³⁻⁵⁵

Our results differ from previous studies for several potential reasons. First, a different anti-VEGF therapy is used. Many studies have demonstrated no significant changes to intraocular cytokine levels following intravitreal bevacizumab.^{13,15,34,49} By contrast, patients in our study received intravitreal ranibizumab, and ranibizumab could have a more potent effect on the intraocular cytokine profile than bevacizumab. Consistent with this hypothesis, the DRCRnet trial^{28,29} has demonstrated a lesser effect of bevacizumab on reducing DME compared with ranibizumab and aflibercept reported in the DRCRnet trial.^{28,29}

Second, the patient cohort may have influenced the results. Studies that have shown an increase, and not decrease, in concentration of cytokines after bevacizumab or ranibizumab therapy only included patients with PDR, with nonclearing vitreous hemorrhage and/or tractional retinal detachment requiring pars plana vitrectomy.^{31,32} Studies demonstrating a significant upregulation of numerous cytokines and chemokines in PDR patients confirm that the vitreous in PDR patients is complex.⁵⁶ It is unclear to what extent vitreous hemorrhage, on top of active PDR, alters the intraocular angiogenic and inflammatory cytokine profile. By contrast, our study cohort comprised patients with NPDR and quiescent PDR following panretinal photocoagulation.

Third, the timing of cytokine analysis may explain the difference in results. In some reports, anti-VEGF therapy was

performed as a pretreatment approximately 1 week before vitrectomy and cytokine levels in aqueous humor were measured at the time of anti-VEGF treatment as well as on the day of surgery.^{31,32} Others measured cytokine levels at the 1-month time point following only a single bevacizumab injection.³⁴ This time frame may be too early to detect changes in inflammatory cytokine concentrations. By contrast, our study measured cytokine concentrations at 2 months following two intravitreal anti-VEGF injections. In many cases, multiple injections are required in eyes with DME to lead to a meaningful improvement in BCVA and CMT.^{17,18,23,29} Thus, a single injection may not be adequate to demonstrate such changes in the cytokine profile. Finally, in contrast to many studies that analyzed only a limited number of cytokines, our study examined a wider range of cytokines, which expands our ability to detect the effects of anti-VEGF therapy.

Our study has a number of limitations. First, aqueous sampling was used, rather than vitreous fluid, as an index of primarily posterior segment pathology. The cytokine levels in the vitreous are usually higher, but aqueous sampling is less invasive than vitreous fluid sampling, hence it was favored in our study group. Second, the number of patients in our study is relatively small. Despite this, the results were statistically significant, and this may only serve to strengthen the results of the study. Third, the multiplex array assay has its limitations if the cytokine levels are very low, hence we had to exclude four cytokines from statistical analysis due to undetectable levels.

In conclusion, although previous studies have shown that intravitreal anti-VEGF therapy only reduces free VEGF levels in the eye and has no influence on other cytokines,^{13,34,49} our study demonstrates an effect of intravitreal ranibizumab on numerous cytokines associated with inflammation. Further studies are required to better understand the complex network of signaling pathways in DME, determine the relative importance of specific cytokines in the pathogenesis of DME, and evaluate the utility of biomarkers in predicting response to treatment.

Acknowledgments

Supported by a grant from Novartis Pharma AG, and National Health and Medical Research Council Early Career Fellowships GNT 1128343 (SSW) and GNT 1109330 (LLL). The Centre for Eye Research Australia receives Operational Infrastructure Support from the Victorian Government.

Disclosure: **S.W. Lim**, None; **E. Bandala-Sanchez**, None; **M. Kolic**, None; **S.L. Rogers**, None; **A.K. McAuley**, None; **L.L. Lim**, None; **S.S. Wickremasinghe**, None

References

- Klaassen I, Van Noorden CJ, Schlingemann RO. Molecular basis of the inner blood-retinal barrier and its breakdown in diabetic macular edema and other pathological conditions. *Prog Retin Eye Res.* 2013;34:19–48.
- Adamis AP. Is diabetic retinopathy an inflammatory disease? *Br J Ophthalmol.* 2002;86:363–365.
- Clermont AC, Cahill M, Salti H, et al. Hepatocyte growth factor induces retinal vascular permeability via MAP-kinase and PI-3 kinase without altering retinal hemodynamics. *Invest Ophthalmol Vis Sci.* 2006;47:2701–2708.
- Johnson MW. Etiology and treatment of macular edema. *Am J Ophthalmol.* 2009;147:11–21.e1.
- Joussen AM, Poulaki V, Le ML, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J.* 2004;18:1450–1452.
- Kaji Y, Usui T, Ishida S, et al. Inhibition of diabetic leukostasis and blood-retinal barrier breakdown with a soluble form of a receptor for advanced glycation end products. *Invest Ophthalmol Vis Sci.* 2007;48:858–865.
- Kim YM, Kim YM, Lee YM, et al. TNF-related activation-induced cytokine (TRANCE) induces angiogenesis through the activation of Src and phospholipase C (PLC) in human endothelial cells. *J Biol Chem.* 2002;277:6799–6805.
- Schram MT, Chaturvedi N, Schalkwijk CG, Fuller JH, Stehouwer CD. Markers of inflammation are cross-sectionally associated with microvascular complications and cardiovascular disease in type 1 diabetes—the EURODIAB Prospective Complications Study. *Diabetologia.* 2005;48:370–378.
- Meleth AD, Agron E, Chan CC, et al. Serum inflammatory markers in diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2005;46:4295–4301.
- Kocabora MS, Telli ME, Fazil K, et al. Serum and aqueous concentrations of inflammatory markers in diabetic macular edema. *Ocul Immunol Inflamm.* 2016;24:549–554.
- Funatsu H, Noma H, Mimura T, Eguchi S, Hori S. Association of vitreous inflammatory factors with diabetic macular edema. *Ophthalmology.* 2009;116:73–79.
- Jonas JB, Jonas RA, Neumaier M, Findeisen P. Cytokine concentration in aqueous humor of eyes with diabetic macular edema. *Retina.* 2012;32:2150–2157.
- Roh MI, Kim HS, Song JH, Lim JB, Kwon OW. Effect of intravitreal bevacizumab injection on aqueous humor cytokine levels in clinically significant macular edema. *Ophthalmology.* 2009;116:80–86.
- Dong N, Xu B, Chu L, Tang X. Study of 27 aqueous humor cytokines in type 2 diabetic patients with or without macular edema. *PLoS One.* 2015;10:e0125329.
- Funk M, Schmidinger G, Maar N, et al. Angiogenic and inflammatory markers in the intraocular fluid of eyes with diabetic macular edema and influence of therapy with bevacizumab. *Retina.* 2010;30:1412–1419.
- Lee WJ, Kang MH, Seong M, Cho HY. Comparison of aqueous concentrations of angiogenic and inflammatory cytokines in diabetic macular oedema and macular oedema due to branch retinal vein occlusion. *Br J Ophthalmol.* 2012;96:1426–1430.
- Massin P, Bandello F, Garweg JG, et al. Safety and efficacy of ranibizumab in diabetic macular edema (RESOLVE Study): a 12-month, randomized, controlled, double-masked, multicenter phase II study. *Diabetes Care.* 2010;33:2399–2405.
- Elman MJ, Aiello LP, Beck RW, et al. Randomized trial evaluating ranibizumab plus prompt or deferred laser or triamcinolone plus prompt laser for diabetic macular edema. *Ophthalmology.* 2010;117:1064–1077.e35.
- Elman MJ, Bressler NM, Qin H, et al. Expanded 2-year follow-up of ranibizumab plus prompt or deferred laser or triamcinolone plus prompt laser for diabetic macular edema. *Ophthalmology.* 2011;118:609–614.
- Elman MJ, Qin H, Aiello LP, et al. Intravitreal ranibizumab for diabetic macular edema with prompt versus deferred laser treatment: three-year randomized trial results. *Ophthalmology.* 2012;119:2312–2318.
- Mitchell P, Bandello F, Schmidt-Erfurth U, et al. The RESTORE study: ranibizumab monotherapy or combined with laser versus laser monotherapy for diabetic macular edema. *Ophthalmology.* 2011;118:615–625.
- Schmidt-Erfurth U, Lang GE, Holz FG, et al. Three-year outcomes of individualized ranibizumab treatment in patients with diabetic macular edema: the RESTORE extension study. *Ophthalmology.* 2014;121:1045–1053.
- Nguyen QD, Brown DM, Marcus DM, et al. Ranibizumab for diabetic macular edema: results from 2 phase III randomized trials: RISE and RIDE. *Ophthalmology.* 2012;119:789–801.
- Do DV, Nguyen QD, Boyer D, et al. One-year outcomes of the da Vinci Study of VEGF Trap-Eye in eyes with diabetic macular edema. *Ophthalmology.* 2012;119:1658–1665.
- Do DV, Schmidt-Erfurth U, Gonzalez VH, et al. The DA VINCI Study: phase 2 primary results of VEGF Trap-Eye in patients with diabetic macular edema. *Ophthalmology.* 2011;118:1819–1826.
- Heier JS, Korobelnik JF, Brown DM, et al. Intravitreal aflibercept for diabetic macular edema: 148-week results from the VISTA and VIVID studies. *Ophthalmology.* 2016;123:2376–2385.
- Korobelnik JF, Do DV, Schmidt-Erfurth U, et al. Intravitreal aflibercept for diabetic macular edema. *Ophthalmology.* 2014;121:2247–2254.
- Wells JA, Glassman AR, Ayala AR, et al. Aflibercept, bevacizumab, or ranibizumab for diabetic macular edema. *N Engl J Med.* 2015;372:1193–1203.
- Wells JA, Glassman AR, Ayala AR, et al. Aflibercept, bevacizumab, or ranibizumab for diabetic macular edema: two-year results from a comparative effectiveness randomized clinical trial. *Ophthalmology.* 2016;123:1351–1359.
- Bressler SB, Qin H, Beck RW, et al. Factors associated with changes in visual acuity and central subfield thickness at 1 year after treatment for diabetic macular edema with ranibizumab. *Arch Ophthalmol.* 2012;130:1153–1161.
- 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.
- Forooghian F, Kertes PJ, Eng KT, et al. Alterations in intraocular cytokine levels following intravitreal ranibizumab. *Can J Ophthalmol.* 2016;51:87–90.
- Shiraya T, Kato S, Araki F, Ueta T, Miyaji T, Yamaguchi T. Aqueous cytokine levels are associated with reduced macular thickness after intravitreal ranibizumab for diabetic macular edema. *PLoS One.* 2017;12:e0174340.
- Sohn HJ, Han DH, Kim IT, et al. Changes in aqueous concentrations of various cytokines after intravitreal triamcinolone versus bevacizumab for diabetic macular edema. *Am J Ophthalmol.* 2011;152:686–694.
- Krohne TU, Liu Z, Holz FG, Meyer CH. Intraocular pharmacokinetics of ranibizumab following a single intravitreal

- injection in humans. *Am J Ophthalmol.* 2012;154:682-686.e2.
36. Xu L, Lu T, Tuomi L, et al. Pharmacokinetics of ranibizumab in patients with neovascular age-related macular degeneration: a population approach. *Invest Ophthalmol Vis Sci.* 2013;54:1616-1624.
 37. Photocoagulation for diabetic macular edema. Early Treatment Diabetic Retinopathy Study report number 1. Early Treatment Diabetic Retinopathy Study research group. *Arch Ophthalmol.* 1985;103:1796-1806.
 38. Huisingh C, McGwin G Jr. An analysis of the use of multiple comparison corrections in ophthalmology research. *Invest Ophthalmol Vis Sci.* 2012;53:4777.
 39. Perneger TV. What's wrong with Bonferroni adjustments. *BMJ.* 1998;316:1236-1238.
 40. Selim KM, Sahan D, Muhittin T, Osman C, Mustafa O. Increased levels of vascular endothelial growth factor in the aqueous humor of patients with diabetic retinopathy. *Indian J Ophthalmol.* 2010;58:375-379.
 41. Galli R, Paone A, Fabbri M, et al. Toll-like receptor 3 (TLR3) activation induces microRNA-dependent reexpression of functional RARbeta and tumor regression. *Proc Natl Acad Sci U S A.* 2013;110:9812-9817.
 42. Hong KH, Ryu J, Han KH. Monocyte chemoattractant protein-1-induced angiogenesis is mediated by vascular endothelial growth factor-A. *Blood.* 2005;105:1405-1407.
 43. Funatsu H, Yamashita H, Ikeda T, Mimura T, Eguchi S, Hori S. Vitreous levels of interleukin-6 and vascular endothelial growth factor are related to diabetic macular edema. *Ophthalmology.* 2003;110:1690-1696.
 44. Aiello LP, Wong JS. Role of vascular endothelial growth factor in diabetic vascular complications. *Kidney Int Suppl.* 2000;77:S113-S119.
 45. Xia L, Dong Z, Zhang Y, et al. Interleukin-4 and granulocyte-macrophage colony-stimulating factor mediates the upregulation of soluble vascular endothelial growth factor receptor-1 in RAW264.7 cells—a process in which p38 mitogen-activated protein kinase signaling has an important role. *J Microbiol Immunol Infect.* 2016;49:344-351.
 46. Oh IK, Kim SW, Oh J, Lee TS, Huh K. Inflammatory and angiogenic factors in the aqueous humor and the relationship to diabetic retinopathy. *Curr Eye Res.* 2010;35:1116-1127.
 47. Hillier RJ, Ojaimi E, Wong DT, et al. Aqueous humor cytokine levels as biomarkers of disease severity in diabetic macular edema. *Retina.* 2017;37:761-769.
 48. Sawada O, Kawamura H, Kakinoki M, Sawada T, Ohji M. Vascular endothelial growth factor in aqueous humor before and after intravitreal injection of bevacizumab in eyes with diabetic retinopathy. *Arch Ophthalmol.* 2007;125:1363-1366.
 49. Wen J, Jiang Y, Zheng X, Zhou Y. Six-month changes in cytokine levels after intravitreal bevacizumab injection for diabetic macular oedema and macular oedema due to central retinal vein occlusion. *Br J Ophthalmol.* 2015;99:1334-1340.
 50. Gupta N, Mansoor S, Sharma A, et al. Diabetic retinopathy and VEGF. *Open Ophthalmol J.* 2013;7:4-10.
 51. Agostini H, Boden K, Unsold A, et al. A single local injection of recombinant VEGF receptor 2 but not of Tie2 inhibits retinal neovascularization in the mouse. *Curr Eye Res.* 2005;30:249-257.
 52. Lorquet S, Berndt S, Blacher S, et al. Soluble forms of VEGF receptor-1 and -2 promote vascular maturation via mural cell recruitment. *FASEB J.* 2010;24:3782-3795.
 53. Dace DS, Khan AA, Kelly J, Apte RS. Interleukin-10 promotes pathological angiogenesis by regulating macrophage response to hypoxia during development. *PLoS One.* 2008;3:e3381.
 54. Apte RS, Richter J, Herndon J, Ferguson TA. Macrophages inhibit neovascularization in a murine model of age-related macular degeneration. *PLoS Med.* 2006;3:e310.
 55. Kelly J, Ali Khan A, Yin J, Ferguson TA, Apte RS. Senescence regulates macrophage activation and angiogenic fate at sites of tissue injury in mice. *J Clin Invest.* 2007;117:3421-3426.
 56. Bromberg-White JL, Glazer L, Downer R, Furge K, Boguslawski E, Duesbery NS. Identification of VEGF-independent cytokines in proliferative diabetic retinopathy vitreous. *Invest Ophthalmol Vis Sci.* 2013;54:6472-6480.

APPENDIX

TABLE A1. Full Study Inclusion and Exclusion Criteria

Inclusion criteria	<ol style="list-style-type: none"> 1. Age \geq18 years 2. Center-involved DME that in the opinion of the investigator, would not benefit from macular laser treatment (e.g., diffuse leak from the capillary bed, disruption of the foveal avascular zone, or perifoveal capillary dropout) as determined by fluorescein angiography 3. BCVA of 17-70 logMAR letters (6/120-6/12) 4. CMT of $>$300 μm as measured by Heidelberg OCT
Exclusion criteria	<p>Systemic</p> <ol style="list-style-type: none"> 1. Uncontrolled blood pressure (systolic $>$180 mm Hg and diastolic $>$110 mm Hg) 2. Chronic renal failure 3. Major surgery within 1 month of study 4. Previous systemic anti-VEGF treatment 5. Women of childbearing potential not using adequate contraception and women who are breast-feeding 6. Intercurrent severe disease such as septicemia <p>Ocular</p> <ol style="list-style-type: none"> 1. Glaucoma that is uncontrolled or is controlled but with glaucomatous visual field defects 2. History of severe steroid response with IOP $>$ 35 mm Hg following steroid treatment 3. Loss of vision due to other causes (e.g., AMD, myopic macular degeneration) 4. Visual acuity of $<$6/60 in the fellow eye 5. Argon laser photocoagulation within 3 months of study entry 6. Previous intraocular surgery within 6 months of study entry 7. Prior use of intravitreal anti-VEGF agents (within 3 months of study entry) or corticosteroids (within 6 months of study entry) 8. Stroke or myocardial infarction less than 3 months before screening 9. Any active periocular or ocular infection or inflammation at screening or baseline