Aqueous Humor Cytokines and Long-Term Response to Anti–Vascular Endothelial Growth Factor Therapy in Diabetic Macular Edema

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• PURPOSE: To determine the association of aqueous humor cytokine concentrations with long-term treatment response to anti–vascular endothelial growth factor (VEGF) agents in diabetic macular edema (DME).

• DESIGN: Retrospective case series.

• METHODS: Pooled data of aqueous humor cytokine concentrations collected at baseline and 2-month follow-up (2 injections) for treatment-naïve eyes with center-involving DME previously enrolled in a prospective study were reviewed. Subjects receiving intravitreal anti-VEGF injections outside of study protocol as per standard of care were classified into Responders versus Nonresponders based on qualitative assessment of optical coherence tomography for persistence of DME at longitudinal follow-up visits.

• RESULTS: Of the 41 eyes, 85% were classified as Responders with a significant decline in baseline central subfield thickness and macular volume (P values $<$ .001), and 15% were identified as Nonresponders to anti-VEGF therapy over 51.4 ± 18.7 months of follow-up. No significant difference in baseline aqueous humor VEGF concentration was noted, while at the 2-month follow-up the Nonresponder group had a significantly higher VEGF concentration compared with the Responder group (451.5 ± 690.9 pg/mL vs 113.7 ± 211.4 pg/mL; P = .02). The Responder group also demonstrated a significant decline from baseline to 2-month follow-up in concentration of intercellular adhesion molecule-1 (P $<$ .001), interleukin-10 (P = .041), monocyte chemotactic protein-1 (P = .046), placental growth factor (P = .027), and transforming growth factor-β2 (P = .017).

• CONCLUSIONS: Aqueous humor cytokine concentrations serve as an early biomarker for long-term response to anti-VEGF therapy and may enable more effective treatment regimens that improve anatomical outcomes in eyes with DME. (Am J Ophthalmol 2019;206:176–183. © 2019 Elsevier Inc. All rights reserved.)

DIABETIC MACULAR EDEMA (DME) IS A LEADING cause of visual loss that is associated with accumulation of excess fluid in the extracellular space of the neurosensory retina.[1] Although the exact pathogenesis remains unknown, increased oxidative stress, inflammation, and ultimately vascular dysfunction have been proposed as contributors to the development of DME.[2,3] Vascular endothelial growth factor (VEGF) is a highly potent vasopermeable agent and an important mediator of DME.[4,5] With the advent of anti-VEGF agents and their validation through the RISE and RIDE phase III clinical trials, the treatment of DME has been revolutionized.[6] Ranibizumab, a monoclonal antibody with high affinity to bind VEGF-A isoforms,[7] was the first anti-VEGF agent approved by the U.S. Food and Drug Administration for the indication of intravitreal therapy in DME. It has been suggested that intravitreal anti-VEGF significantly downregulates VEGF expression shortly after initiation of treatment.[8,9] The regimens for administration of anti-VEGF agents are most commonly based on monthly, pro re nata (PRN), or treat-and-extend approaches established in major clinical trials.[10–15] According to a large Medicare claims analysis, patients receive a mean number of 4.2 intravitreal anti-VEGF injections over the first year of treatment,[16] although more frequent injections may be required in eyes with more advanced disease at baseline.[17]

Despite the efficacy of anti-VEGF agents in improving visual acuity and decreasing retinal thickening,[11,18–20] approximately 32–66% of eyes treated with intensive injection regimens over 6 months or beyond have persistent DME,[6,11,18,21,22] and 30–70% of eyes show minimal improvement in best-corrected visual acuity.[6,22–27] In a recent post hoc analysis of data from the Diabetic Retinopathy Clinical Research (DRCR) Network Protocol

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I study, one-third of eyes showed little to no anatomical improvement after the first 3 ranibizumab injections, while 48% of eyes showed minimal (<20%) reduction in central retinal thickness at 3 years.[28] Alternative treatments with intravitreal corticosteroid agents[22,29,30] have demonstrated effectiveness in reducing inflammation[31] and overcoming anti-VEGF–resistant disease[32] by targeting other inflammatory mediators, such as interleukins (ILs), monocyte chemotactic protein (MCP)-1, and intercellular adhesion molecule (ICAM)-1, which have been implicated in disease progression.[2,33–36] Given the variability in response to anti-VEGF therapy,[28,37] predictors of treatment response have the potential to increase the efficacy of therapeutics, particularly in cases of refractory DME.

In an investigation of aqueous humor cytokine concentrations, our research group previously found that ICAM-1 is associated with severity of DME on spectral-domain optical coherence tomography (OCT) in treatment-naïve eyes.[38] More recently, we suggested that elevated ICAM-1 and lower VEGF aqueous humor concentrations before initiation of treatment serve as predictors of favorable response to intravitreal ranibizumab therapy over a 2-month follow-up period.[39] Previous studies have suggested that aqueous humor VEGF may serve as a predictor of macular edema,[40] supporting the notion that anti-VEGF therapy would not only reduce macular thickness but also result in an expected decline in aqueous humor VEGF concentration.[9] Despite variability in baseline aqueous humor cytokine concentrations, it is speculated that eyes with persistently higher cytokine concentrations will demonstrate suboptimal response to long-term anti-VEGF therapy. Early predictors of anatomical response to anti-VEGF therapy may enable more targeted management strategies and reduce the burden of treatment. The current study aims to determine the association of aqueous humor cytokine concentrations at baseline and at the 2-month follow-up of patients enrolled in our original prospective studies[38,39] with long-term anatomical response to anti-VEGF treatment in eyes with DME.

METHODS

• **STUDY DESIGN:** This is a retrospective case series based on the review of extension data from electronic medical records at St. Michael's Hospital and Sunnybrook Health Sciences Centre, Ontario, Canada between December 22, 2011 and December 30, 2017, from the cohort of patients enrolled in the original prospective study performed by Hillier and colleagues.[38] All study eyes with ≥9 months of follow-up from the original enrollment date (December 22, 2011 to June 13, 2013) were included in the current study. The following clinical data were collected from each patient's medical record: age, sex, baseline best-corrected visual acuity (logarithm of the minimum angle of resolution [logMAR]), baseline hemoglobin A1c, baseline severity of retinopathy, previously collected multiplex immunoassay results, intravitreal treatment type and frequency, total length of follow-up, spectral-domain OCT central subfield thickness (CST) and macular volume (MV). The primary preplanned analysis of the study aimed to explore the association of aqueous humor cytokine concentrations at 2 months of follow-up with long-term anatomical response. The study protocol was approved by institutional reviews through the St. Michael's Healthcare Research Ethics Board and Sunnybrook Health Sciences Centre Research Ethics Board and adhered to the tenets of the Declaration of Helsinki.

The original prospective study's principal eligibility criteria included eyes with treatment-naïve nonproliferative diabetic retinopathy and DME with CST of ≥310 μm on spectral-domain OCT (Cirrus HD-OCT; Carl Zeiss Meditec AG, Jena, Germany).[38] Baseline severity of diabetic retinopathy for eyes enrolled in the initial prospective study was evaluated according to clinical examination using the Early Treatment Diabetic Retinopathy Study severity scale. Monthly intravitreal ranibizumab injections (0.5 mg) were administered at the baseline, 1-month (visit 2), and 2-month (visit 3) follow-up visits. Aqueous humor samples (0.2 mL) were obtained at the baseline and 2-month follow-up visits and underwent multiplex immunoassay (Ciraplex; Aushon Biosystems, Billerica, Massachusetts, USA) for epidermal growth factor, ICAM-1, IL-2, IL-3, IL-6, IL-8, IL-10, IL-17, MCP-1, placental growth factor (PIGF), transforming growth factor (TGF)-β2, vascular cell adhesion molecule (VCAM)-1, and VEGF. Response to treatment in the first 2 months of follow-up was defined as a ≥50% (≥310 μm) reduction in excess CST and/or a ≥10% reduction in MV between baseline and the 3-month follow-up visits. A detailed description of study design and imaging review protocol is outlined elsewhere.[38,39] After completion of the prospective study, patients were followed as per standard of care outside of study protocol and assessed at each visit using best-corrected visual acuity and spectral-domain OCT. Patients were treated with ranibizumab, bevacizumab, or aflibercept based on a PRN treatment strategy according to the discretion of the treating physician. Based on standard practice patterns at the institutions, intravitreal triamcinolone injection was administered in eyes with DME that were refractory to anti-VEGF therapy.

• **ASSESSMENT OF LONG-TERM TREATMENT RESPONSE:** All spectral-domain OCT images from each follow-up visit were retrospectively assessed for the persistence of intraretinal and or subretinal fluid using CST and MV measures. All eyes were classified into Responders or Nonresponders according to anatomical changes on spectral-domain OCT over the total study follow-up period. Responders were identified as those demonstrating reduction or resolution of the intraretinal and/or subretinal fluid after an anti-VEGF injection. Eyes were classified as Nonresponders if the CST and MV were higher than the previous values in ≥2 consecutive

TABLE 1. Baseline Patient and Study Eye Characteristics

Variable	Responders (n = 35)	Nonresponders (n = 6)	*P* Value
Age (y), mean (SD)	62.7 (6.8)	60.3 (10.1)	.466
Male sex, n (%)	28 (80.0)	3 (50.0)	.114
Right eye, n (%)	18 (51.4)	3 (50.0)	.948
Hemoglobin A1c (%), mean (SD)	8.2 (1.8)	7.6 (0.6)	.498
LogMAR[a] visual acuity, mean (SD)	0.6 (0.3)	0.7 (0.4)	.432
Severity of retinopathy, n (%)			.642
Mild	6 (17.1)	2 (33.3)	
Moderate	16 (45.7)	2 (33.3)	
Severe	13 (37.1)	2 (33.3)	
Central subfield thickness (μm), mean (SD); range			
Baseline	480.4 (117.4); 321–832	623.0 (177.8); 384–842	.015[b]
3 months	336.9 (54.6); 242–480	479.7 (176.7); 296–779	<.001[b]
12 months	332.5 (91.5); 196–674	307.0 (62.2); 384–842	.704
Macular volume (mm^3), mean (SD); range			
Baseline	12.6 (2.1); 9.6–19.0	14.3 (3.0); 10.4–17.6	.099
3 months	11.1 (1.1); 9.6–13.9	13.1 (2.7); 10.4–16.8	.003[b]
12 months	10.9 (1.2); 9.6–13.8	10.1 (0.2); 10.0–10.3	.407
No. of injections in first 12 months of follow-up, mean (SD); range	8.2 (3.5); 3–14	9.8 (2.2); 7–12	.406
Total follow-up duration (y), mean (SD); range	4.1 (1.6); 1.2–5.7	5.1 (0.8); 3.9–5.8	.240

SD = standard deviation.
[a]Counting fingers = 2; hand motion = 2.3; light perception = 2.7; no light perception = 3.
[b]Statistically significant ($P < .05$).

visits despite ongoing intravitreal anti-VEGF injections. Eyes with persistent DME that required a change in treatment from anti-VEGF injections to corticosteroid therapy were also defined as Nonresponders.

• **STATISTICAL ANALYSIS:** Descriptive statistics were used to summarize data. Cytokines with low sensitivity for the immunoassay used (epidermal growth factor, IL-2, IL-3, and IL-17) were excluded from analysis because of uncertainty regarding the reliability of these measurements. Comparisons between mean values of various aqueous humor cytokine concentrations at baseline and at 2-month follow-up in the 2 groups, Responder and Nonresponder, were performed using an independent samples *t* test. The difference in aqueous humor cytokine concentrations between the baseline and 2-month follow-up visit was defined as the change over the early 2-month follow-up period and analyzed using a paired samples *t* test. All statistical analyses were performed with SPSS software (Version 25; IBM Corp, Armonk, New York, USA). The overall type I error probability at 5% was used for all statistical analyses.

RESULTS

A TOTAL OF 41 OF THE 48 EYES FROM THE ORIGINAL STUDY were included in the current analysis. The 7 eyes excluded did not have ≥9 months of follow-up. The mean follow-up time was 51.4 ± 18.7 months (range 1.2–5.8 years). The sample consisted of 24.4% (10/41) females, with a mean age of 62.4 ± 7.3 years (Table 1). The baseline CST was 501.3 ± 135.2 μm, MV was 12.9 ± 2.3 mm^3, and severity of diabetic retinopathy was defined as mild in 19.5% (8/41), moderate in 43.9% (18), and severe in 36.6% (15). Mean baseline best-corrected visual acuity was 0.6 ± 0.3 logMAR. The hemoglobin A1c at the time of recruitment was 8.1 ± 1.7% for the 29 patients where these data were available. In the first year of follow-up, the mean number of ranibizumab injections was 8.2 ± 3.5. Of the entire sample, 85.4% (35/41) of eyes were defined as long-term Responders while 14.6% (6/41) of eyes were Nonresponders. In the first year of follow-up, eyes in the Responder group had a significant decline in the baseline CST ($P < .001$) and MV ($P < .001$) while no significant changes were noted in CST ($P = .356$) and MV ($P = .476$) in the Nonresponder group. From the initial prospective 2-month follow-up study,[39] 63.4% (26/41) were identified as early Responders and 36.6% (15) as early Nonresponders. Over the longitudinal follow-up period, 58.5% (24/41) of all eyes remained Responders, 9.8% (4) remained as Nonresponders, 26.8% (11) deemed to be early Nonresponders became long-term Responders, and 4.9% (2) deemed to be early Responders became Nonresponders.

Overall, there was a significant decline from baseline to 2-month follow-up in aqueous humor cytokine

TABLE 2. Aqueous Humor Cytokine Concentrations in the Responder and Nonresponder groups at Baseline and 2-Month Follow-Up

Cytokine	Responders (n = 35)	Nonresponders (n = 6)	*P* Value[a]
ICAM-1 (pg/mL), mean (SD)			
Baseline	1649.3 (1312.1)	1927.3 (819.5)	.651
2 months	1222.7 (788.2)	1817.4 (674.7)	.092
P value[b]	.001[c]	.432	
IL-6 (pg/mL), mean (SD)			
Baseline	744.8 (2061.4)	293.4 (545.5)	.601
2 months	102.2 (124.2)	61.8 (56.6)	.442
P value[b]	.084	.341	
IL-8 (pg/mL), mean (SD)			
Baseline	16.0 (11.6)	16.1 (7.3)	.984
2 months	15.2 (9.3)	15.0 (5.9)	.966
P value[b]	.543	.775	
IL-10 (pg/mL), mean (SD)			
Baseline	0.4 (0.5)	0.4 (0.4)	.880
2 months	0.2 (0.2)	0.2 (0.2)	.979
P value[b]	.041[c]	.377	
MCP-1 (pg/mL), mean (SD)			
Baseline	1582.9 (822.9)	1687.2 (643.4)	.770
2 months	1355.2 (512)	1415.1 (422.8)	.789
P value[b]	.046[c]	.073	
PlGF (pg/mL), mean (SD)			
Baseline	11.0 (29.3)	16.5 (23.4)	.669
2 months	4.9 (14.4)	7.7 (14.2)	.666
P value[b]	.027[c]	.105	
TGF-β2 (pg/mL), mean (SD)			
Baseline	10811.8 (2917.5)	11115.5 (4808.8)	.832
2 months	9530.9 (3304.9)	11801.4 (5558.3)	.172
P value[b]	.017[c]	.460	
VCAM-1 (pg/mL), mean (SD)			
Baseline	51328.9 (26057.5)	55517.5 (13185.2)	.729
2 months	50487.7 (32933.2)	53356.6 (20582.1)	.839
P value[b]	.940	.065	
VEGF (pg/mL), mean (SD)			
Baseline	929.2 (560.2)	1391.4 (906.4)	.097
2 months	113.7 (211.4)	451.5 (690.9)	.022[c]
P value[b]	<.001[c]	.012[c]	

ICAM = intercellular adhesion molecule; IL = interleukins; MCP = monocyte chemotactic protein; PlGF = placental growth factor; TGF = transforming growth factor, VCAM = vascular cell adhesion molecule; VEGF = vascular endothelial growth factor

[a]Comparative analysis between Responder and Nonresponder groups.

[b]Comparative analysis between baseline and 2-month follow-up.

[c]Statistically significant ($P < .05$).

concentrations of ICAM-1 ($P < .001$), IL-10 ($P = .023$), MCP-1 ($P = .018$), PlGF ($P = .008$), TGF-β2 ($P = .037$), and VEGF ($P < .001$) in all eyes. There was no significant change in the concentrations of IL-6 ($P = .067$), IL-8 ($P = .491$), and VCAM-1 ($P = .578$). Within the Responder group, there was a significant decline from baseline to 2-month follow-up in aqueous humor cytokine concentration of ICAM-1 ($P < .001$), IL-10 ($P = .041$), MCP-1 ($P = .046$), PlGF ($P = .027$), TGF-β2 ($P = .017$), and VEGF ($P < .001$; Table 2). Among the Nonresponders, a significant decline was noted in VEGF aqueous humor concentration ($P = .012$). There were no significant differences between any of the baseline aqueous humor cytokine concentrations in the Responder versus Nonresponder groups. At 2-month follow-up, VEGF concentration was significantly lower in the Responder (113.7 ± 211.4 pg/mL) compared with the Nonresponder group (415.5 ± 690.9 pg/mL; $P = .022$; Figure).

A total of 11.4% (4/41) of the eyes received intravitreal triamcinolone because of persistent DME despite anti-VEGF therapy over the follow-up period. The 2-month follow-up aqueous humor VEGF concentration was

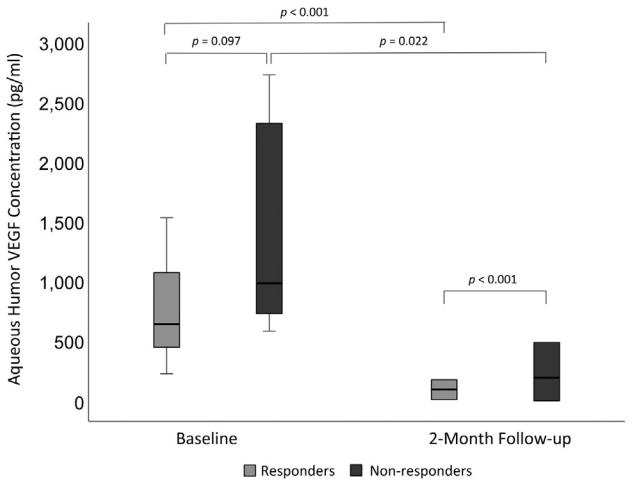

FIGURE. Boxplots comparing baseline and 2-month follow-up aqueous humor cytokine concentrations for the Responder and Nonresponder groups based on long-term anatomical response to anti–vascular endothelial growth factor therapy. *Note*: Box intersection lines represent median, box edges represent the 25th and 75th percentiles, and whiskers represent 5th and 95th percentile.

significantly higher in the eyes that required intravitreal triamcinolone treatment (506.5 ± 876.5 pg/mL) compared with the group that received anti-VEGF therapy only (126.3 ± 215 pg/mL; $P = .031$).

DISCUSSION

OPTIMAL TREATMENT OF DME HAS STRONG IMPLICATIONS on quality of life of patients with diabetes mellitus.[41] Despite the efficacy of anti-VEGF agents,[11,18–20] many patients with DME who are receiving intravitreal anti-VEGF injections as a first line of treatment show minimal improvement, even after multiple injections.[6,11,18,21–28] The current study explored the role of aqueous humor cytokine concentration after 2 intravitreal ranibizumab injections as early indicators of long-term treatment response over 4 years of follow-up. Our results demonstrate a statistically significant relationship between long-term anatomical response of eyes with DME undergoing anti-VEGF therapy and lower aqueous humor VEGF concentration early in the course of treatment. Although there was a significantly lower VEGF concentration in all eyes after 2 months of ranibizumab treatment, the 2-month follow-up VEGF concentration was significantly lower in eyes identified to have optimal long-term response compared with eyes with a poor response to treatment. These findings suggest that aqueous humor cytokine concentrations may serve as an early biomarker of eyes that will likely demonstrate suboptimal response to long-term anti-VEGF therapy.

Patients enrolled in the study on average received 8 injections annually based on a PRN treatment regimen. Despite close follow-up, persistent DME was evident in 15% of eyes in this study, which further attests to the possible role of inflammatory markers other than VEGF in the pathogenesis of DME.[8] In fact, the pathogenesis of DME has been strongly attributed to hyperglycemia-induced oxidative stress and inflammation and subsequent upregulation of various growth factors and cytokines.[2] In this study, ICAM-1 and IL-10 were 2 cytokines that significantly decreased after anti-VEGF therapy in eyes with optimal response. We have previously demonstrated the role of aqueous ICAM-1 and IL-10 with disease severity and vision outcomes, respectively.[38] In line with these results, Funatsu and associates[36] have suggested that VEGF and ICAM-1 have a strong influence on severity of DME. Among the other cytokines investigated in this study, MCP-1, PIGF, and TGF-β2 concentrations also significantly decreased following the 2-month follow-up visit in eyes with optimal response to anti-VEGF therapy. Therefore, in selected cases, treatment options including corticosteroids may be more effective for wider targeting of other inflammatory molecules.

According to post hoc analysis of data from the DRCR Network Protocol I, early anatomical response to ranibizumab has been suggested to be one of the strongest predictors of long-term anatomical prognosis.[28] In addition, post hoc analysis of the RESTORE and the RESTORE-extension studies identified spectral-domain OCT features as strong predictors of therapeutic efficacy.[42] Other predictive biomarkers of patients' response to treatment, including baseline visual acuity, age, and the presence of hard exudates in the macula at the time of treatment initiation, have been previously identified.[37,43] Aqueous humor cytokine concentrations may serve as additional biomarkers of long-term response and guide treatment in consideration of already established prognosticators. Given that a delay in treatment with ranibizumab has been associated with decreased likelihood of long-term vision improvements,[44] anti-VEGF therapy may always be considered the first-line therapy and further adjustments may be made in consideration of the early response to treatment. The role of more potent anti-VEGF agents has also been explored, but these drugs may or may not offer significant clinical benefit to eyes refractory to current therapies.

Early recognition of response to anti-VEGF enables the timely switch of treatments or consideration of alternate therapy for targeting cytokines that play a role in the inflammatory pathogenesis of DME independently of VEGF.[45] A subgroup analysis of the DRCR Network Protocol U trial with intravitreal corticosteroids suggested that there may be a visual acuity benefit in adding dexamethasone to ranibizumab in pseudophakic eyes.[46,47] However, the DRCR Network Protocol U results overall did not show a significant difference in visual acuity from baseline to 24 weeks using ranibizumab therapy with or

without addition of sustained release dexamethasone implant in eyes with persistent DME.[29] In our study, 11% of all eyes over 4 years of follow-up were switched to intravitreal corticosteroids.[48] It is speculated that chronic DME may be driven by inflammatory cytokines, which may be more suited to treatment with intravitreal corticosteroids. Bressler and associates,[49] in the post hoc analysis of the DRCR Network Protocol T clinical trial, cautioned against a switch to alternative therapies earlier than 6 injections as improvements in outcomes may be achieved with continued treatment alone. They suggest that a large proportion of eyes will have substantial (≥2-line) long-term improvement in visual acuity with long-term anti-VEGF therapy of up to 2 years.[49] Aligned with these findings, 27% of all eyes with suboptimal response to anti-VEGF therapy during the early follow-up period in our original prospective study showed resolution of DME with long-term treatment. Nonetheless, 10% of all eyes had early indications of poor anatomical response and remained poor responders over the 4-year follow-up period despite ongoing anti-VEGF treatment. Our group also previously demonstrated an association between lower baseline aqueous humor VEGF concentration and more favorable short-term anatomical response to anti-VEGF therapy. Although no significant differences in baseline aqueous humor cytokine levels among the long-term Responder and Nonresponder groups were noted in the current study, larger studies are required to explore these complex relationships.

There are several limitations in this study that we would like to acknowledge. Long-term response to anti-VEGF therapy in this study was based on spectral-domain OCT features, which provides an accurate and reliable measure of CST and MV and enables quantitative longitudinal monitoring of disease progression in DME.[50] However, we did not take into account functional outcomes, such as long-term visual acuity, due to limitations of this measure in an uncontrolled retrospective study setting. Furthermore, ranibizumab is a monoclonal antibody that is affinity-enhanced for VEGF-A and therefore our aqueous humor cytokine concentration measures may not be applicable to other anti-VEGF agents. Other differences amongst the agents, such as higher likelihood of persistent DME for bevacizumab compared with aflibercept and ranibizumab, were not investigated in this study.[49] The variable follow-up period for patients included in the retrospective study also serves as a limitation. Caution should be taken in drawing conclusions from the results of the subgroup analysis, as this study was not powered to examine the relationship between the Responder and Nonresponder groups.

The current study explored the role of aqueous humor cytokine concentration after 2 intravitreal ranibizumab injections as a biomarker for long-term treatment response over 4 years of follow-up. Our results demonstrate a statistically significant relationship between long-term anatomical response of eyes with DME undergoing anti-VEGF therapy and lower aqueous humor VEGF concentration early in the course of treatment. Although there was a significantly lower VEGF concentration in all eyes after 2 months of ranibizumab treatment, the 2-month aqueous VEGF concentration was significantly lower in eyes identified to have optimal long-term response compared with eyes later proven to have suboptimal response to treatment. The use of aqueous humor cytokine concentrations for timely identification of potential poor responders to anti-VEGF therapy may enable more effective treatment regimens that improve anatomical outcomes in eyes with DME.

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