Aqueous Cytokine Expression and Higher Order OCT Biomarkers: Assessment of the Anatomic-Biologic Bridge in the IMAGINE DME Study

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• PURPOSE: To identify biomarkers for predicting response to anti−vascular endothelial growth factor (VEGF) therapy in diabetic macular edema (DME) and evaluate any links between cytokine expression and optical coherence tomography (OCT) phenotype.

• DESIGN: The IMAGINE is a *post hoc* image analysis and cytokine expression assessment of the Efficacy & Safety Trial of Intravitreal Injections Combined With PRP for CSME Secondary to Diabetes Mellitus (DAVE) randomized clinical trial.

• METHODS: Subjects were categorized as anatomical responders or nonresponders, and within the responder group as rebounders and non-rebounders based on quantitative, longitudinal OCT criteria. Retinal layer and fluid features were extracted using an OCT machine-learning augmented segmentation platform. Responders were further sub-classified by rapidity of response. Aqueous concentrations of 54 cytokines were measured at multiple timepoints. Expression was compared between responder groups and correlated with OCT imaging biomarkers.

• RESULTS: Of the 24 eyes studied, 79% were anatomical responders with 38% super responders, 17% early responders, and 25% slow responders. Twenty-one percent were nonresponders. Super responders had increased baseline vascular endothelial growth factor (VEGF) (880.0 pg/mL vs 245.4 pg/mL; $P = .012$) and decreased monocyte chemotactic protein-1 (MCP-1) (513.3 pg/mL vs 809.5 pg/mL; $P = .0.042$) concentrations compared with nonresponders. Interleukin-6 (−24.9 pg/mL vs 442.8 pg/mL; $P = .032$) concentrations increased among nonresponders during therapy. VEGF concentrations correlated with central subfield thickness ($r = 0.49$; $P = .01$). Panmacular retinal volume correlated with increased interleuckin-6 ($r = 0.47$; $P = .02$) and decreased MCP-1 ($r = -0.45$; $P = .03$). Matrix metallopeptidase-1 correlated with subretinal fluid volume ($r = 0.50$; $P = .01$).

• CONCLUSIONS: OCT imaging biomarkers correlated with both intraocular cytokines and responsiveness to anti-VEGF therapy, which indicated a possible link to underlying pathways and their relevance to DME prognosis. Baseline concentrations of VEGF and MCP-1 are associated with anatomic response to anti-VEGF therapy. (Am J Ophthalmol 2021;222:328–339. © 2020 Elsevier Inc. All rights reserved.)

DIABETIC MACULAR EDEMA (DME) IS THE LEADING cause of visual loss associated with diabetes ahead of proliferative diabetic retinopathy with fluid accumulating in the macula as either intraretinal fluid (IRF) or subretinal fluid (SRF).[1] The pathogenesis of DME has been tied to multiple factors, including increased oxidative stress, perturbation of the blood-retinal barrier, and subsequent vascular permeability dysfunction.[1,2]

Vascular endothelial growth factor (VEGF) is a prominent mediator of DME pathophysiology.[3] Inhibitors of VEGF have become first-line treatment in DME management after multiple clinical trials, including the RISE/RIDE and VISTA/VIVID phase III studies, which demonstrated significant clinical efficacy compared with previous standard therapies, including photocoagulation.[4–7] However, these clinical trials revealed that only 31%-46% of patients who received anti-VEGF therapy gained ≥3 lines of vision, whereas significant proportions of patients had an incomplete response to anti-VEGF therapy anatomically, functionally or both.[4–7] Moreover, these data suggested delays in therapy might be associated with irrecoverable vision loss. An improved understanding of the biologic underpinnings of DME in an individual patient could enable personalized management, which would optimize visual and anatomic outcomes though these might not always be congruent. Specifically, because intraocular cytokines represent secretion of proteins from the retina, a thorough exploration could distinguish between a phenotype driven predominately by VEGF versus multifactorial inflammatory mediators.[1,2]

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This could provide a unique opportunity for correlation with specific imaging or clinical phenotypes that could facilitate more precise therapeutic decision-making.

Previous analyses of aqueous humor cytokines have provided some insights into which patients are likely to respond to anti-VEGF therapy, but there is substantial heterogeneity in their results.[8–10] For VEGF levels alone, previous reports have been conflicting, with only some identifying baseline VEGF differences between eyes as associated with responsiveness or unresponsiveness to anti-VEGF therapy.[9,11] Moreover, previous studies have reported conflicting results on whether increased or decreased aqueous humor VEGF is associated with a response to anti-VEGF therapy. Interestingly, increased levels of other cytokines including intercellular cell adhesion molecule -1, monocyte chemotactic protein (MCP)-1, and interleukin (IL)-6 have been associated with responsiveness to intravitreal ranibizumab.[8] To engender effective, timely personalized treatment regimens for patients with DME, additional cohort analyses on aqueous humor cytokine profiles are required to clarify literature discrepancies and expand knowledge on the role of cytokines, other than VEGF, in disease pathophysiology and responsiveness.

Emerging technology now enables higher order optical coherence tomography (OCT) analysis, including targeted feature extraction through multilayer segmentation and pathologic feature characterization, including panmacular fluid feature volumetric assessment.[12–14] This sophisticated platform generates higher order parameters such as the retinal fluid index (RFI), which has been correlated with visual outcomes in DME in the VISTA study.[13] The use of this technology in tandem with a thorough correlation of aqueous humor cytokines may expand knowledge regarding specific imaging phenotypes, facilitating more precise therapeutic decision-making.

The goals of the present study were to: 1) characterize the longitudinal cytokine profile in patients with DME who received intravitreal anti-VEGF therapy and delineate baseline cytokines that might be predictive of anatomic resolution of macular edema; and (2) evaluate the association of higher order OCT features with underlying intraocular cytokine expression and the link to DME treatment response to anti-VEGF therapy.

METHODS

• **STUDY DESIGN:** The Evaluation of Imaging Biomarkers with Cytokine Profiling in Diabetic Macular Edema and Retinal Venous Occlusive Disease (IMAGINE) study was a *post hoc* study that evaluated aqueous cytokine expression with in-depth assessment of the imaging studies obtained throughout the phase I/II Efficacy & Safety Trial of Intravitreal Injections Combined With PRP for CSME Secondary to Diabetes Mellitus (DAVE) study performed by Brown and colleagues.[15] The endpoint of this study was evaluation of the baseline cytokine profiles in patients with DME for their association with treatment response. The IMAGINE study was determined to be exempt by the Cleveland Clinic's Institutional Review Board and adhered to the tenants of the Declaration of Helsinki.

Briefly, the DAVE study was a 3-year prospective randomized trial that evaluated ranibizumab alone compared with combination therapy with targeted retinal photocoagulation (TRP) to areas of nonperfusion in treatment-naïve eyes with DME. All eyes received 4 doses of monthly 0.3-mg ranibizumab injections before starting monthly visits with as needed retreatment based on disease activity (*pro re nata* [PRN]) for the remainder of the study. Reinjection criteria during the PRN phase was the presence of DME within the foveal depression based on spectral domain (SD)-OCT.[15] TRP was performed at week 1 in the eyes randomized to that treatment arm to areas of retinal capillary nonperfusion outside the macula with possible retreatment at months 6, 18, and 25. Inclusion criteria for the original study included treatment-naïve visually affected patients with DME who were older 18 years of age with severe non-proliferative diabetic retinopathy (NPDR) or early proliferative diabetic retinopathy (PDR). SD-OCT was obtained at baseline and at months 1, 2, 3, 5, 6, and 12. Demographic and clinical information, including sex, age, years with diabetes mellitus, hemoglobin A_{1c} (HbA_{1c}), and diabetic retinopathy severity were collected at baseline. A detailed description of the study protocol has been previously described.[15]

• **CYTOKINE ANALYSIS:** All study eyes with available concurrent aqueous humor samples from baseline were included in the analysis. Aqueous humor samples were obtained when able at baseline and at months 3 and 12 through paracentesis and were then frozen and stored at −80 °C. Samples underwent commercial multiplex enzyme linked immunosorbent assay (RayBioTech, Peachtree Corners, GA, USA), which targeted angiogenesis and inflammatory pathways, including measurement of activin A, agouti-related neuropeptide, angiogenin, angiopoietin-2 (ANG-2), angiopoietin-like 4 (ANGPTL4), basic fibroblast growth factor, epithelial-neutrophil activating peptide, growth-related α protein, heparin-binding EGF-like growth factor, hepatocyte growth factor (HGF), interferon-γ, insulin-like growth factor, IL-1a, IL-2, IL-6, IL-8, IL-17, interferon-γ–induced protein 10, leptin, leukemia inhibitory factor (LIF), monocyte chemotactic protein-1 (MCP-1), platelet-derived growth factor subunit B, placental growth factor (PLGF), C-C motif chemokine ligand 5, transforming growth factor (TGF)-β1, tissue inhibitor of metalloproteinases 1, tissue inhibitor of metalloproteinases 2, angiostatin, C-X-C motif chemokine ligand 16, epidermal growth factor (EGF), fibroblast growth factor-4, follistatin, granulocyte colony stimulating factor, granulocyte-macrophage colony-stimulating factor, C-C

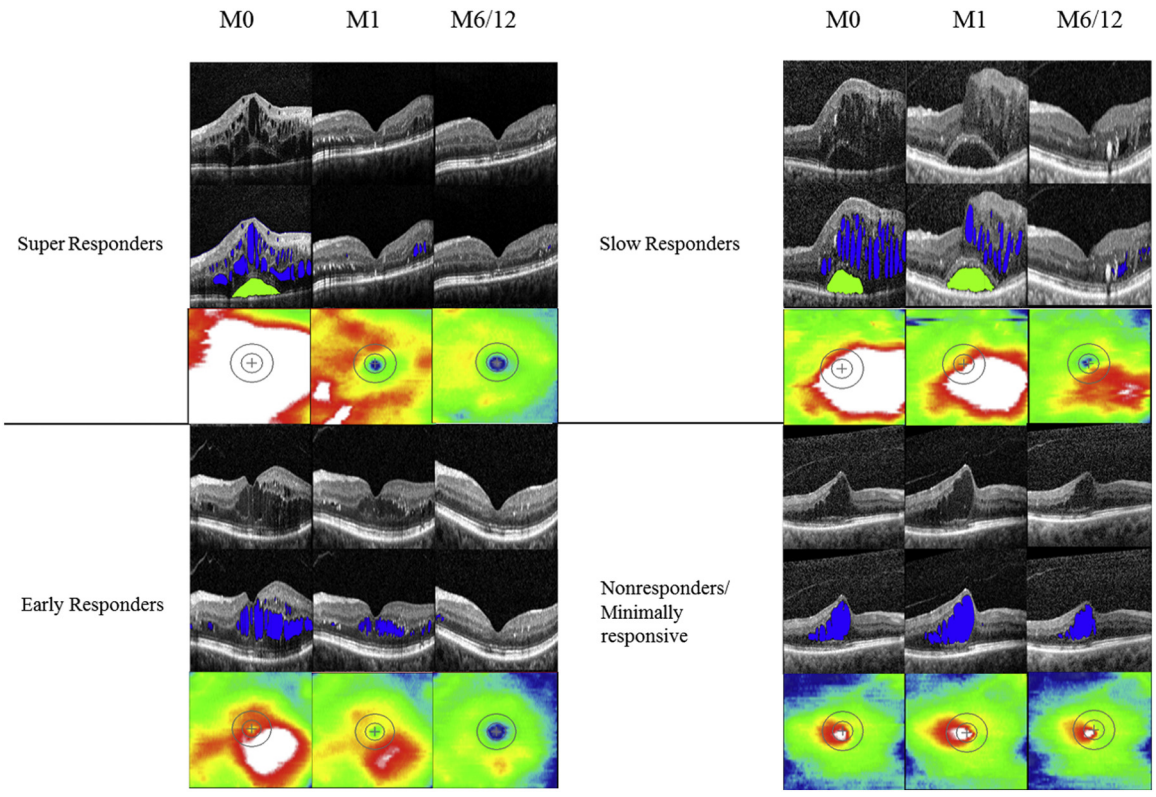

FIGURE 1. Representative optical coherence tomography foveal B-scans from Responder categories without volumetric fluid segmentation (first row), with fluid segmentation (second row), and ILM-RPE thickness map for each category taken at baseline, month 1, and month 6 or 12. Color on B-scan distinguishes intraretinal (blue) and subretinal (green) fluid.

motif chemokine ligand 1, IL-1b, IL-4, IL-10, IL-12p40, IL-12p70, interferon-inducible T cell a-chemoattractant, MCP-2, MCP-3, MCP-4, matrix metallopeptidase (MMP) 1, MMP-9, platelet and endothelial cell adhesion molecule 1 (PECAM-1), TGF-α, TGF-β3, tyrosine kinase with immunoglobulin like and EGF-like domains 1, tyrosine kinase with immunoglobulin-like and EGF-like domains 2, plasminogen activator, urokinase receptor (uPAR), and VEGF. Concentrations of the cytokines were measured at each time point in quadruplicate, averaged, normalized, and values below the limit of detection for each cytokine were forced to 0. For this report, only cytokines with a detectable level in at least ≥20% samples were included in analysis.

• **CATEGORIZATION OF EYES BY ANATOMICAL RESPONSE AND REBOUND PROFILES:** SD-OCT macular cube scans (Spectralis, Heidelberg, Germany) were uploaded into a previously described software platform that enables linear, area, and volumetric features of multiple imaging biomarkers.[12–14] Using automated analysis, scans underwent fluid feature extraction and multilayer segmentation that evaluated IRF, SRF, and various retinal layer thickness parameters, such as the internal limiting membrane−retinal pigment epithelium and the ellipsoid zone (EZ)−retinal pigment epithelium with manual correction as needed. An image analyst reviewed each B-scan for segmentation accuracy in retinal layers and fluid segmentation. A secondary quality control pass was performed to evaluate imaging and segmentation consistency.

Several quantitative metrics were exported for cytokine correlation, including retinal thickness parameters (internal limiting membrane−retinal pigment epithelium) and outer retina parameters (EZ−retinal pigment epithelium) that evaluated panmacular and central subfield regions. In addition, intraretinal and subretinal volumes were extracted with similar regional stratification. Previously described RFIs that calculated the ratio of fluid to tissue in either the entire macular cube or subfield were also exported.[13]

Eyes were categorized into anatomical responder groups by the following criteria: 1) super responders were those eyes in which IRF volume was reduced by >80% or to <0.001 mm^3 after 1 injection and/or an 80% reduction of excess thickening of the central subfield thickness (CST). Excess thickening was defined as CST >300 μm; 2) early responders were those eyes that met these criteria after the third injection at month 3; 3) slow responders were those eyes that met this criteria between months 3 and 12; 4) nonresponders/minimal responders were those eyes

TABLE 1. Demographic Characteristics of All Eyes, Responders, Including Subtypes and Nonresponders

Parameter	All (n = 24)	Responders (n = 19)	Super Responders (n = 9)	Early Responders (n = 4)	Slow Responders (n = 6)	Nonresponders (n = 5)
Age (y)	54.6 ± 9.1	51.5 ± 8.5	47.2 ± 7.8	55.5 ± 10.5	55.2 ± 5.8	66.6 ± 5.7
Male sex	17 (85.0)	17 (89.5)	9 (100)	3 (75)	5 (83)	2 (40.0)
Right eye	10 (50)	7 (36.8)	3 (33)	1 (25%)	3 (50)	3 (60.0)
Hemoglobin A1c (%)	8.2 ± 2.2	8.4 ± 2.1	8.5 ± 2.3	8.7 ± 3.0	8.2 ± 2.4	7.3 ± 2.5
ETDRS BCVA	58 ± 13.0	60 ± 11.5	53 ± 12.6	65 ± 17.1	61 ± 7.0	50 ± 16.3
Severity of retinopathy						
Mild NPDR	1 (4.2)	0 (0)	0	0 (0)	0 (0)	1 (20)
Moderate NPDR	3 (12.5)	3 (15.8)	2 (22.2)	0 (0)	1 (16.7)	0 (0)
Severe NPDR	12 (50.0)	10 (52.6)	2 (22.2)	4 (100)	4 (66.7)	2 (40)
PDR	8 (3.3)	6 (31.5)	5 (55.6)	0 (0)	1 (16.7)	2 (40)

BCVA = best-corrected visual acuity; NPDR = non-proliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy.
Values are n (%) and mean ± SD.

that maintained >50% of CST excess and/or IRF >50% of initial volume by 12 months of treatment; and 5) indeterminate were those eyes that met none of the preceding criteria (Figure 1).

Eyes that were within the anatomical super responder, early responder, or slow responder groups were treated as rebounders if they experienced worsening of 50% of resolved IRF volume or 50% of maximum CST reduction over the treatment course.

• **STATISTICAL ANALYSIS:** Due to several cytokines having non-normal distributions assessed by Shapiro-Wilk tests and small group sample sizes, nonparametric testing was used throughout to calculate *P* values. Longitudinal changes in aqueous humor cytokine concentrations from baseline to months 3 or 12 were compared using Wilcoxon signed-rank tests. Comparisons between mean cytokine levels within responder and rebounder groups were performed using Mann-Whitney U tests. Spearman's correlation coefficients were used to measure the association between exported imaging features and cytokine concentrations at baseline. The effect of ranibizumab monotherapy versus combination therapy ranibizumab with TRP and baseline diabetic profiling (HbA_{1c}) were evaluated for their impact on aqueous cytokine dynamics using Mann-Whitney U testing and Spearman's correlation, respectively. A $P < .05$ was considered significant. All statistical analyses were conducted using R (R Statistical Foundation, Vienna, Austria).

RESULTS

• **BASELINE CLINICAL CHARACTERISTICS AND DEMOGRAPHICS:** Of the total 40 eyes studied in the DAVE trial, 24 eyes from 20 patients (17 men, 3 women) who had sufficient baseline aqueous humor samples for analysis were included in the study (Table 1). The mean age at presentation was 54.6 ± 9.1 years (range: 31-75 years). Eleven eyes (46%) received anti-VEGF monotherapy with ranibizumab, whereas 13 eyes (54%) received ranibizumab and TRP. The average HbA_{1c} pretreatment was 8.2 ± 2.2%, whereas 13.0 ± 8.1 was the mean years with diabetes mellitus. Twelve of the patients had severe NPDR (50%), 4 had mild to moderate NPDR (17%), and 8 had PDR (33%). The mean baseline best-corrected visual acuity was 58 ± 13, measured by ETDRS letters. In the first 12 months of treatment that this study evaluated, patients received ranibizumab injections in 244/261 visits (93%).

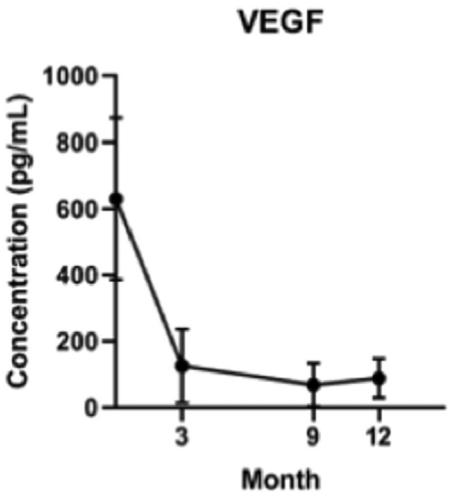

FIGURE 2. Longitudinal mean vascular endothelial growth factor (VEGF) concentrations across all eyes from baseline to month 12 of treatment showing a significant drop in VEGF concentrations after treatment initiation. Error bars represent 95% confidence intervals.

TABLE 2. Longitudinal Cytokine Concentrations Among All Patients

Cytokine (pg/mL)	Baseline	Month 3	Month 9	Month 12
AgRP	3.64 ± 4.0	3.32 ± 2.94	4.79 ± 3.91	4.32 ± 4.54
ANG-1	36.67 ± 56.76	26.82 ± 43.43	29.43 ± 46.17	58.78 ± 90.58
Angiogenin	6,249.07 ± 991.63	6,514.71 ± 1,359.58	6,499.98 ± 845.77	6,629.58 ± 1,057.5
ANGPTL4	1,198.25 ± 1,637.5	1,136.1 ± 2,836.2	478.6 ± 574.31	1,686.06 ± 3,677.1
bFGF	1.4 ± 2.53	1.82 ± 3.54	0.78 ± 2.41	1.55 ± 2.11
CXCL16	642.4 ± 316.77	557.18 ± 304.51	654.13 ± 368.06	712.51 ± 348.36
EGF	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.09
FGF-4	49.59 ± 169.01	78.58 ± 229.75	44.19 ± 101.79	0 ± 0
G-CSF	20.03 ± 20.99	13.36 ± 16.59	32.48 ± 92.76	29.26 ± 65.21
HGF	245.71 ± 176.41	232.47 ± 255.51	204.79 ± 135.58	309.55 ± 355.96
I-309	142.28 ± 188.53	79.8 ± 144.52	117.51 ± 166.88	118.59 ± 179.54
IL-12p40	6.07 ± 5.18	6.23 ± 6.09	6.3 ± 5.5	7.1 ± 5.21
IL-12p70	1.04 ± 1.59	0.72 ± 1.36	0.82 ± 1.75	0.82 ± 1.9
IL-6	18.86 ± 44.09	32.14 ± 110.23	54.01 ± 83.71	125.15 ± 447.91
IP-10	9.74 ± 11.83	15.81 ± 32.11	14.5 ± 19.43	41.39 ± 65.38
Leptin	35.94 ± 89.92	41.9 ± 93.19	174.02 ± 464.2	123.04 ± 338.02
LIF	24.87 ± 32.34	29.68 ± 34.18	18.82 ± 32.87	30.07 ± 39.13
MCP-1	490.63 ± 562.15	382.72 ± 433.31	366.18 ± 426.89	400.68 ± 428.24
MCP-4	2.4 ± 2.97	3.44 ± 8.03	3.54 ± 5.12	1.51 ± 2.6
MMP-1[a]	25.82 ± 91.91	57.13 ± 129.03	56.88 ± 139.53	166.13 ± 318.19
MMP-9	95.78 ± 160.13	100 ± 264.61	88.05 ± 153.67	109.05 ± 198.23
PECAM-1	2,443.11 ± 2,015.2	1,702.11 ± 2,090.6	2,166.63 ± 2,477.4	1,902.03 ± 2,290.5
TGF-á	0.04 ± 0.05	0.05 ± 0.06	0.04 ± 0.04	0.06 ± 0.08
TIMP-1	1,5571.16 ± 3,122.9	1,5241.85 ± 4,259.78	15,432.98 ± 2,852.99	16,573.56 ± 2,142.5
TIMP-2	10,864.71 ± 3,914.5	9,262.07 ± 4,341.2	10,537.22 ± 4,007.2	11,527 ± 4,083.5
uPAR	355.8 ± 200.2	361.65 ± 263.06	396.66 ± 389.01	485.65 ± 478.72
VEGF[a]	631.09 ± 578.52	126.44 ± 218.03	68.67 ± 135.22	88.69 ± 131.15

AgRP = agouti-related neuropeptide (AgRP); ANG-1 = angiopoietin-1; ANGPTL4 = angiogenin, angiopoietin like 4; bFGF = basic fibroblast growth factor; CXCL16 = C-X-C motif chemokine ligand 16; EGF = epidermal growth factor; FGF-4 = fibroblast growth factor-4; G-CSF = follistatin, granulocyte colony stimulating factor; HGF = hepatocyte growth factor; I-309 = C-C motif chemokine ligand 1; IL = interleukin; IP-1 = interferon-γ–induced protein 10; LIF = leukemia inhibitory factor; MCP = monocyte chemotactic protein; MMP = matrix metallopeptidase; PECAM-1 = platelet and endothelial cell adhesion molecule 1; TGF-α = transforming growth factor-α; TIMP = tissue inhibitor of metalloproteinases; uPAR = plasminogen activator, urokinase receptor; VEGF = vascular endothelial growth factor.

[a]A significant change in concentration in Wilcoxon signed rank testing from baseline at $P < .05$.

• **BASELINE AND LONGITUDINAL CYTOKINE DYNAMICS:** Of the 54 cytokines evaluated, 27 had levels above detection threshold across all visits. Across all eyes, mean VEGF concentrations were significantly lower at month 3 (n = 16; $P < .001$) and month 12 (n = 15; $P < .001$) following initiation of treatment (Figure 2). Mean MMP-1 increased at 12 months following treatment ($P = .036$), whereas interferon-γ–induced protein 10 trended toward a significant elevation at month 12 ($P = .065$) and mean MCP-1 trended toward decreased levels ($P = .073$). There were no significant differences or trends observed among the concentrations of the other detected cytokines (Table 2).

At month 12, subjects who received combination therapy (n = 13) with TRP had increased mean uPAR ($P = .05$) and TGF-α ($P = .026$) compared with ranibizumab alone (n = 11). There was no significant difference in reduction in mean VEGF between the groups at month 12 ($P = .10$). HbA_{1c} at baseline correlated with increased levels of C-C motif chemokine ligand 1 ($P = .044$), increased MCP-4 ($P = .013$), and decreased tissue inhibitor of metalloproteinases-1 ($P = .013$). There were no differences between HbA_{1c} at baseline between anatomical responders and nonresponders.

• **BASELINE OCT IMAGING BIOMARKERS AND CYTOKINE EXPRESSION:** VEGF correlated with CST ($r = 0.49$; $P = .01$) and trended toward significance with multiple fluid parameters, including the macular IRF index and central macular IRF index ($P < .1$) (Figure 3). Panmacular retinal volume correlated with increased IL-6 ($r = 0.47$; $P = 0.02$) and decreased MCP-1 ($r = -0.45$; $P = .03$). Increased PECAM-1 was associated with decreased IRF central subfield volume ($r = -0.42$; $P = .04$), a decreased central

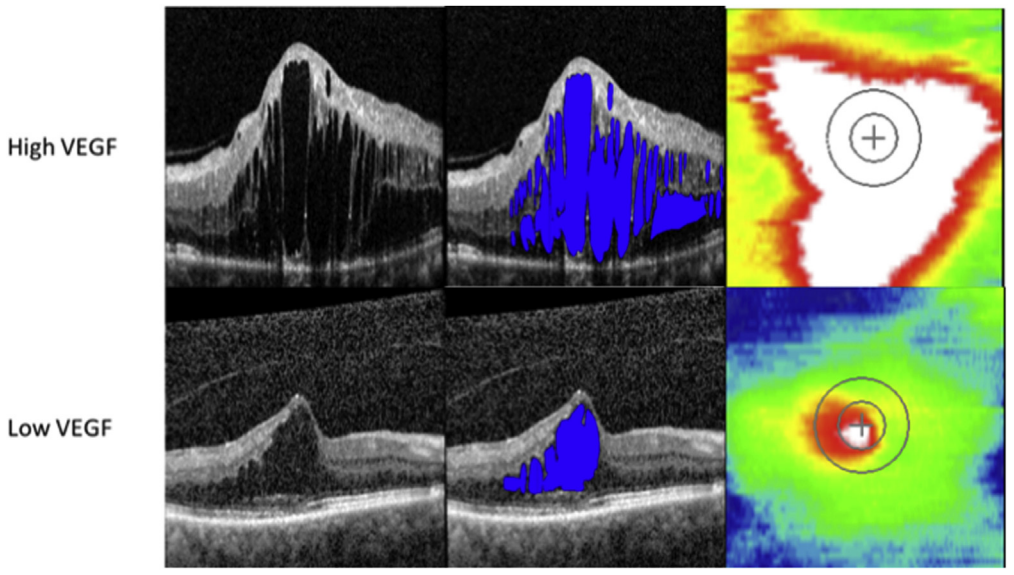

FIGURE 3. Representative optical coherence tomography foveal B-scans from high and low vascular endothelial growth factor (VEGF) eyes without volumetric fluid segmentation (first column), with fluid segmentation (second column), and retinal thickness maps (third column) taken at baseline.

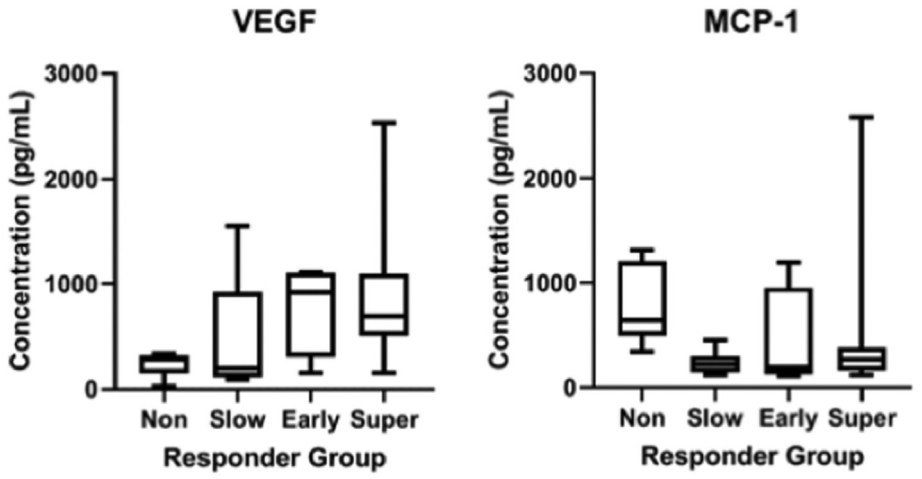

FIGURE 4. Box and whisker plots of baseline vascular endothelial growth factor (VEGF) and monocyte chemotactic protein-1 (MCP-1) concentrations by anatomical responder category visualizing increased VEGF and decreased MCP-1 among anatomical responders compared with nonresponders.

macular IRF index (r = −0.42; $P = .04$), and a decreased central subfield total RFI (r = −0.44; $P = .03$). In addition, there was a trend toward an association between IRF central subfield volume with IL-6 ($P = .13$). Multiple SRF parameters were correlated with MMP-1, including the total macular SRF index (r = 0.50; $P = .01$), the central macular SRF index (r = 0.48; $P = .02$), the central subfield SRF index (r = 0.45; $P = .03$), and the SRF central subfield volume (r = 0.45; $P = .03$).

Multiple cytokines were correlated with panmacular EZ attenuation, including ANGPTL4 (r = 0.57; $P = .003$), basic fibroblast growth factor (r = 0.48; $P = .02$), FGF-4 (r = −0.48; $P = .02$), LIF (r = 0.46; $P = .02$), and hepatocyte growth factor (r = 0.45; $P = .03$). In addition, increased central subfield EZ–retinal pigment epithelium volume was correlated with decreased C-X-C motif chemokine ligand 16 (r = −0.51; $P = .01$), hepatocyte growth factor (r = −0.44; $P = .03$), and VEGF-A (r = −0.44; $P = .04$). Panmacular EZ–retinal pigment epithelium volume was similarly correlated with decreased ANGPTL4 (r = −0.48; $P = .02$), uPAR (r = −0.41; $P = .05$), and C-X-C motif chemokine ligand 16 (r = −0.41; $P = .05$).

• **ANATOMICAL RESPONDERS AND NONRESPONDERS:** Of the 24 eyes studied, 79% (19/24) of eyes were anatomical responders split between 38% super responders (9/24), 17% early responders (4/24), and 25% slow responders (6/24), whereas 21% (5/24) of eyes were nonresponders. There were no eyes in the indeterminate group. At baseline, anatomical super responders had significantly greater average VEGF (880.0 pg/mL vs 245.4 pg/mL; $P = .012$) and lower average MCP-1 (513.3 pg/mL vs 809.5 pg/mL; $P = .042$) concentrations compared with nonresponders (Figure 4). Aggregating anatomical super responders with early responders and comparing these to nonresponders revealed a similar greater baseline mean VEGF (848.2 pg/mL vs 245.4 pg/mL; $P = .014$), in addition to lower mean MCP-1 (486.4 pg/mL vs 809.5 pg/mL, 0.035) concentrations. When comparing all anatomical responders to nonresponders, average VEGF concentration was higher among responders, although this difference did not reach statistical significance (732.6 pg/mL vs 245.4 pg/mL; $P = .10$), whereas baseline average MCP-1 remained significantly higher in the nonresponders (406.7 pg/mL vs 809.5 pg/mL; $P = .0093$). Evaluating anatomical super and early responders against slow responders and nonresponders showed increased mean VEGF (848.2 pg/mL vs 374.5 pg/mL; $P = .018$) and increased mean ANGPTL4 (1677.4 pg/mL vs 632.0 pg/mL; $P = .023$) in the former groups. Multiple other cytokines had sizeable effect size differences in this analysis, including leptin and agouti-related neuropeptide, which did not reach statistical significance (Supplemental Table 1).

Following 3 months of treatment, no significant differences in cytokine changes comparing anatomical super responders versus nonresponders and all anatomical responders versus nonresponders in paired analyses were detected. However, at 12 months, super responders had a greater reduction in mean VEGF (−737.8 pg/mL vs −103.9 pg/mL; $P = .009$) and mean LIF (−37.5 pg/mL vs 30.1 pg/mL; $P = .028$) concentrations compared with nonresponders. Comparing all anatomical responders to nonresponders at 12 months, the change in mean concentration of VEGF was significantly greater (−683.9 pg/mL vs −103.9 pg/mL; $P = 0.005$) among the responders, whereas mean LIF (−17.7 pg/mL vs 30.1 pg/mL; $P = .028$) and IL-6 (−24.9 vs pg/mL s 442.755 pg/mL; $P = .032$) concentrations increased among nonresponders (Supplemental Table 2).

• **OCT CHARACTERISTICS OF ANATOMICAL RESPONSE GROUP:** When compared against nonresponders, anatomical super responders at baseline had increased panmacular retinal volume (16 mm^3 vs 11 mm^3; $P = .004$), increased macular total RFI (12% vs 4%; $P = .02$), increased mean retinal CST (614 μm vs 360 μm; $P = .03$), increased IRF volume (2.0 mm^3 vs 0.44 mm^3; $P = .04$), and an increased macular IRF index (11% vs 4%; $P = .04$). Comparing all anatomical responders against nonresponders, responder eyes were associated with increased panmacular retinal volume (15 mm^3 vs 11 mm^3; $P = .003$), mean retinal CST (557 μm vs 360 μm; $P = .009$), and several fluid parameters, including IRF volume (1.6 mm^3 vs 0.44 mm^3; $P = .02$), central subfield total RFI (43% vs 12%; $P = .01$), and macular total RFI (11% vs 4%; $P = .01$) (Supplemental Table 3).

• **REBOUNDERS AND NON-REBOUNDERS:** Among the 19 responders, 5 eyes exhibited rebounder behavior as defined in this study. At baseline, multiple cytokines differentiated the rebounders from the non-rebounders, including increased mean concentrations of TGF-α (0.11 pg/mL vs 0.029 pg/mL; $P = .008$), LIF (58.6 pg/mL vs 13.5 pg/mL; $P = .015$), and uPAR (562.2 vs 289.1; $P = .034$). Of the rebounder eyes, 4 had cytokines available at a follow-up time point associated with disease rebounding. There were no statistically significant differences detectable between the cytokines at baseline and at the rebound time point. Mean VEGF levels were elevated compared with a follow-up time point associated with anatomic response (37.5 pg/mL vs 918.1 pg/mL; $P = .125$).

Baseline SD-OCT parameters were similar between rebounders and non-rebounders. However, early macular RFI volatility, defined as an increase macular RFI of >2.5 points between months 1 and 2 (during the loading phase), was associated with an increased risk of rebounding with transition to PRN treatment. In the rebounder group, 50% of eyes demonstrated early macular RFI volatility compared with 0% of eyes in the non-rebounder group ($P = .02$).

DISCUSSION

THIS STUDY EVALUATED THE LONGITUDINAL DYNAMICS OF aqueous humor cytokine concentrations in eyes that underwent anti-VEGF therapy with ranibizumab, as well as the link between cytokine levels and therapeutic response defined by reduction in macular edema. In this analysis, anatomical super responders and early responders to anti-VEGF therapy had significantly higher intraocular mean VEGF at baseline than nonresponders, as well as decreased mean MCP-1. Distinct longitudinal cytokine dynamics were observed between anatomical responders and nonresponders for VEGF, LIF, and IL-6, with both LIF and IL-6 increasing in nonresponders.

In addition, this study investigated the link between higher order imaging biomarkers from OCT in predicting treatment response in eyes that received anti-VEGF therapy, as well as the biological underpinnings of the imaging parameters through correlations with aqueous humor cytokine concentrations. Anatomical super responders and responders overall had greater IRF, RFI, and retinal thickness throughout the entire macula and within the central

subfield compared with nonresponders at baseline. Panmacular retinal volume correlated with increased VEGF and IL-6 and decreased MCP-1; PECAM-1 correlated with reduced IRF feature parameters, and MMP-1 correlated with increased SRF parameters. Levels of TGF-α, uPAR, and LIF at baseline differentiated eyes more likely to experience rebounding of disease. Glycemic control as measured by HbA_{1c} correlated with C-C motif chemokine ligand 1, MCP-4, and tissue inhibitor of metalloproteinases-1. These findings supported the possible usefulness of baseline aqueous humor cytokine levels in optimizing treatment selection in DME management for predicting anatomic resolution.

Expanding knowledge of biomarkers for treatment response in DME may be important for patient outcomes, treatment burden, and cost effectiveness. The gold standard treatment of DME validated by numerous clinical trials is anti-VEGF therapy, yet substantial proportions of patients with DME do not respond completely functionally or anatomically to this treatment, represented by 21% of the patients in the present analysis.[4,5] Previous studies reported up to 31%-46% of patients with incomplete functional improvement with anti-VEGF therapy.[4,6] Time to treatment with anti-VEGF therapy was associated with extent of visual recovery.[16] The cost of ophthalmic use of anti-VEGF therapy to the Medicare Part B budget alone is >$2 billion annually.[17] A thorough understanding of an individual patient's disease phenotype through pretreatment intraocular cytokine evaluation might engender optimal treatment regimen selection, reducing a less effective and costly treatment burden.

Although several studies assessed the predictive value of aqueous humor cytokines in determining clinical response to anti-VEGF therapy in DME, these reports yielded heterogeneous and often conflicting results. Although Shimura et al[8] and Udaondo and associates[11] reported, similarly to the present analysis, that increased mean VEGF concentration at baseline predicted treatment response, both Felfeli and associates[18] and Kwon and associates[9] did not identify baseline VEGF as a predictive factor. Moreover, Hillier and associates[10] reported that decreased mean VEGF levels were associated with favorable anatomic response to ranibizumab. The causes of these inconsistencies were likely multifactorial, including variability in responder categorization, baseline disease severity, analytical power, and heterogeneity in disease phenotypes.

Udaondo and associates[11] raised the possible impact of baseline and treatment course glycemic control on anti-VEGF therapy response and levels of pre-treatment VEGF. In the present set of patients, there was no significant increase in HbA_{1c} at baseline between the anatomical responders and nonresponders. Instead, the nonresponders had a reduced mean HbA_{1c} at baseline compared with responders, although this difference was not statistically significant. In contrast to both Udaondo and associates[11] and Shimura and associates,[8] whose baseline mean VEGF were <200 pg/mL, this cohort overall had a mean VEGF concentration of 631 pg/mL, which was more similar to the cohorts of Hillier and associates[10] and Felfeli and associates,[18] with higher average VEGF levels that yet yielded differing conclusions.

The present study also evaluated cytokine dynamics related to eyes that initially responded to therapy but proceeded to experience a clinically meaningful amount of rebound DME when transitioned to a PRN treatment schedule. At baseline, this work identified multiple cytokines involved in inflammation and/or interacted with VEGF that differentiated eyes associated with DME rebound, including uPAR and LIF. The uPAR protein was implicated as a regulator in VEGF-mediated blood retinal barrier disruption.[19] VEGF levels at the rebound time points had a substantial effect size difference compared with VEGF levels at time points associated with anatomic response, although the small sample size impeded the meeting of significance thresholds using nonparametric statistics. Additional investigations into the underpinnings of DME rebounding involving larger numbers of patients may be able to provide more clarity on which patients may be able to be shifted from fixed monthly injections to less frequent injection, as needed re-treatment.

Nearly two-thirds of patients may not achieve a clinically meaningful vision improvement with anti-VEGF therapy, which highlights the mixture of drivers underlying the pathophysiology of DME.[4–7] In the IMAGINE analysis, the anatomical responders and the super responders represented eyes with a more VEGF driven phenotype, whereas the nonresponders, and to a lesser extent, the slow responders likely represented a more multifactorial, inflammation-driven phenotype that would benefit from alternate therapy. These results provided evidence of this because anatomical nonresponders had increased baseline MCP-1, a mediator of inflammation that was implicated in DME disease progression.[20] Moreover, nonresponders had statistically significant increases in IL-6 and LIF. The former is one of the key activators of proinflammatory pathways, whereas LIF was implicated in both inflammation and VEGF modulation.[21,22]

The present study found mixed concordance with previous data on inflammatory drivers. Shimura and associates[8] found good responders had increased IL-6 and MCP-1 at baseline compared with poor responders, which contrasted with our results, whereas Felefil and associates[18] demonstrated a significant decrease in MCP-1 following therapy. Although MCP-1 decreased in this dataset following treatment, this difference was not statistically significant. In contrast, Kwon and associates[9] reported that although VEGF did not predict treatment response to anti-VEGF therapy, IL-8 levels did. Multiple studies also identified the possible role of intercellular cell adhesion molecule-1 as an early biomarker of treatment response, although this work did not detect intercellular cell adhesion molecule-1 in sufficient quantities for analysis.[8,10]

Previous investigations into the predictive capacity of OCT in DME outcomes after anti-VEGF therapy highlighted several imaging features of interest. These included the association between ganglion cell layer thinning and the presence of hyper-reflective foci.[23,24] The depth of information available for phenotype characterization through OCT imaging is rich, and deep learning models have been applied, with some success, in predicting response to treatment in DME.[25,26] To date, no investigation has combined higher order quantitative OCT image analysis with intraocular cytokine assessment.

This study explored the association between imaging biomarkers and underlying signaling molecules at baseline. Several cytokines were associated with either an increased retinal thickness or fluid parameters. In contrast to a previous study,[27] VEGF concentrations correlated with baseline CST and trended toward association with fluid indexes. IL-6 increased with worsened edema, whereas MCP-1 decreased. Both were associated with DME pathogenesis through an increase in retinal permeability, and both IL-6 and MCP-1 levels correlated with response to treatment in a previous study.[8,20] Hyperglycemia led to loss of PECAM-1 in the retinal endothelium, which aligned with these data because worse disease by fluid parameters were correlated with decreased PECAM-1, whose functions include maintenance of endothelial cell junction integrity.[28] MMPs were hypothesized to mediate that specific effect and to contribute to blood-retinal-barrier disruption.[1] MMP-1 in this study correlated with increased SRF, with no correlation to IRF providing possible evidence of anatomically localized pathogenic activity.

Aligning with multiple previous studies, the present work affirmed the importance of IRF, particularly within the fovea, as an important marker for response to anti-VEGF.[26,29,30] In addition, previous studies have highlighted the importance of micro-localization and morphology of IRF in relation to predicting impact on responsiveness; IRF height and localization to the outer nuclear layer around the fovea were strongly predictive. The present report added to these findings by emphasizing the possible usefulness of IRF indexes both throughout the macula and within the central subfield in predicting which eyes will anatomically respond to anti-VEGF. RFI was shown to correlate with functional outcomes in previous work, and this study further demonstrated its baseline predictive capacity.[13] In addition, affirming previous investigations using both qualitative and quantitative methodologies, SRF trended toward differentiation of anatomical responders and nonresponders.[26,29,31]

In our review of the literature, no study to date correlated outer retina integrity parameters with intraocular cytokines. In this analysis, using quantitative EZ features, several cytokines correlated with increased disruption to EZ integrity, including VEGF, ANGPTL4, LIF, and hepatocyte growth factor. These cytokines, in particular, were previously implicated in DME pathogenesis, often cooperating with VEGF to promote microvascular permeability.[21,32,33] One cytokine with functions in cell survival and cell migration, basic fibroblast growth factor, correlated with improved EZ integrity. Whether these correlations were the result of a more general inflammatory perturbation to the outer retina was unclear, but IL-6 and several other mediators of inflammation did not correlate with any EZ parameter. Although no literature was found regarding intraocular cytokines, serum levels of VEGF, anti-myeloperoxidase antibody, and ICAM-1 correlated with EZ disruption in diabetic retinopathy.[34,35] The serum VEGF correlation with EZ disruption aligned with aqueous humor findings in this study. Further investigations into these associations could continue to provide additional insights into DME pathophysiology.

Some eyes also experienced rebounding of disease as treatment durations increased. In contrast to tortuosity metrics from ultra-widefield fluorescein angiography,[36] OCT features at baseline in this study were unable to distinguish these eyes. However, higher order assessment of early fluid volatility on OCT, specifically an increase in macular IRF index between months 1 and 2, might differentiate eyes unable to tolerate treatment interval increases. This aligned with a previous analysis of patients enrolled in the VISTA trial.[37] Exploring the mechanisms behind this early treatment response volatility would require a more comprehensive sampling of intraocular cytokines than was performed in this investigation. Specifically, evaluating cytokine dynamics between months 1 and 2 and their association with RFI volatility could provide clinically meaningful insights. This intriguing finding should be further explored in additional datasets as a potential marker for predicting treatment burden and for exploring new therapeutic alternatives in those eyes that did not tolerate treatment interval extension.

• **STUDY LIMITATIONS:** Although the present study reported multiple findings of interest, there were a number of limitations worth acknowledging. First, the use of aqueous humor cytokine assessment might not completely reflect changes in the posterior segment. However, several studies previously showed correlation with vitreous and aqueous humor cytokine levels.[38–40] As with most of the current reports in the literature that evaluated aqueous cytokine data, this study was limited by available sample size, and therefore, had limited statistical power, especially in subgroup assessments. In particular, there were only 5 nonresponders. For example, ANGPTL4, a molecule that cooperates with VEGF in inducing DME, had a significant effect size difference between

anatomical responders and nonresponders that did not achieve significance using nonparametric testing.[32] Similarly there were insufficient eyes to perform multivariable regression that could control for possible confounding parameters. The predefined categorization of treatment response generated responder groups with significantly more fluid volume than the nonresponders. Whether this reflected increased fluid on presentation in VEGF-driven DME or was a source of bias was unclear. There was some evidence against this being a source of bias because the cohort in the study by Felfeli and associates[18] found anatomical nonresponders had increased macular volume compared with responders. The categorization system also defined response status solely by anatomic features that might not have consistently correlated with visual recovery if persistent macular edema caused irrecoverable vision loss. In addition, not all eyes had cytokines available at each time point. The DAVE trial used ranibizumab, which might have limited the generalizability of these findings to other VEGF targeting therapies, such as aflibercept and bevacizumab.[15,18] Relatedly, multiple isoforms and/or subtypes of VEGF exist, as well as heterogeneity in an assay isoform detection method, because the assay used in this study specifically detected the VEGF-A isoforms, VEGF165 and VEGF121. Differences in the isoform specificity of detection platforms, like the multiplexed enzyme-linked immunosorbent assay used in this study versus multiplexed bead-based assay methodologies, could explain some of the observed variability in the literature. However, even using the same general detection approach did not account for contrasting literature; for example, both this study and Hillier and associates[10] used a similar multiplex enzyme-linked immunosorbent assay—based approach with distinct results. Whether VEGF-A bound to ranibizumab was detected by this assay remains unclear, which is another important limitation, although there was a significant and intuitive drop in detected VEGF after treatment initiation.

CONCLUSIONS

OVERALL, THIS WORK ASSESSED AQUEOUS HUMOR CYTOkine expression as predictive biomarkers for anatomic treatment response to intravitreal ranibizumab in DME. These results indicated both VEGF and MCP-1 pretreatment concentrations differed between eyes likely to experience anatomic response to anti-VEGF therapy. This study further characterized the anatomic-biologic bridge that identified correlations between signaling molecules and higher order imaging features from OCT, such as RFI. Further research is needed to validate and to provide enhanced characterization of the link between OCT features and underlying cytokine expression. The characterization of imaging biomarkers that provide a link to the primary underlying biologic phenotype for a given patient's DME could engender cost-effective, timely personalized treatment regimens for eyes with DME as additional therapeutic options become available.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

JOSEPH R. ABRAHAM: FORMAL ANALYSIS, INVESTIGATION, Writing - original draft, Project administration. **Charles C. Wykoff:** Resources, Investigation, Supervision, Writing - review & editing. **Sruthi Arepalli:** Formal analysis, Writing - review & editing. **Leina Lunasco:** Formal analysis, Investigation, Project administration, Writing - review & editing. **Hannah J. Yu:** Investigation, Writing - review & editing. **Ming Hu:** NA. **Jamie Reese:** Supervision, Project administration. **Sunil.K. Srivastava:** Resources, Writing - review & editing. **David M. Brown:** Resources, Investigation, Writing - review & editing. **Justis P. Ehlers:** Conceptualization, Resources, Methodology, Software, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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