Angiogenic and Inflammatory Vitreous Biomarkers Associated With Increasing Levels of Retinal Ischemia

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Purpose. To characterize the angiogenic and inflammatory vitreous biomarker profiles in a spectrum of ischemic retinopathies, including neovascular glaucoma.

Methods. This institutional review board–approved study retrospectively analyzed 80 undiluted vitreous samples obtained during pars vitrectomy. The specimens were frozen (−80°C) and sent for concentration analysis of 34 proteins by Bio-Plex Pro assays. Specimens were divided into four groups: patients undergoing epiretinal membrane (ERM) peeling and/or macular hole (MH) surgery with no history of diabetes (non-DM group), patients undergoing ERM peeling, and/or MH surgery with a history of diabetes (DM group), patients with proliferative diabetic retinopathy (PDR group), and patients with neovascular glaucoma (NVG group). Parametric and nonparametric analyses of demographics and cytokine levels were performed using SPSS.

Results. There were no significant differences in demographics among cohorts. Numerous proteins were significantly elevated between non-DM and DM (G-CSF, sCD40L, Endoglin, IL-6, placental growth factor [PlGF], VEGF-D), DM and PDR (leptin, IL-8, PlGF, VEGF-A), and PDR and NVG (G-CSF, leptin, TIE-2, sCD40L, EGF, HB-EGF, IL-6, IL-8, PlGF, TNF-α). Only PlGF was significantly elevated between each successive cohort. The most potent drivers of NVG were PlGF, VEGF-A, IL-6, and IL-8.

Conclusions. While the role of angioproliferative growth factors is well documented in ischemic retinopathy, our study delineates the importance of inflammatory and previously underreported angiogenic proteins. It also demonstrates a significant incremental increase in certain factors with increasing levels of ischemia. Both of these findings may guide the development of future therapies for ischemic retinopathies.

Keywords: retinal ischemia, angiogenic, inflammatory, vitreous, neovascular glaucoma

The increase in the prevalence of diabetes mellitus over the past few decades has led to a commensurate rise in the number of diabetic ocular complications.[1] Given this increasingly severe disease burden, developing a better understanding of the molecular mechanisms underlying ischemic retinopathy is a critical objective in the fight to prevent vision loss. The basis of current therapies rests largely upon specific protein-targeted therapy; however, this modality has been largely limited to a single family of growth factors to date. Flushing out a complete profile of vitreous proteins involved across a spectrum of ischemic retinopathies, including neovascular glaucoma (NVG), may serve to direct future research and therapies.

Diabetic retinopathy is one of the world's leading causes of blindness, whether through the leakage of fluid in diabetic macular edema or through angioproliferative disease resulting in vitreous hemorrhage and traction retinal detachments.[2] The pathophysiology of proliferative diabetic retinopathy (PDR) has been well investigated to date, with great emphasis on the VEGF family as chief mediators.[3,4] However, there have been significantly fewer studies comparing the protein composition of eyes with nonproliferative disease (NPDR), despite it constituting another, less severe, form of ischemic retinopathy. Neovascular glaucoma, the dreaded outcome of the severe retinal ischemia, can have a diverse array of underlying pathologies including PDR, retinal vein occlusions (RVO), and ocular ischemia. Like NPDR, NVG has been less thoroughly analyzed beyond the VEGF family of proteins.[5,6] By analyzing this broader spectrum of ischemic retinopathies, it may be possible to develop greater insight into the pathophysiology that results in vision loss in each condition and specific protein drivers that lead to NVG.

Most prior studies on ischemic retinopathy pathophysiology have focused on identifying the importance of growth factors, emphasizing the family of VEGF-related proteins. Those studied include VEGF-A, -B, -C, -D, and placental growth factor (PlGF).[7] While there has been great improvement in disease outcomes with the use of anti-VEGF therapies, disease recurrence following initial treatment courses is common, and the long-term consequences of anti-VEGF therapy is still unknown.[4] By

focusing on other contributory proteins, which may relate directly to VEGF or act independently, a novel focus for therapeutic intervention may be identified.

Besides assessing the role of growth factors, many researchers have also studied the role of inflammatory proteins in a broad spectrum of ischemic vitreoretinal diseases.[8] However, diabetic retinopathy remains the most widely covered disease entity, and many researchers have already investigated the inflammatory mechanisms inherent to the pathogenesis of both retinal vascular leakage as well as capillary nonperfusion.[9,10] As such, in establishing potential targets for future intervention in retinal ischemic disease it is essential to include a profile of inflammatory mediators.

While many of the aforementioned proteins have been elucidated through animal models, aqueous sampling, or operative tissue specimens, the list of proteins assessed via human vitreous specimens is even less conserved. A literature search provided the following partial list of proteins as elevated in vitreous samples from PDR patients: FGF-2, MIP-1β, VEGF, FLT-3L, GRO, IL-1α, IL-6, IL-7, IL-8, IL-10, IP-10, GM-CSF, IL-12p40, sCD40L, MCP-1, MCP-3, MDC, and IFNα2.[11–16] As opposed to aqueous sampling, vitreous specimens are likely to give a better approximation of the cytokine levels that are actually associated with retinal disease pathogenesis.

Despite the notable recent attention to protein levels in ischemic disease there have been some gaps in the knowledge generated so far. Most notable is the absence of nonproliferative ischemic disease cohorts and significant sample sizes of patients with severe ischemic disease resulting in neovascular glaucoma. While the exact mechanism of disease in macular holes and epiretinal membranes certainly may include local ischemia as part of their disease pathogenesis, in the present study we included this group as a comparative cohort against diseases with known global microvascular ischemia as a driver of their pathogenesis, including early diabetic retinopathy even prior to developing proliferative disease. The aims of the present study were multifold. First, we sought to contribute to the growing body of knowledge regarding the cytokine milieu associated with ischemic retinal disease by assessing the aforementioned under-represented cohorts. We also sought to delineate critical proteins, which to date have not been assessed in vivo despite their theorized role in disease pathogenesis. Finally, we aimed to delineate the presence of angiogenic and inflammatory protein biomarkers, particularly in patients with NVG, which may help better guide future treatment strategies and investigations.

Methods

Study Population

This study was a retrospective analysis of undiluted vitreous samples from 80 eyes from 80 patients undergoing pars plana vitrectomy by a single surgeon between November 2010 and September 2012 at Beth Israel Deaconess Medical Center (BIDMC; Harvard Medical School, Boston, MA, USA). Approval was received from the BIDMC institutional review board for the establishment of a repository for the collection of the vitreous samples and for the biomarker analysis performed in this paper. All research adhered to the tenets of the Declaration of Helsinki. Inclusion criteria included all patients undergoing pars plana vitrectomy with single surgeon (JA) between the above dates for indications of macular hole or epiretinal membranes, complications of PDR, or complications of NVG secondary to PDR, and retinal artery or retinal vein occlusion. Patients were excluded from analysis if they were undergoing vitrectomy for rhegmatogenous retinal detachments or for chronic or recurrent detachments, any retinal detachment in patients without PDR or NVG, choroidal detachments, retinoschisis, retinal artery or vein occlusion in any non-NVG patient, history of radiation (with or without clinical diagnosis of radiation retinopathy), asteroid hyalosis, dislocation or subluxation of the intraocular lens, ocular trauma, or pretreatment with intravitreal or periocular steroids. Patients with traction retinal detachments secondary to neovascular membranes were included in the PDR and NVG groups as this was thought to be secondary to their disease pathogenesis. Patients with NVG or PDR receiving prior treatment with intravitreal bevacizumab were included for comparison within their respective cohorts with the untreated eyes. Clinical consents were obtained from each patient, but because this was a retrospective study, a waiver of informed consent for sample collection was approved. Clinical data from each patient was recorded via manual chart review.

Vitreous Collection, Preparation, Storage, and Analysis

During surgery, undiluted vitreous were collected from patients using a previously described technique.[17] In brief, prior to turning on the infusion and beginning the vitrectomy, the vitreous cutter was used to obtain a 1- to 2-cc undiluted vitreous specimen. A 3-cc syringe on a three-way stopcock was used to provide manual aspiration of the specimen. Once a sufficient volume of vitreous was obtained, the infusion was turned on and the stopcock was rotated to continue with the vitrectomy. The undiluted vitreous sample was initially centrifuged if there was evidence of red blood cells or other confounding cell material and immediately transferred to a cold storage freezer, where they were maintained at –80°C until being sent for analysis. Samples were sent to Schepens Eye Research Institute (Boston, MA, USA) for analysis between December 2012 and April 2013. There the frozen samples were analyzed using a Bio-Plex Pro Human Cancer Biomarker Assay (with one analysis by ELISA).

Bio-Plex Pro human cancer biomarker assays are a unique blend of magnetic bead–based assays designed to produce robust and reproducible measurements of 34 biomarkers involved in disease processes such as angiogenesis, metastasis, cell proliferation, cell adhesion/migration, apoptosis, and inflammation. Proteins analyzed include Angiopontin-2, EGF, Endoglin, FGF, Follistatin, GCSF, HB-EGF, HGF, IGFBP, IL-6, IL-8, IL-18, Leptin, Osteopontin, PAI-1, PDGF, PECAM-1, PlGF, Prolactin, sCD40L, SCF, sEGFR, sFASL, sHER2/neu, sIL-6Ra, sVEGF-R1, sVEGF-R2, TGF-α, TIE-2, TNF-α, uPA, VEGFa, VEGFc, and VEGFd. Results were reported in picograms per milliliter. Supplementary Appendix S1 provides this assays sensitivity and working range, as well as detailed instructions on sample preparation.

Prior to running the assay, the vitreous samples were thawed, the Bio-Plex machine was calibrated, and the machine's lasers were warmed for 4 hours. The standards were reconstituted with 781 μL standard diluent, and the controls were reconstituted with 250 μL standard diluent. After vortexing the bottles for 5 seconds, they were iced for 30 minutes. During this 30-minute icing period, the 6.5-μm magnetic beads were prepared for the assay. The beads were vortexed for 30 seconds in a foil-covered vial, and then 5472 μL Assay Buffer and 288 μL beads were added to a 15-mL tube, and each well was filled with 50 μL of this solution and shaken. After the 30-minute icing period, the samples were diluted with a 4-fold standard dilution series and 50 μL standards, blanks, controls, and samples were added to each. This plate was then covered with adhesive plate sealer and foil and was shaken while incubating for 1 hour. Ten minutes prior to the completion of this incubation, the detection antibodies were

Table 1. Demographic Information for Subjects Included in Study

Demographic	Non-DM, $n = 29$	DM Group, $n = 10$	PDR Group, $n = 29$	NVG Group, $n = 12$	P Value*
Age, mean ± SD	70.1 ± 9.9	71.5 ± 11.1	65.0 ± 13.6	68.5 ± 9.5	0.285
Sex, M:F	11:18	6:4	12:17	9:3	0.129
Eye type, OD:OS	14:15	6:3†	12:17	2:9†	0.212
Pretreatment	0	0	4	1	

* All analyses were conducted with SPSS ANOVA tests.
† All samples did not include eye type.

vortexed for 5 seconds and 145 μL detection Assay Buffer was added along with 2755 μL Assay Buffer diluent. Once the 1-hour incubation was complete and the plate was washed three times, 25 μL of this diluted detection antibody solution was added to each well and the plate was once again covered and shaken while incubating for 30 minutes.

The Stretavidin-PE (SA-PE) stock solution was prepared 10 minutes prior to the completion of this incubation period. The SA-PE solution was covered with foil and vortexed for 5 seconds prior to adding 60 μL SA-PE as well as 5940 μL Assay Buffer to a separate vial. After the incubation period, the plate was washed with antibodies three times and 50 μL diluted, vortexed SA-PE solution was added to each well. The plate was once again incubated for 10 minutes then washed three times with antibodies. The magnetic beads were resuspended in 125 μL Assay Buffer and added to each well of the plate, which was then shaken for 30 seconds. After removing the plate sealer, the Bio-Plex Machine then read the plate to record measurements for each of the 34 biomarkers.

All multiplex assay components, including validation kit, calibration kit, and human Bio-Plex Pro Human Cancer Biomarker Assay Panels 1 and 2 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Human vitreous samples (50 μL) were run cleanly, and the results reflect the dilution factor of 1. Samples, standards, blank, and controls were run in duplicate on the multicytokine suspension array system (Bio-Plex; Bio-Rad Laboratories), as suggested by the manufacturer's protocol. Vortex-diluted (×1) magnetic coupled beads were first added to each well of the assay plate, followed by a plate washing (×2) with wash buffer (Bio-Rad Laboratories). Samples, standards, blank, and controls (50 μL) are added to each well according to the plate layout, and incubated in the dark for an hour at room temperature with vigorous shaking. Between each incubation period, washing of the plate follows immediately (×3). Detection antibodies (×1) are added to the plate, with a 30-minute incubation period, followed by the addition of Streptavidin-PE (×1) for 10 minutes with shaking. After the final wash, the magnetic beads are resubmerged in assay, incubated, and shook for 30 seconds before the plate is analyzed via the Bio-Plex Manager Software (Bio-Plex; Bio-Rad Laboratories).

Data Analysis

Samples were divided into four groups: (1) those who had undergone vitrectomy for macular hole and/or epiretinal membrane peeling with no history of diabetes mellitus (non-DM group, $n = 29$); (2) eyes undergoing vitrectomy for macular hole or epiretinal membrane peeling with a history of diabetes or nonproliferative diabetic retinopathy (DM group, $n = 10$); (3) eyes with a history of proliferative diabetic retinopathy (PDR group, $n = 29$); and (4) eyes with neovascular glaucoma (NVG group, $n = 12$). All data organization and analysis was performed with SPSS software version 12.0 for Windows (SPSS, Inc., Chicago, IL, USA). All data was tested for normal distribution, and if given a skewed distribution of data, a nonparametric test (ANOVA test) was performed. Demographic information collected and compared included sex, age, eye, and pretreatment with bevacizumab. These were analyzed using χ^2 and Mann-Whitney U tests, respectively. Mann-Whitney U tests were used to compare individual biomarker levels between groups, as well as between pretreated and untreated eyes within the PDR and NVG groups. For all of the aforementioned tests a 2-tailed P value of less than 0.05 was deemed to be significant.

Results

Demographic

Vitreous samples of 80 eyes from 80 patients were analyzed. Table 1 displays the distribution of samples for each of the four cohorts and a comparison of demographic information. In all, 29 non-DM eyes, 10 DM eyes, 29 PDR eyes, and 12 NVG eyes were included in the study. There were no significant differences in age or sex between any of the cohorts ($P = 0.436$, $P = 0.178$). None of the patients in the non-DM or DM group received anti-VEGF treatment prior to collecting samples. There was one patient in the NVG group and four in the PDR group who received intravitreal bevacizumab within 30 days prior to surgery.

Comparative Biomarker Levels

Tables 2 and 3 display a list of all proteins analyzed with their respective Mann-Whitney U test P values comparing the mean concentrations between various subgroups. Several proteins levels were noted to be significantly elevated when comparing the non-DM group with the DM group (growth factors: G-CSF, PlGF, VEGF-D; inflammatory proteins: sCD40L, IL-6; Endoglin), the DM with the PDR group (growth factors: PlGF, VEGF-A; inflammatory proteins: IL-8; other biomarkers: leptin), and the PDR with the NVG group (growth factors: EGF, G-CSF, HB-EGF, PlGF; inflammatory proteins: IL-6, IL-8, sCD40L, TNF-α; other biomarkers: Leptin); though some showed an inverse relationship between protein level and ischemia severity. Comparison of the PDR group with the controls showed significant elevations of (1) growth factors: Angiopontin-2, EGF, Endoglin, GCSF, HB-EGF, HGF, PDGF, PlGF, sHER2/neu, sVEGF-R1, VEGF-A, and VEGF-D, (2) inflammatory proteins: IL-6, IL-8, IL-18, PECAM-1, SCF, sFASL, sIL-6Ra, and TNF-α, and (3) other biomarkers: leptin, osteopontin, PAI-1, and uPA. Comparison of the NVG group with the non-DM showed significant differences in levels of (1) growth factors: Angiopontin-2, EGF, Endoglin, GCSF, HB-EGF, HGF, IGFBP, PlGF, TIE-2, sVEGF-R1, VEGF-A, VEGF-C, and VEGF-D; (2) inflammatory proteins: IL-6, IL-8, IL-18, sCD40L, sFASL, and TNF-α; and (3) other biomarkers: leptin, osteopontin, PAI-1. Comparison of the NVG with the DM samples revealed significant differences in levels of (1) growth factors: Angiopontin-2, HGF, PlGF, VEGF-A; (2) inflammatory proteins: IL-8, IL-18, and TNF-α; and (3) other biomarkers: PAI-1. Samples with pretreatment with bevacizumab were compared against untreated samples within the same cohort using Mann-Whitney U tests. There were four

Table 2. Mean Biomarker Concentrations Arranged by Group, With SD

Biomarker Group	Biomarker	Non-DM	DM	PDR	NVG
Growth factor	Angiopoietin	128.3 ± 88.3	260.7 ± 262.0	1,389.7 ± 2,800.9	2,312.0 ± 3,277.1
	EGF	2.5 ± 0.9	3.3 ± 1.5	3.2 ± 1.1	7.7 ± 6.6
	Endoglin	17 ± 13	36 ± 23	42 ± 30	52 ± 30
	FGF-Basic	76.0 ± 21.5	83.1 ± 46.6	73.7 ± 24.8	70.1 ± 26.2
	G-CSF	15.2 ± 5.8	110.9 ± 241.4	29.8 ± 57.8	181.5 ± 232.9
	HB-EGF	1.1 ± 0.5	3.2 ± 2.6	2.9 ± 2.4	3.9 ± 1.9
	HGF	5,399.5 ± 2,917.5	5,070.0 ± 3,886.5	8,711.5 ± 4,504.2	10,230 ± 5,526.3
	IGFBP-1	410.0 ± 525.0	713.5 ± 1,387.0	584.0 ± 602.5	653.5 ± 414.0
	PDGF	23.2 ± 5.4	25.6 ± 11.3	31.9 ± 19.8	31.9 ± 18.7
	PLGF	3.5 ± 2.4	13.8 ± 16.2	42.8 ± 42.4	310.2 ± 361.6
	sEGFR	2,212.3 ± 814.4	2,994.0 ± 1,983.0	2,679.6 ± 1,168.6	2,597.0 ± 959.0
	sHER2/neu	289.1 ± 188.5	485.3 ± 379.4	466.7 ± 266.7	422.0 ± 229.0
	sVEGFR-1	1,887.0 ± 1,881.5	3,118.2 ± 5,346.8	840.4 ± 742.8	505.9 ± 506.0
	sVEGFR-2	2,506.0 ± 1,601.5	3,506.5 ± 3,378.0	2,272.5 ± 1,329.5	2,455.4 ± 1,499.8
	TGF-α	9.5 ± 17.0	3.4 ± 2.2	4.4 ± 4.3	4.8 ± 4.6
	TIE-2	1,722.6 ± 488.5	1,522.4 ± 519.6	1,512.8 ± 376.0	1,261.2 ± 342.0
	VEGF-A	36.0 ± 63.3	260.8 ± 640.4	518.8 ± 653.3	2,864.4 ± 4,531.6
	VEGF-C	73.4 ± 30.7	100.9 ± 61.4	96.4 ± 45.6	167.9 ± 132.4
	VEGF-D	59.0 ± 19.5	87.0 ± 33.0	91 ± 51.5	157.0 ± 105.0
Inflammatory proteins	IL-18	7.6 ± 5.6	11.7 ± 12.4	39.4 ± 87.4	54.3 ± 72.7
	IL-6	17.3 ± 19.7	579.7 ± 1,259.8	170.3 ± 370.5	2,858.4 ± 4,111.6
	IL-8	5.7 ± 8.8	56.4 ± 147.5	34.3 ± 43.6	171.6 ± 290.0
	PECAM-1	376.3 ± 90.3	414.1 ± 138.7	486.8 ± 158.9	474.6 ± 185.1
	sCD40L	6.9 ± 4.0	19.3 ± 14.0	18.2 ± 9.3	30.0 ± 17.0
	SCF	46.2 ± 16.5	61.2 ± 39.9	58.2 ± 23.7	61.8 ± 28.6
	sFASL	10.3 ± 2.8	18.1 ± 13.0	19 ± 15.7	27.5 ± 16.4
	sIL-6Ra	277.0 ± 191.0	541.0 ± 387.0	605.5 ± 433	393.0 ± 149.5
	TNF-α	2.2 ± 1.4	3.6 ± 2.0	4.6 ± 3.5	7.0 ± 5.2
Other biomarkers	Follistatin	121.6 ± 94.7	199.7 ± 147.6	137.5 ± 76.7	162.7 ± 141.9
	Leptin	669.3 ± 1,107.1	1,018.6 ± 1,635.0	3,143.1 ± 4,000.8	750.8 ± 377.9
	Osteopontin	51,884.0 ± 35,898.6	31,918.0 ± 18,520.9	33,134.1 ± 28,557.6	24,572.4 ± 8,529.9
	PAI-1	4,557.4 ± 4,402.2	9,337.2 ± 9,685.0	15,890.8 ± 10,302.0	20,926.6 ± 14,577.4
	Prolactin	478 ± 115	487 ± 203	481 ± 128	521 ± 256
	uPA	181.9 ± 111.3	265.7 ± 144.6	386.8 ± 368.1	339.0 ± 359.1

samples in the PDR group that were pretreated with intravitreal bevacizumab within 35 days prior to vitrectomy (mean 7.5 days prior, range, 5–14). There was one sample in the NVG with pretreatment with intravitreal bevacizumab 4 days prior to vitrectomy. There was no significant difference between those receiving pretreatment and those without within both the PDR and NVG cohorts.

Biomarker Levels Within Each Group

Scatterplots of protein levels for each group were also generated to create visual representations of the intergroup comparisons. Figure 1 shows PlGF and VEGF levels, but only PlGF had statistically significant elevations between each successive subgroup. Figure 2 shows protein levels for IL-6 and IL-8.

Discussion

Delineation of Biomarkers in NVG

The results of the present study show exponentially increasing levels of certain angiogenic and inflammatory proteins moving from non-DM to NPDR to PDR to NVG groups. The present study is one of the first to thoroughly delineate and contextualize a substantial cohort of NVG patients among a spectrum of patients with other ischemic retinopathies, including PDR, which had their samples collected and run concurrently. Few other studies have looked at protein levels

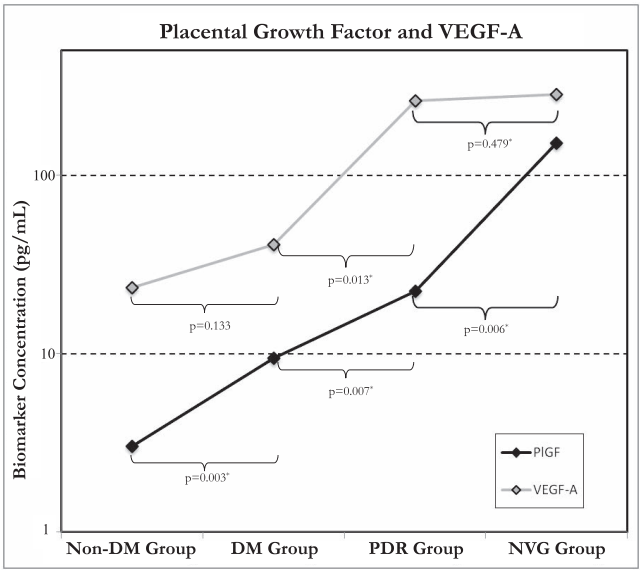

Figure 1. Comparison of biomarker concentrations (log scale in pg/mL) of PlGF and VEGF-A between non-DM, DM, PDR, and NVG groups. *Statistical significance ($P < 0.05$) as assessed by Mann-Whitney U Test.

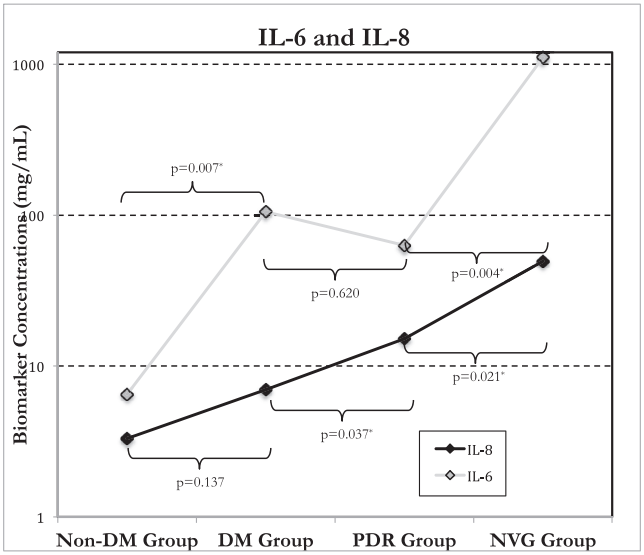

FIGURE 2. Comparison of biomarker concentrations (log scale in mg/mL) of IL-6 and IL-8 between non-DM, DM, PDR, and NVG groups. *Statistical significance ($P < 0.05$) as assessed by Mann-Whitney U Test.

with the majority of samples devoid of pretreatment with bevacizumab as well. In the present study, we had a small number of samples pretreated with bevacizumab within 35 days prior to vitrectomy, a period after which we would not expect to see significant influence of the agent, and these samples had no significant difference in their biomarker levels compared with untreated eyes within the PDR and NVG cohorts.[18,19] There have been numerous recent studies on the efficacy of bevacizumab in controlling the complications of NVG, however these focus on the clinical outcomes or levels of VEGF alone rather than delineating complete vitreous protein profiles.[20–22] Many of the studies delineating specific proteins in severe ischemic eye diseases, and specifically NVG, only assessed levels in human aqueous samples[23,24] or were performed in nonhuman models.[25] This study demonstrated trends in a number of proteins, however the following discussion will focus on some of the most notable findings.

PlGF and the VEGF Family

As expected, with increasingly severe levels of ischemic retinopathy there were significantly elevated levels of growth factors such as VEGF. Interestingly, the only protein found to have a statistically significant elevation between each successive group was PlGF. Placental growth factor is from the VEGF family of growth factors, and is known to act through a variety of modalities linked directly to VEGF and its receptors.[26] Mitamura et al.[27] found elevated levels of both PlGF and VEGF in eyes with PDR compared with non-DM eyes, hypothesizing also that PlGF's role is to augment the VEGF pathway in proliferative disease. Rakic et al.[28] looked at surgically extracted neovascular membranes and found PlGF deficiency to be associated with significantly decreased rates and severity of neovascular membrane growth. They concluded PlGF significantly increases rates and severity of neovascular disease, but, unlike VEGF, it is not essential to normal development. Others have similarly specified the presence and role of VEGF-B, VEGF-C, and PlGF in human choroidal neovascular membranes in addition to the more commonly described VEGF-A.[29] Furthering this notion, Tjwa et al.[30] reviewed the specific interaction of PlGF with VEGF-R1 on a molecular level while other studies have also demonstrated that PlGF can form heterodimers with VEGF-A,[31] activate VEGF-R1 in the absence of VEGF,[32] and even displace VEGF-A from VEGF-R1.[33] All of these mechanisms direct PlGF's mechanism of action through the same pathway dictated by VEGF and its receptors.

However, many other studies have clearly defined that PlGF has an alternative pathway through which it can act independently from VEGF,[34] though there may be a cooperatively synergistic effect as well.[21,35] Placental growth factor has been shown to promote the chemotaxis of RPE cells, in the presence of TGF-β and ultimately in the progression of fibrovascular membranes associated with diabetic retinopathy.[36] Besides the stimulation of proliferative disease PlGF has been shown to play a role in subretinal fluid accumulation and opening of the tight junctions between RPE cells, inducing retinal edema formation.[37] Outside of the retina, PlGF can mobilize bone marrow cells and inflammatory cells, helping to drive some of the inflammatory findings that we and other researchers have found to be associated with ischemic disease.[24] As such, despite the evidence that PlGF also acts through the VEGF pathway, it might be worthwhile targeting PlGF specifically given its alternative mechanisms of action in the pathogenesis of ischemic disease.

While PlGF has been delineated in eyes with PDR,[38] it has not been fully delineated across a spectrum of ischemic retinal disease until the present study. Given the literature suggesting PlGF works independently during ischemic disease pathogenesis, specifically targeting this protein may prove to be efficacious for advanced ischemic disease. Aflibercept is a more recently available antiangiogenic treatment, and unlike its anti–VEGF-A counterparts bevacizumab, an antibody, and ranibizumab, an antibody fragment, it contains domains from VEGF-R1 and VEGF-R2, which bind to PlGF in addition to VEGF-A and VEGF-B.[39,40] Given our findings of the significant elevations of PlGF in successively advancing ischemic retinopathy the use of this medication may be warranted in cases of advanced disease that are at high risk of developing NVG. Further investigation regarding the possible role of this medication in such cases is certainly needed.

Inflammation in Ischemic Retinal Disease

Our study also noted concurrent statistically significant elevations in inflammatory proteins with increasing ischemic disease as well. Some of these proteins have been documented in other studies to date, notably IL-6, IL-8, and MCP-1.[41–45] Cohen et al.[42] demonstrated an association between the inflammatory factor IL-6 and VEGF, showing IL-6 may function upstream of VEGF. Interleukin-8 has also been identified as a key mediator in ocular inflammation and ischemic disease.[46–48] Jonas et al.[49] looked at both growth factors and inflammatory proteins in patients with diffuse diabetic macular edema and found increased levels of ICAM-1, which correlated with macular thickness. Our results also indicate IL-18 levels significantly increased between the non-DM and each of the ischemic groups and between diabetic patients and NVG patients, again consistent with other published data.[50] Merendino et al.[51] and Park et al.[52] found IL-18 upregulates vascular molecule adhesion and provides for angiogenesis signaling. Similarly, in a model of ischemia and reperfusion inflammation, Qi et al.[53] found that TLR4 was a major driver of Caspase-1 activation and by extension release of IL-18 and IL-1β. Our findings are consistent with this model of ischemia/reperfusion leading to release of IL-18. As suggested by Yoshimura et al.,[8] it is possible that these inflammatory mediators are also responsible for increased vascular permeability, unlike growth factors, which primarily drive proliferative disease and a then subsequent increase in the expression of inflammatory mediators. However, despite much of this

TABLE 3. *P* Values From Comparison of Biomarker Levels Between Groups Using Mann-Whitney *U* Test

Biomarker Group	Biomarker	Non-DM vs. DM	DM vs. PDR	PDR vs. NVG	Non-DM vs. PDR	Non-DM vs. NVG	DM vs. NVG
Growth factor	Angiopontin-2	0.166	0.223	0.091	**0.000***	**0.000**	**0.008**
	EGF	0.180	0.777	**0.014**	**0.009**	**0.001**	0.075
	Endoglin	**0.013**	0.676	0.288	**0.000**	**0.000**	0.263
	FGF	0.850	0.832	0.658	0.811	0.547	0.696
	GCSF	**0.005**	0.167	**0.009**	**0.043**	**0.001**	0.241
	HB-EGF	0.052	0.620	**0.030**	**0.000**	**0.000**	0.051
	HGF	0.668	0.052	0.330	**0.003**	**0.002**	**0.030**
	IGFBP	0.973	0.167	0.194	0.069	**0.025**	0.051
	PDGF	0.460	0.490	0.595	**0.011**	0.492	0.546
	PlGF	**0.003**	**0.007**	**0.006**	**0.000**	**0.000**	**0.001**
	sEGFR	0.571	0.777	0.953	0.114	0.229	0.804
	sHER2/neu	0.440	0.860	0.637	**0.003**	0.063	0.915
	sVEGF-R1	0.605	0.254	0.215	**0.007**	**0.001**	0.097
	sVEGF-R2	0.850	0.887	0.813	0.714	0.688	0.915
	TGF-α	0.855	0.851	0.817	0.673	0.380	0.699
	TIE-2	0.295	0.915	0.051	0.064	**0.005**	0.337
	VEGFa	0.133	**0.013**	0.479	**0.000**	**0.000**	**0.021**
	VEGFc	0.304	0.939	0.095	0.063	**0.008**	0.177
	VEGFd	**0.021**	0.958	0.053	**0.001**	**0.000**	0.145
Inflammatory protein	IL-18	0.483	0.080	0.205	**0.002**	**0.002**	**0.034**
	IL-6	**0.007**	0.620	**0.004**	**0.000**	**0.000**	0.082
	IL-8	0.137	**0.037**	**0.021**	**0.000**	**0.000**	**0.012**
	PECAM-1	0.345	0.416	0.585	**0.001**	0.160	0.594
	sCD40L	**0.004**	0.983	**0.025**	**0.000**	**0.000**	0.123
	SCF	0.571	0.915	0.906	**0.048**	0.109	0.859
	sFASL	0.073	0.750	0.099	**0.038**	**0.003**	0.204
	sIL-6Ra	0.061	0.645	0.316	**0.001**	0.017	0.499
	TNF-α	0.085	0.366	**0.041**	**0.001**	**0.000**	**0.028**
Other biomarkers tested	Follistatin	0.154	0.167	1.000	0.170	0.456	0.749
	Leptin	0.525	**0.026**	**0.039**	**0.000**	**0.048**	0.188
	Osteopontin	0.172	0.937	0.442	**0.038**	**0.039**	0.749
	PAI-1	0.255	0.073	0.273	**0.000**	**0.000**	**0.036**
	Prolactin	0.797	0.887	0.929	1.000	0.774	0.972
	uPA	0.096	0.524	0.516	**0.002**	0.080	0.972

Bolded values indicate statistical significance.

prior research, no studies have delineated a clear exponential growth pattern in the levels of inflammatory proteins across a full spectrum of ischemic retinopathies, including patients with NVG, as was demonstrated in the present study.

Despite the literature suggesting the direct role in actively driving the pathogenesis of ischemic disease there is still ongoing debate as to the actual role of these inflammatory proteins in ischemic retinal disease. While in this study we have speculated as to the role of some of these proteins in driving the disease pathogenesis, based in part of some of the above research, there is certainly a body of knowledge suggesting otherwise. Sanchez et al.[54] hypothesized a protective effect of IL-6 on retinal ganglion cells in the setting of retinal ischemic reperfusion injury, finding both upregulation in microglia cells and phagocytic cells as well as assessing the role of exogenously added IL-6. Besides inflammatory markers, even VEGF-A has been hypothesized to play a role in neuroprotection during ischemic injury.[55] Further research to better clarify these findings is needed, but readers should also note that the present study serves only to delineate associations and not causality in the context of ischemic disease.

Clinical Relevance of Retinal Inflammation

The direct relationship between levels of certain inflammatory proteins and increasingly severe ischemic disease established in this study, notably with elevations between successive cohorts and prominently the NVG group in IL-6 and IL-8 among other proteins, suggests a role for the use of steroids or other anti-inflammatory agents in conjunction with current anti-growth factor therapies. It is logical that the use of glucocorticoids might help decrease the inflammation associated with the increasing ischemic disease, despite a dearth of evidence demonstrating efficacy in reducing interleukin levels.[56] However, Arjamaa et al.[57] identified that despite elevated levels of IL-6 in patients with PDR, there was not a concurrent upregulation of NF-kB or HIF-1α, which suggests that in such ischemic diseases perhaps a molecular target of the proteins might work better than glucocorticoids. One nonglucocorticoid and nonmolecular approach could be the use of minocycline, which has recently been investigated for its anti-inflammatory properties in the retina during ischemia-reperfusion and branch retinal vein occlusion (BRVO) via the inhibition of microglial activation and apoptosis inhibition.[58,59] Minocycline has also been shown to reduce many of the side effects of diabetic ischemic disease thought to be associated with the aforementioned inflammatory cascade.[60,61] Specifically targeting inflammatory cytokines, whether through any of the above mechanisms, may yield improved clinical outcomes in severe ischemic disease.

Despite the significant findings, the present study is not without its limitations. It must be noted that the cytokines

measured represent only a single data point in a dynamic and likely wide-ranging disease process for each individual. Furthermore, lumping groups of patients into disease cohorts likewise represents a similarly oversimplified representation of each disease process, which may have been sampled at differing stages of their disease. As was aforementioned in the discussion of inflammatory biomarkers like IL-6, the present study only serves to correlate levels of biomarkers with various stages of disease rather than delineating causal relationships. Further research certainly needs to be conducted to more clearly elucidate what role these various biomarkers may actually play in the various stages of ischemic retinal disease.

While there were a substantial number of eyes sampled, these were not evenly distributed amongst the four cohorts. Statistical analysis would have been better served by greater numbers of DM and NVG patients, and more samples in general. The NVG cohort also was not composed of just eyes from patients only with progression of their PDR, but rather also included eyes with NVG due to central retinal vein occlusion (CRVO) and central retinal artery occlusion (CRAO). Though they exist on a spectrum of ischemic retinal disease, ideally future studies would be able to include only patients with NVG secondary to specific retinal diseases. Finally, a few of the eyes in this study were treated preoperatively with anti-VEGF agents (though no steroids), though except for VEGF-A levels in the NVG group this did not appear to significantly affect cytokine levels. While this increases the heterogeneity of the study cohort being analyzes, it does in many ways create cohorts more reflective of actual practices. Future prospective studies would be best served by creating larger homogenous cohorts for comparison.

In conclusion, there are a number of novel findings from the delineation of the biomarkers associated with NVG, including the prominence of inflammatory proteins and PlGF in severe ischemic disease as reported by this paper. Further studies are necessary to confirm our findings and to test therapeutic treatment strategies in cases of severe retinal ischemia and NVG.

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