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Serial Aqueous Humor Proteomics in Diabetic Macular Edema

Correlation of Novel Targets with Retinal Fluid Volume

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Objective: Diabetic macular edema (DME) with incomplete response to anti-VEGF therapy is a significant challenge. Here, we integrated advanced proteomics and an artificial intelligence (AI)-driven platform to elucidate molecular mechanisms associated with resistance to anti-VEGF treatment.

Design: Translational longitudinal study.

Subjects: Patients with DME (n = 30) under treatment with anti-VEGF therapy who had reached their plateau phase.

Methods: Aqueous humor samples were obtained at the time of 6 consecutive anti-VEGF injections. OCT scan fluid volumes were assessed by an AI-based platform (Discovery OCT Fluid and Biomarker Detector, RetinAI). Proteomics analysis of the samples collected during the first 3 time points was performed by label-free liquid chromatography-tandem mass spectrometry. Confirmatory enzyme-linked immunosorbent assay (ELISA) was performed for afamin and VEGF for time points 4 to 6.

Main Outcome Measures: Differentially expressed aqueous humor proteins and their correlation with AI-quantified OCT fluid volumes.

Results: Proteomics identified 202 proteins in ≥70% of the samples. Ten proteins were consistently differentially regulated in patients with substantial edema versus no edema. Upregulated proteins included afamin (highest fold change 1.8 ± 0.4), alpha-1B-glycoprotein, and angiotensinogen, whereas cystatin-C, clusterin, apolipoprotein E, epidermal growth factor-containing fibulin-like extracellular matrix protein, Ig lambda chain V-I, latent transforming growth factor beta-binding protein 2, and reelin were downregulated. Afamin levels correlated with fluid volumes (total, inner retinal, inner macular ring). The upregulation of afamin was confirmed by ELISA.

Conclusions: In this hypothesis-generating study using serial aqueous humor proteomics, multiple novel targets were identified that correlated with retinal fluid volumes. Specifically, afamin, a wingless/integrated (Wnt)-signaling carrier protein and established biomarker of type 2 diabetes, was consistently upregulated in eyes with anti-VEGF-refractory DME. Whether this reflects active Wnt pathway involvement in eyes demonstrating anti-VEGF treatment resistance or vascular dysfunction warrants additional study. Concurrent downregulation of cystatin-C and clusterin suggests impaired endogenous anti-inflammatory pathways in eyes with refractory DME.

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Diabetic macular edema (DME) is an important cause of visual loss and may occur in any stage of diabetic retinopathy (DR).[1–3] The global prevalence of diabetes is projected to reach 600 million by 2030, and the number of DME cases is expected to grow substantially in the coming decades.[4] Diabetic macular edema is a significant burden due to its recurrent nature and the need for frequent retreatments and follow-up visits. In general, patients with DME have multiple contacts with the health care system.[5] Intravitreal inhibitors of VEGF are most often used as first-line therapy in the management of DME, whereas intravitreal corticosteroid such as dexamethasone and fluocinolone implants can be a suitable alternative.[6] Persisting DME despite anti-VEGF therapy is a meaningful and common clinical challenge.[7] An increasing body of evidence supports the use of quantitative and qualitative OCT features to predict visual and anatomic outcomes with treatment.[8] For example, a number of OCT features including hyperreflective foci and subretinal fluid have been proposed to be indicators of an inflammatory response in DME.[8]

In addition to OCT biomarkers, integration of proteomic analyses of biofluids may be able to move the field forward by identifying novel potential markers of disease activity

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and facilitating a more personalized approach to DME management. The aqueous humor is a key biofluid for studying intraocular disease mechanisms because it reflects the local ocular microenvironment[9] and can be obtained via anterior chamber paracentesis. In DME, aqueous humor biomarkers can be used to identify inflammatory, angiogenic, and metabolic pathways implicated in treatment resistance or responsiveness. Proteins altered by chronic hyperglycemia (advanced glycation end-products, cytokines, protein-kinase C, hypoxia-inducible factor-alpha) may contribute to anti-VEGF resistance via inflammation, oxidative stress, and VEGF-independent pathways.[10–12] Addressing these mechanisms with adjunctive therapies may be able to improve outcomes in patients with refractory DME.

Proteomics studies aim to identify and quantify the complete set of proteins in a given body fluid or tissue.[13,14] Previous studies using proteomics approaches have identified a multitude of proteins associated with treatment naïve DME,[15,16] but little is known about proteome changes associated with DME with incomplete response to anti-VEGF therapy. Artificial intelligence (AI)-derived software has great potential in providing precise quantification of OCT biomarkers in retinal disease.[17–19] Despite significant advances, no previous reports have integrated AI-derived OCT analyses in DME with proteomics. Here, we combined the 2 techniques to elucidate biological processes associated with incomplete response to anti-VEGF therapy in DME. We aimed to identify proteins associated with DME that are refractory to anti-VEGF treatment and hypothesized that proinflammatory proteins and those related to glycemic control would be differentially expressed.

Methods

Patient Characteristics

The study was approved by the Advarra Institutional Review Board, located in Columbia, Maryland (Pro00057096). A signed informed consent was obtained from each patient before inclusion, and the research adhered to the Declaration of Helsinki. All patients were recruited from Retina Consultants of Texas. All patients had DME and were undergoing intravitreal anti-VEGF therapy with 4- to 6-week intervals and were in their plateau phase (Table 1).

Aqueous humor samples were obtained at 6 consecutive intravitreal injections, where aqueous sampling was performed before each anti-VEGF injection. Volumes of 50 to 100 μL of aqueous humor were collected and immediately stored at −80°C until analysis.

Spectral domain OCT was performed at each visit before aqueous sampling and intravitreal injection. OCT was performed by Heidelberg Spectralis OCT and analyzed by an AI-based platform (Discovery OCT Fluid and Biomarker Detector, RetinAI AG)[17–19] that was validated against the manual grading by expert human graders with expertise and experience in annotating anatomical structures on OCT and fundus images.[20,21] The software was used to assess the total fluid volume, intraretinal, and subretinal fluid as well as foveal center and inner macular ring fluid volumes were calculated.

Patients were categorized into 3 groups based on DME fluid volume measurements. These anatomically defined phenotypes included (1) substantial edema (n = 9), defined as mean macular ring fluid volume >150 nL and total fluid volume >200 nL; (2) no-to-minimal edema (n = 11), defined as macular ring fluid volume <30 nL and total fluid volume <250 nL; and (3) intermediate edema (n = 10) for remaining cases.

Samples collected at the first 3 time points were used for proteomics analysis by mass spectrometry, whereas samples collected at the last 3 timepoints were used for enzyme-linked immunosorbent assay (ELISA).

Mass Spectrometry

The volume of the aqueous humor samples was measured, and an equal volume of 2× lysis buffer (10% sodium dodecyl sulfate, 100 mM triethylammonium bicarbonate, pH 8.5) was added. The total amount of protein was estimated using infrared spectrometry (Direct Detect). Of each sample, up to 100 μg protein was digested with trypsin using the S-Trap micromethod (Protifi). The amount of generated peptide was estimated by tryptophan fluorescence. Peptides were finally dissolved in 0.1% formic acid to a concentration of 0.2 μg/μL. Of each sample, 0.5 μg (2.5 μL) was injected into the mass spectrometer. Generally, duplicate injections were performed with several days to weeks of intermission except for 1 sample that was analyzed 3 times. The samples were analyzed using the universal method on an Orbitrap Fusion Tribrid mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano liquid chromatograph (Thermo Fisher Scientific Instruments Inc). The total analysis generated 226 samples, which were entered into MaxQuant[22] version 1.6.5.0 for search in the *Homo sapiens*[23] database downloaded on August 10, 2022. The average of the median coefficient of variation among the analyzed samples was 12.9%. Filtration of the proteomics data was performed as previously described,[24] requiring successful quantification in ≥70% of the samples in each group.

ELISA

Aqueous samples were thawed and divided into aliquots of 50 μL and stored at −80°C until analysis. Proteins were quantified by sandwich ELISA, according to the manufacturer's protocols. Samples were diluted 500-fold for afamin (ELH-AFM-1, RayBiotech) and 2-fold for VEGFA (DVE00, R&D Systems). Values below the limit of detection were excluded.

Statistical Analysis

In Table 1, clinical variables with numeric values were tested for normality and significant differences in standard deviations. Based on this, 1-way analysis of variance was applied to normally distributed values with equal variances (age, body mass index), whereas a chi-square test was used for categorical or nominal data. For comparison of fluid volumes (Tables 1 and 3), Kruskal-Wallis tests were used to assess differences among all groups, whereas Dunn multiple comparison tests were used for comparisons between no edema and substantial edema. All mass spectrometry data were log2-transformed, and differences between no edema and substantial edema were tested with an unpaired *t* test. Enzyme-linked immunosorbent assay data were not transformed, and differences were tested with a Mann-Whitney test. A P value <0.05 was considered statistically significant. Spearman rank correlation analysis was used to investigate significant correlations between protein concentrations and the amount of fluid. Moderate correlations were defined as coefficients between 0.400 and 0.599, and strong correlations >0.600. Venn diagrams were generated with a

Table 1. Patient Characteristics Categorized on Level of Edema, Based on the First 3 Time Points

Characteristic	No Edema (N = 11)	Intermediate (N = 10)	Substantial (N = 9)	*P* Value	*P* Value No vs Substantial
Age, median (Q1 to Q3), yrs	57.9 (55.8 to 67.1)	59.3 (48.4 to 64.7)	57.9 (46.7 to 72.7)	0.741	0.999
Sex				0.935	
Female (%)	4 (36)	4 (40)	4 (44)		
Diabetes mellitus				0.597	
Type 1 (%)	1 (9)	1 (10)	0		
Type 2 (%)	9 (82)	9 (90)	9 (100)		
Prediabetes (%)	1 (9)	0	0		
Duration of DM until sample 1, median (Q1 to Q3), yrs*	24 (21 to 31)	26 (25 to 31)	20 (16 to 33)	0.771	0.826
HbA1c, median (Q1 to Q3), mmol/mol†	7.1 (6.3 to 7.2)	6.9 (6.7 to 7.3)	6.8 (6.8 to 7.0)	0.935	0.938
BMI, median (Q1 to Q3)	31.3 (27.7 to 34.9)	33.8 (30.3 to 33.9)	29.8 (26.3 to 32.4)	0.741	0.768
DR type				0.526	
NPDR (%)	4 (36)	6 (60)	5 (56)		
PDR (%)	7 (64)	4 (40)	4 (44)		
Anti-VEGF treatment				0.235	
Aflibercept 2 mg (%)	9 (82)	5 (50)	7 (78)		
Faricimab (%)	2 (18)	5 (50)	2 (22)		
Panretinal photocoagulation				0.014‡	>0.999
No (%)	10 (91)	4 (40)	8 (89)		
Yes (%)	1 (9)	6 (60)	1 (11)		
Fluid volumes, median (Q1 to Q3), nL					
Total fluid volume	20 (5 to 27)	117 (70 to 170)	642 (544 to 713)	<0.001‡	<0.001‡
Inner retina (IRF)	12 (4 to 21)	91 (70 to 151)	635 (544 to 708)	<0.001‡	<<0.001‡
Subretinal (SRF)	0	0	0 (0 to 5)	0.591	0.979
PED (nL)	0	0 (0 to 1)	0	0.667	>0.999
Foveal center	1 (0 to 5)	13 (8 to 16)	76 (40 to 122)	0.001‡	<0.001‡
Inner macular ring	5 (1 to 14)	63 (41 to 96)	319 (262 to 410)	<0.001‡	<0.001‡

BMI = body mass index; DM = diabetes mellitus; DR = diabetic retinopathy; HbA1c = glycated hemoglobin; NPDR = nonproliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy; PED = pigment epithelial detachment; PRP = panretinal photocoagulation; Q1 and Q3 = first and third quartile values.
*In group with no and intermediate edema, respectively, 1 and 3 with unknown duration.
†In group with substantial edema, 1 value unknown.
‡*P* values <0.05 are statistically significant.

freely available online tool of the University of Gent, Belgium (https://bioinformatics.psb.ugent.be/webtools/Venn/).

Results

Patient Characteristics

Among 30 patients with DME receiving anti-VEGF therapy, serial aqueous humor samples were collected and analyzed (Table 1). Patients were divided into 3 groups based on intraretinal fluid volumes (Fig 1): no edema (n = 11), intermediate edema (n = 10), and substantial edema (n = 9). No differences were found between groups for age, sex, type of diabetes, body mass index, DR severity, duration of diabetes mellitus, and glycated hemoglobin levels (Table 1). The intermediate-edema group had more cases treated with panretinal photocoagulation compared with the other groups, whereas no difference in the prevalence of panretinal photocoagulation was observed between the no-edema and substantial-edema groups. Fluid volumes were significantly different between groups for total fluid, inner retina fluid, foveal center fluid, and inner macular ring fluid. Fluid volumes for subretinal fluid and pigment epithelial detachment fluid were negligible in all 3 groups.

Identification of Proteins with Differential Levels between Patients with No Edema versus Substantial Edema

The groups with substantial edema and no edema could generally be separated based on their protein profiles. The overall macular status assessed as total fluid volume and macular ring volume was widely reflected in the aqueous humor proteome. We started with the analysis of proteins in aqueous humor samples of the first 3 time points by mass spectrometry. A total of 202 proteins were identified and quantified in ≥70% of the samples in each group. A total of 26, 48, and 43 proteins were significantly differentially regulated at time points 1, 2, and 3, respectively (Table S1–S3, available at www.ophthalmologyscience.org). A total of 10 proteins were found to be consistently differentially regulated in substantial edema versus no edema at all time points (Fig 2, Table 2). Upregulation was observed for 3 proteins: afamin, alpha-1B-glycoprotein, and angiotensinogen (Table 2, Fig 3A). Downregulation was observed for 7 proteins: cystatin-C, clusterin,

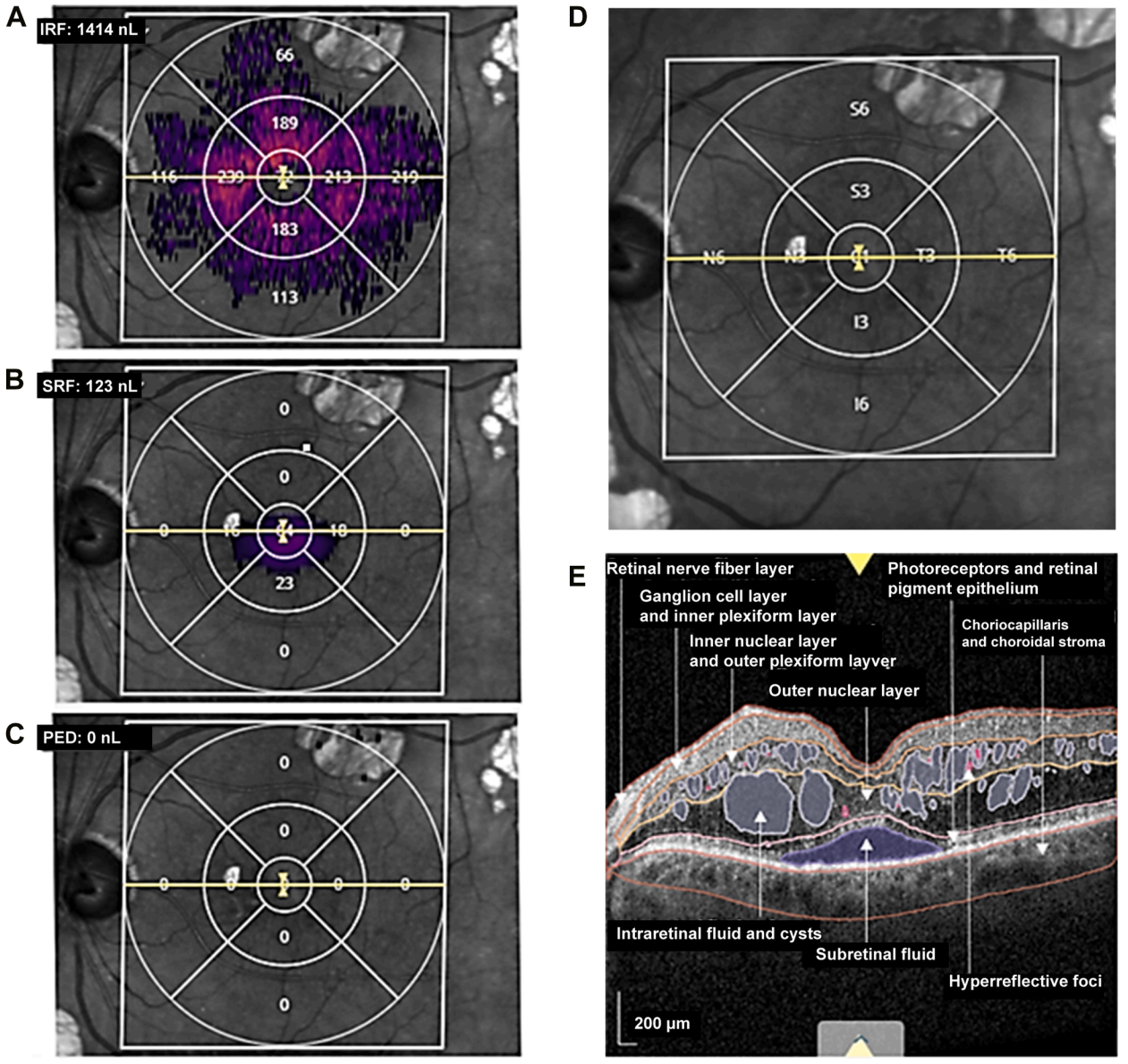

Figure 1. RetinAI Discovery platform. OCT images were analyzed using a segmentation model that quantified fluid volumes and categorized them as **(A)** intraretinal fluid (IRF), **(B)** subretinal fluid (SRF), or **(C)** pigment epithelial detachment (PED). **D,** The standard ETDRS grid divided the retina into 9 regions, and the values for the central (C1), inner macular ring (C3 + S3 + T3 + N3 + I3), and total (IRF + SRF + PED) fluid volumes were used to assess fluid quantities. **E,** The RetinAI segmentation model was trained using high-quality, manually annotated OCT images to segment key retinal layers, and these annotations served as inputs to a convolutional neural network, enabling the development of an automated segmentation model that accurately measures thickness, volume, and defects. Structures and layers in the retina are indicated. The yellow triangles indicate the position of the fovea.

apolipoprotein E, epidermal growth factor-containing fibulin-like extracellular matrix protein, Ig lambda chain V-I, latent transforming growth factor beta-binding protein 2, and reelin (Table 2, Fig 3B).

Verification of Afamin Protein Levels

Because the difference in fold change between substantial and no edema was highest for afamin, we then pursued verification of our results by ELISA. For this assay, we used aqueous humor samples from the subsequent 3 consecutive clinical time points. OCT-based retinal fluid volumes were again significantly different between the patient groups (Table 3). Increased concentrations of afamin in the substantial edema groups as compared with the no-edema group were confirmed by ELISA (Fig 3C). In addition, VEGFA levels were assessed by ELISA in these samples to determine whether afamin levels were related to

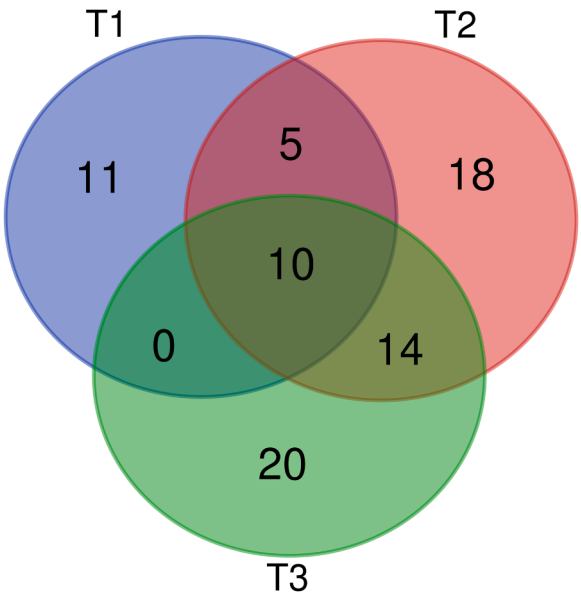

Figure 2. Overlap of significantly regulated proteins across the first 3 time points. Venn diagram showing proteins with significantly different levels between no edema and substantial edema groups at each time point. Ten proteins were common to all 3 time points (listed in Table 2).

VEGFA levels, and no statistically significant difference in VEGFA levels between the substantial edema and no edema groups was observed (Fig 3D).

Correlation of Protein Levels with Retinal Fluid Volumes

Protein levels of afamin were then compared with the different types of fluid volumes. Weak correlations were found for afamin with total retinal fluid, intraretinal fluid, and foveal fluid volumes, and a moderate correlation with macular ring fluid volume in the first 3 time points together (Fig 3, Table 4). Among the other proteins that were significantly differentially regulated at all time points, Ig lambda chain V-I showed a weak negative correlation with macular ring fluid volume (Fig 4, Table 4). In the subsequent 3 time points together, a statistically significant but weak correlation for afamin with total retinal fluid ($\rho = 0.286$; $P = 0.020$) and intraretinal fluid ($\rho = 0.287$; $P = 0.019$) was found, whereas no significant correlation was identified between VEGFA and any of the fluid volumes.

Discussion

In the current study utilizing serial proteomics analyses of aqueous humor samples from patients receiving anti-VEGF therapy for DME, 2 major phenotypes were studied: one group with substantial and persistent intraretinal fluid despite anti-VEGF therapy, and another with near complete resolution of DME with anti-VEGF therapy. Across both groups, aqueous humor VEGF levels (approximately 100 pg/mL in both groups) showed no correlation with OCT parameters and aligned with published baseline values in untreated DME.[25] Although our measurements, taken 1 month postinjection during the plateau phase of management, did not assess the degree or rate of VEGF change from baseline, and although the equilibrium between anterior chamber and vitreous cavity VEGF levels remains incompletely defined, the observed disparity in anatomic responses implies VEGF-independent mechanisms being relevant to the persistent intraretinal fluid observed in the current cases. Specifically, proteins involved in alternative pathways including inflammatory mediators likely contribute to persistent edema, highlighting critical pathways to consider in order to understand and potentially address anti-VEGF-A therapeutic resistance.

Afamin was upregulated at all time points in patients with substantial macular edema despite anti-VEGF therapy. An increased level of afamin has previously been reported in aqueous humor samples from DME patients,[15] but an increased level of afamin has not previously been reported in eyes with DME that are refractory to anti-VEGF therapy. Afamin is a human plasma glycoprotein of the albumin family[26] and has emerged as a significant biomarker in diabetes research, particularly for type 2 diabetes (T2D) and metabolic dysfunction.[27–29]

Table 2. Overlapping Proteins with Significant Differences between No Edema and Substantial Edema in the First 3 Time Points. Fold Changes Are Presented per Time Point (T1 to T3)

Gene Name	Protein Name	T1	T2	T3
AFM	Afamin	1.70	2.25	1.37
A1BG	Alpha-1B-glycoprotein	1.57	1.64	1.56
AGT	Angiotensinogen	1.52	1.60	1.38
CST3	Cystatin-C	0.66	0.56	0.53
CLU	Clusterin	0.66	0.59	0.71
APOE	Apolipoprotein E	0.63	0.64	0.69
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	0.56	0.62	0.63
IGLV1	Ig lambda chain V-I	0.54	0.51	0.30
LTBP2	Latent transforming growth factor beta-binding protein 2	0.29	0.27	0.42
RELN	Reelin	0.17	0.26	0.29

EGF = epidermal growth factor.

Table 3. Summary of Fluid Volumes for T4-6, Categorized on Level of Edema

Characteristic	No (N = 11)	Intermediate (N = 10)	Substantial (N = 9)	P Value No vs. Intermediate	P Value No vs Substantial
Fluid volumes, median (Q1 to Q3), nL					
Total fluid volume	16 (5 to 47)	108 (35 to 149)	432 (410 to 537)	<0.001*	<0.001
Inner retina (IRF)	11 (3 to 29)	89 (35 to 148)	429 (410 to 533)	<0.001*	<0.001*
Subretinal (SRF)	0	0	0	0.693	>0.999
PED (nL)	0	0	0	0.767	>0.999
Foveal center	0 (0 to 10)	6 (0 to 12)	39 (20 to 136)	0.021*	0.024*
Inner macular ring	3 (1 to 17)	28 (17 to 104)	214 (138 to 273)	<0.001*	<0.001*

IRF = intraretinal fluid; PED = pigment epithelial detachment; Q1 and Q3 = first and third quartile values; SRF = subretinal fluid.
*P values <0.05 were considered statistically significant.

Kollerits et al[28] measured afamin levels in 8 prospective cohort studies involving >20 000 patients and identified afamin as a marker of prevalent T2D and a predictor of incident T2D. Serum afamin concentrations were higher in individuals with longstanding diabetes compared with those with a shorter disease duration. Additionally, afamin levels correlated with waist circumference, small-dense low-density lipoprotein, fasting blood sugar, and insulin resistance.[30] Afamin has also been implicated in gestational diabetes, suggesting a role in glucose metabolism during pregnancy.[31,32] Supporting these findings, transgenic mice that overexpress afamin exhibited increased body weight, elevated cholesterol and triglyceride levels, and hyperglycaemia.[29] Collectively, these observations indicate that afamin contributes to lipid accumulation, metabolic dysfunction, and the development of insulin resistance and T2D.

Although our study identified afamin as a potential marker of persistent DME, its possible mechanistic role in DME remains to be elucidated. Afamin functions as an extracellular chaperone, stabilizing lipid-modified wingless/integrated (Wnt) proteins (e.g., Wnt3a, Wnt5a) in a 1:1 complex.[33] This interaction preserves Wnt solubility and biological activity, facilitating binding to cell-surface Frizzled receptors and subsequent gene activation.[33]

Notably, inhibition of Wnt signaling with intravitreal injection of the Wnt inhibitor DKK1 was reported to reduce retinal inflammation, vascular leakage, and pathological neovascularization.[34] More recently, EYE101, a tetravalent, biologic Wnt agonist and Norrin-mimetic, was reported to demonstrate efficacy in eyes with DME in the absence of VEGF inhibition, and this therapeutic is being studied in an ongoing global phase III program (ClinicalTrials.gov identification numbers: NCT06957080 and NCT06571045). Additional studies will be required to explore if afamin has a mechanistic role in DME and potential as a therapeutic target.

Although afamin's involvement in Wnt signaling is well-established, its potential contribution to DME development and treatment resistance remains unclear. Several key questions deserve investigation: whether afamin plays a pathogenic role in DME, functions as a compensatory factor, or simply represents an epiphenomenon of vascular leakage. The observed elevation of afamin in the serum of T2D patients, coupled with the absence of evidence for local ocular production, suggests rather a secondary effect.

Nine additional proteins showed differential expression between the groups with substantial versus no-to-minimal edema. A number of protective proteins including cystatin-C and clusterin were reduced in the substantial edema group. Previous proteomics studies suggest that the downregulation of cystatin-C and clusterin reflects a failed compensatory response in retinal vascular disease rather than a mere depletion through anti-VEGF therapy. In aqueous humor from patients with treatment naïve central retinal vein occlusion, we previously identified a down-regulation of cystatin-C and clusterin, which both correlated negatively with best-corrected visual acuity and the severity of macular edema.[24] In a follow-up study integrating proteomics with AI-driven OCT biomarker analysis, cystatin-C was found to correlate negatively with the intraretinal fluid volume, whereas clusterin correlated negatively with the volume of subretinal fluid.[35]

Cystatin-C is a protease inhibitor, primarily expressed in retinal pigment epithelium,[36] that protects against vascular damage. Cystatin-C knockout mice show exacerbated ischemia/reperfusion injury with degenerate capillaries and disrupted inner blood-retinal barrier via zonula occludens-1 downregulation. In primary human retinal endothelial cells grown under hyperglycemic conditions, cystatin-C reduces permeability and inflammatory mediators.[37] Clinically, aqueous humor cystatin-C levels inversely correlate with DR severity and central retinal thickness in DME[38] and with macular edema severity in central retinal vein occlusion.[15] These findings are in agreement with our results and suggest cystatin-C deficiency may contribute to treatment-refractory DME through impaired vascular permeability regulation.

Clusterin, an extracellular chaperone protein, may protect against retinal vascular disease. In oxygen-glucose–deprived retinal endothelial cells, it preserves tight junctions and reduces cell death.[39] Its levels are reduced in vitreous humor from proliferative DR[40] and aqueous humor from central retinal vein occlusion, where it inversely correlates with macular edema severity and best-corrected visual acuity.[24] This downregulation may contribute to anti-VEGF-refractory DME by impairing vascular stability.

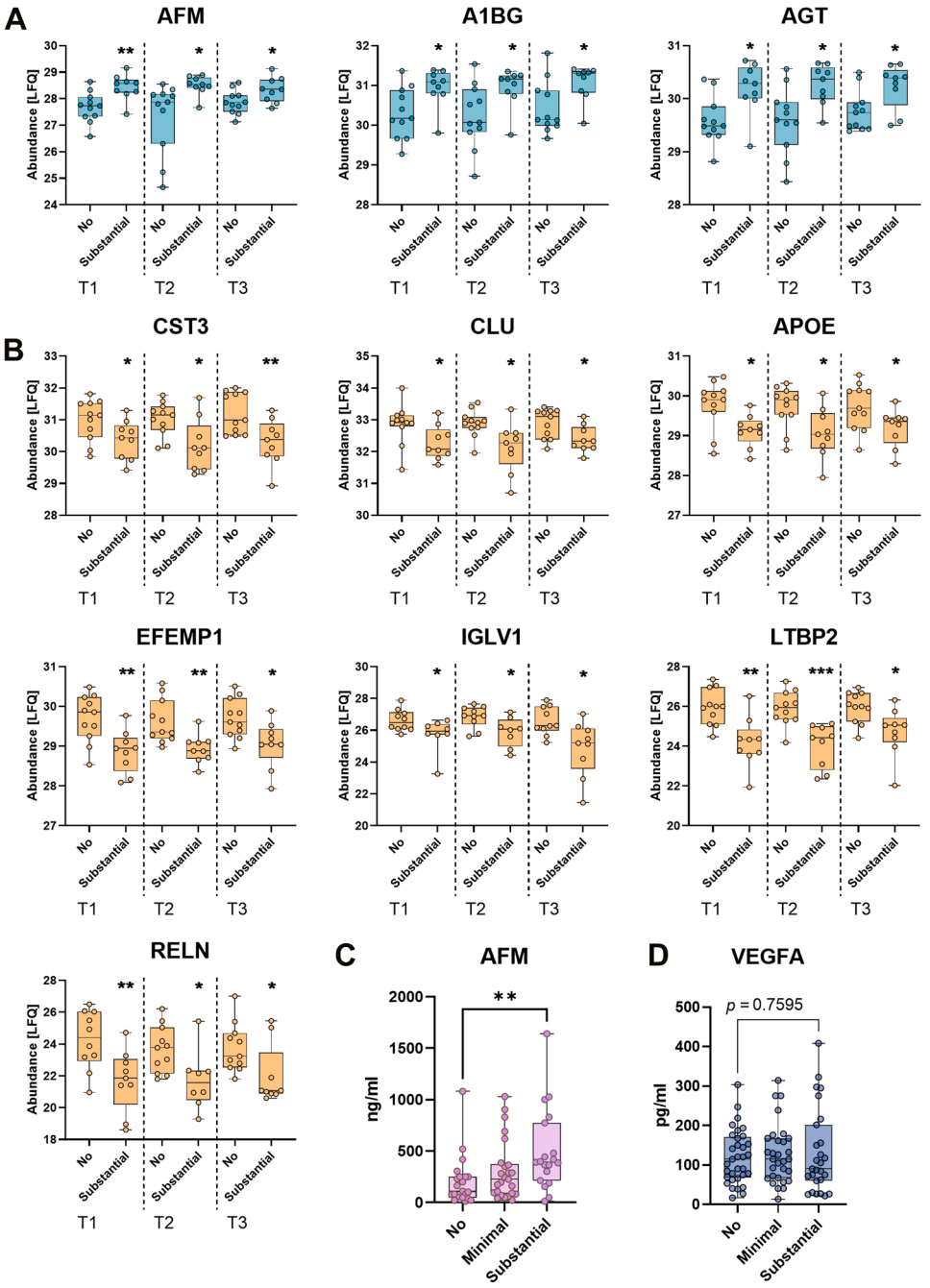

Figure 3. Abundance of proteins significantly associated with incomplete response to anti-VEGF therapy. A total of 10 proteins were found to be significantly regulated in substantial edema versus no edema. **A,** Upregulated proteins determined by mass spectrometry at time points 1 to 3 (T1-T3) included afamin (AFM), alpha-1B-glycoprotein (A1BG), and angiotensinogen (AGT). **B,** Downregulated proteins included cystatin-C (CST3), clusterin (CLU), apolipoprotein E (APOE), epidermal growth factor-containing fibulin-like extracellular matrix protein (EFEMP1), Ig lambda chain V-I (IGLV1), latent transforming growth factor beta-binding protein 2 (LTBP2), and reelin (RELN). **C,** The increased level of afamin was confirmed by ELISA. **D,** VEGFA concentration assessed by ELISA. There was no significant difference in VEGFA between the substantial edema and no edema groups. Box plots indicate median, min and max and first and third quartiles. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ELISA = enzyme-linked immunosorbent assay; LFQ = Label-Free Quantification.

Although reelin's association with retinal vascular disease is established,[15,24] its role in DR remains unclear. This extracellular matrix protein has been reported to be downregulated in aqueous humor samples from treatment-naïve DME patients versus controls[15] and in central retinal vein occlusion, where it inversely correlates with macular edema severity and best-corrected visual acuity.[24] Further studies are required to elucidate whether reelin has protective effects in retinal vascular disease.

Table 4. Correlations with Fluid Volumes for T1 to T3. Spearman Rho and *P* values Are Given

Protein		Total Fluid	IRF	SRF	PED	Foveal Center	Inner Macular Ring
AFM	Rho	0.301*	0.341*	0.107	−0.083	0.279*	0.429*
	P value	0.004*	<0.001*	0.318	0.438	0.008*	<0.001*
A1BG	Rho	0.096	0.164	0.026	−0.065	0.000	0.076
	P value	0.370	0.123	0.806	0.542	0.998	0.474
AGT	Rho	0.124	0.190	0.140	−0.106	0.059	0.130
	P value	0.244	0.072	0.189	0.319	0.581	0.223
CST3	Rho	−0.040	−0.071	−0.206	−0.057	−0.032	−0.121
	P value	0.707	0.504	0.051	0.591	0.763	0.255
CLU	Rho	−0.116	−0.162	−0.114	0.022	−0.008	−0.073
	P value	0.275	0.126	0.283	0.835	0.940	0.495
APOE	Rho	−0.100	−0.121	−0.146	0.130	−0.102	−0.112
	P value	0.346	0.255	0.171	0.223	0.336	0.292
EFEMP1	Rho	−0.006	−0.073	−0.131	0.169	−0.052	−0.135
	P value	0.958	0.491	0.217	0.112	0.629	0.204
IGLV1	Rho	−0.071	−0.097	−0.008	−0.073	−0.179	−0.240*
	P value	0.516	0.377	0.944	0.507	0.101	0.027*
LTBP2	Rho	−0.002	−0.101	0.036	0.049	−0.040	−0.171
	P value	0.984	0.342	0.740	0.646	0.708	0.107
RELN	Rho	−0.032	−0.101	−0.094	0.037	−0.078	−0.139
	P value	0.766	0.354	0.386	0.736	0.474	0.199

AFM = afamin; AGT = angiotensinogen; A1BG = alpha-1B-glycoprotein; APOE = apolipoprotein E; CLU = clusterin; CST3 = cystatin-C; EFEMP1 = epidermal growth factor-containing fibulin-like extracellular matrix protein 1; IGLV1 = Ig lambda chain V-I; IRF = inner retinal fluid; LTBP2 = latent transforming growth factor beta-binding protein 2; PED = pigment epithelial detachment fluid; RELN = reelin; SRF = subretinal fluid.
*Statistically significant correlations.

Apolipoprotein E, expressed in multiple retinal cell types, participates in extracellular matrix (ECM) remodeling, and protects against ECM impairment.[41] Although serum apolipoprotein E levels serve as risk factors for DR incidence and severity,[42] its role appears complex. Apolipoprotein E influences DR progression through lipid metabolism, inflammation, and genetic polymorphisms, although reported effects remain inconsistent.[42]

Angiotensinogen is a precursor protein of the renin-angiotensin-aldosterone system (RAAS), which plays a vital role in systemic vascular control. The retina is one of the few tissues with local RAAS secretion, supporting the hypothesis that RAAS contributes to DR development and progression.[43] Indeed, RAAS inhibitors have been shown to reduce DR risk, and vitreous angiotensinogen levels are elevated in proliferative DR patients compared with diabetic controls without proliferative DR.[44–46]

Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 is implicated in vascular permeability regulation, particularly in the retina, through multiple mechanisms involving VEGF signaling, ECM integrity, and complement system interactions.[47–49]

IGLV1-derived lambda light chains (e.g., IGLV1-40, -44, -36) are implicated in vascular permeability in POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal plasma cell disorder, and skin changes)

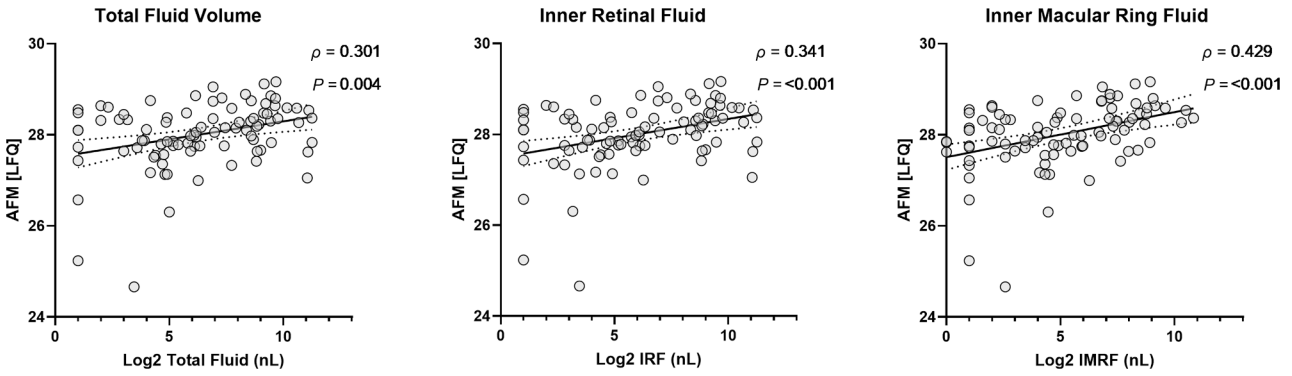

Figure 4. Correlation of afamin concentrations with fluid volumes. Correlation between total fluid volume, inner retinal fluid (IRF), and inner macular ring fluid (IMRF) with concentrations of AFM at timepoints 1 to 3 together (n = 90) is shown. A best-fit linear regression line with 95% confidence bands (dotted line) is shown. Spearman correlation coefficient (ρ) and *P* values (*P*) are given, with $P < 0.05$ being statistically significant. AFM = afamin; LFQ = Label-Free Quantification.

syndrome, a rare paraneoplastic disorder. Monoclonal plasma cells producing these light chains drive VEGF overproduction, leading to vascular leakage that manifests as volume overload and papilledema.[50] The IGLV1 light chains exhibit restricted clonality and disease-progressive mutations. Though not yet documented in retinal vasculature, their systemic permeability effects suggest potential relevance to retinal pathology.[50]

Latent transforming growth factor beta-binding protein 2 does not seem to have a direct, well-documented connection to vascular permeability or DR. Latent transforming growth factor beta-binding protein 2 is primarily associated with ECM integrity and microfibril organization.[51]

Our study represents a hypothesis-generating early discovery stage in the search for proteins associated with incomplete response to anti-VEGF therapy. We applied the predefined criteria of consistent and statistically significant regulation across 3 consecutive timepoints. This requirement excluded the majority of proteins and reduced the likelihood of findings identified by chance. There is very limited availability of consecutively obtained aqueous humor samples from DME patients with incomplete response to anti-VEGF therapy. Within the confines of the sample size of the current hypothesis-generating study, the application of traditional correction for multiple hypothesis testing would introduce a real risk of resulting in failure to identify true positives.[52] This challenge has been described in detail in experimental proteomics settings.[53] Analogously, comparison of large, complex data sets such as that in the current study implies a risk of falsely identified findings that seem "significant" without being relevant or biologically plausible. To address this risk, the current work intentionally focused on evaluating each identified protein and pathway within the context of existing literature, biological plausibility, and internal consistency across analyses, rather than broad statistical correction across all tests. Our approach was modeled after the approach that was recommended in Kenneth Rothman's seminal paper on this topic.[54]

Successful biomarker discovery or establishment of novel therapeutic targets involves several studies of independent cohorts.[55,56] Proteomics studies are typically followed up by studies of larger independent cohorts where a few selected proteins are quantified by immunoassays or targeted mass spectrometry.[57]

Wolf et al[9] recently conducted a study on aqueous humor and vitreous samples using the aptamer-based proteomics assay (SomaScan Assay v4.1, Somalogic),[58] which is based on short single-stranded, chemically modified DNA molecules, specifically binding to their protein targets. The proteomic setup was combined with single-cell RNA sequencing. Using this multi-omics approach, Wolf et al[9] identified 1920 cell-type marker proteins providing the expression in their respective cell types. The approach identified angiogenic proteins associated with DR and could be used to bring further insights into DME with incomplete response to anti-VEGF therapy. Likewise, the approach described by Wolf et al[9] could potentially be used to validate the proteins in a larger cohort.

Conclusion

Our serial aqueous humor proteomic analysis identified 202 proteins consistently detected across the samples, with 10 proteins showing significant dysregulation among patients with anti-VEGF-refractory DME. Among these, afamin demonstrated the most pronounced upregulation (1.8-fold increase), correlating strongly with intraretinal fluid accumulation, a relationship confirmed by ELISA and independent of VEGF concentrations. Our study identified afamin as a potential marker of persistent DME, but the mechanistic role of afamin in DME remains to be established. Notably, the concurrent downregulation of cystatin-C and clusterin points to a possible loss of endogenous anti-inflammatory and vascular-protective mechanisms in refractory DME. Together, these findings highlight distinct protein signatures associated with persistent edema despite anti-VEGF therapy, offering new insights into the molecular underpinnings of treatment resistance. Given the hypothesis-generating nature of the study, validation in larger, independent cohorts is essential to substantiate our findings.

Footnotes and Disclosures

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Data Availability: Mass spectrometry data can be obtained by contacting the authors.

HUMAN SUBJECTS: Human subjects were included in this study. The study was approved by the Advarra Institutional Review Board, located in Columbia, Maryland (Pro00057096). A signed informed consent was obtained from each patient before inclusion and the research adhered to the Declaration of Helsinki.

No animal subjects were used in this study.

Author Contributions:

Conception and design: Cehofski, Klaassen, Wykoff

Data collection: Cehofski, Klaassen, Cao, Zhou, Ørskov, Vorum, Wykoff, Honoré

Analysis and interpretation: Cehofski, Klaassen, Cao, Zhou, Ørskov, Vorum, Wykoff, Honoré

Obtained funding: Cehofski, Wykoff, Honoré

Overall responsibility: Cehofski

Abbreviations and Acronyms:
AI = artificial intelligence; **DME** = diabetic macular edema; **DR** = diabetic retinopathy; **ECM** = extracellular matrix; **ELISA** = enzyme-linked immunosorbent assay; **RAAS** = renin-angiotensin-aldosterone system; **T2D** = type 2 diabetes; **Wnt** = wingless/integrated.

Keywords:
Afamin, Anti-VEGF therapy, Diabetic retinopathy, Retinal thickness, Wnt signaling.

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