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Proteomic Analysis of Autoimmune Retinopathy Implicates Neuronal Cell Adhesion Molecule as a Potential Biomarker

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Purpose: To identify vitreous molecular biomarkers associated with autoimmune retinopathy (AIR).

Design: Case-control study.

Participants: We analyzed 6 eyes from 4 patients diagnosed with AIR and 8 comparative controls diagnosed with idiopathic macular holes (IMHs) and epiretinal membranes (ERMs).

Methods: Vitreous biopsies were collected from the participants and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) or multiplex enzyme-linked immunoassay (ELISA).

Main Outcome Measures: Protein expression changes were evaluated by 1-way analysis of variance (significant P value < 0.05), hierarchical clustering, and pathway analysis to identify candidate protein biomarkers.

Results: There were 16 significantly upregulated and 17 significantly downregulated proteins in the vitreous of 3 patients with AIR compared with controls. The most significantly upregulated proteins included lysozyme C, zinc-alpha-2-glycoprotein, complement factor D, transforming growth factor-ß (TGF-ß)—induced protein, beta-crystallin B2, and alpha-crystallin A chain. The most significantly downregulated proteins included DIP2C, retbindin, and amyloid beta precursor-like protein 2. Pathway analysis revealed that vascular endothelial growth factor (VEGF) signaling was a top represented pathway in the vitreous of patients with AIR compared with controls. In comparison with a different cohort of 3 patients with AIR analyzed by multiplex ELISA, a commonly differentially expressed protein was neuronal cell adhesion molecule (NrCAM) with P values of 0.027 in the LC-MS/MS dataset and 0.035 in the ELISA dataset.

Conclusions: Protein biomarkers in the vitreous, such as NrCAM, may eventually help diagnose AIR. *Ophthalmology Science 2022;2:100131 Published by Elsevier on behalf of the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).*

Autoimmune retinopathy (AIR) is a rare inflammation-mediated retinopathy that leads to retinal degeneration and results in vision loss.[1-4] The clinical presentation of AIR includes symptoms of photoreceptors' dysfunction, including decline in visual acuity, central and midperipheral ring scotomas, loss of contrast sensitivity, nyctalopia, impaired dark adaptation, photopsias, and peripheral visual field defects.[3,4] However, ocular examination is usually unremarkable initially and can show signs of retinal degeneration in later stages, such as retinal pigment epithelium changes, diffuse retinal atrophy, retinal vascular attenuation, and optic disk pallor.[3,4] Electroretinography is abnormal and shows irregularities of rod and cone responses.[3] Autoimmune retinopathy is divided into paraneoplastic, cancer-associated retinopathy, melanoma-associated retinopathy, and non-paraneoplastic AIR.[4]

Antiretinal antibodies (e.g., anti-recoverin and anti-enolase antibody) are detected in the serum of patients with AIR and are thought to lead to apoptotic retinal cell death.[3,4] However, the pathogenicity of most antibodies has not been well established.[4,5] In addition, these antibodies are not specific for AIR and can be detected in healthy individuals, in patients with systemic autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis, inflammatory bowel disease, and Behcet's disease, and in patients with other eye diseases, such as inherited retinal degenerations (retinitis pigmentosa and cone-rod dystrophy), acute zonal occult outer retinopathy, posterior uveitis, and age-related macular degeneration.[3,4,6] Furthermore, different laboratories use different techniques to detect antiretinal antibodies,[3] and concordance analysis of testing results between 2 laboratories showed only 60% concordance for any antibody detection and 36% antibody-specific concordance.[5] Therefore, diagnosing AIR is challenging and frequently delayed, which may lead to severe vision loss.

These multiple issues limit the ability of antiretinal antibodies to function as a diagnostic biomarker for AIR and make the search for additional diagnostic biomarkers, which can be used alongside these antibodies and other diagnostic tests, a necessity.[4,6] Proteomic profiling of human vitreous is a powerful tool for identifying new biomarkers of

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inflammatory of retinal diseases. In a study of various posterior segment uveitides,[7] we used a multiplex enzyme-linked immunosorbent assay (ELISA) platform and identified interleukin (IL)-23 subunit alpha, platelet-derived growth factor receptor ß, E-cadherin, triggering receptor expressed on myeloid cells 1, and IL-1 receptor type 1 as potential biomarkers of AIR. In this study, we used an unbiased proteomic platform liquid chromatography-tandem mass spectrometry (LC-MS/MS), to identify additional candidate biomarkers associated with AIR.

Methods

Study Approval

The study was approved by the Stanford University and University of Iowa Institutional Review Board and adhered to the tenets set forth in the Declaration of Helsinki (Institutional Review Board: 201803853). All participants provided written informed consent.

Clinical Examination

Subjects underwent eye examinations that included slit-lamp examination, dilated retinal bio-microscopy, indirect ophthalmoscopy, OCT, and electroretinography. Vitreous biopsies were obtained from 3 eyes of 2 patients with AIR undergoing Retisert (fluocinolone acetonide implant; Bausch & Lomb) implantation. Each patient in our study underwent an extensive inflammatory and infectious laboratory workup that included complete blood cell count, complete chemistry panel, urinalysis, erythrocyte sedimentation rate, levels of C-reactive protein, antinuclear antibodies, antiphospholipid antibody, rheumatoid factor, interferon-γ release, human leukocyte antigen B27, titers for Lyme disease, syphilis antibody screen, hepatitis B virus panel, hepatitis C surface antigen, and chest x-ray. Genetic testing was performed with retinal testing panels (Blueprint) and by whole exome sequencing.[8] Additionally, we used the AIR diagnostic criteria set forth by Fox et al.[9] On the basis of these guidelines, patients enrolled in our study met the following criteria: (1) no history or examination findings indicative of another apparent cause of visual function abnormality; (2) the presence of synaptic signaling abnormalities on electroretinography; (3) the presence of serum antiretinal antibodies; and (4) the absence of overt intraocular inflammation. Additional supportive criteria included signs/symptoms of photoreceptor dysfunction, personal/family history of autoimmune disease or uveitis, and the rapid onset of vision changes.[9] Vitreous biopsies were also obtained from 3 control subjects with idiopathic macular hole (IMH) and analyzed using mass spectrometry as detailed later in the "Methods" section. We reanalyzed our previously published data of a separate group of 3 AIR eyes and 5 control subjects with epiretinal membranes (ERMs) and IMHs. Vitreous samples of this cohort were analyzed using multiplex ELISA as previously described (Table 1).[7]

Sample Collection

All participants provided written informed consent to undergo a vitrectomy that included collection of undiluted vitreous samples, and there were no vitreous biopsy-related complications. Pars plana vitrectomy was performed using a single-step transconjunctival 23 or 25-gauge trocar cannular system (Alcon Laboratories Inc.), and an undiluted 0.5-ml sample of the vitreous was manually aspirated into a 3-ml syringe. Vitreous samples were immediately centrifuged in the operating room at 15 000*g* for 5 minutes at room temperature to remove impurities and stored at −80°C, as previously described.[10]

Mass Spectrometry

Mass spectrometry−based measurements were performed in duplicate for control and AIR vitreous samples (Table 1; Cohort 1). An LC-MS/MS approach was used for the relative quantitation and simultaneous identification of proteins. Vitreous protein concentration was measured using the Qubit Protein Quantification assay (Thermo Fisher), and 20 μg protein per sample was precipitated in chloroform-methanol and dissolved in 50 mM ammonium bicarbonate with 0.1% Rapigest detergent. Trypsin was then added to each sample at a ratio of 1:40 enzyme/protein and digested overnight at 37°C. The reaction was quenched by adding 90% formic acid to a final concentration of 2%. A total of 1 μg of trypsin-digested peptide per sample (5 μl) was then injected into a 10-cm ChromXP C18 reverse phase analytical column. High-performance liquid chromatography was performed on a NanoLC Eskigent high-performance liquid chromatography pump with a linear gradient of Buffer B (0.1% formic acid, 98% acetonitrile) to Buffer A (5% acetonitrile, 0.1% formic acid) at a flow rate of 0.2 μl/min. Mass spectrometry was performed on a Q Exactive HF Hybrid Quadrupole-Orbitrap (Thermo Fisher) with a nano-LC electrospray ionization source (ThermoFinnigan). Full mass spectrometry data were recorded over a 400 to 100 m/z range (positive ion mode). Data-independent acquisition was used to generate mass spectrometry data within a 25 Da fixed window. Biognoysis Spectronaut Pulsar was used to search the data-independent approach data. The human Uniprot database was used in the database search. Positive identification was set at 1% peptide false discovery rate. K-Nearest Neighbor imputation was for missing values. The mass spectrometry proteomics data have been deposited to Mendeley Data with the dataset identifier 10.17632/t7yrm5x3ws.1.

Multiplex ELISA

We analyzed a separate group of 3 AIR eyes and 5 control subjects with ERMs and IMHs (Table 1; Cohort 2). Vitreous samples of this cohort were analyzed using multiplex ELISA as previously described.[7] Briefly, vitreous cytokine signaling proteins were measured using the Human Cytokine Quantibody Array 4000 (RayBio) per the manufacturer's protocol. This array concurrently detected and processed 200 human proteins. First, the array chips were incubated with sample diluents for 30 minutes at room temperature to act as a block. Vitreous sample volumes under 500 μl were diluted with 1× lysis buffer (RayBiotech) to reach a required sample volume. Vitreous (100 μl; 4 technical replicates per sample) was then added to the wells of the array and incubated overnight at 4 C. A standard protein dilution was added to the wells of the array to determine protein concentrations. For signal detection, 80 μl of Cy3-streptavidin was added to each well, rinsed, and visualized by laser scanner. The RayBio Analysis Tool (RayBio) was used for protein classification. Final protein concentrations (in pg/ml) were corrected for sample dilution.

Statistical and Bioinformatics Analysis

Results were saved in Excel as .txt format and were uploaded into the Partek Genomics Suite 6.5 software package. The data were normalized to log base 2 and compared using 1-way analysis of variance. For better interpretation, all proteins with nonsignificant or less significant ($P > 0.05$) changes were eliminated from the analysis. The significant values were mapped using the cluster based on significant genes' visualization function with the standardization option chosen. Gene ontology analysis was performed in PANTHER.[11,12] Pie charts were created for the visualization of

Table 1. Patient Demographics

Patient	Sex	Age, yrs*	Eye	Eye Disease(s)	Systemic Disease(s)	ARA(s)	Symptom Duration	Additional Criteria	Genetic Testing	Laboratory Testing	Treatment before PPV	PPV Indication
Cohort 1 (LC-MS/MS)												
1†	F	61	OD	AIR, possible CAR, high myopia	Metastatic colon cancer	α-CAII, α-GAPDH, α-HSP27	1.5 yrs	Abnormal ERG, mild a-wave reduction, delayed b-waves; VF depression	Negative	Negative	IVT	Retisert implantation
	F	62	OS								Ozurdex	Retisert implantation
2	F	64	OD	AIR, ERM, recurrent CME	Hypothyroidism	α-Arrestin	3 yrs	Abnormal ERG, diminished cone response	Negative	Negative	IVT	Retisert implantation, recurrent CME
3	F	65	OD	IMH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Macular hole
4	M	66	OD	IMH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Macular hole
5	M	68	OD	IMH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Macular hole
Cohort 2 (Multiplex ELISA)												
1	F	64	OD	AIR, ERM, recurrent CME	Hypothyroidism	α-Arrestin	3 yrs	Abnormal ERG, diminished cone response	Negative	Negative	IVT	Retisert implantation, recurrent CME
2	F	83	OS	AIR, dry AMD, geographic atrophy	N/A	α-Enolase	1 yr	Abnormal ERG, diminished rod response; VF depression	Negative	Negative	IVT	Retisert implantation
3	M	77	OD	AIR, POAG	History of cutaneous melanoma s/p excision, additional cancer workup negative	α-Enolase, α-CAII	2 mos	Abnormal ERG, mildly decreased mfERG signal; VF depression	Negative	Negative	IVT	Retisert implantation
4	M	68	OS	ERM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Membrane peeling
5	F	74	OD	ERM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Membrane peeling
6	F	74	OS	ERM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Membrane peeling
7	M	65	OS	IMH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Macular hole
8	M	61	OS	IMH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Macular hole

AIR = autoimmune retinopathy; AMD = age-related macular degeneration; ARA = anti-retinal antibody; CAR = cancer-associated retinopathy; CAII = carbonic anhydrase II; CME = cystoid macular edema; ELISA = enzyme-linked immunosorbent assay; ERG = electroretinogram; ERM = epiretinal membrane; IMH = idiopathic macular hole; IVT = intravitreal triamcinolone; LC-MS/MS = liquid chromatography-tandem mass spectrometry; mfERG = multi-focal electroretinogram; N/A = not available; OD = right eye; OS = left eye; POAG = primary open-angle glaucoma; PPV = pars plana vitrectomy; VF = visual field.

*At the time of surgery.

†Multiple samples from the same patient.

gene ontology distributions within the list of proteins under the Batch ID search menu. Pie charts were created for each gene ontology term category including biological process, molecular function, and cellular component. Reactome Pathway Analysis software was used to determine the most significant pathways.[13]

Results

Case Summary

A total of 6 eyes from 4 patients diagnosed with AIR underwent proteomic analysis using 2 separate platforms (i.e., mass spectrometry and multiplex ELISA). The clinical and demographic information are described in Table 1. Briefly, the average patient age was 71.3 years, and the AIR cohort was 75% female. Each patient reported subjective vision loss or nyctalopia. Participants underwent an extensive clinical and laboratory workup that excluded infectious, inherited, or other autoinflammatory etiologies of posterior uveitis and met the AIR diagnostic criteria set forth by Fox et al ("Methods" section),[9] including detection of abnormal electroretinography. Fluorescein angiography studies showed no evidence of retinitis, choroiditis, retinal vasculitis, or optic neuritis. One patient with AIR had metastatic colorectal cancer. Each patient reported subjective improvement in vision after administration of sub-Tenon's triamcinolone and later elected to undergo surgical implantation of Retisert (fluocinolone acetonide intravitreal implant) in lieu of systemic immunosuppression. No patient had been on oral immunosuppressive medications, and there was a washout period of at least 6 weeks between the last intraocular corticosteroid treatment and surgery. Vitreous biopsies were performed at the time of surgery. Comparative control samples were collected from patients undergoing vitrectomy for IMH or ERM repair. No patients in the control group had a history of cancer.

Mass Spectrometry Reveals Differential Protein Expression in AIR Vitreous Compared with Controls

The vitreous samples of Cohort 1 underwent trypsinization and liquid chromatography before analysis by tandem mass spectrometry (LC-MS/MS). A data-independent approach was used for simultaneous quantitation and identification of proteins for both patient groups.[14] In control samples, there were 343 ± 69 unique proteins (mean ± standard deviation; n = 3). The AIR samples presented with 412 ± 54 unique proteins (mean ± standard deviation; n = 3). Protein intensities from the LC-MS/MS data were analyzed by 1-way analysis of variance to identify significantly differentially expressed proteins (Fig 1A, B). A total of 33 proteins were differentially expressed between AIR and control samples (16 upregulated, 17 downregulated; $P < 0.05$). The most significantly upregulated proteins include lysozyme C, zinc-alpha-2-glycoprotein, complement factor D, and transforming growth factor-ß (TGF-ß)—induced protein. Four crystallins (beta-crystallin A3, beta-crystallin B1, beta-crystallin B2, and alpha-crystallin A chain) were also upregulated. The most significantly downregulated proteins included DIP2C, retbindin, and amyloid beta precursor like protein 2. DIP2C is a member of the disconnected DIP2 family with 2 other isoforms: DIP2A and DIP2B. Although little is known about DIP2C, its isoform DIP2A is expressed in retinal ganglion cells (RGCs), retinal nerve fiber layer (RNFL), inner nuclear layer, and outer nuclear layer, and has been thought to play a role in synapse formation and axon guidance.[15]

Comparative Dataset Analysis Confirms Depletion of NrCAM in AIR Vitreous

Different proteomic platforms typically identify nonoverlapping protein biomarkers due to the different detection methods. To further characterize the proteomics of AIR, we interrogated our prior proteomics data on AIR vitreous from a separate cohort (Table 1; Cohort 2), which was analyzed by multiplex ELISA (Fig 1C, D).[7] The reanalysis of "omics" data in the context of new comparative datasets is an important method, especially for rare diseases, like AIR. The only commonly differentially expressed protein between the 2 datasets was neuronal cell adhesion molecule (NrCAM) with P values of 0.027 in the LC-MS/MS dataset and 0.035 in the ELISA dataset (Fig 2). We reviewed other vitreoretinal diseases, such as proliferative vitreoretinopathy, infectious endophthalmitis, lens-induced uveitis, and intermediate uveitis, that used the same proteomic platforms and found that NrCAM was not significantly downregulated in these conditions (Table 2). Neuronal cell adhesion molecule facilitates cell—cell communication via interacting with several molecules extracellularly and intracellularly[16] and plays an important role in the development of RGCs.[17,18] Antibodies against enolase, a protein expressed in RGCs, are frequently detected in patients with AIR,[3] suggesting that RGCs may be one of the cell types impaired in AIR.

Gene ontology analysis was conducted to classify differentially expressed proteins by their biological processes, molecular functions, and cellular compartments.[19] Gene ontology analysis was further separated into upregulated and downregulated proteins to better characterize the changes in the vitreous due to AIR (Fig 3A). When differentially expressed proteins in the mass spectrometry dataset were classified by biological process, the most represented category was "cellular process." Within this category were proteins involved in cell adhesion, such as TGF-ß—induced protein ig-h3 and spondin-1. Metabolic process proteins that were downregulated included triosephosphate isomerase and carboxypeptidase E. Proteins related to binding were both upregulated and downregulated, yet proteins related to structural molecular activity and molecular transducer activity were only downregulated. Molecular pathway analysis was used to further identify functionally linked proteins in AIR vitreous (Fig 3B, C). When analyzing the differentially expressed upregulated proteins from AIR samples, the top represented pathways were blood coagulation ($P = 0.022$) and vascular endothelial growth factor (VEGF) signaling ($P = 0.033$; Fig 2B). Antithrombin-III and alpha-crystallin

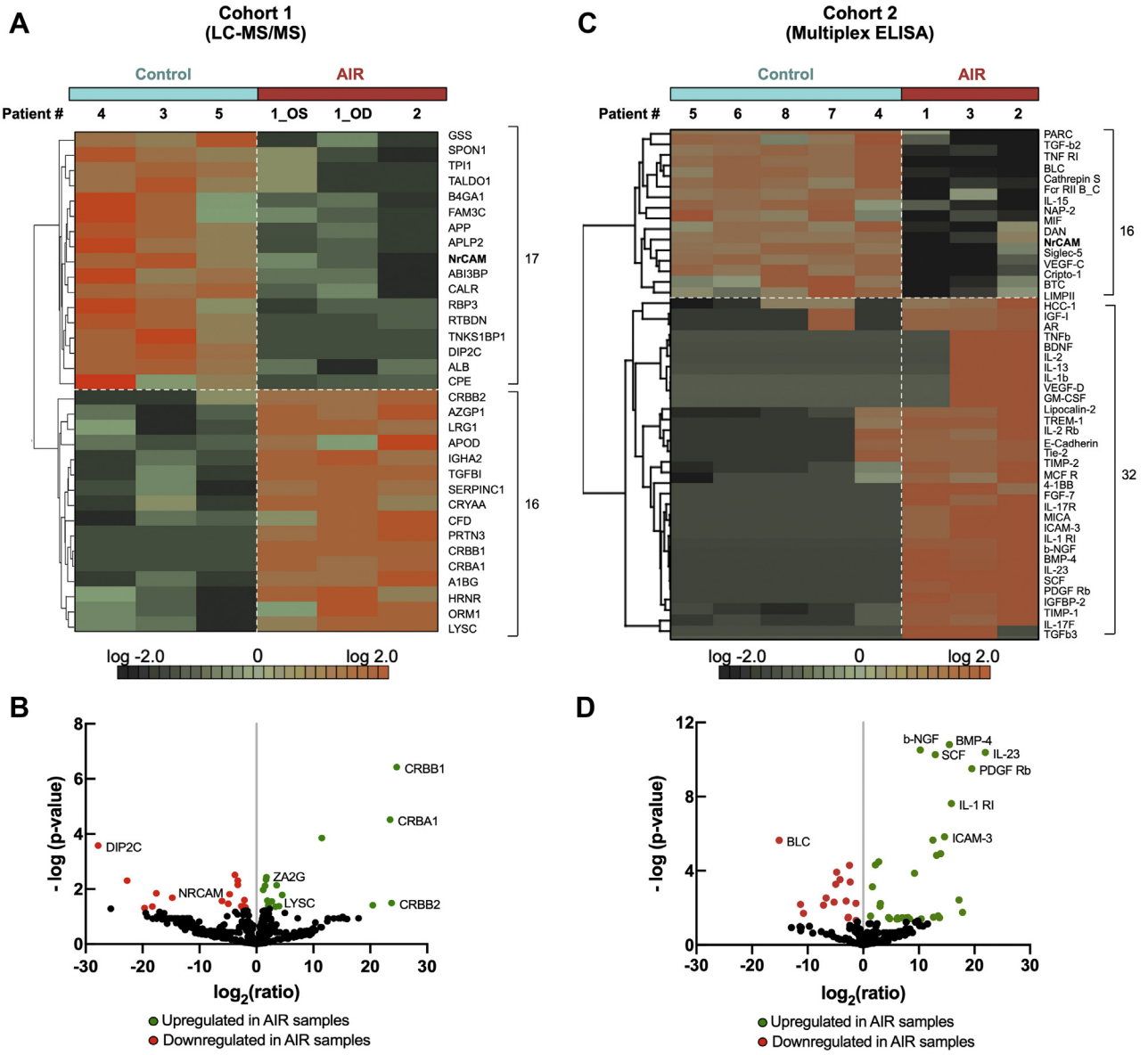

Figure 1. Proteomic analysis of autoimmune retinopathy (AIR) vitreous reveals differentially expressed proteins: differentially expressed proteins in AIR vitreous using mass spectrometry. Protein intensities were compared using 1-way analysis of variance. **A,** Hierarchal clustering of proteins differentially expressed in our AIR samples compared with controls. Results are represented as a heatmap and display protein expression levels on a logarithmic scale. Orange indicates high expression, and dark green/black indicates low or no expression. **B,** Protein fold-changes are represented as a volcano plot. The horizontal axis (x-axis) displays the log2 fold-change value, and the vertical axis (y-axis) displays the noise-adjusted signal as the -log10 (P value) from the analysis. A total of 16 proteins were upregulated, and a total of 17 proteins were downregulated ($P < 0.05$). **C,** Differentially expressed proteins in a second cohort of AIR vitreous using multiplex enzyme-linked immunosorbent assay (ELISA). Protein concentrations (pg/ml) were analyzed using 1-way analysis of variance and hierarchal clustering. **D,** Protein fold-changes are represented as a volcano plot, a total of 32 proteins were upregulated, and a total of 16 protein were downregulated ($P < 0.05$). LC-MS/MS = liquid chromatography-tandem mass spectrometry; OD = right eye; OS = left eye.

A chain were the proteins identified in these pathways, respectively.

Discussion

Autoimmune retinopathy represents a diagnostic challenge due to multiple limitations in the currently available diagnostic tests. The detection of serum antiretinal antibodies has low specificity, and such antibodies are often found in patients without AIR.[6] Therefore, additional disease biomarkers are needed. This proteomics study investigated and identified several candidate protein biomarkers that potentially can, alongside antiretinal antibodies and other clinical tests, aid in the diagnosis of AIR. Although LC-MS/MS and multiplex ELISA are 2 different methods for analyzing protein expression levels, both methods showed that NrCAM was downregulated in the vitreous of patients

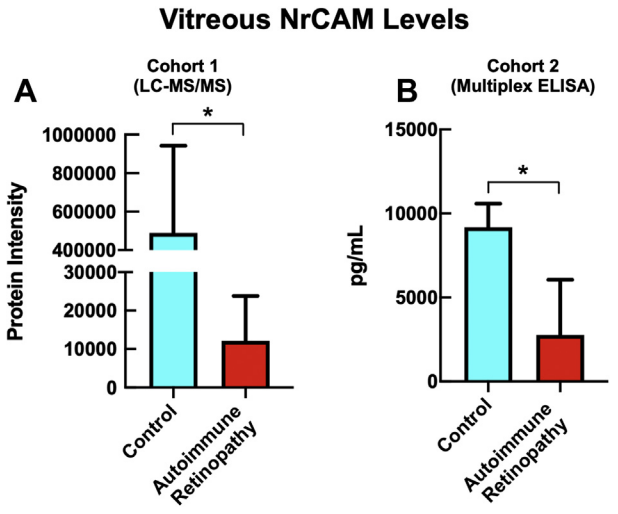

Figure 2. The neuronal cell adhesion molecule (NrCAM) levels are depleted in autoimmune retinopathy vitreous: **(A)** Differential expression of NrCAM in Cohort 1 (liquid chromatography-tandem mass spectrometry [LC-MS/MS]) and **(B)** Cohort 2 (multiplex enzyme-linked immunosorbent assay [ELISA]). Results are displayed as a bar graph and represent the mean ± standard deviation. Asterisks denote statistical significance with *P* values of 0.027 and 0.035 in the LC-MS/MS and ELISA datasets, respectively.

with AIR, which suggests that this protein may be an important biomarker for AIR. Neuronal cell adhesion molecule is a neuronal cell adhesion protein that belongs to the L1 family of immunoglobulin super family.[16] It plays multiple roles in nervous system development, including cell proliferation and differentiation, axon growth and guidance, synapse formation, and formation of the myelinated nerve structure.[16] Cerebrospinal fluid levels of NrCAM are decreased in Alzheimer's disease, and NrCAM has been suggested as a biomarker for this disease and mild cognitive impairment.[20] The decreased levels of NrCAM in the vitreous of patients with AIR could be due to axonal damage or other unknown mechanisms.

The loss of NrCAM protein expression suggests potential loss of RGC and RNFL. Although earlier imaging studies showed that the thickness of only the outer retinal layers is decreased in patients with AIR,[21,22] a more recent study by Sepah et al[23] showed that the thickness of RNFL and RNFL + ganglion cell layers are also decreased in patients with AIR and that greater RNFL tissue loss is observed in patients who were positive for the anti-enolase antibody, one of the most frequently detected antibodies in patients with AIR.[3] Enolase is expressed in RGCs and plays a role in their function.[24] It was not possible to evaluate the longitudinal change in the thickness of RGC and RNFL in our patients with AIR because images were obtained using different machines and protocols, and at different time points. Further studies are needed to evaluate the thickness of RGC and RNFL in patients with AIR, which could help determine if molecular biomarkers and imaging biomarkers correlate.

Photoreceptor and immune-signaling biomarkers were differentially expressed in the vitreous of patients with AIR and may be molecular links to disease mechanisms. BIGH3, or TGF-ß—induced protein, is an extracellular matrix protein expressed in retinal pigment epithelium cells, and its disruption is involved in photoreceptor survival.[25,26] Destabilized variants of lysozyme lead to disruption in the development of the eye and retina,[27] and early apoptosis of rod photoreceptors in mice.[28] Lysozyme C is a cornerstone of innate immunity and plays an essential role in immune modulation in both physiological and pathological conditions.[29] Complement factor D is one of the serine proteases that regulates the activation of the alternative complement system in multiple autoimmune diseases.[30,31] Zinc alpha-2 glycoprotein is a protein that structurally possesses a class I major histocompatibility complex fold and may be a biomarker of proliferative diabetic retinopathy.[32] Crystallins are heat shock proteins expressed in the lens and retina, and play an important role in suppressing protein denaturation and aggregation in the retina to maintain its homeostasis.[33] Antibodies against heat shock protein 70 are one of the retinal autoantibodies detected in patients with AIR.[3] Our study revealed increased levels of both beta-crystallin B2 and alpha-crystallin A chain. The multiplex ELISA dataset in our study showed that the levels of IL-23 subunit alpha, platelet-derived growth factor receptor beta, E-cadherin, triggering receptor expressed on myeloid cells 1, and IL-1 receptor type 1 were elevated. A prior study showed differential expression of multiple cytokines in the plasma of patients

Table 2. Comparative Vitreous Expression of NrCAM in Other Disease States

Disease	Proteomic Platform	No. of Eyes (n)		NrCAM Expression Compared with Controls		
		Control	*Disease*	*Fold-change*	*Description*	P *Value*
AIR	LC-MS/MS	3	3	−65.5212	Downregulated	0.027*
	ELISA	5	3	−6.74481	Downregulated	0.035*
PVR	ELISA	3	7	−2.66245	Downregulated	0.321
Endophthalmitis	LC-MS/MS	4	6	−8.63231	Downregulated	0.138
LIU	LC-MS/MS	4	9	−6.75834	Downregulated	0.293
NIIU	LC-MS/MS	4	4	−6.84121	Downregulated	0.212

AIR = autoimmune retinopathy; ELISA = enzyme-linked immunosorbent assay; LC-MS/MS = liquid chromatography-tandem mass spectrometry; LIU = lens-induced uveitis; NIIU = noninfectious intermediate uveitis; NrCAM = neuronal cell adhesion molecule; PVR = proliferative vitreoretinopathy.
*$P < 0.05$.

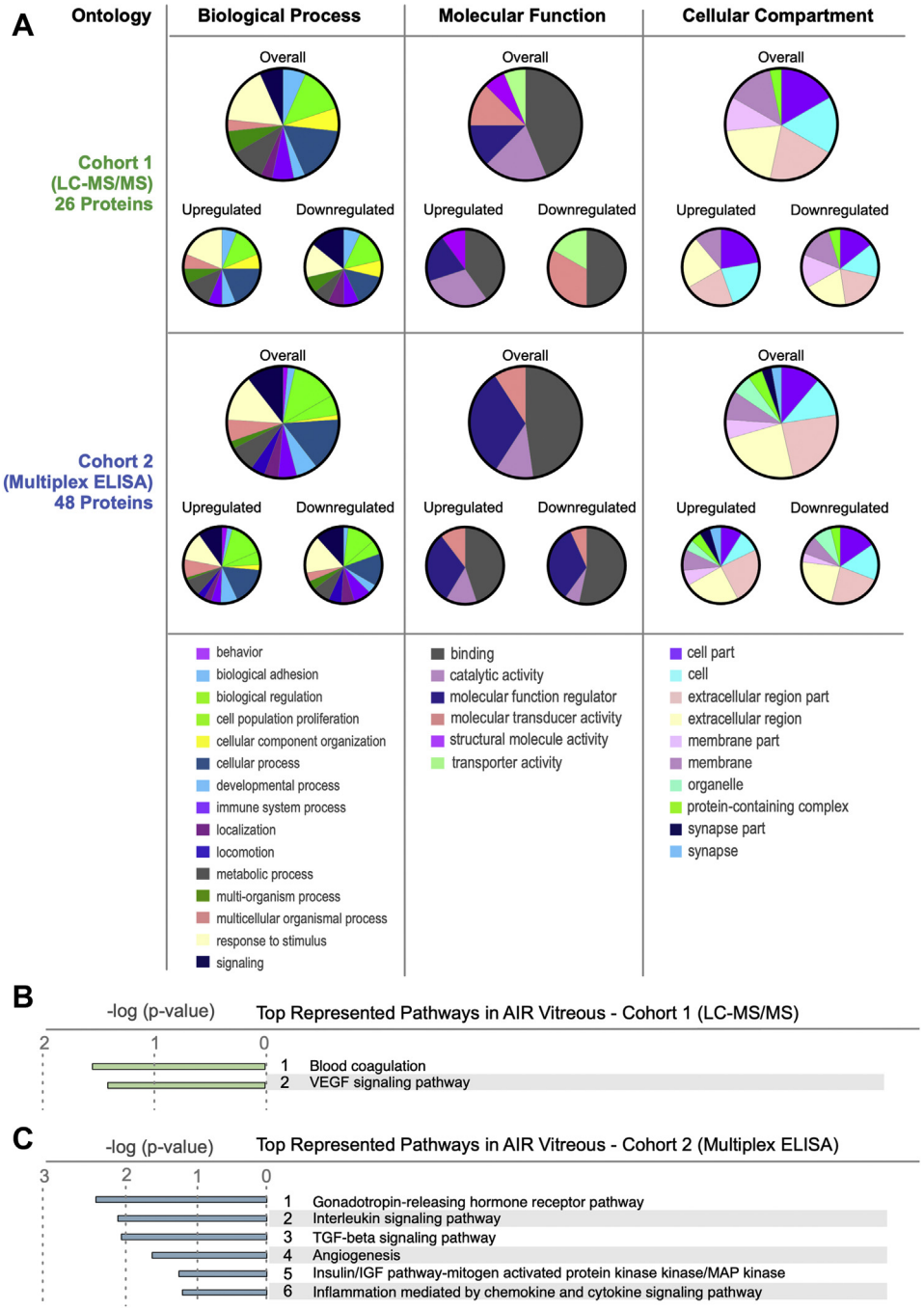

Figure 3. Gene ontology and pathway analysis of autoimmune retinopathy (AIR) vitreous proteins analyzed by mass spectrometry and multiplex enzyme-linked immunosorbent assay (ELISA). **A,** Differentially expressed proteins in Cohort 1 (LC-MS/MS) and Cohort 2 (multiplex ELISA) were categorized by their biological process, molecular function, and cellular compartment. Some quantitative data were preserved by the separation of upregulated and downregulated proteins. **B,** Pathways represented by upregulated proteins in Cohort 1 ($P < 0.05$). **C,** Pathways represented by upregulated proteins in Cohort 2 ($P < 0.05$). LC-MS/MS = liquid chromatography-tandem mass spectrometry; VEGF = vascular endothelial growth factor.

with AIR compared with controls and found that IL-23 is decreased in the plasma of patients with AIR.[34] Our study confirms differential expression of some cytokines in the vitreous of patients with AIR.

Among the differentially expressed molecular pathways in AIR vitreous, VEGF signaling and angiogenesis pathways were among the top represented pathways in the LC-MS/MS and ELISA multiplex datasets, respectively. This suggests that components of VEGF signaling pathway may be involved in the pathogenesis of AIR. Because of the complexity of this signaling pathway and its multiple roles in the physiological and pathological state of the retina,[35] it could be involved in other aspects of the pathogenesis of AIR and further studies are needed for such an evaluation.

A prior study showed that the elevated systemic levels of VEGF in some forms of cancers may trigger the loss of retinal pericytes via VEGF receptor 1,[36] which leads to the increased permeability and vascular attenuation that are clinically seen in patients with cancer-associated retinopathy,[19] a subtype of AIR.[37] However, to our knowledge, VEGF signaling has not been implicated in nonparaneoplastic AIR. The other differentially expressed pathways in AIR identified in our study (e.g., IL and TGF-ß signaling pathways) shed light on other possible mechanisms and cellular responses in this disease, a finding that may have implications for its treatment.

Study Limitations

There are limitations to the current study. Because it is not ethical to collect vitreous from nondiseased eyes, ophthalmic proteomic studies commonly use comparative controls, such as samples from patients with IMHs or ERMs.[7,10,38] Although imperfect, these comparative controls allow for clinical and molecular comparisons to conditions different from AIR and to other vitreoretinal diseases.[10,39] Furthermore, it is rare to obtain AIR vitreous samples because the prevalence for the disease is very low. Despite the limitation of the small sample size, descriptive semiquantitative analysis can provide insight into underlying disease pathways and uncover putative biomarkers in rare patient populations, and there are no human AIR proteomic reports to date.[40] Further studies are required to prospectively validate these candidate biomarkers and correlating autoantibodies in larger patient cohorts.

In conclusion, proteomic analysis is a powerful tool for studying the molecular basis of different retinopathies and identifying novel biomarkers that could help in the diagnosis and management of these diseases.[7,38,41] This proteomic study identified possible protein biomarkers in the vitreous for AIR. In addition to proteins and signaling pathways related to the regulation of innate immunity and response to cellular stress, we demonstrated that the RGC protein NrCAM is differentially expressed. Along with the history and clinical examination, ancillary testing, and retinal autoantibodies, these proteomics and imaging biomarkers could eventually help in diagnosing this challenging condition and point to potential targets for treatment.

Footnotes and Disclosures

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Author Contributions:
Conception and design: Bassuk, Tsang, Mahajan
Data collection: Al-Moujahed, Velez, Vu, de Carvalho, Levi
Analysis and interpretation: Al-Moujahed, Velez, Vu, Bassuk, Sepah, Mahajan
Obtained funding: Mahajan
Overall responsibility: Al-Moujahed, Velez, Tsang, Mahajan

Abbreviations and Acronyms:
AIR = autoimmune retinopathy; **ELISA** = enzyme-linked immunosorbent assay; **ERM** = epiretinal membrane; **IL** = interleukin; **IMH** = idiopathic macular hole; **LC-MS/MS** = liquid chromatography-tandem mass spectrometry; **NrCAM** = neuronal cell adhesion molecule; **RGC** = retinal ganglion cell; **RNFL** = retinal nerve fiber layer; **TGF-ß** = transforming growth factor beta; **VEGF** = vascular endothelial growth factor.

Keywords:
Autoimmune retinopathy, NrCAM, Proteomics, Retina, Vitreous.

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References

1. Heckenlively JR, Ferreyra HA. Autoimmune retinopathy: a review and summary. *Semin Immunopathol*. 2008;30:127−134.
2. Grange L, Dalal M, Nussenblatt RB, Sen HN. Autoimmune retinopathy. *Am J Ophthalmol*. 2014;157:266−272 e1.
3. Grewal DS, Fishman GA, Jampol LM. Autoimmune retinopathy and antiretinal antibodies: a review. *Retina*. 2014;34:827−845.
4. Bagheri S, Sobrin L. Autoimmune retinopathy: challenges in diagnosis and management. *Adv Ophthalmol Optom*. 2018;3:375−387.
5. Faez S, Loewenstein J, Sobrin L. Concordance of antiretinal antibody testing results between laboratories in autoimmune retinopathy. *JAMA Ophthalmol*. 2013;131:113−115.
6. Chen JJ, McKeon A, Greenwood TM, et al. Clinical utility of antiretinal antibody testing. *JAMA Ophthalmol*. 2021;139:658−662.
7. Velez G, Roybal CN, Colgan D, et al. Precision medicine: personalized proteomics for the diagnosis and treatment of idiopathic inflammatory disease. *JAMA Ophthalmol*. 2016;134:444−448.
8. Li AS, Velez G, Darbro B, et al. Whole-exome sequencing of patients with posterior segment uveitis. *Am J Ophthalmol*. 2021;221:246−259.
9. Fox AR, Gordon LK, Heckenlively JR, et al. Consensus on the diagnosis and management of nonparaneoplastic autoimmune retinopathy using a modified Delphi approach. *Am J Ophthalmol*. 2016;168:183−190.
10. Velez G, Tang PH, Cabral T, et al. Personalized proteomics for precision health: identifying biomarkers of vitreoretinal disease. *Transl Vis Sci Technol*. 2018;7:12.
11. Thomas PD, Campbell MJ, Kejariwal A, et al. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res*. 2003;13:2129−2141.
12. Mi H, Muruganujan A, Thomas PD. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic Acids Res*. 2013;41(Database issue):D377−D386.
13. Fabregat A, Sidiropoulos K, Viteri G, et al. Reactome pathway analysis: a high-performance in-memory approach. *BMC Bioinformatics*. 2017;18:142.
14. Rolli V, Macquin C, Kokten T, et al. Zinc-Alpha-2-glycoprotein in inflammatory bowel disease. *Inflamm Bowel Dis*. 2018;24:e10.
15. Zhang L, Mabwi HA, Palange NJ, et al. Expression patterns and potential biological roles of Dip2a. *PLoS One*. 2015;10:e0143284.
16. Sakurai T. The role of NrCAM in neural development and disorders–beyond a simple glue in the brain. *Mol Cell Neurosci*. 2012;49:351−363.
17. Williams SE, Grumet M, Colman DR, et al. A role for Nr-CAM in the patterning of binocular visual pathways. *Neuron*. 2006;50:535−547.
18. Zelina P, Avci HX, Thelen K, Pollerberg GE. The cell adhesion molecule NrCAM is crucial for growth cone behaviour and pathfinding of retinal ganglion cell axons. *Development*. 2005;132:3609−3618.
19. Gonzalez CD, Resnik R, Vaccaro MI. Secretory autophagy and its relevance in metabolic and degenerative disease. *Front Endocrinol (Lausanne)*. 2020;11:266.
20. Hu WT, Chen-Plotkin A, Arnold SE, et al. Novel CSF biomarkers for Alzheimer's disease and mild cognitive impairment. *Acta Neuropathol*. 2010;119:669−678.
21. Abazari A, Allam SS, Adamus G, Ghazi NG. Optical coherence tomography findings in autoimmune retinopathy. *Am J Ophthalmol*. 2012;153:750−756, 756 e1.
22. Pepple KL, Cusick M, Jaffe GJ, Mruthyunjaya P. SD-OCT and autofluorescence characteristics of autoimmune retinopathy. *Br J Ophthalmol*. 2013;97:139−144.
23. Sepah YJ, Sadiq MA, Hassan M, et al. Assessment of retinal structural and functional characteristics in eyes with autoimmune retinopathy. *Curr Mol Med*. 2015;15:578−586.
24. Magrys A, Anekonda T, Ren G, Adamus G. The role of anti-alpha-enolase autoantibodies in pathogenicity of autoimmune-mediated retinopathy. *J Clin Immunol*. 2007;27:181−192.
25. Kheir V, Cortes-Gonzalez V, Zenteno JC, Schorderet DF. Mutation update: TGFBI pathogenic and likely pathogenic variants in corneal dystrophies. *Hum Mutat*. 2019;40:675−693.
26. Allaman-Pillet N, Oberson A, Bustamante M, et al. Tgfbi/Bigh3 silencing activates ERK in mouse retina. *Exp Eye Res*. 2015;140:159−170.
27. Kumita JR, Helmfors L, Williams J, et al. Disease-related amyloidogenic variants of human lysozyme trigger the unfolded protein response and disturb eye development in Drosophila melanogaster. *FASEB J*. 2012;26:192−202.
28. Metrailler S, Schorderet DF, Cottet S. Early apoptosis of rod photoreceptors in Rpe65(-/-) mice is associated with the upregulated expression of lysosomal-mediated autophagic genes. *Exp Eye Res*. 2012;96:70−81.
29. Ragland SA, Criss AK. From bacterial killing to immune modulation: recent insights into the functions of lysozyme. *PLoS Pathog*. 2017;13:e1006512.
30. Park DH, Connor KM, Lambris JD. The challenges and promise of complement therapeutics for ocular diseases. *Front Immunol*. 2019;10:1007.
31. Thurman JM, Frazer-Abel A, Holers VM. The evolving landscape for complement therapeutics in rheumatic and autoimmune diseases. *Arthritis Rheumatol*. 2017;69:2102−2113.
32. Garcia-Ramirez M, Canals F, Hernandez C, et al. Proteomic analysis of human vitreous fluid by fluorescence-based difference gel electrophoresis (DIGE): a new strategy for identifying potential candidates in the pathogenesis of proliferative diabetic retinopathy. *Diabetologia*. 2007;50:1294−1303.
33. Slingsby C, Wistow GJ. Functions of crystallins in and out of lens: roles in elongated and post-mitotic cells. *Prog Biophys Mol Biol*. 2014;115:52−67.
34. Stansky E, Biancotto A, Dagur PK, et al. B cell anomalies in autoimmune retinopathy (AIR). *Invest Ophthalmol Vis Sci*. 2017;58:3600−3607.
35. Penn JS, Madan A, Caldwell RB, et al. Vascular endothelial growth factor in eye disease. *Prog Retin Eye Res*. 2008;27:331−371.
36. Cao R, Xue Y, Hedlund EM, et al. VEGFR1-mediated pericyte ablation links VEGF and PlGF to cancer-associated retinopathy. *Proc Natl Acad Sci U S A*. 2010;107:856−861.
37. Shildkrot Y, Sobrin L, Gragoudas ES. Cancer-associated retinopathy: update on pathogenesis and therapy. *Semin Ophthalmol*. 2011;26:321−328.

38. Roybal CN, Velez G, Toral MA, et al. Personalized proteomics in proliferative vitreoretinopathy implicate hematopoietic cell recruitment and mTOR as a therapeutic target. *Am J Ophthalmol*. 2018;186:152−163.
39. Mahajan VB, Skeie JM. Translational vitreous proteomics. *Proteomics Clin Appl*. 2014;8:204−208.
40. Velez G, Mahajan VB. Molecular surgery: proteomics of a rare genetic disease gives insight into common causes of blindness. *iScience*. 2020;23:101667.
41. Velez G, Roybal CN, Binkley E, et al. Proteomic analysis of elevated intraocular pressure with retinal detachment. *Am J Ophthalmol Case Rep*. 2017;5:107−110.