Molecular Surgery: Proteomics of a Rare Genetic Disease Gives Insight into Common Causes of Blindness

Gabriel Velez1,2,3 and Vinit B. Mahajan1,2,4,*

SUMMARY

Rare diseases are an emerging global health priority. Although individually rare, the prevalence of rare “orphan” diseases is high, affecting approximately 300 million people worldwide. Treatments for these conditions are often inadequate, leaving the disease to progress unabated. Here, we review the clinical features and pathophysiology of neovascular inflammatory vitreoretinopathy (NIV), a rare inflammatory retinal disease caused by mutations in the CAPN5 gene. Although the prevalence of NIV is low (1 in 1,000,000 people), the disease mimics more common causes of blindness (e.g. uveitis, retinitis pigmentosa, proliferative diabetic retinopathy, and proliferative vitreoretinopathy) at distinct clinical stages. There is no cure for NIV to date. We highlight how personalized proteomics helped identify potential stage-specific biomarkers and drug targets in liquid vitreous biopsies. The NIV vitreous proteome revealed enrichment of molecular pathways associated with common retinal pathologies and implicated superior targets for therapeutic drug repositioning. In addition, we review our pipeline for collecting, storing, and analyzing ophthalmic surgical samples. This approach can be adapted to treat a variety of rare genetic diseases.

INTRODUCTION: NEOVASCULAR INFLAMMATORY VITREORETINOPATHY

Intraocular inflammation (uveitis) poses a diagnostic and therapeutic challenge to health care professionals because patients often have non-specific concerns and unremarkable clinical examination findings. Although often restricted to the eye, uveitis can be an early symptom of debilitating systemic disease with a prevalence of 1 in 4,500 people (Jabs, 2008; Mattapallil et al., 2008; Pascolini and Mariotti, 2012; Pascolini et al., 2004; Resnikoff et al., 2004) and should be aggressively treated to prevent significant visual morbidity and blindness. Diagnostic workup for non-syndromic uveitis can be prohibitively expensive and has low yield, leading to delays in diagnosis and unnecessary vision loss (Velez et al., 2016). Uveitis also presents a mechanistic challenge to basic and translational researchers due to patient scarcity. Genetic mutations associated with non-syndromic uveitis have only recently been identified, and only a handful of genes are linked to retinal neovascularization and fibrosis (Berger et al., 2010; Li et al., 2020; Wallace and Niemczyk, 2011). More than half of the uveitis cases remain idiopathic, and genetic susceptibility is poorly understood (Gritz and Wong, 2004; Lee et al., 2014; Rodriguez et al., 1996). Therefore, we need more reliable diagnostic testing and personalized therapies to distinguish the various causes of uveitis. Even when a genetic cause has been determined, the molecular mechanisms behind the phenotypic manifestations are often unexplored, leaving treatment options scarce and inadequate. We care for a patient population that offers an opportunity to overcome these challenges, a poorly understood autoinflammatory genetic disease of the retina, neovascular inflammatory vitreoretinopathy (NIV; OMIM #193235), for which there is no effective treatment.

Neovascular inflammatory vitreoretinopathy is a rare and devastating eye disease that develops slowly over the lifetime of the patient, leading to significant visual morbidity and blindness. Patients with NIV suffer from non-syndromic vision loss due to severe inflammatory uveitis, retinal degeneration, neovascularization, and intraocular fibrosis (Figure 1)(Bennett et al., 1990; Mahajan et al., 2012). The disease is inherited in an autosomal dominant fashion and has been extensively characterized in three families. Although a majority of patients with NIV do not have associated systemic autoimmune or inflammatory disease, there are reports of patients with NIV with hearing loss and developmental delay in addition to aggressive visual
symptoms (Velez et al., 2018a; Wang et al., 2018b). Most patients are asymptomatic for the first two decades of life and do not exhibit clinical manifestations of the disease. NIV has five distinct stages and each mimics common eye diseases that together account for a significant fraction of visual morbidity and blindness (e.g., uveitis, retinitis pigmentosa, proliferative diabetic retinopathy, and proliferative vitreoretinopathy) (Bennett et al., 1990; Mahajan et al., 2012). Stage I is clinically indistinguishable from non-infectious posterior uveitis and is characterized by mild intraocular inflammation and retinal synaptic signaling defects (i.e., reduced b-wave on electroretinography [ERG] Figure 1A). Stage II progresses to retinal photoreceptor degeneration, cataract, moderate intraocular inflammation, and macular edema (Figure 1B). Later stages exhibit retinal neovascularization, vitreous hemorrhage, pan-uveitis, vitreoretinal fibrosis, and tractional and rhegmatogenous retinal detachment (RD) (Figures 1C and 1D) (Bennett et al., 1990; Tang et al., 2018). Ultimately, NIV disease culminates in blindness and phthisis bulbi by the fifth decade of life (Figure 1E). NIV phenocopies each of these common eye diseases at different stages,

Figure 1. Disease Stages of NIV Phenocopy Common Vitreoretinal Conditions
(A) In Stage I disease, there are mild cells (white dots) in the vitreous (orange) and a reduced b-wave on electroretinography (ERG).
(B) In Stage II disease, the anterior chamber (blue) shows mild inflammatory cells and there is early development of cataract. The posterior segment shows moderate cells, retinal pigmentary changes, and some edema in the macula or optic nerve head. There is selective loss of the b-wave in the scotopic bright flash ERG.
(C) In Stage III disease, the anterior segment shows moderate cells, progressive cataract, and iris synechiae. Progressive inflammation in the posterior segment shows development of vitreous bands and epiretinal membranes and more posterior retinal pigmentary changes. There is reduction of the a-wave on ERG.
(D) In Stage IV disease, inflammation in the anterior segment causes neovascular and angle closure glaucoma. Neovascularization develops in the retina with vitreous hemorrhage and progressive retinal detachment that may include features of anterior or posterior proliferative vitreoretinopathy. The ERG becomes non-recordable.
(E) In Stage V disease, the eye becomes phthisical. Blue bar, anterior chamber features; orange bar, posterior chamber features; black bar, ERG features. Graphical illustrations by Alton Szeto and Vinit Mahajan.
Figure adapted with permission from PLoS Genetics (Mahajan et al., 2012. Calpain-5 mutations cause autoimmune uveitis, retinal neovascularization, and photoreceptor degeneration, 8(10): e1003001. Copyright 2012, Mahajan, et al.).
suggested a potential mechanistic overlap between NIV and other retinal pathologies. For this reason, we often refer to NIV as a “Rosetta Stone” disease; if the mechanisms underlying NIV are better understood, the knowledge gained from these studies could be applied to other eye diseases.

**HYPERACTIVATING CAPN5 MUTATIONS CAUSE NIV**

Whole-exome sequencing showed that three NIV kindreds harbored a unique missense mutation in the CAPN5 gene. CAPN5 encodes calpain-5 (CAPN5), a Ca^{2+}-activated cysteine protease that is broadly expressed in the body, including retinal photoreceptors and the central nervous system (Schaefer et al., 2016; Singh et al., 2014). This discovery provided a working model for studying the molecular basis of non-syndromic uveitis. CAPN5 is a member of a large family of cysteine proteases that mediate limited proteolysis in the cell. Three-dimensional (3D) modeling of the CAPN5 protein structure placed three NIV mutations (p.R243L, p.L244P, and p.K250N) on a flexible loop that gates access to the active site (Bassuk et al., 2015). Modeling suggested how structural changes that regulate catalysis might be induced by Ca^{2+} and why CAPN5 mutations would hyperactivate the protease: NIV mutations disrupt a Ca^{2+}-sensitive gating loop, leaving the active site always un-gated and open to substrates, even in the absence of Ca^{2+}. In testing this idea, NIV mutations were introduced into purified recombinant CAPN1/5 hybrid molecules and confirmed that NIV mutations dramatically increased calpain catalytic activity (Vert et al., 2015). However, the physiologic function and natural substrates of CAPN5 are poorly understood. Thus, the mechanisms by which CAPN5 hyperactivity in the retina leads to disease are unknown. A detailed understanding of how CAPN5 mutations cause uveits will aid in the development of Precision Medicine strategies to treat inflammatory retinal disease in humans.

Numerous studies have demonstrated that calpains are aggravating factors in neurodegenerative and neuroinflammatory diseases, including retinal degeneration (Azuma and Shearer, 2008). For example, CAPN1 and -2 are activated in the retina under hypoxic conditions. The hypoxia-induced Ca^{2+} influx in retinal cells leads to hyperactivation of calpain and subsequent proteolysis of microtubule-associated proteins (e.g., tau), spectrin, pro-caspase-3 (to 30-kDa caspase-3), and p35 (to p25) (Sakamoto et al., 2000; Tamada et al., 2005). Calpain-mediated breakdown of these neuronal proteins leads to the activation of necrotic and apoptotic pathways and subsequent cell death. For example, conversion of p35 to p25 leads to prolonged cyclin-dependent kinase 5 (CDK5) activation, resulting in hyper-phosphorylation of tau. Hyperphosphorylated tau aggregates and forms insoluble filaments that slow down neuronal processes and cause cell retinal death (Azuma and Shearer, 2008). A similar calpain-mediated mechanism of pathogenesis has been proposed for neurodegenerative diseases like Alzheimer disease (Mahaman et al., 2019). The contribution of calpain in retinal disease pathology is not surprising because the retina contains some of the highest calcium concentrations in the body (levels as high as 16 mM have been reported in ischemic whole rat retinas) (Sakamoto et al., 2000). Thus, it can be reasoned that a hyperactivated CAPN5 would lead to the retinal cell death and accumulated tissue damage seen in patients with NIV.

**MECHANISTIC INSIGHTS INTO NIV PATHOGENESIS**

Since the discovery of the first NIV-causing mutations, efforts have been taken to understand the structure, function, targets, and downstream signaling effectors of the CAPN5 protease (Bassuk et al., 2015; Gakhar et al., 2016; Mahajan et al., 2012; Schaefer et al., 2016; Wert et al., 2014, 2015, 2019). Our group solved the structure of the CAPN5 protease core domain (termed CAPN5-PC), a finding that furthers the study of NIV disease mechanisms (Velez et al., 2020). The wild-type CAPN5-PC structure revealed unique structural features compared with classical calpain cores and provided clues as to how NIV mutations would increase enzymatic activity (Figure 2A). Calpain activation is tightly regulated, and there are several conserved structural mechanisms in place to prevent aberrant calpain activity in the cell (Campbell and Davies, 2012). It is postulated that NIV mutations disrupt different critical regulatory motifs depending on their location on the CAPN5 structure. For example, the hyperactivating (i.e., gain-of-function) mutations p.R243L, p.L244P, and p.K250N are located on the G1 gating loop, a regulatory loop that is believed to block the access of substrates into the active site in the absence of calcium (Bassuk et al., 2015). Disruption of this mechanism, either by altering the charge or stability of this regulatory loop, would leave the active site groove always open to substrate, leading to dysregulated CAPN5 activity (Figures 2B–2E). Our CAPN5-PC structure revealed that the p.G267S mutation is located on the PC2L2 loop. Although the function of this loop is unknown, transfer of the unique PC2L2 loop (with the hyperactivating p.G267S mutation) onto rat CAPN1 led to hyperactivity in a CAPN1/5 hybrid (Figure 2F). This finding suggests that the PC2L2 loop can remotely regulate calpain activity independent of its non-catalytic domains (Velez et al.,
The p.R289W mutation is associated with the most aggressive NIV phenotype and is located on the G2 loop, which houses the critical tryptophan “wedge” residue (Trp286). Disruption of this regulatory motif might allow for the formation of the catalytic triad in the absence of calcium (Velez et al., 2018a) (Figure 2G).

Future structural studies on CAPN5 mutants should elucidate the mechanisms by which NIV mutations lead to hyperactivity.

To examine the specific allelic mutations leading to NIV in patients, mouse models were generated by our laboratory for the CAPN5 c.728G > T, p.R243L mutation found in the first two NIV families. Viral gene transfer of this mutant CAPN5 allele and a transgenic mouse expressing mutant human CAPN5 in the retina (hCAPN5R243L mouse) recapitulated key features of human NIV: loss of ERG b-wave, immune cell infiltration, photoreceptor degeneration, and inflammatory gene expression (Wert et al., 2014, 2015). In both model systems, expression of mutant CAPN5 was driven by an opsin promoter, and therefore limited to the rod photoreceptor cells. Rod expression alone of mutant human CAPN5 was able to drive the NIV clinical phenotype. However, these phenotypes were either not transferred through generations or the allele was bred out (unpublished observation). Thus, these systems offer limited utility for in vivo studies or therapeutic testing. Attempts to generate more physiologically relevant murine models using gene knockin of...
the CAPN5R243L allele and CRISPR have resulted in no detectable phenotype up to 12 months (unpublished observation). Wang et al. published an NIV murine model generated with gene knockin of the CAPN5R289W allele (Wang et al., 2018b). This mouse model displayed proliferation of the retinal pigmented epithelium (RPE) layer but failed to recapitulate other key features of NIV disease (e.g., pigmentary degeneration, inflammatory cell infiltration, and synaptic signaling defects) (Wang et al., 2018b). Thus, the generation of useful mouse model for in vivo studies remains a challenge. As is in the case of most rare diseases, there is often no good animal or tissue culture model from which to derive mechanistic insight. However, we have the ability to collect surgical tissue and fluid biopsies from patients and perform detailed molecular analysis. In the case of NIV, clinical treatments can benefit from the ability to localize them to the region of the eye, and in particular, the retina.

MOLECULAR SURGERY: A PRECISION MEDICINE STRATEGY FOR DIAGNOSING AND TREATING INFLAMMATORY DISEASE

Organ-specific inflammatory diseases often occur as a result of complex genetic and environmental factors. The inheritance of susceptibility to these disorders is often polygenic and poorly understood (Rose-Zerilli et al., 2011). Thus, many affected patients are labeled as idiopathic, leaving them with limited treatment options. Diagnosis and treatment of organ-specific inflammatory diseases is most often empirical, relying heavily on clinical examination. Exploration of human eye disease is entering an era of molecular surgery. In ophthalmology, microsurgical techniques can be used to extract highly compartmentalized fluids and tissues safely to identify disease molecules. The same surgical techniques can be used to deliver various molecular therapies in the clinic and operating room. Physicians are becoming more and more aware of the exact molecular targets of the drugs they are giving patients. Newer drugs are biologics (e.g., anti-vascular endothelial growth factor [VEGF] antibodies) and engineered small molecules. It is common now to directly inject into the eye antibodies that bind to a specific protein target. The same is true for a variety of immune diseases. Often, laboratory models for human eye disease do not exist, but human eye fluid biopsies can determine the exact molecular cause of a patient’s disease. Analyzing the proteins in only a few drops of eye fluid has led to diagnosis and selection of the right drugs at the right time. It has also helped explain why some clinical trials have failed, and it has revealed the new molecules ophthalmologists should be targeted. Recasting eye surgery in molecular terms will allow ophthalmologists to take innovative approaches to curing blindness.

Advances in proteomics and genomics are leading to the development of more precise diagnostic tools and interventions. Proteomic analysis is becoming an attractive and powerful method for characterizing the molecular profiles of diseased tissues (Velez et al., 2018c). Screening for systemic biomarkers is straightforward. However, organ-specific proteins released from the diseased tissue are often substantially diluted or degraded once they enter the systemic circulation. In addition, the serum contains a large proportion of albumin and wide dynamic range of abundance of other proteins, making it difficult to reliably quantify the organ-specific biomarkers. Proteomic changes in the serum may represent systemic changes to inflammation rather than being derived from the diseased tissue itself (Geyer et al., 2017). In the case of organ-specific diseases, sampling fluid compartments near the diseased tissue (e.g., synovial fluid, urine, cerebrospinal fluid) may be a more reliable source of potential diagnostic and therapeutic biomarkers than the serum (Di Meo et al., 2017; Rosengren et al., 2003; Shankar et al., 2017).

Retinal photoreceptors are non-dividing and terminally differentiated. Thus, sampling the neurosensory retina in living patients for the purpose of diagnostic testing would result in unacceptable visual morbidity (Cole et al., 2008). Rather, the adjacent vitreous humor is more advantageous to measure protein biomarkers. The vitreous is an optically transparent extracellular matrix (ECM) that fills the space between the retina and lens. Its primary known purpose is to help develop the shape of the eyeball in utero and thereafter allow light to reach the retina. The composition of the human vitreous is estimated to be over 90% water. The vitreous contains a dilute network of unbranched heterotypic collagen fibers (including type II, V, IX, and XI), and hyaluronan, with type II collagen being the most abundant (Le Goff and Bishop, 2008). These collagenous structural proteins and albumin make up a majority of the proteins found in the vitreous, whereas non-collagenous and soluble proteins are less abundant (Figure 3A). Despite the biochemical and structural properties of an ECM, we found a significant number of unique intracellular proteins in the human vitreous (Skeie et al., 2015). It is well documented that dynamic changes in the retina, during disease and aging processes, are closely linked to biochemical changes in the adjacent vitreous (Le Goff and Bishop, 2008). Previous proteomic analysis of the non-diseased human vitreous by our group
identified over 1,000 non-redundant intracellular proteins with diverse molecular functions (Figure 3B). Our research has also demonstrated that retinal, RPE, and choroidal proteins are deposited into the vitreous, and that proteomic analysis of liquid vitreous biopsies can detect molecular changes in the adjacent retina, uncover potential diagnostic biomarkers, and identify protein targets for therapy (Skeie et al., 2015; Wert et al., 2020). This supports the concept that the vitreous is not only an ultrafiltrate of the blood but also represents its surrounding ocular tissues. In fact, as of 2019, there have been over 60 published proteomic studies on human vitreous biopsies in the context of several disease states (Figure 3C). We have demonstrated a proteomic Precision Medicine strategy to be effective for inflammatory eye diseases, including NIV. Vitreous biopsies can be obtained from living patients through pars plana vitrectomy or fine needle aspiration, and we found that clinical proteomic analysis is highly dependent on the quality of the sample (Skeie et al., 2012, 2014). These techniques are extensively reviewed in Velez et al. (2018c). Thus, proteomic analysis of vitreous biopsies is a promising Precision Health strategy for characterizing and treating numerous complex vitreoretinal diseases.

PROTEOMICS OF EARLY-STAGE NIV: INSIGHTS TO RETINAL INFLAMMATION AND DEGENERATION

Patients with Stage I NIV display loss of ERG function and intraocular inflammation that is clinically indistinguishable from non-infectious posterior uveitis. Once the patients progress to Stage II, they develop pigmentary photoreceptor degeneration, similar to retinitis pigmentosa. This unique phenotype prompted us to compare NIV proteomic profiles to our previously published proteomic datasets for other vitreoretinal disorders. We sought to identify any overlapping potential biomarkers between these disease categories that may explain their phenotypic similarities. There were several proteins commonly elevated in the vitreous of patients with NIV and posterior segment uveitis: tissue inhibitor of metalloproteinases (TIMP-1 and -2) and Fas ligand (FasL). Metalloproteinases (MMPs) and their post-translational regulation by endogenous inhibitors (TIMPs) play a key role in the remodeling of the retinal ECM. Dysregulation of the MMP-TIMP axis, characterized by excessive MMP activation, has been implicated in the processes of intraocular inflammation, angiogenesis, and fibrosis (Singh and Tyagi, 2017). The elevated levels of

Figure 3. The Vitreous as a Surrogate for Retinal Biopsy

(A) Relative abundance (mean spectral counts) of human vitreous proteins with several illustrative proteins highlighted in red. Identification and relative quantitation of vitreous proteins was performed using LC-MS/MS (Skeie, et al., 2015). The 750 most abundant proteins are shown.

(B) Gene ontology analysis of the 2,062 proteins identified in the human vitreous. Proteins are grouped into subcategories based on their respective cellular localization (left) and molecular function (right). Most proteins were intracellular.

(C) Publications describing proteomic analysis of human vitreous samples (red) compared with the total number of proteomics publications (divided by 100) in gray (left). Overview of the percentage of studies utilizing MS-based methods and their focus (right).
TIMP-1 and -2 present in NIV and uveitis vitreous may represent a response to aberrant MMP activation in the inflamed retina, although we did not detect significantly elevated MMP levels in either patient group. FasL is constitutively expressed in the eye and is hypothesized to play an important role in maintaining ocular immune privilege by inducing the apoptosis of infiltrating inflammatory cells (Griffith et al., 1995). However, this role is controversial as FasL has been demonstrated to have both pro- and anti-inflammatory properties (Ferguson and Griffith, 2006). Changes in the local immune microenvironment and FasL expression may in fact lead to FasL-mediated activation of the innate immune system and termination of ocular immune privilege (Gregory et al., 2002). Indeed, innate immune effectors (e.g., acute phase response proteins and complement) have been previously implicated in NIV and uveitis pathogenesis (Rosenbaum and Kim, 2013; Velez et al., 2019).

As different proteomic platforms can give different results, we obtained a more global view of the NIV vitreous proteome using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Velez et al., 2019). We also compared our previously published MS-based analysis of Stage II NIV vitreous to proteomes from other etiologies of intraocular inflammation such as autoimmune retinopathy, idiopathic posterior uveitis, and lens-induced uveitis (LIU; unpublished data; Figure 4A). Our comparative analysis revealed commonly upregulated proteins such as S100-A8 and beta-crystallins (A3 and B1; Figure 4B). S100A8 is an inflammatory modulator that stimulates leukocyte recruitment and induces the secretion of cytokines (Wang et al., 2018a). Crystallin proteins are known calpain substrates (Shearer et al., 1996). It is not known whether CAPN5 hydrolyzes these lens proteins. However, the presence of elevated beta crystallin in NIV vitreous suggests an overlapping mechanism to LIU, in which foreign lens protein fragments lead to the formation of anti-lens antibodies triggering an inflammatory response (Wirostko and Spalter, 1967). Patients with NIV have not yet been tested for anti-lens antibodies. Several proteins were uniquely upregulated in Stage II NIV vitreous: serine protease HTRA1, complement factors (e.g., C3, C5, C6, C7, C8, and CFH), and coagulation factors (9 and 12; Figure 4B). Interestingly, polymorphisms leading to altered HTRA1 and CFH expression are associated with age-related macular degeneration (AMD) (Landowski et al., 2019; Lin et al., 2018). Among the commonly downregulated proteins were versican and pyruvate kinase (PKM). Versican deficiency is known to cause hereditary vitreoretinopathy in humans (Tang et al., 2019).

The loss of the ERG b-wave in patients with Stage I NIV indicates the presence of early synaptic signaling defects, akin to retinitis pigmentosa (RP) (Tang et al., 2018). LC-MS/MS analysis revealed that synaptic signaling proteins (e.g., neurexin-2, glutamate receptor 4, neurofascin, neuronal growth regulator 1, and oligodendrocyte-myelin glycoprotein) were depleted in NIV vitreous compared with non-inflammatory controls (Velez et al., 2019). It is possible that the loss of the identified synaptic signaling proteins contributes to the early ERG defect that is characteristic of NIV (Velez et al., 2019; Wert et al., 2020). Whether CAPN5 directly targets these proteins in the retina (or their upstream effectors) is still not known. Additionally, we observed depletion of oxidative stress defense proteins in NIV vitreous (e.g., superoxide dismutase and peroxiredoxin), a finding associated with other retinal degenerative diseases (e.g., RP and AMD) (Velez et al., 2018b). Interestingly, proteomic analysis of end-stage human RP vitreous by our group similarly revealed depletion of several of these synaptic and antioxidant proteins compared with controls, suggesting overlapping pathogenic mechanisms (Figures 4C–4E). Two of these antioxidant proteins (peroxiredoxin-2 and -6) were identified as candidate retinal substrates for CAPNS (unpublished observation), suggesting that CAPNS-mediated cleavage of these substrates may contribute to the depletion of antioxidant stress proteins in NIV vitreous. Taken together, these comparative analyses suggest unique and overlapping mechanism of NIV pathogenesis to other inflammatory and degenerative retinal disorders.

PROTEOMICS OF LATE-STAGE NIV: INSIGHTS INTO PROLIFERATIVE RETINOVASCULAR DISEASE

Late-stage (Stages III and IV) NIV is characterized by retinal neovascularization, cystoid macular edema, vitreoretinal fibrosis, and rhegmatogenous and tractional RDs similar to patients with proliferative diabetic retinopathy (PDR) and those with proliferative vitreoretinopathy (PVR). The NIV vitreous proteome displays characteristic enrichment of proteins and pathways associated with PDR and PVR. Notably, patients with PDR and NIV display high vitreous levels of VEGF), which promotes retinal neovascularization by inducing endothelial cell proliferation (Penn et al., 2008). Anti-VEGF therapy has become a mainstay in PDR management and was similarly demonstrated to reduce retinal neovascularization and clear vitreous hemorrhages in patients with NIV (Velez et al., 2017). However, the management of intraocular fibrosis and RD in patients with NIV has remained a challenge. PVR is a rare vision-threatening complication of RD repair. Following
Retinal reattachment surgery, patients with PVR develop retinal fibrotic membranes that can re-open previously repaired retinal tears as well as form new ones (Pastor et al., 2016; Roybal et al., 2017). Early PVR (grades A and B) is amenable to surgical repair, whereas late PVR (grade C) often requires complex surgery with poor visual outcomes (Roybal et al., 2017). Patients with late-stage NIV similarly experience aggressive intraocular fibrosis that is unresponsive to surgery and conventional immunosuppressive medications. To identify cytokine signals driving intraocular fibrosis in these patients, we compared our previously published proteomic datasets on NIV and PVR vitreous (Figure 5A). We previously reported that T cell signaling pathways (e.g., mTOR signaling) were highly represented in early PVR vitreous, whereas monocyte signatures prevailed in late PVR. Interestingly, the proteomic signature of late-stage NIV highly resembled that of late PVR (Figures 5B and 5C). Late-stage NIV similarly displayed elevated levels of monocyte-inducible factors (MIF-1α, -1b, and -1d) and monocyte chemotactic protein (MCP-1), whereas T cell signatures (e.g., IL-17 signaling) were downregulated (Figure 5D). Pro-fibrotic cytokines, namely, IL-6, IL-13, and platelet-derived growth factor, were elevated in all groups. Taken together, these results suggest that similar

Figure 4. Synaptic Signaling and Antioxidant Proteins are Depleted in Early NIV
(A) The abundance of identified proteins differed significantly between control and Stage 2 NIV (left), autoimmune retinopathy (middle left), idiopathic posterior segment uveitis (middle right), and lens-induced uveitis (right). Results are represented as a pie chart indicating the proportion of proteins with an absolute abundance ratio greater than 2-fold. (B) Comparative analysis of upregulated proteins (compared with controls) using Venn diagrams. A total of 39 proteins were commonly upregulated among the four disease groups. (C) Comparative analysis of downregulated proteins (compared with controls) using Venn diagrams. A total of 83 proteins were commonly downregulated among the four disease groups. (D) Differentially expressed proteins between NIV and control vitreous (detected by LC-MS/MS) 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). (E) Differentially expressed proteins between autosomal recessive RP and control vitreous (detected by LC-MS/MS). The x axis displays the unadjusted fold-change value, and the y axis displays the noise-adjusted signal as the -log10 (p value). Red circles, downregulated synaptic signaling proteins; blue circles, downregulated antioxidant proteins.
inflammatory and fibrotic pathways drive these two diseases. However, it is unclear if activation of these pathways occurs as a direct result of CAPN5 hyperactivity in the retina. Nevertheless, the proteomic detection of immune mediators in NIV vitreous has provided some insight into the downstream signaling events that could be targeted for therapeutic purposes.

PROTEOMICS FOR DRUG TARGET IDENTIFICATION

Current treatments for patients with NIV have focused on controlling specific downstream clinical symptoms, but these therapies have not been able to inhibit all aspects of the disease phenotype or progression. For instance, local immunosuppression with steroids can treat early inflammatory symptoms of NIV. However, the later fibrotic response and retinal degeneration persist in these patients, ultimately resulting in blindness (Tlucek et al., 2013). Rare diseases like NIV pose a financial barrier to the development of new medications. High drug development costs for rare diseases are difficult to overcome because the intended market is often small, leaving affected patients with inadequate treatment options. One way to overcome this barrier is through drug repositioning, which is the application of existing and approved drugs toward new indications. It is often difficult to determine which drugs to reposition, especially in genetically heterogeneous patient populations that seem to be affected by the same disorder. The application of drug repositioning strategies

Figure 5. Late-Stage NIV Displays a Similar Proteomic Signature to Proliferative Vitreoretinopathy

(A) The abundance of 200 measured cytokines differed significantly between control and late-stage NIV (stage 3 and 4; left), early PVR (middle), and late PVR (right), respectively. Results are represented as a pie chart indicating the proportion of proteins with an absolute abundance ratio greater than 2-fold.

(B) Comparative analysis of upregulated proteins (compared with controls) using Venn diagrams. A total of 47 proteins were commonly upregulated among the three disease groups.

(C) Comparative analysis of downregulated proteins (compared with controls) using Venn diagrams. No proteins were commonly downregulated among the three disease groups.

(D) Enriched pathways represented in NIV and PVR vitreous. Pathways are ranked by their $-\log(p\text{-value})$ obtained from the right-tailed Fisher’s exact test.
to well-characterized rare diseases can help to overcome this barrier, by limiting genetic heterogeneity in the desired treatment group. The ability of proteomics to guide drug repositioning for rare diseases is exemplified in a prior study by our group, where analysis of vitreous biopsies identified new protein targets for NIV therapy (Velez et al., 2017). Patients with NIV had previously been treated with non-specific immunosuppressive medications, such as oral corticosteroids and infliximab (anti-TNF-\(\alpha\)). Our characterization of the NIV vitreous proteome revealed that levels of TNF-\(\alpha\) were indistinguishable from control vitreous, explaining why infliximab therapy previously failed. Instead, we detected elevated levels of VEGF, T cell proliferative markers (e.g., mTOR and PI3K signaling effectors), and IL-6. We therefore repositioned bevacizumab (anti-VEGF), intravitreal methotrexate (T cell inhibitor), and tocilizumab (anti-IL-6) for the treatment of NIV. These therapies successfully halted neovascularization and cleared vitreous hemorrhages, reduced inflammatory cell infiltration, and mitigated intraocular fibrosis in these patients (Velez et al., 2017). These findings highlight the importance of profiling the adjacent fluid compartments to determine the best therapy, especially to avoid targeting proteins that are absent. Although CAPN5 activity currently cannot be pharmacologically inhibited, many downstream effectors and immune mediators can be targeted by available drugs. These findings have allowed us to generate a model of NIV pathogenesis based on CAPN5’s structure and downstream signaling events (Figure 6).

With the advances in bioinformatic analysis of proteomic data, researchers and clinicians can begin to identify signaling and metabolic pathways that are perturbed in the disease state. Instead of targeting single molecules, proteomic-guided drug repositioning strategies can also be applied to target entire pathways. Our group has recently demonstrated this potential in another rare disease population. Retinal dystrophies display high genetic heterogeneity and can be caused by mutations in over 270 genes (Ziccardi et al., 2019). Inherited mutations in genes result in the production of dysfunctional proteins that lead to irreversible photoreceptor damage and vision loss (Hurley and Chao, 2020; Tsang et al., 2008). Patients with retinal dystrophy often do not display visual symptoms until a significant fraction of their photoreceptors have died, making the timing of therapeutic intervention difficult (Wert et al., 2020). Furthermore, the genetic heterogeneity of these patients makes gene therapy and CRISPR-based interventions unfeasible in the near term (DiCarlo et al., 2018; Takahashi et al., 2018; Xu et al., 2018). An alternative approach to monitoring and treating retinal dystrophy is to use vitreous biomarkers that predict the onset of photoreceptor degeneration and identify candidate therapeutic targets before patients begin to experience vision loss. Our
group evaluated the vitreous proteome of two patients with end-stage autosomal recessive retinitis pigmentosa (arRP) harboring mutations in the PDE6A gene. We identified many proteins with substantial fold changes in arRP vitreous compared with controls, notably elevated levels of fatty acid synthase (FASN). However, the scarcity of vitreous samples from these patients prevented further evaluation of biomarker expression at different disease stages in humans. To achieve statistical power, our group analyzed vitreous and retinal protein expression in a murine model of arRP disease (Pde6a<sup>D670G</sup> mice) at early-, mid-, and late-stage disease (Wert et al., 2020). We observed a significant redistribution of proteins between vitreous at early arRP, indicating that dying retinal cells had leaked proteins into the adjacent vitreous, signaling the onset of photoreceptor degeneration. A significant proportion of these proteins (including FASN) were involved in energy metabolism, which pointed to metabolite-based therapies that could be repurposed to slow the neurodegenerative process. Oral supplementation of α-ketoglutarate alone was demonstrated to be taken up by the retina by desorption electrospray ionization mass spectrometry and was sufficient to slow retinal degeneration in arRP mice (Wert et al., 2020). This nutrient-based therapy influenced the distribution of retinal metabolites in treated mice and provided a basis for its neuroprotective effects on photoreceptor function.

PROTEOMICS FOR CLINICAL TRIAL DESIGN

For rare diseases like uveitis, deciding which patients to enroll in clinical trials is critical. The success of many clinical trials for uveitis has been beset by small patient cohorts and the inclusion of patients with a variety of different conditions under the umbrella term “uveitis.” Although all these conditions are of an inflammatory etiology, their pathogenesis is unlikely to be driven by the same inflammatory pathways (Kim et al., 2015). As we have described for NIV, the targeting of a single cytokine (i.e., TNF-α) was misguided because the patients did not even express the molecule (Velez et al., 2017). Personalized proteomics may guide the segregation and qualification of patients for precise interventions better than non-specific clinical classifications or genetic testing. Before enrollment in clinical trials, patients could be tested to ensure that they express the drug target and that they have similar proteomic signatures (i.e., disease stage). Even if a protein biomarker is convincingly elevated in diseased tissue, its functional role (or lack thereof) in driving the disease process may still be unclear. For example, several proteomic studies on PVR vitreous have reported elevated VEGF levels despite the fact that PVR is not characterized by vasoproliferation (Citirik et al., 2012; Roybal et al., 2017). Historical-control pilot studies investigating the outcomes of intra-silicone bevacizumab (anti-VEGF) injections in eyes undergoing PVR-related RD repair did not show increased reattachment rates or improved final visual acuity (Hsu et al., 2016). Our proteomic analysis of PVR vitreous revealed the presence of elevated IL-17F, TIMP-2, and E-cadherin, known inhibitors of endothelial cell angiogenesis. Thus, despite the presence of elevated VEGF, the proteomic signature of PVR suggests a functional halt in endothelial cell proliferation. Additionally, we identified elevated levels of IL-13 in PVR vitreous, a cytokine that has been demonstrated to make monocytes resistant to glucocorticoids and reduce their suppressive effects on pro-fibrotic IL-6 production (Roybal et al., 2017; Spahn et al., 1996). Thus, a basic understanding of immunological processes underlying vitreoretinal diseases using proteomics can better guide the selection of patients and interventions for clinical trials.

SURGICAL SAMPLE COLLECTION, STORAGE, AND ANALYSIS

To advance Precision Health approaches for intraocular inflammation, our group designed and implemented a novel device and software that allows for immediate and point-of-care processing of liquid biopsy specimens, the Stanford Biorepository for Eye and Surgical Tissue (BEST; Figure 7). A first-in-class web-based relational database for tracking phenotypes and surgical specimens was created and used successfully in the operating room. The Stanford BEST system has several key components: a mobile operating-room cart with a flat laboratory-bench surface (termed the Mobile Operating Room Lab Interface, MORLI), a computer with secure access to a sample database, a barcode scanner, and drawers with laboratory supplies for specimen collection and processing away from the surgical field (Figure 7A) (Skeie et al., 2014). Once liquid biopsies are collected during the procedure, they are passed to a clinical research coordinator or surgical fellow who centrifuges the sample and transfers it to barcoded cryotube (Figure 7B). The use of a 2D-barcoded tube eliminates the need for patient identifiers on the tubes. The sample is immediately flash-frozen in dry ice or liquid nitrogen, and the barcode and corresponding patient phenotypic data are entered into electronic database (Figures 7C–7E).

The Stanford BEST database and specimen tracking system was created using REDCap software. While in the operating room, clinical data corresponding to the collected specimen (e.g., patient...
demographics, phenotype data, and sample features) are entered into the sample submission form (Figure 8). The user interface is designed to resemble an electronic surgical note and allows for efficient entry of data by the clinical team. Additional clinical data are often required to select comparable specimens for proteomic analysis. To ensure detailed patient histories and phenotypic data are captured in our database, our group developed patient consent and protection measures that allow us to link patient identifiers to surgical samples. These consents allow our team to mine the clinical charts in the future as new hypotheses are formed. Previously, this information was explained to patients during the preoperative consent. Our team now administers an electronic consent form using iPads before the patient enters the operating room. After samples are processed in the operating room, they are transferred to the laboratory where the tube is stored in the biorepository (i.e., −80°C freezers). The location of each sample in the biorepository (e.g., box position and freezer row) is then entered into the database for efficient sample retrieval.

Figure 7. The Stanford Biorepository for Eye and Surgical Tissue (BEST)
(A) The Stanford BEST unit provides the necessities for sample collection and processing in the operating room. These items include a computer equipped with a scanner for reading tube barcodes. It is also equipped with sterile pipette tips, pipettors, tubes, and a dry-ice container for immediate snap-freezing following sample collection.
(B) After a sample has been collected, it is transferred to a barcoded tube. Next the sample barcode is scanned into the database using the scanner on the bench top.
(C) The sample is flash frozen in dry ice using a magnetized base container to preserve the temperature.
(D) Samples are processed on the bench top, organized, transported to the laboratory for storage.
(E) Samples are stored in a −80°C freezer in barcoded boxes. The location of each sample in the biorepository (e.g., box position and freezer row) is then entered into the database for efficient sample retrieval.

The initial unbiased identification and semi-quantification of proteins in banked specimens is termed the “discovery phase” of the biomarker discovery pipeline. The “product” of this phase is a list of proteins that
are differentially expressed between diseased and control tissues (e.g., the vitreous). Once ophthalmic surgical specimens are properly collected and stored, their protein content can be analyzed using a variety of unbiased and targeted proteomic platforms. These analytical methods are extensively reviewed in Velez et al. (2018c) and can yield different results. MS is a powerful, unbiased analytical technique that can catalog the hundreds to thousands of proteins and metabolites in a complex biological sample. MS ionizes molecular species and sorts the ions based on their mass-to-charge ratio ($m/z$). Before analysis, samples are proteolytically digested (to generate a peptide mixture) and separated by liquid chromatography (i.e., LC-MS/MS) (Zhang et al., 2013). The separated peptides are then ionized to form gas-phase ions, which enter the mass spectrometer. In the mass spectrometer, isolated peptides produce a fragmentation pattern that yields spectra that can be bioinformatically matched to their corresponding protein (Bjornson et al., 2008; Geer et al., 2004; Perkins et al., 1999; Yen et al., 2009). The identified peptides can then be quantified by their relative abundance in the MS data or by an exogenous label. Overwhelmingly abundant proteins in the sample (e.g., albumin in plasma and vitreous) can suppress the signal from lower abundance ions, making it difficult to less-abundant proteins. The use of targeted platforms, such as multiplex ELISA (enzyme-linked immunosorbent assay) arrays, are quantitative and can enrich the analysis by detecting

Figure 8. Stanford BEST Sample Submission Form and Database

Screenshot of the specimen submission form. The form can be accessed remotely anywhere in the world so samples can be securely entered into the database as soon as they are collected. Fields to be filled out at the time of entry include: (A) information about the person collecting the sample, (B) patient demographic information, (C) surgical procedure information, as well as (D) information about the sample type and collection. Tracking information including the medical record number and the sample barcode are recorded at this time to ensure that the sample is not lost after it leaves the operating room.
less-abundant vitreous proteins that might be otherwise missed by unbiased MS methods (Tighe et al., 2015). However, these platforms are limited to the detection of a finite number of proteins represented on the array. As the human vitreous contains over 2,000 non-redundant proteins, LC-MS/MS and multiplex ELISA arrays should be used concurrently in the discovery phase to maximize the number of potential biomarkers for verification and validation studies. This difference in the use of these analytical platforms is exemplified in our study of the NIV vitreous proteome.

Despite its complexity, MS datasets are extremely versatile and offer information on protein expression levels, post-translational modifications, metabolites, and pharmacokinetics. MS experiments have been increasingly utilized in recent years and produce large volumes of highly complex data. Raw MS data are typically deposited to the PRIDE Archive via the ProteomeXChange Consortium (Figure 9A) (Deutsch et al., 2017; Perez-Riverol et al., 2016; Vizcaino et al., 2016). Many software pipelines exist for analyzing raw MS data, but proprietary mass spectrometer file formats frequently change and the ability to search proteomic data or compare results between experiments remains highly inefficient (Sharma et al., 2012). Additionally, these files are often poorly annotated with the experimental details and software search parameters. Ideally proteomic data would be accessible and searchable by the casual user. Due to the increased interest by ophthalmologists and scientists to interrogate proteomics data for their own research, there is an increased need for dissemination of MS data and a centralized proteomics database for public use. Our group created the Ocular Proteome Database (http://www.proteomics-eye.mahajanlab.org/), a searchable public database that details regional protein expression in the human retina, RPE, and vitreous (Figure 9B). Datasets are also available for the C57BL6/J mouse retina and vitreous. Researchers and clinicians can search the database for their protein of interest (using the protein name or Ensembl ID) as well as download the processed MS datasets for their own interrogation (Figure 6C). The database is expected to expand as our group continues to collect data on human and animal tissues, as well as cultured cells.

CONCLUSIONS AND FUTURE DIRECTIONS

CAPN5 is a Ca²⁺-activated regulatory protease that is ubiquitously expressed in human tissues and is sequentially conserved across vertebrates. Despite CAPN5’s expression in a wide range of organ systems, hyperactivating mutations in the CAPN5 gene typically manifest as non-syndromic inflammatory disease of the eye (NIV) (Mahajan et al., 2012; Schaefer et al., 2016; Velez et al., 2018a). In this review, we discussed many components that contribute to how NIV disease is caused, with an emphasis on the downstream sequelae of aberrant CAPN5 activity in the human vitreous. It should be noted that although the list of potential biomarkers for NIV is exhaustive, it is by no means complete as evidenced by the identification of larger lists of proteins with more sensitive mass spectrometers (Angel et al., 2012; Timp and Timp, 2020). Additionally, the proteomic signature that we identify in the vitreous is dynamic and is the net effect of all metabolic activity happening in the eye at that time of surgery (Mahajan and Skeie, 2014; Velez et al., 2018a).
Thus, not all proteins identified in a proteomics experiment are necessarily related to the disease process. Drawing a causal relationship between these potential therapeutic targets or biomarkers and NIV (or other diseases in question) needs to be done after careful validation. CAPN5 is a member of the calpain family of proteases, which contribute to a variety of human disease processes. Efforts to selectively inhibit different calpains have largely been unsuccessful due to the conserved nature of their active site (among calpain orthologs and other families of cysteine proteases, e.g., papain and cathepsins), which leads to off-target effects, poor bioavailability, and unacceptable side effect profiles (Ono et al., 2016). Thus, to specifically target CAPN5 hyperactivity, the structure and unique features of CAPNS, a non-classical calpain, must be better understood to design precise therapies for retinal disease. We were successful in solving the structure of the wild-type human CAPNS-PC in its inactive state and performed detailed characterization of its activity and biophysical properties. In addition to solving the CAPNS-PC structure, we established that the NIV causing mutations cause calpain hyperactivity. Our group is currently utilizing this knowledge in the design and testing of therapeutic inhibitors for NIV.

Despite these discoveries, understanding the structure of CAPNS does little to help patients with NIV in the near term. Drug development often takes several years and millions of dollars before patients can be treated. We therefore used personalized proteomics to characterize the downstream sequelae of CAPNS hyperactivity in the vitreous to identify proteins that could be targeted by available drugs. FDA-approved drugs already have demonstrated bio-availabilities and well-characterized side effect profiles. Although this approach did not inform us on the direct targets of CAPNS in the eye, we were able to gain some insight into downstream immune signaling pathways that were perturbed as a result of CAPNS hyperactivity. The success of these repurposed drugs highlights the power of personalized proteomics to guide treatment strategies for inflammatory diseases. Although not curative, these treatments will help to delay and prevent blindness in patients with NIV while we develop a CAPNS-specific inhibitor. Further characterization of the NIV vitreous proteome using LC-MS/MS allowed us to capture signaling events that were not represented on our multiplex ELISA array. The NIV vitreous proteome displayed characteristic enrichment and depletion of proteins and pathways associated with more common causes of blindness. NIV phenocopies each of these common eye diseases at different stages, suggesting a potential mechanistic overlap between NIV and other retinal pathologies.

Another critical component to understanding NIV pathology is determining the retinal substrates of CAPNS. Because the calpain family is large and the members are expressed in an overlapping set of tissues, it has been difficult to determine which calpain might execute various biological processes and what their protein substrates might be (Sorimachi et al., 2012). In past studies, the strict calcium dependence of calpains, the multiplicity of targets, and cross-proteolysis of substrate proteins have made it difficult to study calpain proteolysis in anything but highly purified systems in vitro. Despite these challenges some substrates have been identified for certain calpains. Nevertheless, none of the targets identified have been shown to recognize a consensus motif for proteolytic cleavage, leaving open the question of what exactly calpains recognize. These complications leave calpain substrates nearly impossible to predict and calpain pathologies intransigent to treatment. Our laboratory has started to identify the retinal substrates of CAPNS and dissect the sequential determinants of CAPNS specificity using proteomics. The identification of these substrates would further elucidate the mechanistic role of perturbed signaling pathways to NIV pathogenesis. Additional forthcoming biochemical, structural, and pharmacological studies in the years ahead will provide us with a more complete understanding of CAPNS’s role in the development of intraocular inflammation and guide the design of precise, targeted strategies for NIV.

Although individually rare, it is estimated that over 30 million people living in the United States are affected by one of 7,000 rare diseases (Whicher et al., 2018). Despite advances in our understanding of the underlying mechanisms for these rare diseases, only a small fraction (5%) of these have effective treatments (Whicher et al., 2018). A majority of the remaining patients are managed by non-specific treatments, often driven by trial and error. Furthermore, genetic testing information often does little to direct or improve treatment for rare diseases. In contrast, proteomics analysis provides a snapshot of a patient’s disease state by characterizing the real-time effectors of the expressed genes in surrounding tissues. These proteomic signatures can then guide the diagnosis or staging of a patient’s disease as well as identify drug targets that were not previously considered. Our previous research has demonstrated the success of this approach in treating blinding eye diseases, such as NIV and non-infectious posterior uveitis (Velez et al., 2016, 2017). Personalized proteomics and targeted treatments hold the key to advancing treatment for these disabling
diseases. In the future, fluid compartments near the diseased tissue could be routinely sampled and tested for proteomic biomarkers that will point to the best, personalized therapy for patient’s disease.

ACKNOWLEDGMENTS
We thank Teja Chemudupati, Daniel Machlab, and Ted Paulson for technical assistance. Funding/Support: V.B.M. is supported by NIH grants (R01EY024665, R01EY025225, R01EY024698, R21AG050437, and P30EY02687) and Research to Prevent Blindness, New York, NY. G.V. is supported by NIH grants (F30EYE027986 and T32GM007337).

AUTHOR CONTRIBUTIONS
V.B.M. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drafting of the manuscript: G.V. Critical revision of the manuscript for important intellectual content: V.B.M. Statistical analysis: G.V. and V.B.M. Obtained funding: V.B.M. Administrative, technical, and material support: V.B.M. Study supervision: V.B.M.

DECLARATION OF INTERESTS
Conflict of Interest Disclosures: None reported. Role of the Sponsor: The funding organizations had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

REFERENCES
Angel, T.E., Aryal, U.K., Hengel, S.M., Baker, E.S., Kelly, R.T., Robinson, E.W., and Smith, R.D. (2012). Mass spectrometry-based proteomics: existing capabilities and future directions. Chem. Soc. Rev. 41, 3912–3928.

Azuma, M., and Shearer, T.R. (2008). A new technique of proliferative vitreoretinopathy. Ophthalmic Res. 40, 16–23.

Bassuk, A.G., Yeh, S., Wu, S., Martin, D.F., Tsang, S.H., Gakhar, L., and Mahajan, V.B. (2015). Structural modeling of a novel CAPN5 mutation that causes uveitis and neovascular retinal detachment. PLoS One 10, e0122552.

Bennett, S.R., Folk, J.C., Kimura, A.E., Russell, S.R., Stone, E.M., and Raphts, E.M. (1990). Autosomal dominant neovascular inflammatory vitreoretinopathy. Ophthalmology 97, 1125–1135.

Berger, W., Kloeckener-Gruissem, B., and Neidhardt, J. (2010). The molecular basis of human retinal and vitreoretinal diseases. Prog. Retin. Eye Res. 29, 335–375.

Bjornson, R.D., Carriero, N.J., Colangelo, C., Shifman, M., Cheung, K.H., Miller, P.L., and Williams, K. (2008). AlTandem, an improved method for running XTandem in parallel on collections of commodity computers. J. Proteome Res. 7, 293–299.

Campbell, R.L., and Davies, P.L. (2012). Structure-function relationships in calpains. Biochem. J. 447, 335–351.

Czirik, M., Kabatas, E.U., Bateman, C., Akin, K.O., and Kabatas, N. (2012). Vitreous vascular endothelial growth factor concentrations in proliferative diabetic retinopathy versus proliferative vitreoretinopathy. Ophthalmic Res. 47, 7–12.

Cole, C.J., Kwan, A.S., Laidlaw, D.A., and Aylward, G.W. (2008). A new technique of combined retinal and choroidal biopsy. Br. J. Ophthalmol. 92, 1357–1360.

Deutsch, E.W., Cordas, A., Sun, Z., Jamuzczak, A., Perez-Riverol, Y., Tement, T., Campbell, D.S., Bernal-Llanoes, M., Okuda, S., Kawano, S., et al. (2017). The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. Nucleic Acids Res. 45, D1100–D1106.

Di Meo, A., Bartlett, J., Cheng, Y., Pastic, M.D., and Yousef, G.M. (2017). Liquid biopsy: a step forward towards precision medicine in urologic malignancies. Mol. Cancer 16, 380.

DiCarlo, J.E., Mahajan, V.B., and Tsang, S.H. (2018). Gene therapy and genome surgery in the retina. J. Clin. Invest. 128, 2177–2188.

Ferguson, T.A., and Griffith, T.S. (2006). A vision of cell death: Fas ligand and immune privilege 10 years later. Immunol. Rev. 213, 228–238.

Gakhar, L., Bassuk, A.G., Velez, G., Khan, S., Yang, J., Tsang, S.H., and Mahajan, V.B. (2016). Small-angle X-ray scattering of calpain-5 reveals a highly open conformation among calpains. J. Struct. Biol. 196, 309–318.

Geer, L.Y., Markey, S.P., Kowalak, J.A., Wagner, L., Xu, M., Maynard, D.M., Yang, X., Shi, W., and Bryant, S.H. (2004). Open mass spectrometry search algorithm. J. Proteome Res. 3, 958–964.

Guyer, P.E., Holdt, L.M., Teupser, D., and Mann, M. (2017). Revisiting biomarker discovery by plasma proteomics. Mol. Syst. Biol. 13, 942.

Gregory, M.S., Repp, A.C., Holbaum, A.M., Saff, R.R., Marshak-Rothstein, A., and Ksnander, B.R. (2002). Membrane Fas ligand activates innate immunity and terminates ocular immune privilege. J. Immunol. 169, 2727–2735.

Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R., and Ferguson, T.A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. Science 270, 1189–1192.

Gritz, D.C., and Wong, I.G. (2004). Incidence and prevalence of uveitis in northern California: the northern California epidemiology of uveitis study. Ophthalmology 111, 491–500.

Hsu, J., Khan, M.A., Sheih, W.S., Chiang, A., Maguire, J.J., Park, C.H., Garg, S.J., Ho, A.C., and Kaiser, R.S. (2016). Effect of serial intrascleral oil bevacizumab injections in eyes with recurrent proliferative vitreoretinopathy retinal detachment. Am. J. Ophthalmol. 161, 65–70.e61–62.

Hurley, J.B., and Chao, J.R. (2020). Retinal disease: how to use proteomics to speed up diagnosis and metabolomics to slow down degeneration. EBioMedicine 53, 102687.

Jabs, D.A. (2008). Epidemiology of uveitis. Ophthalmic Epidemiol. 15, 283–284.

Kim, J.S., Knickelbein, J.E., Nussenblatt, R.B., and Sen, H.N. (2015). Clinical trials in noninfectious uveits. Int. Ophthalmol. Clin. 55, 79–110.

Landowski, M., Kelly, U., Klingeborn, M., Groelle, M., Ding, J.D., Grigsby, D., and Bowes Rickman, C. (2019). Human complement factor H Y402H polymorphism causes an age-related macular degeneration phenotype and lipoprotein dysregulation in mice. Proc. Natl. Acad. Sci. U.S.A. 116, 3703–3711.

Le Golf, M.M., and Bishop, P.N. (2008). Adult vitreous structure and postnatal changes. Eye (Lond) 22, 1214–1222.

Lee, K., Bajwa, A., Freitas-Neto, C.A., Metzinger, J.L., Wentworth, B.A., and Foster, C.S. (2014). A comprehensive review and update on the non-biologic treatment of adult noninfectious uveitis part I. Expert Opin. Pharmacother. 15, 2141–2154.

Li, A.S., Velez, G., Darbro, B., Toral, M.A., Yang, J., Tsang, S.H., Ferguson, P.J., Folk, J.C., Bassuk, A.G., Yeh, S., Wu, S., Martin, D.F., Tsang, S.H., Gakhar, L., and Mahajan, V.B. (2015). Clinical trials in noninfectious uveits. Int. Ophthalmol. Clin. 55, 79–110.
Electrophoresis identification by searching sequence databases

Perkins, D.N., Pappin, D.J., Creasy, D.M., and Hull, C. (1999). \textit{Electrophoresis} 20, 3551–3567.

Resnikoff, S., Pascolini, D., Etya'ale, D., Kocur, I., Pararajasegaram, R., Pokharel, G.P., and Mariotti, S.P. (2004). Global data on visual impairment in the year 2002. \textit{Bull. World Health Organ.} 82, 844–851.

Rodriguez, A., Calonge, M., Pedraza-Seres, M., Akova, Y.A., Messmer, E.M., D'Amico, D.J., and Foster, C.S. (1996). Referral patterns of uveitis in a tertiary eye care center. \textit{Arch. Ophthalmol.} 114, 593–599.

Rose-Zerilli, M.J., Davis, S.A., Holgate, S.T., and Holloway, J.W. (2013). The genetics of allergic disease and asthma. \textit{In Pediatric Allergy: Principles and Practice, Second Edition}, D.Y.M. Leung, H.A. Sampson, R. Geha, and S.J. Szelifer, eds. (Elsevier), pp. 22–39.

Rosenbaum, J.T., and Kim, H.W. (2013). Innate immune signals in autoimmune and autoinflammatory uveitis. \textit{Int. Rev. Immunol.} 32, 68–75.

Rosengren, S., Firestein, G.S., and Boyle, D.L. (2003). Measurement of inflammatory biomarkers in synovial tissue extracts by enzyme-linked immunosorbent assay. \textit{Clin. Diagn. Lab. Immunol.} 10, 1002–1010.

Roybal, C.N., Velez, G., ToraL, M.A., Tsang, S.H., Bassuk, A.G., and Mahajan, V.B. (2017). Personalized proteomics in proliferative vitreoretinopathy implicate hematopoietic cell recruitment and mTOR as a therapeutic target. \textit{Am. J. Ophthalmol.} 186, 152–163.

Sakamoto, Y.R., NakaJima, T.R., Fukugre, C.R., Sakai, O.R., Yoshida, Y.R., Aezuma, M.R., and Shearer, T.R. (2000). Involvement of calpain isoforms in ischemia-reperfusion injury in rat retina. \textit{Curr. Eye Res.} 21, 571–580.

Schaefer, K.A., ToraL, M.A., Velez, G., Cox, A.J., Baker, S.A., Borchering, N.C., Colgan, D.F., Bondada, V., Mashburn, C.B., Yu, C.G., et al. (2016). Calpain-5 expression in the retina localizes to photoreceptor synapses. \textit{Invest. Ophthalmol. Vis. Sci.} 57, 2509–2521.

Shank, G.M., Balaj, L., Stott, S.L., Nahed, B., and Carter, B.S. (2017). Liquid biopsy for brain tumors. \textit{Expert Rev. Mol. Diagn.} 17, 943–947.

Sharma, V., Eng, J.K., Mccoss, M.J., and Riffle, M. (2012). A mass spectrometry proteomics data management platform. \textit{Mol. Cell Proteomics} 11, 824–831.

Shearer, T.R., Shih, M., Mizuno, T., and David, L.L. (1996). Crystallins from rat lens are especially susceptible to calpain-induced light scattering compared to other species. \textit{Curr. Eye Res.} 15, 860–865.

Singh, M., and Tyagi, S.C. (2017). Metalloproteinasmes as mediators of inflammation and the eyes: molecular genetic underpinnings governing ocular pathophysiology. \textit{Int. J. Ophthalmol.} 10, 1308–1319.

Singh, R., Brewer, M.K., Mashburn, C.B., Lou, D., Bondada, V., Graham, B., and Geddes, J.W. (2014). Calpain 5 is highly expressed in the central nervous system (CNS), carries dual nuclear localization signals, and is associated with nuclear promyelocytic leukemia protein bodies. \textit{J. Biol. Chem.} 289, 19383–19394.

Skeie, J.M., Brown, E.N., Martinez, H.D., Russell, S.R., Birkholz, E.S., Folk, J.C., Boldt, H.C., Gehrs, K.M., Stone, E.M., Wright, M.E., et al. (2012). Proteomic analysis of vitreous biopsy techniques. \textit{Retina} 32, 2141–2149.

Skeie, J.M., Roybal, C.N., and Mahajan, V.B. (2015). Proteomic insight into the molecular function of the vitreous. \textit{PLoS One} 10, e0127567.

Skeie, J.M., Tsang, S.H., Zande, R.V., Fickbohm, M.M., Shah, S.S., Vallone, J.G., and Mahajan, V.B. (2014). A biorepository for ophthalmic surgical specimens. \textit{Proteomics Clin. Appl.} 8, 209–217.

Sormachi, H., Namitsuka, H., and Ono, Y. (2012). Understanding the substrate specificity of conventional calpains. \textit{Biochim. Biophys. Acta} 1824, 853–871.

Spahn, J.D., Sfeir, S.J., Surs, W., Doherty, D.E., Nimmsgadda, S.R., and Leung, D.Y. (1996). A novel action of IL-13: induction of diminished monocyte glucocorticoid receptor-binding affinity. \textit{J. Immunol.} 157, 2654–2659.

Takashaki, V.K.L., Takiuti, J.T., Jauregu, R., and Tsang, S.H. (2018). Gene therapy in inherited retinal degenerative diseases, a review. \textit{Ophthalmol. Genet.} 39, 560–568.

Tamada, Y., NakaJima, E., NakaJima, T., Shearer, T.R., and Aezuma, M. (2005). Proteolysis of neuronal cytoskeletal proteins by calpain contributes to rat retinal cell death induced by hypoxia. \textit{Brain Res.} 1050, 148–155.

Tang, P.H., Kinncick, T.R., Folk, J.C., Mahajan, M., Bassuk, A.G., Tsang, S.H., and Mahajan, V.B. (2018). Progression of scotopic single-flash electroretinography in the stages of CapN5 vitreoretinopathy. \textit{Retin. Cases Brief. Rep.} https://doi.org/10.1016/j.crbi.2018.09.001.

Tang, P.H., Velez, G., Tsang, S.H., Bassuk, A.G., and Mahajan, V.B. (2019). \textit{VCAN} canonical splice site mutation is associated with vitreoretinal degeneration and disrupts an MMP proteolytic site. \textit{Invest. Ophthalmol. Vis. Sci.} 60, 282–293.

Tighe, P.J., Ryder, R.R., Todd, I., and Fairclough, L.C. (2015). EUISA in the multiplex era: potentials and pitfalls. \textit{Proteomics Clin. Appl.} 9, 406–422.

Timp, W., and Timp, G. (2020). Beyond mass spectrometry, the next step in proteomics. \textit{Sci. Adv.} 6, eaax8978.

Tlueck, P.S., Folk, J.C., Sabol, W.M., and Mahajan, V.B. (2013). Surgical management of fibrotic encapsulation of the fluocinolone acetonide implant in CAPN5-associated proliferative vitreoretinopathy. \textit{Clin. Ophthalmol.} 7, 1093–1098.

Tsang, S.H., Tsui, I., Chou, C.L., Zarnant, J., Haamer, E., Iramnashesh, R., Tosi, J., and Allikmets, R. (2008). A novel mutation and phenotypes in phosphodiesterase 6 deficiency. \textit{Am. J. Ophthalmol.} 146, 780–788.

Velez, G., Bassuk, A.G., Colgan, D., Tsang, S.H., and Mahajan, V.B. (2017). Therapeutic drug repositioning using personalized proteomics of liquid biopsies. \textit{JCI Insight} 2, e97818.

Velez, G., Bassuk, A.G., Schaefer, K.A., Brooks, B., Gakhkar, L., Mahajan, M., Kahn, P., Tsang, S.H., Ferguson, P.J., and Mahajan, V.B. (2018a). A novel de novo CANP5 mutation in a patient with
inflammatory vitreoretinopathy, hearing loss, and developmental delay. Cold Spring Harb. Mol. Case Stud. 4, a002519.

Velez, G., Machlab, D.A., Tang, P.H., Sun, Y., Tsang, S.H., Bassuk, A.G., and Mahajan, V.B. (2018b). Proteomic analysis of the human retina reveals region-specific susceptibilities to metabolic- and oxidative stress-related diseases. PLoS One 13, e0193250.

Velez, G., Machlab, D.A., Tang, P.H., Sun, Y., Tsang, S.H., Bassuk, A.G., and Mahajan, V.B. (2018b). Proteomic analysis of the human retina reveals region-specific susceptibilities to metabolic- and oxidative stress-related diseases. PLoS One 13, e0193250.

Velez, G., Roybal, C.N., Colgan, D., Tsang, S.H., Bassuk, A.G., and Mahajan, V.B. (2016). Precision medicine: personalized proteomics for the diagnosis and treatment of idiopathic inflammatory disease. JAMA Ophthalmol. 134, 444–448.

Velez, G., Sun, Y.J., Khan, S., Yang, J., Herrmann, J., Chemudupati, T., MacLaren, R.E., Gakhar, L., Wakatsuki, S., Bassuk, A.G., et al. (2020). Structural insights into the unique activation mechanisms of a non-classical calpain and its disease-causing variants. Cell Rep. 30, 881–892 e885.

Velez, G., Tang, P.H., Cabral, T., Cho, G.Y., Machlab, D.A., Tsang, S.H., Bassuk, A.G., and Mahajan, V.B. (2018). Personalized proteomics for precision health: identifying biomarkers of vitreoretinal disease. Transl. Vis. Sci. Technol. 7, 12.

Velez, G., Yang, J., Li, A.S., Tsang, S.H., Bassuk, A.G., and Mahajan, V.B. (2019). Proteomic insight into the pathogenesis of CAPN5-vitreoretinopathy. Sci. Rep. 9, 7608.

Vizzacino, J.A., Csordas, A., del-Toro, N., DiCesare, J.A., Gross, J., Lavdas, I., Mayer, G., Perez-Riverol, Y., Reisinger, F., Tement, T., et al. (2016). 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 44, D447–D456.

Wallace, G.R., and Niemczyk, E. (2011). Genetics in ocular inflammation–basic principles. Ocul. Immunol. Inflamm. 19, 10–18.

Wang, S., Song, R., Wang, Z., Jing, Z., Wang, S., and Ma, J. (2018a). Stabilization of A9 in inflammation. Front. Immunol. 9, 1298.

Wang, Y., Li, H., Zhang, S., Li, F., Chen, Y., Zhang, X., Song, Z., Peng, Q., and Gu, F. (2018b). Photoreceptor cell-derived CAPN5 regulates retinal pigment epithelium cell proliferation through direct regulation of SLIT2 cleavage. Invest. Ophthalmol. Vis. Sci. 59, 1810–1821.

Wert, K.J., Bassuk, A.G., Wu, W.H., Gakhar, L., Coglan, D., Mahajan, M., Wu, S., Yang, J., Lin, C.S., Tsang, S.H., et al. (2015). CAPN5 mutation in hereditary uveitis: the R243L mutation increases calpain catalytic activity and triggers intraocular inflammation in a mouse model. Hum. Mol. Genet. 24, 4584–4598.

Wert, K.J., Koch, S.F., Velez, G., Hsu, C.W., Mahajan, M., Bassuk, A.G., Tsang, S.H., and Mahajan, V.B. (2018a). Personalized proteomics for precision health: identifying biomarkers of vitreoretinal disease. Transl. Vis. Sci. Technol. 7, 12.

Wert, K.J., Skeie, J.M., Bassuk, A.G., Olivier, A.K., Tsang, S.H., and Mahajan, V.B. (2014). Functional validation of a human CAPN5 exome variant by lentiviral transduction into mouse retina. Hum. Mol. Genet. 23, 2656–2677.

Wert, K.J., Velez, G., Kanchustambham, V.L., Shankar, V., Evans, L.P., Sengillo, J.D., Zare, R.N., Bassuk, A.G., Tsang, S.H., and Mahajan, V.B. (2020). Metabolite therapy guided by liquid biopsy proteomics delays retinal neurodegeneration. EBioMedicine 52, 102636.

Whicher, D., Philbin, S., and Aronson, N. (2018). An overview of the impact of rare disease characteristics on research methodology. Orphanet J. Rare Dis. 13, 14.

Wisniewski, R., and Tomanek, R. (1967). Lens-induced uveitis. Arch. Ophthalmol. 78, 1–7.

Xu, C.L., Cho, G.Y., Sengillo, J.D., Park, K.S., Mahajan, V.B., and Tsang, S.H. (2018). Translation of CRISPR genome surgery to the bedside for retinal diseases. Front. Cell Dev. Biol. 6, 46.

Xie, Y., Meyer-Arendt, K., Eichelberger, B., Sun, S., Houel, S., Old, W.M., Knight, R., Ahn, N.G., Hunter, L.E., and Resing, K.A. (2009). A simulated MS/MS library for spectrum-to-spectrum searching in large scale identification of proteins. Mol. Cell Proteomics 8, 857–869.

Zhang, Y., Fonslow, B.R., Bhan, B., Baez, M.C., and Yates, J.R., 3rd (2013). Protein analysis by shotgun/bottom-up proteomics. Chem. Rev. 113, 2343–2394.

Ziccardi, L., Cordeddu, V., Gaddini, L., Matteucci, A., Parravano, M., Malchiardi-Albedi, F., and Varano, M. (2019). Gene therapy in retinal dystrophies. Int. J. Mol. Sci. 20, 5722.