Integration of Tear Fluid Biomarkers and Machine Learning for the Early Detection of Orbital Inflammatory Disorders

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DOI: https://dx.doi.org/10.51244/IJRSI.2025.1210000336

Received: 06 November 2025; Accepted: 13 November 2025; Published: 22 November 2025

ABSTRACT

The integration of tear fluid biomarkers and machine learning holds great promise for early detection and prognostication of orbital inflammatory disorders (OID) such as Graves' orbitopathy and nonspecific orbital inflammation..

A hybrid diagnostic framework that combines proteomic analysis of tear fluid with AI-driven imaging enables improved sensitivity and specificity in identifying, staging, and predicting the progression of OID. This method utilizes non-invasive tear sampling to identify disease-specific molecular signatures and employs machine learning to differentiate inflammatory and non-inflammatory states using imaging data, bringing precision medicine to the forefront of orbital disease management.

Introduction

Orbital inflammatory disorders are characterized by a heterogeneous clinical course and lack sensitive, non-invasive biomarkers for early diagnosis and risk stratification. Traditional diagnostic approaches rely on subjective clinical grading and imaging, often failing to distinguish active inflammation from chronic, fibrotic stages. Tear fluid biomarkers, identifiable through proteomic analysis, offer molecular specificity, while artificial intelligence (AI) can augment imaging interpretation for objective assessments.

Methods

Tear Proteomics

Tear samples are collected from OID patients and analyzed using mass spectrometry and multi-omic techniques to identify proteins associated with inflammatory activity—such as extracellular matrix components, immune mediators, and metabolic markers. Validation follows a phased approach, progressing from pilot cohorts to multicenter studies for reproducibility.

Machine Learning Algorithms

AI models, including support vector machines, deep learning, and AutoML, are trained on imaging datasets (such as ultrasound) to differentiate OID, predict disease activity, and stage inflammatory changes.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue X October 2025

Performance metrics include precision-recall curves, ROC analysis, and confusion matrices.

Integration

Tear biomarker signatures are incorporated as features in machine learning models alongside imaging-based features to generate composite risk scores and staging predictions. The workflow is validated against conventional modalities.

Results

- Biomarker panels from tear fluid can discriminate OID from non-inflammatory orbitopathies and predict disease activity.
- AI imaging models trained on curated datasets achieve high precision and recall (PR AUC ≈ 0.98), reliably distinguishing inflammatory from non-inflammatory cases.
- The integrated workflow outperforms standalone modalities, providing improved sensitivity, specificity, and prognostic accuracy.

Discussion

Significance

This integrated approach enables earlier and more accurate diagnosis, personalized risk prediction, and precise selection for targeted therapies. Tear sampling is a safe and repeatable procedure; AI-enhanced imaging reduces operator dependency and subjectivity in interpretation. The framework supports advanced response monitoring and is extensible to other autoimmune and orbital inflammatory conditions.

Clinical Impact and Limitations

Early intervention and tailored management improve outcomes in OID. Limitations include the need for large-scale biomarker validation, potential operator variability in imaging, resource constraints for omics and AI implementation, and the dynamic expression of disease biomarkers requiring longitudinal analysis.

Ethics and Future Directions

IRB approval, patient consent, and strict privacy protocols are mandatory. Ongoing development will focus on external validation, standardized protocols, real-world data integration, platform scaling, and streamlining biomarker panels for broader application.

Conclusion

Integrating tear fluid biomarkers with machine learning-powered imaging represents an innovative solution for early detection and precision management of orbital inflammatory disorders, addressing unmet clinical needs and advancing the field of ophthalmic diagnostics.

Keywords: Tear Proteomics, Graves Orbitopathy, Orbital Inflammatory Diseases, Eye Proteins/analysis, Tear Biomarkers/metabolism, Molecular Diagnostic Techniques/methods.

INTRODUCTION

Recent developments in the accessibility and sensitivity of proteomic assays have led to the examination of tear fluids as a potential source for biomarker analysis. Tear sampling provides a convenient and non-invasive

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue X October 2025

method for analyzing an accessible body fluid to investigate biomarkers in predictive, preventive, and personalized medicine. In addition, as a complex mixture, tears offer the possibility of discovering not only proteins but also RNA, lipid, and metabolite biomarkers that could complement the traditional clinical tools available for ophthalmologists. Orbital inflammations include a broad spectrum of orbital diseases that can be idiopathic, infectious, or from primary and secondary inflammatory processes. The most common orbital inflammatory disease is Graves' ophthalmopathy (GO), also known as thyroid eye disease (TED). Being able to diagnose and manage these orbital diseases promptly can prevent permanent vision loss and potentially save a patient's life. Standard history, physical examination, laboratory testing, and radiologic studies are utilized in the evaluation of these orbital disorders, but limitations in diagnosis and monitoring remain.

Tear film proteomics offers powerful analytical tools for studying the proteins involved in ocular diseases. Using these tools, we aim to establish the interaction of these proteins in the underlying pathophysiological processes and provide diagnostic biomarkers. The study of differential protein expression in complex biofluids such as the tear film requires highly reproducible and accurate quantification.

Specific Aim:

To analyze the tear proteomes longitudinally in patients with GD, with and without orbitopathy, and compare with patients with other orbital inflammatory disorders, including Sarcoidosis, IgG4-related orbitopathy, granulomatosis with polyangiitis (GPA), Sjogren's syndrome, and nonspecific orbital inflammation (NSOI).

We hypothesize that each form of orbital disease will demonstrate unique pathogenic markers:

1) The tear proteomes of patients with GD and no orbitopathy will differ from the proteomes of those with orbitopathy (GO).

2) The proteomes of tears from patients with GO differ from the proteomes of patients with other types of orbital inflammation

Significance

Graves' disease leads to Orbitopathy: Unmet needs

Graves' disease (GD) is an autoimmune thyroid disease that affects 1% of the population. About 50% of patients with GD develop orbitopathy, of which only 10% have symptomatic disease. When there is a threat of vision loss from optic nerve compression, vascular compromise with the risk of infarction from vasculitis or orbital apex or cavernous sinus involvement, or significant disability due to pain, intractable diplopia, or ocular surface damage from exposure, there is a great impetus to quickly and accurately diagnose and treat these diseases.

Establishing a diagnosis of GO is usually straightforward because there are characteristic clinical findings, such as bilateral exophthalmos, scleral show, and lid lag; laboratory findings such as elevated serum thyroid-stimulating immunoglobulin (TSI); and characteristic imaging results such as bilateral disease and extraocular muscle swelling with sparing of the tendon. Performing a comprehensive medical history detailing the timeline and acuity, along with a complete physical examination, and laboratory and radiologic testing, will help narrow the differential diagnosis. Blood tests should be guided by clinical suspicion, which includes complete blood counts, erythrocyte sedimentation rate (ESR), C-reactive protein, antinuclear antibody (ANA), cytoplasmic antineutrophil cytoplasmic antibodies (C-ANCA), rheumatoid factor (RF), serum protein electrophoresis, angiotensin-converting enzyme (ACE), and thyroid function studies. Radiologic orbital evaluation commonly involves a computerized tomography scan (CT) or magnetic resonance imaging (MRI) with intravenous contrast, which is invaluable in narrowing down the differential diagnoses and assessing the location and extent of the disease process.

Insulin-like growth factor (IGF) is also known as somatomedin C. It can bind to the insulin receptor or IGF-receptor 1 or 2. 6 separate binding proteins modify its activity. IGF receptor 1 is expressed on most cells. The

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue X October 2025

receptor is a tyrosine kinase. Immunostaining on orbital fibroblasts indicates that the IGF receptor co-localizes with the thyroid-stimulating hormone receptor, which is activated by thyroid-stimulating immunoglobulin, a characteristic feature of Graves' disease. The binding of IGF-1 to this receptor leads to the activation of two main pathways, AKT/Phosphatidyl inositol 3-kinase or ERK. Many proteins are downstream of these pathways, as discussed in the preliminary data below.

Treatment of GO has been relatively ineffective. A recent, promising clinical advance is the use of a monoclonal antibody to the insulin-like growth factor (IGF)-1 receptor. Diplopia and extraocular muscle fibrosis have no definitive treatment except partial benefit from lens prisms or surgery to correct muscle imbalance. Fibrosis is a significant clinical finding in several medical conditions, including idiopathic pulmonary fibrosis (IPF), cirrhosis, scleroderma, scarring with wound healing, Dupuytren's contracture, and IgG4 disease. Two drugs, pirfenidone and nintedanib, are now FDA-approved to slow the progression of fibrosis in IPF. Identifying the mediators of fibrosis and understanding who is at risk for fibrosis in orbital disease should allow early and more successful treatment of this major complication. There are multiple unmet needs for GO. Although many classify GO as having an inflammatory and then a fibrotic phase, there is no biochemical correlation to these phases, and no definition exists as to when one phase ends and the other begins. Despite the autoimmune nature of thyroid disease, we have noted a relative absence of inflammatory mediators, such as mRNA for cytokines, in the orbital adipose tissue affected by GO. It remains unclear as to why some with Graves develop GO and others do not. There are no biomarkers to predict who will respond to specific therapies or who is at risk of developing complications such as fibrosis.

"Tear proteomics: potential utility in GO."

Tears offer a biofluid that can be obtained non-invasively and that reflects ocular and systemic pathologies. For example, tears have specific alterations in macular degeneration, glaucoma, anterior uveitis, Sjogren's syndrome, and graft versus host disease. The major tear proteins can be grouped into two categories: proteins produced by the lacrimal gland and serum proteins that leak from the conjunctival capillaries. The major tear proteins, lysozyme (LYZ), lactotransferrin, secretory immunoglobulin A (IgA), lipocalin, albumin, and lipophilin constituted about 80-90 % of the total amount of tear proteins. These proteins are secreted by the lacrimal glands, meibomian glands, and conjunctival goblet cells or derived from plasma leakage. Several studies have reported the presence of numerous proteins in tears, while other studies have demonstrated that the tear fluid proteome is closely related to ocular health. A wide range of techniques have been applied to study the tear proteome, including enzyme-linked immunosorbent assays (ELISA), one- and two-dimensional electrophoresis, and chromatographic methods

DISCUSSION

According to available sources, tear proteins can be classified into the following groups. (Figure 1)

(a) Proteins secreted by the main lacrimal gland, Meibomian glands, goblet cells, and accessory lacrimal glands of the ocular surface: This group includes major tear proteins, such as lysozyme, lactoferrin, secretory immunoglobulin A (sIgA), lipocalin-1 (previously called tear-specific pre-albumin, LCN-1), lipophilin, lacritin, and proline-rich proteins, etc., high abundance end (mg/ml to mg/ml)

(b) Ocular cell/tissue leakage products: These proteins usually function with the ocular surface cells but can be released into the tear fluid as a result of normal secretion or cell damage and death. Middle abundance end (mg/ml to ng/ml)

(c) Aberrant secretions: These proteins are usually released from diseased tissues and may be used as diagnostic biomarkers. low abundance end (ng/ml to pg/ml).

(d) Foreign Proteins: These proteins have another source, such as an infectious organism that releases materials into the tear fluid. Mixed abundance end

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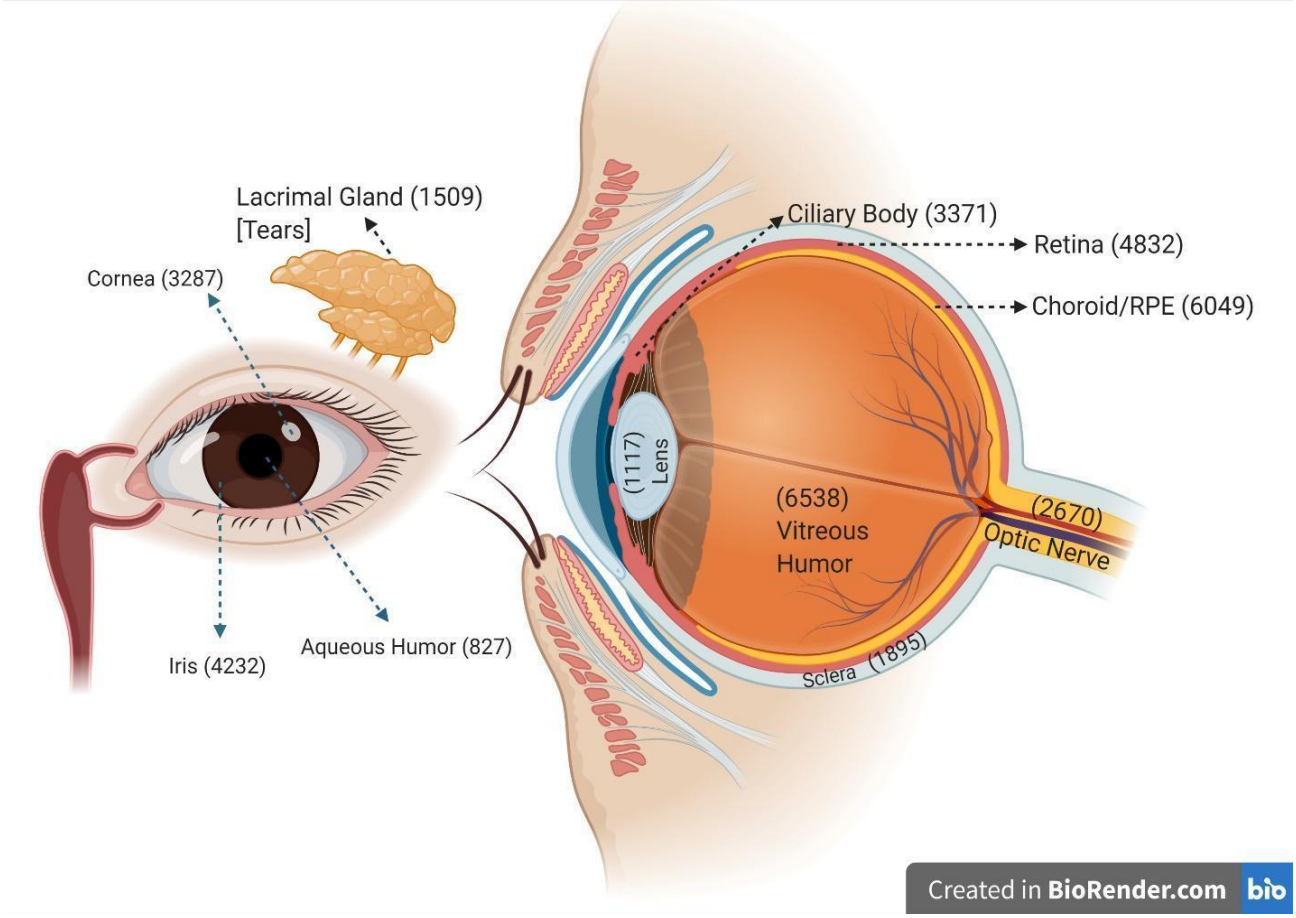

Figure 1: Schematic diagram of the human eye with the number of nonredundant proteins identified in various tissues and biofluids shown below (Courtesy of Danesh Khazaei-Artist)

According to their abundance, tear proteins can be divided into three classes: major tear proteins at the high abundance end (mg/ml to mg/ml), proteins secreted from ocular surface tissue or cells, or cell signaling molecules in the middle (mg/ml to ng/ml), such as cytokines and growth factors at the low abundance end (ng/ml to pg/ml).

The two main components of tears that we are looking for in proteomic analysis are proteinaceous secretions of the Lacrimal gland and secretions from the Conjunctival accessory glands and adnexal tissue, including caruncle and lid margins. Secretions from the lacrimal glands are responsible for different pathways, including the inflammatory and the IGF pathways. We aim to establish a relationship between these two pathways and orbital inflammation.

The first type of secretion is directly affected by the clinical active scoring and the stage of the disease, particularly when the patient is in the early active phase with a high clinical active score associated with severe proptosis. Lacrimal gland secretions consist of a type of protein that is abundant in cases of orbital inflammatory disorders detected by mass spectrometry.

The second type of secretion consists of a cytokine-induced immune response. This is primarily seen in ocular surface disorders, but the stage of orbital inflammatory disease does not influence cytokine secretion. If we are looking for a method to stage the activity of the disease during an early phase, using the ELISA test to detect cytokines is more feasible than doing mass spectrometry.

If we are looking at collecting tears during this stage, we need to avoid using any additional topical medication, including proparacaine and steroids. Tears should be collected using a non-contact, non-stimulating technique known as the pinch technique or capillary tube, which reveals the type of immune response but does not represent the stage of the disease.

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It is advisable to use a proparacaine technique for tear collection if we are looking for lacrimal gland secretion, and the pinch technique if we're looking for cytokines, which can give us a definitive immune response and stage of the disease.

The mechanical compression on the orbital lacrimal gland, as well as the inflammatory component, causes the lacrimal gland to shut down. At this stage, collecting tears may be difficult due to low flow, and so far, all experiments conducted during this early stage show a severe decrease in tear production. We recommend not collecting tears during this phase of the disease and instead look for ultrasonic findings to stage the disease.

After examining the different stages of the disease in cases of orbital inflammation, it was noted that cases without orbitopathy have ample tear secretion.

In these cases, we hypothesize that the amount of protein secreted by the lacrimal gland provides more information than the stage of disease where the lacrimal gland shuts down. The disease can be at a very early stage without active components like proptosis or at a late stage where the proptosis has reduced and the lacrimal gland returns to secreting normally. In our preliminary data, we have observed that the tear component is abundant with inflammatory proteins during very early non-active cases of orbital inflammation without orbitopathy and later stages where the proptosis has reduced. It doesn't matter if we reduce the active phase using medication or surgery because it's a mechanical effect that causes the lacrimal gland to shut down.

If the degree of proptosis is reduced, the lacrimal gland secretion returns to normal, indicating why higher amounts of these proteins are present during the very early phase without orbitopathy and in the later stages, where the eye returns to its normal position. If we are looking for a specific pathway in the inflammatory process response, we suggest collecting tears during the very early stages of the disease without orbitopathy or at a later stage when the orbitopathy has reduced, but not at a stage when the patient has developed dry eye - this is when the fibrosis has become established. The tear collection at this point is of no use.

In a very active stage characterized by proptosis, high clinical activity scoring of orbital inflammation, chemosis, and conjunctival congestion, tear collection will mainly contain cytokine components; therefore, it's not recommended to perform mass spectrometry during this stage. Elisa's test to assess the cytokines does provide much information. The staging and classification can be confirmed using ultrasonic tests.

When the patient is in the active phase, it's too late to intervene. Intervention is recommended in the pre-active phase of Graves without orbitopathy or the early phase of orbital inflammation before it becomes active. The other option is the late phases, where treatment has been given and orbitopathy has reduced.

The next question is whether orbital inflammatory disorders cause proptosis and other activity in the orbit, or if it is a mechanical compression of the veno-lymphatic drainage of the orbit. Most literature mentions that the orbit is devoid of lymphatic drainage; however, there is evidence that the orbital structure, including the eyeball itself, drains into a lymphatic system through the orbital tissue, ultimately draining into the cervical lymph nodes(1). The question is the pathophysiology of orbital disorder. Is it because of the inflammatory process, or is it due to the mechanical compression?

This raises a big question. If we decompressed the orbits, would the inflammation subside? This is something we observe in soft tissue decompression or bony decompression of the orbit, or even optic nerve fenestration. We know the inflammation has reduced, although we are not targeting inflammatory cells, by just decompressing the orbit. It will cause an endolymphatic drainage of the orbital tissue, thereby reducing the disease activity. This is an important aspect of the treatment. In the case of orbital inflammatory disorders, we have the option to treat the orbital compartment syndrome, which reduces the inflammatory cells in the orbit responsible for the disease activity.

We have demonstrated that a simple canthotomy or soft tissue orbital decompression achieved through the removal of orbital fat or optic sheath fenestration can reduce inflammation to a certain extent, which is likely due to improved lymphatic drainage. This also explains why, after doing decompression, the intraocular pressure decreases. Is mechanical decompression causing pressure reduction, or is it because we are improving

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the lymphatic drainage? This is a big question that can explain the mechanism of primary open-angle glaucoma.

In one of our experiments on orbital lymphatic drainage, we utilized photoacoustic tomography to demonstrate the drainage of interocular fluorescence material through the Vortex vein to the orbital lymphatic and blood vessels to the cervical lymph nodes. This is not published data, but it indicates that even intraocular pressure can be monitored and reduced by improving the lymphatic drainage of the orbit. How can we demonstrate this clinically? We use a component of anti-inflammatory agents to improve the lymphatic drainage, resulting in a reduction in the intraocular pressure.

The question is, when do we stop the treatment, and how much and for how long can we use these agents? The answer could be in the proteomics analysis of the tear. If we can identify the specific protein responsible for the lymphatic drainage obstructions, we can improve the stage of the disease or potentially treat the disease. The procedures and methods employed for tear collection are a crucial first step that can significantly impact the results of tear analysis.

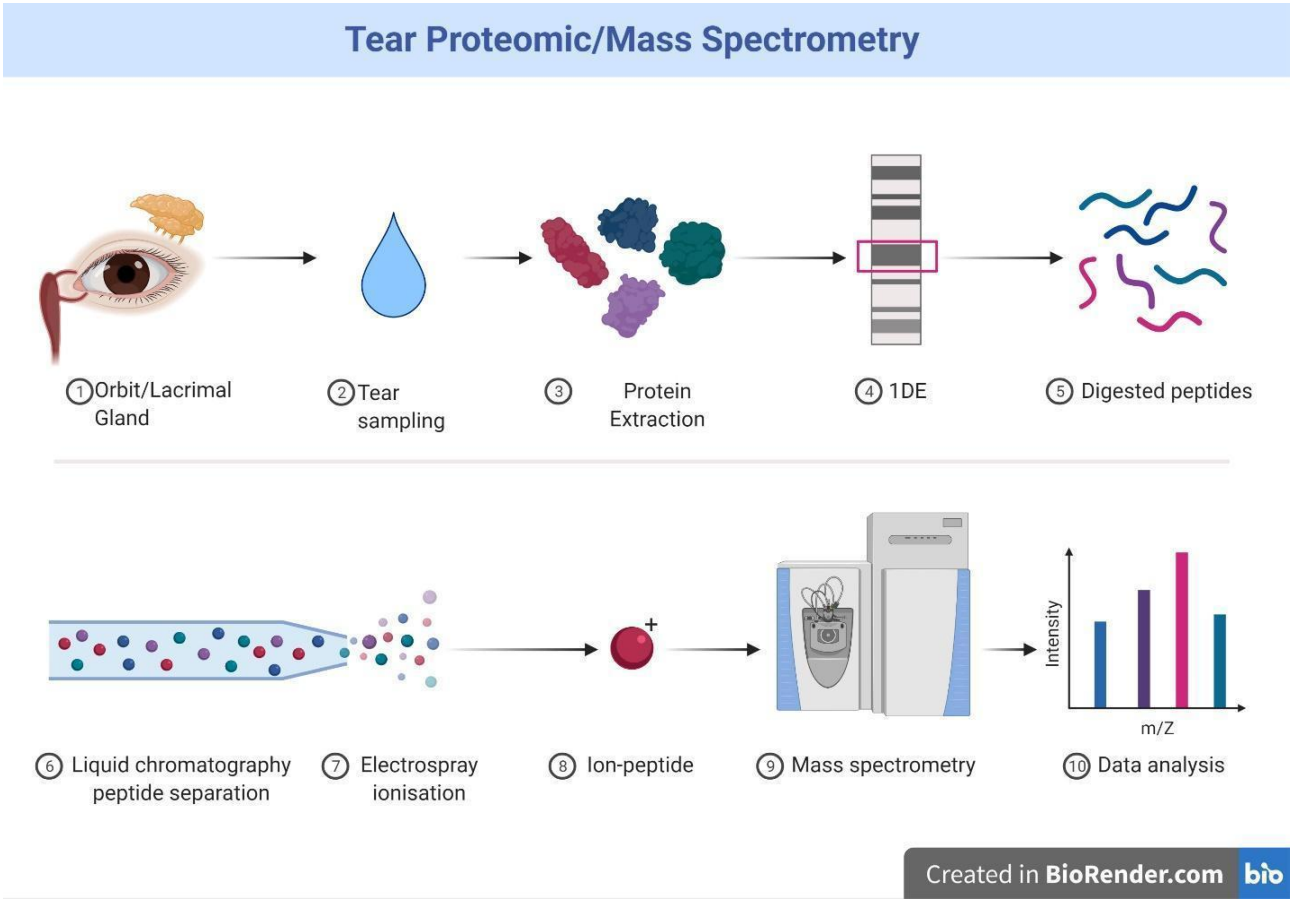

Figure 2: Tear Protein Digestion and Tandem Mass Tag (TMT) Labeling, Mass Spectrometric Analysis, and Protein Identification

Collection of tear fluid for clinical proteomics (Figure 2)

As the tears represent the “proximal fluid”, the final output of the lacrimal functional unit (Stern et al., 1998; Stern et al., 2004), variations in tear composition are an ideal source for discovering biomarkers associated with the various components of the functional unit and due to the close relationship of tears to disease sites, offers greater specificity in understanding the underlying pathology. Tear fluid can be obtained using several established collection methods, yet different sampling methods are known to affect the quality of tear samples and, consequently, the results of tear proteome analysis (2). The three most adopted sampling methods for tear fluid are cellulose sponges, Schirmer's strips, and capillary tubes. These sampling methods are non-invasive and do not require local anesthesia.

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A cellulose sponge may be used for tear collection by placing it into the lower conjunctival sac for ~1 min. It has been commonly adopted due to its high effectiveness in collecting tears, even from patients with low tear volume. This method is non-irritating and is generally well-tolerated by patients (3). Additionally, the sponge sampling method enables the standardization of the tear collection volume (4). Nevertheless, a variety of sponges and extraction buffers have been used in different studies, thus making it difficult to compare their results directly (4-5). In addition, some cytokines, including interleukins and gamma-interferon, bind tightly to the sponge, making the recovery and extraction of these proteins more difficult (6).

Schirmer's strips

Schirmer's strips are used in the Schirmer's test for dry eye assessment (7). For tear protein analysis, the strip is placed in the inferior conjunctival sac and left in place until it has been wetted to the control line. Later incubation in buffer solution to rehydrate the strip allows proteins or metabolites to be extracted for further molecular tests. This technique yields higher recovery of interleukins compared to samples collected with cellulose sponges (8) and improved protein identification compared with tear fluid collected with a capillary tube (9). Although Schirmer's strips have been considered a convenient and easy method for performing tear collection, their use can cause strong irritation, leading to reflex tearing that may result in unwanted dilution of tear proteins (10). Additionally, improper handling can also affect protein content (11). The estimation of the tear protein loss during sample manipulation at the diffusion-based protein extraction stage ranged from 2% (LYZ) to 41.2% (mucin 4) (11).

Both Schirmer's strip and cellulose sponge methods utilize absorptive materials that have contact with the conjunctiva, which can potentially damage the ocular surface. An increase in the number of certain proteins in the conjunctiva has been reported following mechanical trauma(12,13). Hence, extra care should be taken to minimize the trauma-induced stimulation of proteins during sample collection.

Capillary tube

To overcome the drawbacks of absorptive materials, capillary tube or pipette sampling can be employed. The tear fluid is drawn from the inferior temporal tear meniscus near the external canthus of the eyes to a disposable borosilicate glass microcapillary tube by simple capillary force (14). Compared to the use of absorptive materials, this method is less invasive, as it avoids reflex tearing and results in less protein disruption during the sample recovery process (15). However, it is time-consuming, requires precise handling, and may not be suitable for anxious or uncooperative patients and children (4). Improper handling of capillary tubes can induce reflex tears due to contact between the tube and the conjunctiva. In general, capillary tube sampling is not always practical and feasible in clinical studies that require reproducible data from large cohorts, particularly when children are involved (4). Furthermore, the collectible sample volume is limited. To overcome the limited tear volume of samples, pooling tears from multiple subjects can be useful in research. However, it is undesirable in clinical studies, as individual characteristics cannot be determined (16).

A study by Stuchell et al. (1984) showed that the levels of proteins of serum origin (albumin, IgG and transferrin) were significantly higher when the Schirmer’s filter paper strip was used to collect tears compared to the capillary tube while no significant differences were observed between the levels of proteins of lacrimal origin (lactoferrin and lysozyme) with the two collection methods. Recently, a detailed proteomic study compared the tear proteome using Schirmer’s test strips and glass capillary tubes (Green-Church et al., 2008). From 1D and 2D SDS PAGE gels, serum albumin was present at much higher levels when using Schirmer’s strip tear collection method. They also demonstrated that some proteins, such as a-enolase and S100 calcium-binding proteins, were observed using Schirmer’s method but not from capillary-collected tears (Green-Church et al., 2008).

Tear Protein Digestion and Tandem Mass Tag (TMT) Labeling

Fragments of the Schirmer strip will be thawed, and 33.3 μL 100-mM triethyl ammonium bicarbonate (TEAB), 2 μL 1% ProteaseMax detergent (Promega, Madison, WI, USA), and 1 μL 0.5 M dithiothreitol will be

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added. Following vortexing, the samples will be heated at 56°C for 20 minutes, then alkylated by the addition of 2.7 µL 0.55 M iodoacetamide and incubated in the dark at room temperature for 15 minutes. Proteins will be digested by the addition of 1 µL 1% ProteaseMax detergent and 10 µL 0.1 µg/µL trypsin (Pierce, MS Grade; Thermo Scientific, Waltham, MA, USA). After shaking at 37°C overnight, the solution will be removed from the threads by transferring to another 0.5-mL centrifuge tube, and 5 µL 10% trifluoroacetic acid will be added. The samples will be stored at room temperature for 1 hour and spun at 16,000*g* for 5 minutes; following this, the supernatant will be removed. Peptides will be solid phase extracted using MicroSpin columns (The Nest Group, Southborough, MA, USA) and dried by vacuum centrifugation. Each digest will then be dissolved by vigorous shaking for 10 minutes in 20 µL 100 mM TEAB, and 200 µg TMT 10-plex reagent (Thermo Scientific), and then dissolved in 12 µL anhydrous acetonitrile. Samples will then be incubated for 1 hour at room temperature, and 2 µL 5% hydroxylamine will be added for an additional 15-minute incubation.

Mass Spectrometric Analysis

Then, 2 µL of each reaction mixture will be combined, dried by vacuum centrifugation, and dissolved in 5% formic acid. Approximately 2 µg of peptide will be analyzed, as previously described, to normalize the total reporter ion intensity of each multiplexed sample and to check labeling efficiency. Based on these results, the remaining samples will be combined with volumes adjusted to produce equal total reporter ion intensities, and samples dried down in preparation for two-dimensional liquid chromatography–mass spectrometry (LC-MS) analysis. One-half of the multiplexed samples, corresponding to approximately 60 µg of peptides, will be separated using an automated two-dimensional (2D) nano–reverse phase/reverse phase chromatography system and Orbitrap Fusion mass spectrometer (Thermo Scientific) using synchronous precursor isolation for MS3-based reporter ion measurement as previously described, except using 14%, 17%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 50%, and 90% acetonitrile elution steps during the first-dimensional separation. Due to re-equilibration times after each LC-MS run, the entire 2D LC-MS/MS method will have 34 hours of instrument time.

Protein identification

Raw instrument files from the 17 fractions will be simultaneously searched with the SEQUEST HT search engine within Proteome Discoverer (PD) version 1.4.1.14 (Thermo Scientific). A manually annotated UniProtKB/Swiss-Prot database containing 7925 entries and common contaminants will be used to minimize the number of shared peptides from homologous proteins. SEQUEST HT search parameters will be identical to those previously used, and a reversed-sequence decoy strategy will be employed to control peptide false discovery, followed by validation using Percolator software within PD. Search results and TMT reporter ion intensities will be exported from PD as text files and processed using in-house Python scripts. Only peptide spectral matches (PSMs) uniquely matching a single protein entry with q scores ≤ 0.05, accurate masses within 10 parts per million, and trimmed average reporter ion intensity peak heights greater than 600 will be used for quantification. TMT reporter ions with zero values will be replaced with intensities of 150 to avoid artifacts. The individual reported ion intensities from all PSMs will be summed to create total protein intensities.

METHODOLOGY

This study aimed to determine the actual effect of an anesthetic (proparacaine) on tear production.

It also compared the quantity of tear proteins before and after the instillation of the local anesthetic. The result of this research will enable ocular health workers to know the exact effect of proparacaine on tear production and take necessary precautions with its use. Tears can be sampled without an anesthetic by pinching the lower lid to create a small pool of tears, which can be absorbed quickly with a Schirmer strip. We used edgeR for the statistical testing. (Figure 3)

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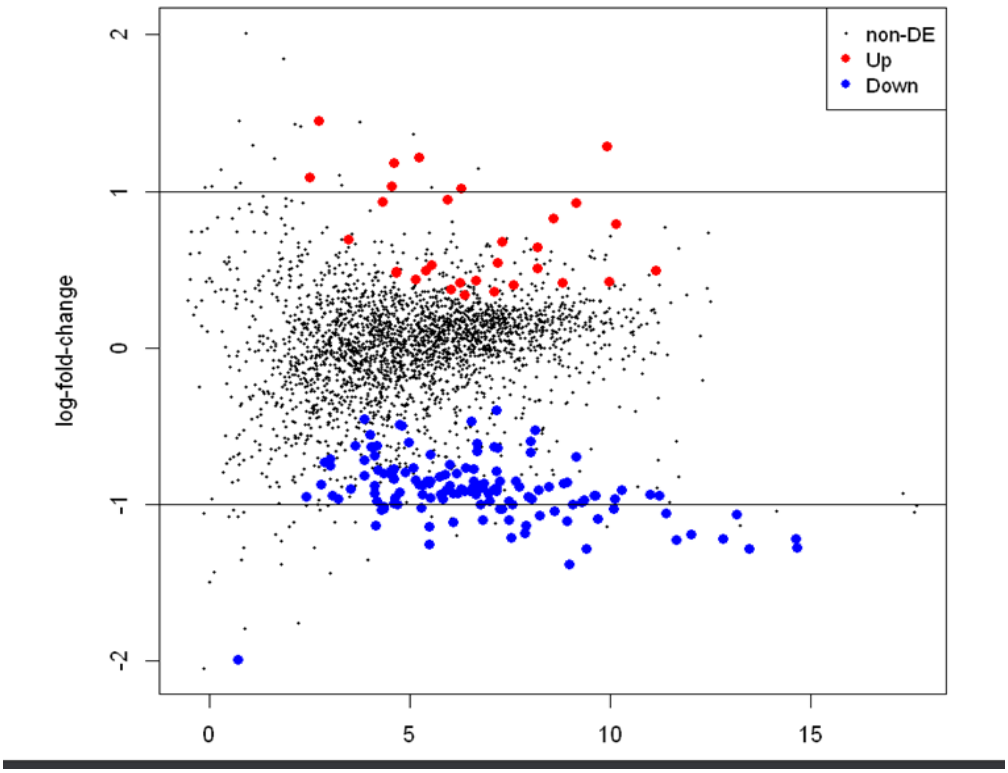

Figure 3: DPIN (Right eye using Pinch test), SPRO (Left eye using proparacaine 1% drop)

DPIN vs SPRO comparison

The test flagged about 170 candidates with an FDR of 0.05 or lower. This is a reasonable number of candidates based on what was expected from the MDS Plot. The p-value distribution exhibits a pronounced peak at low p-values and remains relatively flat at higher p-values(Figure 4)

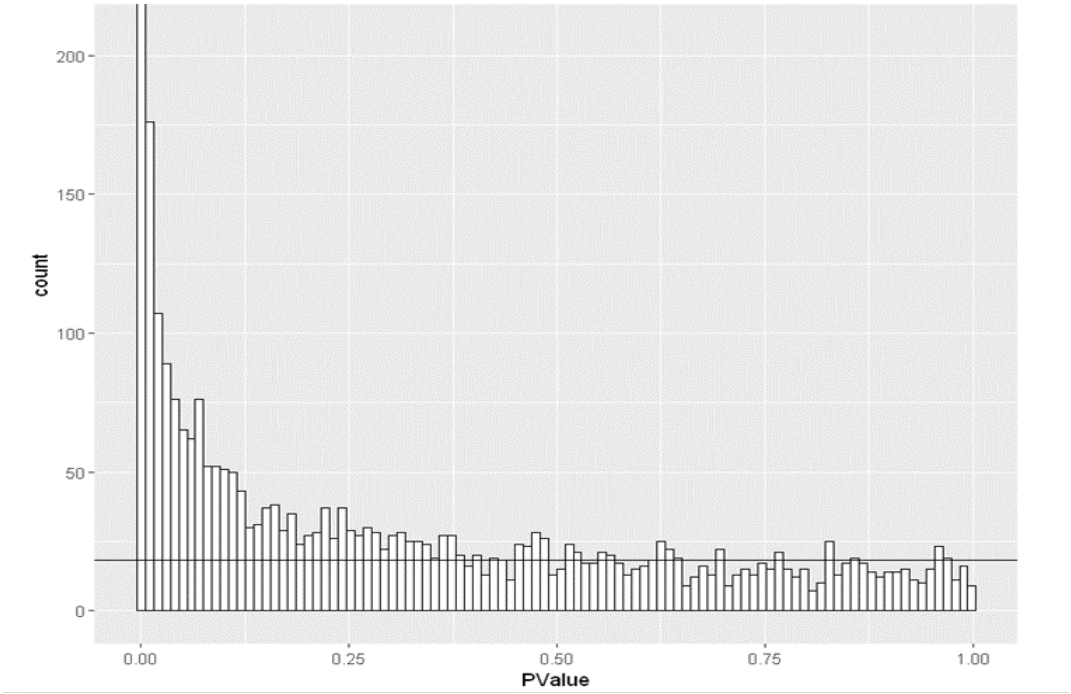

Figure 4: An MDS plot is created to see if the samples cluster by condition. When sample groups cluster on the plot, the tests typically yield larger numbers of differential candidates.

A degree of separation is visible on the graph below. The DPIN samples are mostly to the left of the SPRO samples, though there is some overlap. The spike in low p-values is a good indicator of a population of

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differentially expressed proteins and matches nicely with the cluster of blue-highlighted data points on the log fold-change plot(Figure 5-A&B). A multiple testing correction was performed as part of the test, and the corrected p-values were used to determine candidate status (0.05 or lower for the corrected value). All the statistics and visualizations used appear to be from the paired 6v6 tested, so they considered all 12 samples. However, the samples are paired to control somewhat for pair-specific variation. (Figures 6 & 7)

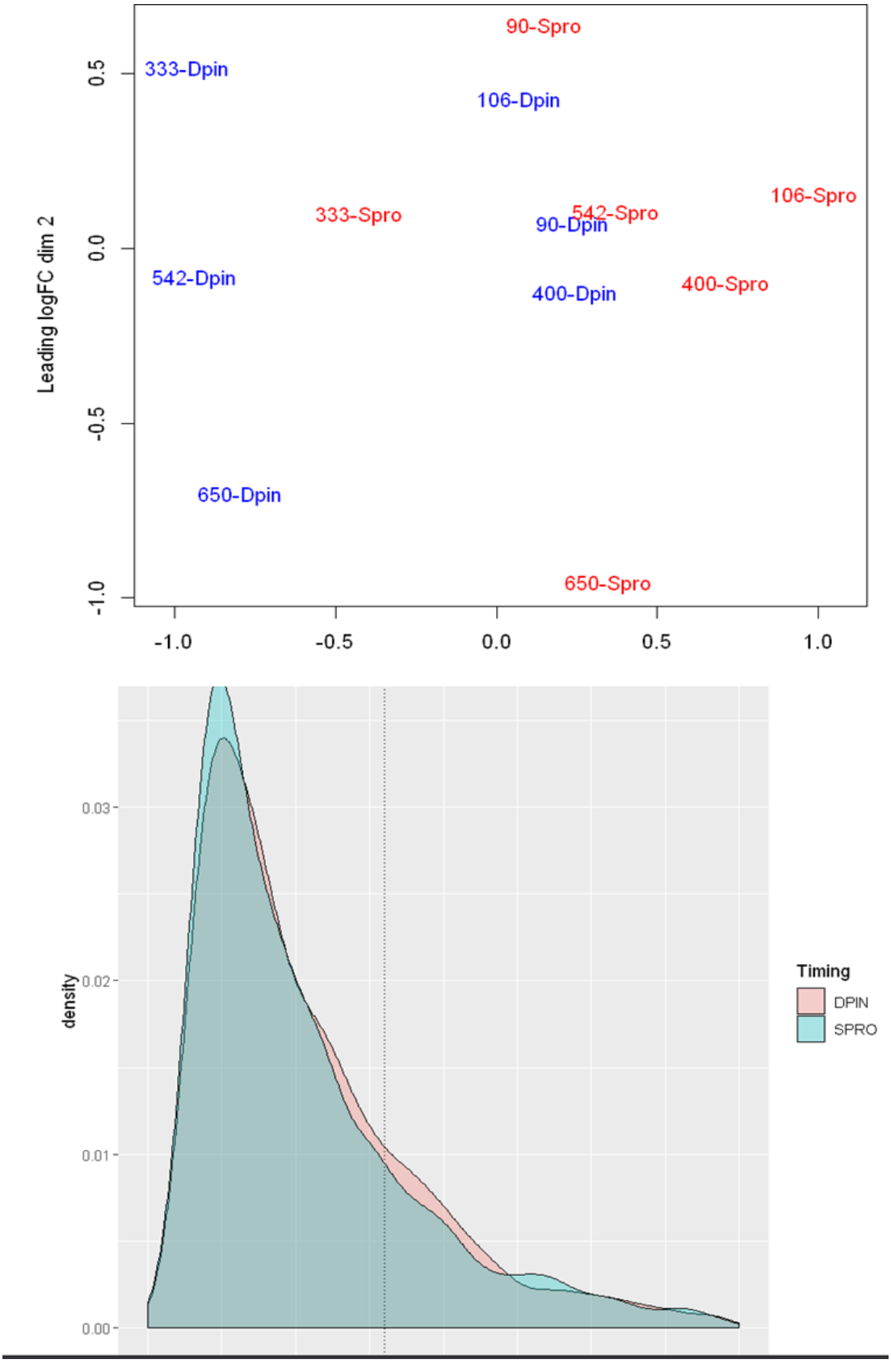

Figure 5(A, B): DPIN (Right eye using Pinch test), SPRO (Left eye using proparacaine 1% drop)

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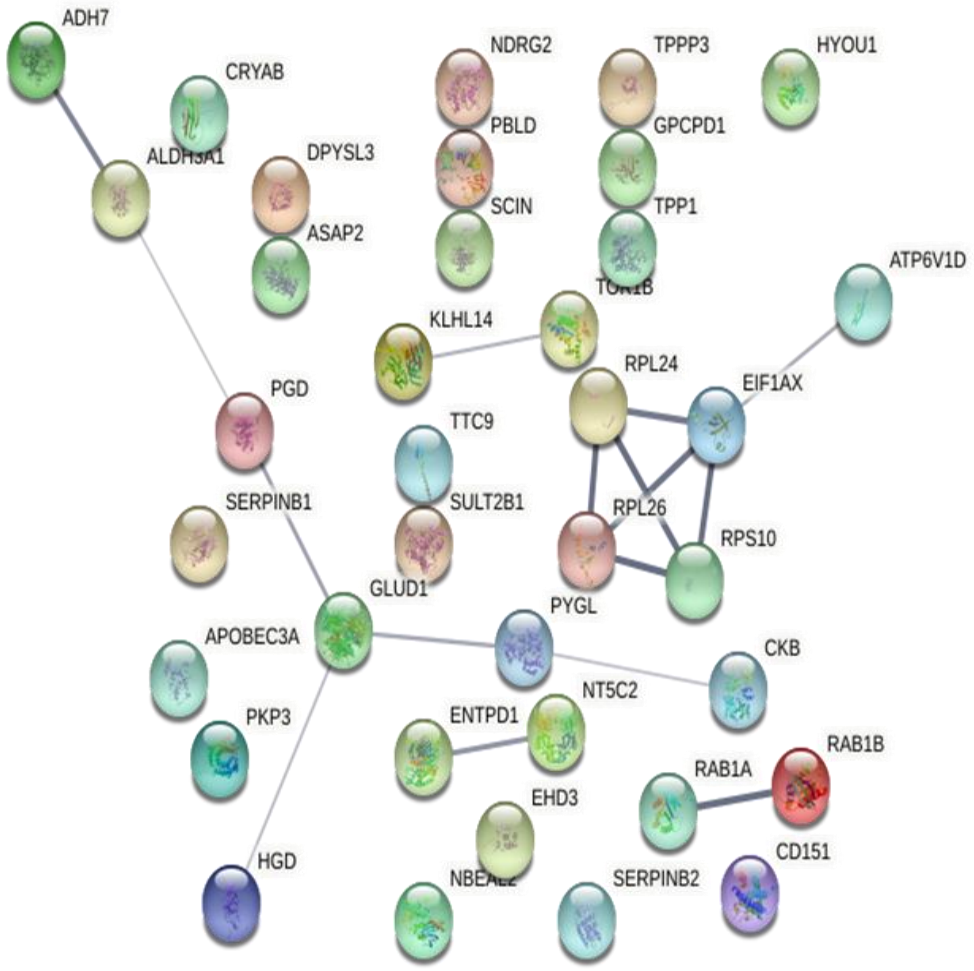

PATHWAY HIGHLIGHT

Miscellaneous transport and binding events

Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)

Neutrophil degranulation

Cell surface interactions at the vascular wall

Initial triggering of complement

Classical antibody-mediated complement activation

Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell

Cell surface interactions at the vascular wall

FCGR activation

Regulation of actin dynamics for phagocytic cup formation

Role of phospholipids in phagocytosis

Scavenging of heme from plasma

Fc epsilon receptor (FCERI) signaling

Role of LAT2/NTAL/LAB on calcium mobilization

FCERI mediated MAPK activation

FCERI mediated Ca+2 mobilization

FCERI mediated NF-kB activation

CD22 mediated BCR regulation

FCGR3A-mediated IL10 synthesis

FCGR3A-mediated phagocytosis

Regulation of Complement cascade

Antigen activates B Cell Receptor (BCR) leading to generation of second messengers

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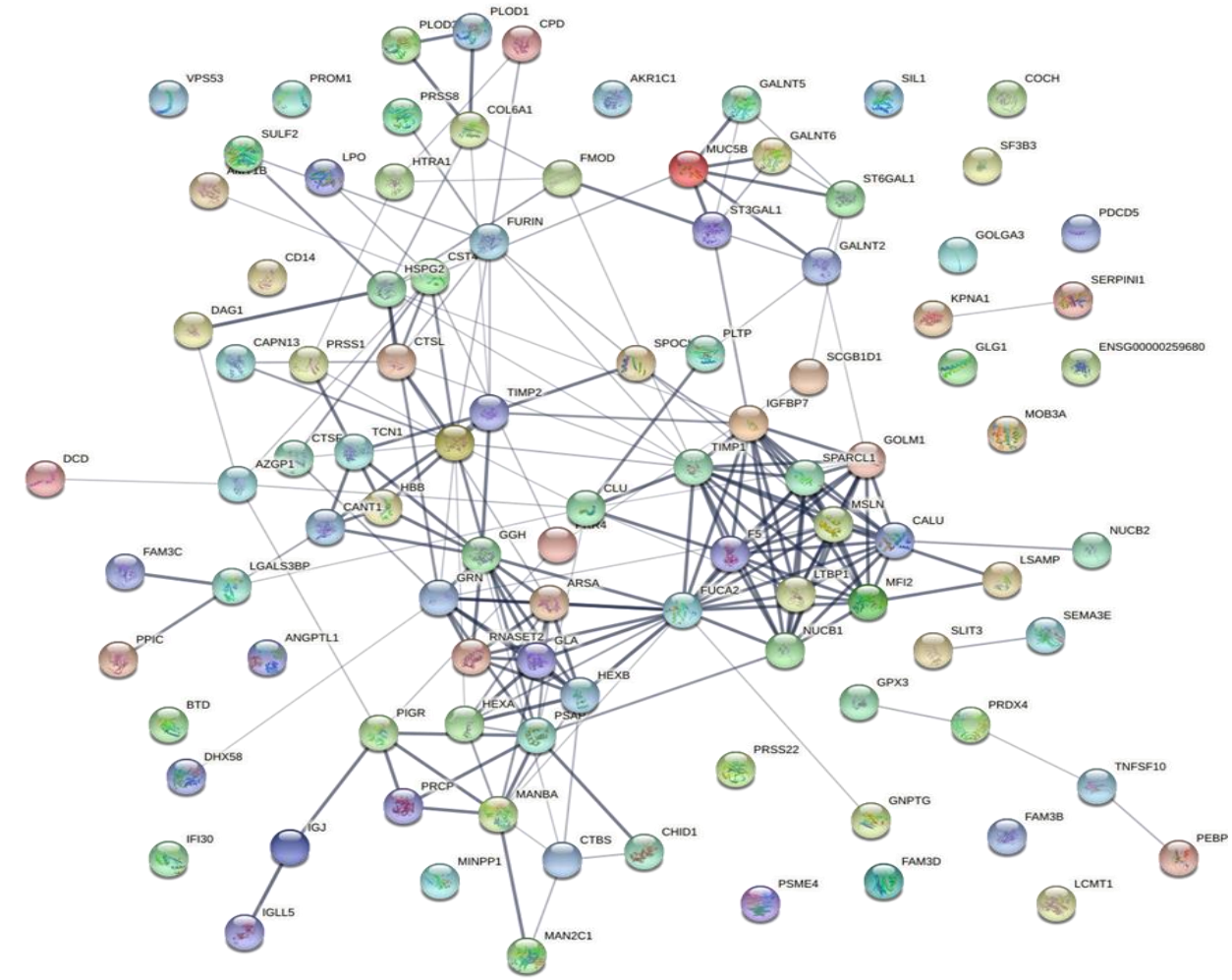


Figure 6 & 7: The data analysis flagged around 170 differential candidates between 6 pairs of OD-Pinch (DPIN) and OS-Proparacaine (SPRO) samples:

Albumin, Complement C3c alpha' chain fragment 2, Serotransferrin (Transferrin), Short peptide from AAT Truncated apolipoprotein A-I. Mesothelin, cleaved form. Protein disulfide-isomerase (PDI). Fibrinogen alpha chain Ceruloplasmin. Antithrombin-III (ATIII). Alpha-2-HS-glycoprotein chain B, Fibrinogen gamma chain, Nucleobindin-1 Apolipoprotein B-48 (Apo B-100; Apo B-48), Endoplasmin, Cystatin-C, Golgi membrane protein 1 DnaJ homolog subfamily C member 3, SPARC-like protein 1, Plasmin light chain, Thrombin heavy chain Reticulocalbin-1, Calumenin, Low molecular weight growth-promoting factor

DISCUSSION

Anesthetics are drugs that induce the state of anesthesia, and anesthesia is a technique that produces insensitivity to external stimuli or a reversible lack of awareness, which can be general or local (17). Local anesthetics act on any part of the nervous system and on every type of nerve and fiber. Different sensations are lost according to the size of the axon serving them. Proparacaine, due to its moderate properties, is one of the most versatile agents in anesthesia and is often used by ophthalmologists in different clinical procedures.

When applied in an effective concentration to nerve tissue, local anesthetics reversibly block the conduction of impulses through nerve fibers. The primary action is to prevent the conduction of sensory impulses. However, they will also block motor nerves in higher concentrations than are typically obtained by topical instillation. Examples of local anesthetics include amethocaine, lignocaine, oxybuprocaine, and proparacaine (18).

These major findings go a long way towards confirming that local anesthetics measure only basic secretion, thus reducing normal tear production/secretion, which is both reflex and basic (19). This could be attributed to

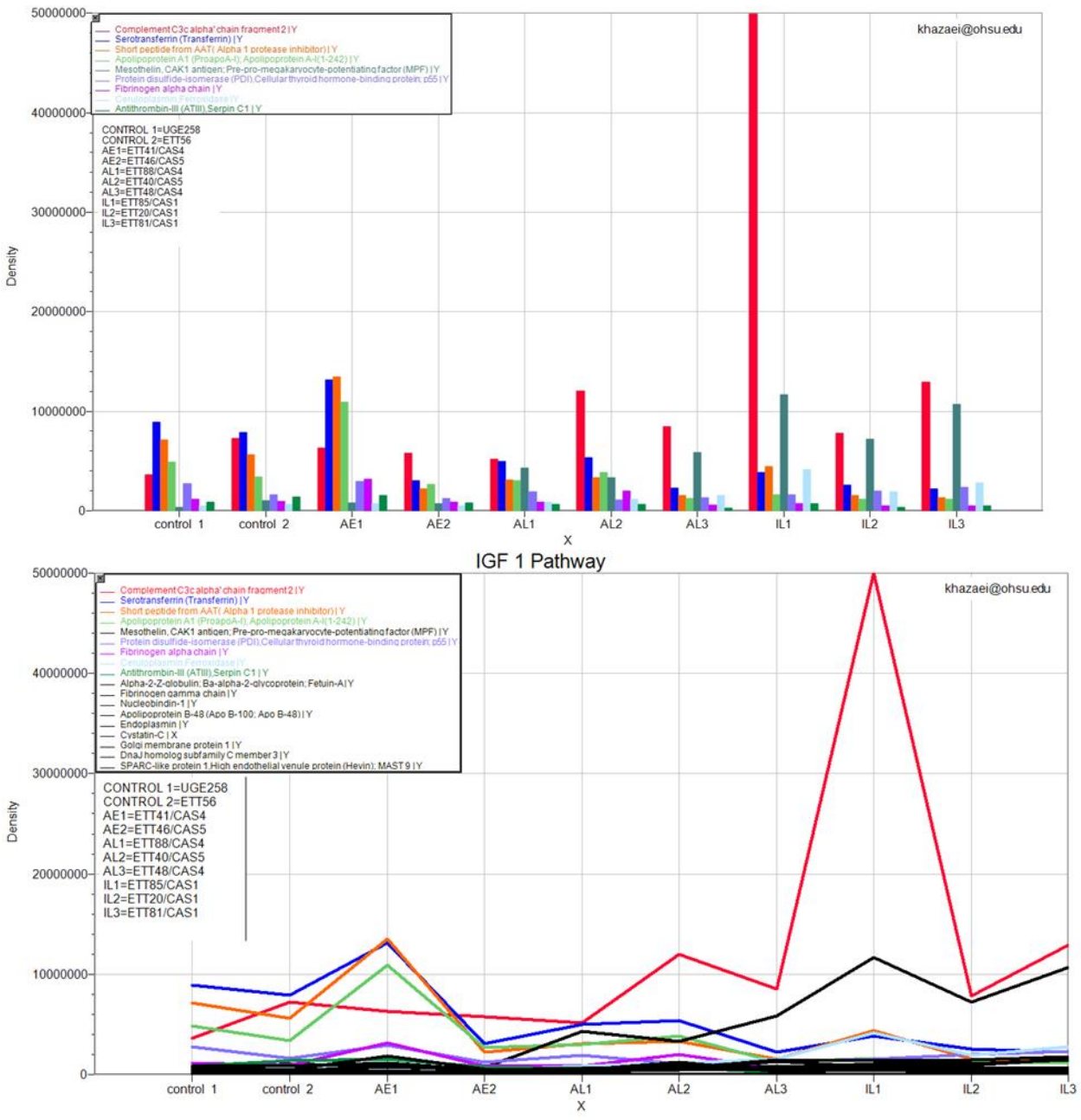
ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue X October 2025

the fact that local anesthetics have an adrenergic potentiating effect. Because lacrimal fluid receives a preganglionic parasympathetic supply from lacrimal muscles and leaves the facial nerve to synapse in the sphenopalatine ganglion before running into the lacrimal gland, its stimulation produces secretion of tears (reflex secretor) (20).

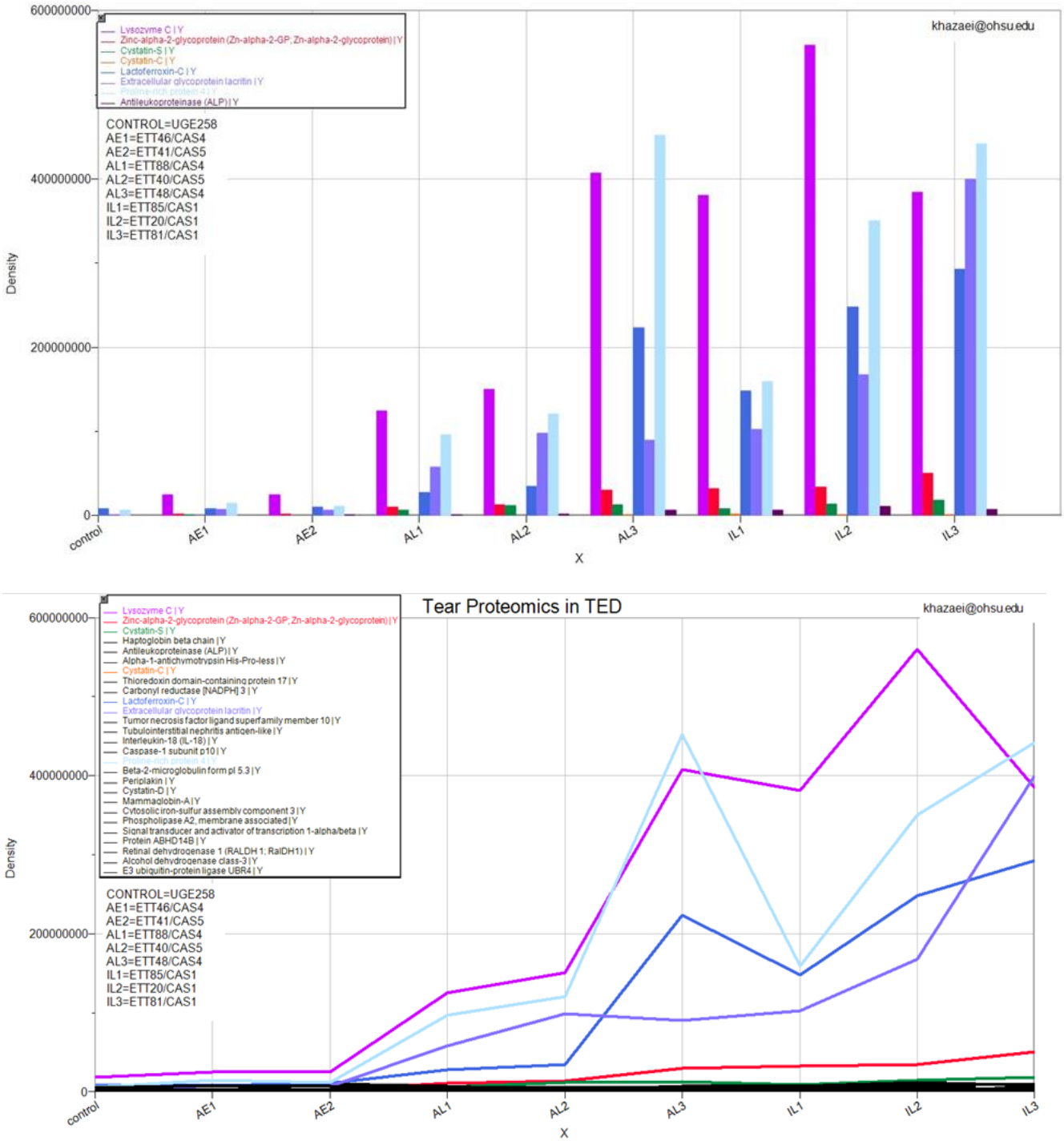
In a study by NWAGI. et al, tear production was measured using Schirmer's technique before and after the installation of a local anesthetic (Lignocaine 2%) in fifty subjects(both males and females) within the age range of 18 -35 years. Results from data collected showed that Lignocaine caused a slight reduction in tear production, with a mean percentage reduction of 29.06%. Statistical analysis also revealed that the effect of lignocaine on tear production was significant ($P<0.05$).

In brief, it is important to select the appropriate collection method for each specific study. For example, when a large sample volume is required, Schirmer's strips are preferable, but if dry eye patients with low-tear menisci are involved, cellulose sponges are preferred (21,22). Notably, the results of proteomics studies using different tear fluid collection methods are not directly comparable, and it is important to consider the potential impact of the collection method on protein concentration and expression (23).

Figure 8 : Data analysis(A,B,C,D)



ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue X October 2025



Exploratory analyses included descriptive statistics, principal component analysis, multidimensional plots, and heat maps. Data was normalized by median sweeping. Differential protein abundances between groups were determined by comparing the normalized intensities using the Bioconductor package limma. By incorporating proper blocking factors, limma can also handle longitudinal data. We analyzed the trend within a patient and compared differences in trends in protein abundance between groups. P-values will be adjusted by the false discovery rate (FDR) (30). Differential abundance with a 1.5-fold change or higher and FDR < 0.05 will be considered significant. To predict who will develop GO, a random forest algorithm supervised ensemble learning was employed to build a prediction/classification system based on differentially abundant proteins by cross-validation. The performance of the system was evaluated by a confusion matrix that includes overall accuracy, no information rate, sensitivity, specificity, etc. In addition, the receiver operating characteristic (ROC) curve was analyzed. UniProt accession numbers of identified proteins were used for further downstream analyses, such as pathway analyses and functional annotation. We used NIH DAVID and Reactome pathways for these analyses. All statistical computations were performed in the R statistical language (http://www.r-project.org).

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue X October 2025

LIMITATIONS OF PROTEOMICS

GRAVES' orbitopathy may be a multifarious illness with a wide spectrum of clinical, radiological, and histopathological presentations. By accurately diagnosing and managing them promptly, we can avoid permanent vision loss and even save a patient's life. Additionally, in-depth knowledge of pathological processes holds promise that improved therapies supporting this molecular understanding have both practical and theoretical implications.

Adherence to strict criteria for inclusion/exclusion is essential for enhancing the understanding of the pathophysiology of eye disease and for the initiation of new preventative and therapeutic modalities. One key factor is whether those tear biomarkers are specific to certain diseases. Most proteins in tears originate from the lacrimal gland; however, they may also be released from epithelial cells that are shed or leaked from blood vessels during inflammation, injury, or irritation.

CONCLUSIONS AND FUTURE IMPACT

Human Tear proteomics has recently gained prominence as a new source of biomarkers and can become a promising and innovative clinical test in orbital diseases. As tear sampling is a noninvasive and rapid method, tear-based tests hold promise for future diagnostic methods and can permit opportunities for a better understanding of inflammatory orbital diseases. Additionally, as a complex mixture, tears offer the potential to discover not only proteins but also RNA, lipid, and metabolite biomarkers that could complement the conventional clinical tools accessible to ophthalmologists.

Over the past decade, advances in mass spectrometry (MS) have considerably improved our understanding of the chemical composition of tears. The biochemical changes in tear fluids have been investigated in several eye diseases, applying the 'Omics' approach over the last decade. Proteomic studies have consistently found that levels of pro-inflammatory proteins indicating ocular surface inflammation were marked by well-known inflammation-related proteins. Soon, these proteomic studies will simply be translated into antibody-based assays for clinical use and can be converted into workplace devices for wider access. Tear 'omics' analysis over the past decade has undeniable long-run applications of tear biomarkers for patient stratification, or what is typically mentioned as precision medicine. Biomarker measurements can help explain the empirical results of clinical studies by relating the effects of interventions on molecular and cellular pathways to clinical responses.

To summarize, unmet needs include discerning the relationship between thyroid disease and orbital disease, determining how the pathogenesis of GD differs from other orbitopathies, predicting the development of orbitopathy, and identifying the mediators of inflammation and fibrosis, which are potential targets for therapy.

ACKNOWLEDGEMENTS

We would like to express our sincere appreciation to Dr. James Rosenbaum and Professor Faryar Etesami, who provided invaluable input and guidance throughout this work. Their mentorship, thoughtful feedback, and encouragement greatly strengthened the quality and clarity of this project.

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