



## OPEN High throughput tear proteomics with data independent acquisition enables biomarker discovery in allergic conditions

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The search for pathological biomarkers in biological fluids that can provide valuable insight into an individual's health status, is a relevant area of research for multiple pathologies. Currently, the use of proteomics for the identification of differences in protein expression profiles between samples from healthy subjects and patients, has emerged as a powerful strategy to improve the current diagnosis of various pathologies or propose novel therapeutic approaches. Among the biological fluids from which new pathological biomarkers can be identified, tear secretion is highly attractive, since it can be collected non-invasively and could better concentrate proteins that sensitively reflect allergic responses, owing to their exposure to environmental factors and its connection to the respiratory system. Despite its potential, tear fluid remains underexplored, offering significant research opportunities. In this study, we collected human tear samples using the Shirmer Test from healthy and allergic individuals. Our optimized workflow, combining sample preparation and high-throughput proteomics using the data-independent acquisition (DIA) strategy, identified 2542 proteins and enabled the successful differentiation of the two groups. We identified 99 differentially expressed proteins. Our results show the feasibility of protein analysis in human tear samples, highlighting tears as a highly sensitive fluid for detecting health conditions. Data are available via ProteomeXchange with identifier PXD067099.

**Keywords** Tear secretion, Biomarkers, High-throughput proteomics, DiaPASEF

Early diagnosis constitutes a cornerstone of modern healthcare strategies, enabling better patient and family preparation, as well as timely therapeutic interventions that can delay or reduce disease symptoms<sup>1</sup>. This early diagnosis can be defined as the ability to identify patients who, although asymptomatic, exhibit molecular alterations critical to disease progression.

In this context, the search for molecular biomarkers in various biological fluids that can provide early information about health status constitutes a promising area of research with the potential to improve diagnostic accuracy<sup>2</sup>. Broadly speaking, molecular biomarkers are biomolecules such as proteins, RNAs, miRNAs, or metabolites, whose production or levels change in response to physiological or pathological conditions<sup>3</sup>. Advances in sensitive and high-throughput analytic techniques, such as proteomics, capable of detecting differences in protein expression profiles between samples from healthy subjects and patients, have enabled the identification of biomarkers across a wide range of pathologies<sup>4,5</sup>.

Mass spectrometry has rapidly advanced in the past five years, driven by the integration of innovative technologies. Notably, 4D proteomics, enabled by timsTOF mass spectrometers, combines trapped ion

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mobility spectrometry (TIMS) with parallel accumulation-serial fragmentation (PASEF). This synergy allows for unprecedented proteome resolution and coverage<sup>6,7</sup>. In this context, data-independent acquisition (DIA) strategies, particularly diaPASEF, have become established as powerful quantitative tools<sup>8</sup>. The ability of diaPASEF to separate ions based on their shape, size, and charge through ion mobility provides a more robust solution for the analysis of complex biological fluids such as plasma<sup>6</sup>.

The biological fluid most used for biomarker identification is plasma/serum, due to its easy access and the possibility of obtaining sufficient volume in a safe manner<sup>9</sup>. However, other fluids, with similar safety and non-invasive obtention may offer better protein profiles, such as tear secretion. Lacrimal or tear secretion fulfills lubricating and protective functions for the ocular surface against physical, chemical and biological insults, and is made up of aqueous and lipid layers<sup>10</sup>. Although primarily composed of water and salts, tear fluid also contains lipids, mucins and proteins, which differ significantly between healthy individuals and patients with ocular or systemic diseases, providing an opportunity for biomarker discovery<sup>11</sup>.

Proteomic analysis using liquid chromatography coupled to mass spectrometry (LC-MS), have identified 1300 to 1500 proteins in tear samples from healthy individuals<sup>12,13</sup>. Among these, the most abundant proteins are associated with anti-inflammatory and antibacterial activity<sup>14</sup>. Different studies have demonstrated that ocular alterations such as uveitis, dry eye syndrome and diabetic retinopathy alter the protein composition of tear secretion<sup>15–18</sup>.

Despite this evidence, tear secretion remains underexplored as a diagnostic or monitoring tool for non-ocular diseases, representing an important opportunity. This study aims to evaluate the feasibility of isolating enough high-quality proteins from tear fluid, using the Shirmer Test to collect them and to develop a robust proteomic analysis workflow for profiling protein expression.

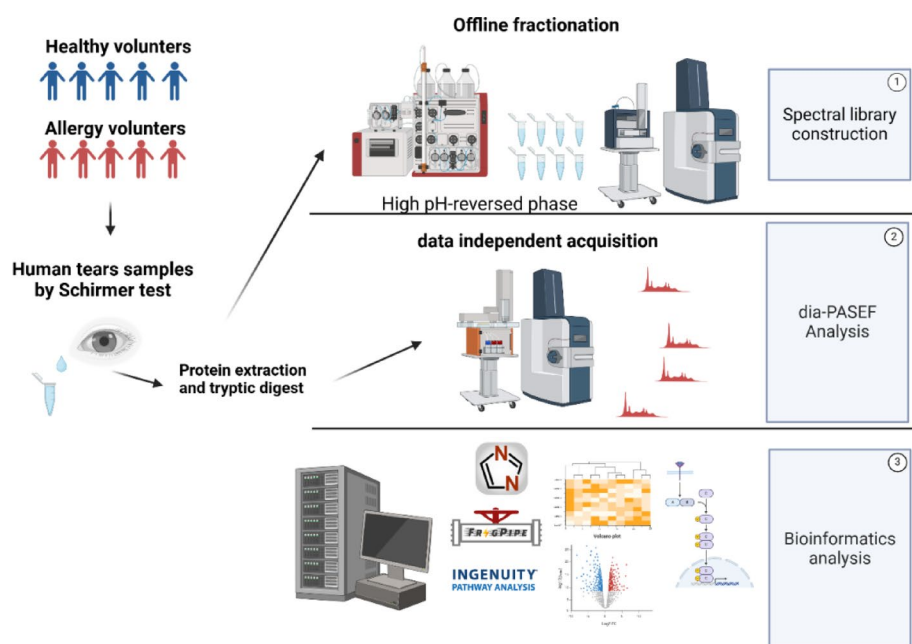
We successfully isolated a substantial quantity of high-quality proteins from tear fluid in both healthy and allergic individuals, which were subsequently analyzed using mass spectrometry. In addition, we established an efficient and reliable strategy for high-throughput proteomic analysis based in DIA methodology that distinguishes between healthy and allergic individuals. Our findings highlight the potential of tear fluid as a valuable biological fluid for biomarker discovery and the detection of systemic and allergic conditions.

## Results

### Quantitative proteomic analyses in human tears samples

Tear samples from healthy and allergic adult subjects were collected using Shirmer Test and processed for further proteomic analysis (Fig. 1). The demographic and clinical characteristics of the participants are summarized in Table 1.

Proteomic analysis of human tears samples was done to identify proteins altered in allergic subjects. Using mass spectrometry, 2542 proteins were identified in all individuals per group, with a false discovery rate (FDR) of 0.01 (Supplementary Files 1 and 2). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>19</sup> partner repository with the dataset identifier PXD067099". The quality of proteomic data was evaluated by assessing protein abundance in each human tear sample (Supplementary Fig. 1A) and analyzing the similarity of individual proteomic signatures using a Pearson correlation matrix (Supplementary Fig. 1B). The correlation coefficients ranged from 0.7 to 1.0, indicating a high



**Fig. 1.** Experimental design to study the proteomic profile of the human tear's samples using Nc-MS/MS timsTOF PRO 2.

|                               | Controls      | Allergic      |
|-------------------------------|---------------|---------------|
| Age (years)                   | 21.0 ± 0.8    | 27.5 ± 4.1    |
| Gender                        |               |               |
| Male                          | 1             | 2             |
| Female                        | 3             | 2             |
| Schirmer left eye (mm)        | 26 ± 12.8     | 28 ± 9.1      |
| Schirmer right eye (mm)       | 29.6 ± 9.9    | 26.4 ± 10.2   |
| Protein concentration (µg/ml) | 435.7 ± 184.6 | 552.7 ± 155.5 |

**Table 1.** Demographic characteristics of controls and cases. Sample characteristics from controls and allergic subjects. The statistical analysis shows the average and standard deviation for each group.

degree of global similarity in the human tears’ proteome of healthy and allergic individuals. We also assessed reproducibility by visualizing the relationships between protein measurements across multiple replicates for each sample (Supplementary Fig. 1C).

To explore the differences in protein expression profiles between healthy controls and allergic subjects, a principal component analysis (PCA) was performed (Fig. 2A). The PCA results revealed two distinct sample clusters, indicating a clear separation between the tear proteomes of healthy and allergic individuals, where PC1 on the X-axis explained 56.12% of total variability, while PC2 on the Y-axis explained 14.25%. Differentially expressed proteins (DEPs) analysis identified 99 proteins with significant differences in expression between the two groups (Supplementary File 3). The PCA findings were further supported by unsupervised hierarchical clustering, visualized in a heatmap plot. This heatmap demonstrated a clear clustering of samples into healthy and allergic groups, as well as the relative expression levels of the 99 DEPs, across samples and groups (Fig. 2B). Of the 99 DEPs, 47 proteins were up-regulated and 52 were down-regulated in allergic tear samples compared to healthy controls. A volcano plot (Fig. 2C) was used to categorize the down and up-regulated proteins according to their fold change ( $\log_2$  FC) and  $-\log_{10}$  (p-value). Also, the volcano plot highlights the top 5 up-regulated and down-regulated proteins in allergic subject tear samples; the up-regulated proteins included Zinc Finger, FYVE Domain Containing 19 (ZFYVE19), HLA class II histocompatibility antigen, DRB5 beta chain (HLA-DRB5), TRNA Nucleotidyl Transferase 1 (TRNT1), IgGFC-binding protein (FCGBP), and Ras-related protein 25 (RAB25). While down-regulated proteins were keratin 80 (KRT80), filaggrin (FLG), DDB1 and CUL4 Associated Factor 7 (DCAF7), Inter-Alpha-Trypsin Inhibitor Heavy Chain 3 (ITIH3) and Elongator Acetyltransferase Complex Subunit 5 (ELP5).

Gene ontology enrichment analysis for pathways in tears human samples

The gene ontology analysis of the 99 significant proteins identified in human tear samples revealed their involvement in 14 enriched pathways, according to IPA software (Fig. 3). Ingenuity Pathway Analysis (IPA) results showed enrichment in *seven pathways related to protein translocation*, such as eukaryotic translation initiation, elongation, and termination; SRP-dependent co-translational protein targeting to membrane; ribosomal quality control; processing of CAP-introns-containing pre-mRNA, and response of EIF2AK4 to amino acid deficiency. In addition, IPA showed enrichment in *two pathways involved in RNA metabolism*, which included nonsense-mediated decay and rRNA processing; as well as *one pathway related to selenoamino acid metabolism*. However, an important difference with the results obtained by Reactome (data not shown) was that IPA was able to find *four pathways related to immune system* responses downregulated, such as binding and uptake of ligands by scavenger receptors, complement cascade, Fc epsilon receptor (FCERI) signaling, and immunoregulatory interactions between a lymphoid and a non-lymphoid cell (Fig. 3).

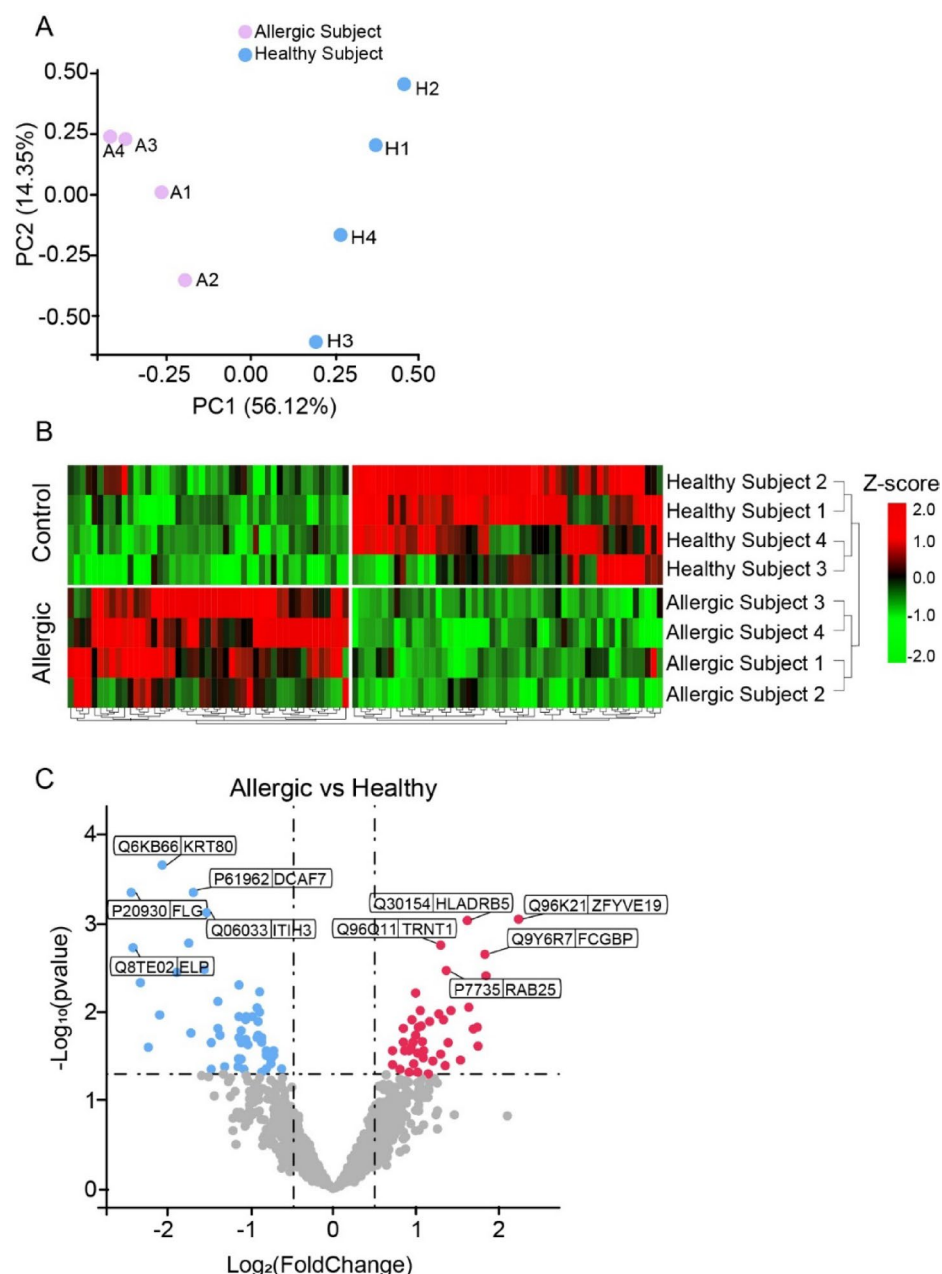
Network analysis by IPA gave us information regarding potential master regulators and regulatory mechanisms involving the DEPs of our tear samples. Of the eight networks identified (Supplementary File 4), ordered from highest to lowest score, three were linked to immunological responses. The first one was related to dermatological, immunological and inflammatory diseases, the third network was related to immune cell trafficking, and the sixth one was related to immunological diseases.

Predicted transcription factors regulating the expression of DEPs

To investigate which transcription factors could be involved in the regulation of the 99 DEPs, we utilized the functional enrichment analysis tool FunRich. Our results identified two transcription factors, CREB1 and SPDEF, as significantly enriched (adjusted p-value < 0.05) in human tears samples from allergic subjects (Table 2). Furthermore, the top five transcription factors with the highest percentage of target proteins were SP1, KLF7, EGR1, SP4 and GABPA (Supplementary Table 1).

Discussion

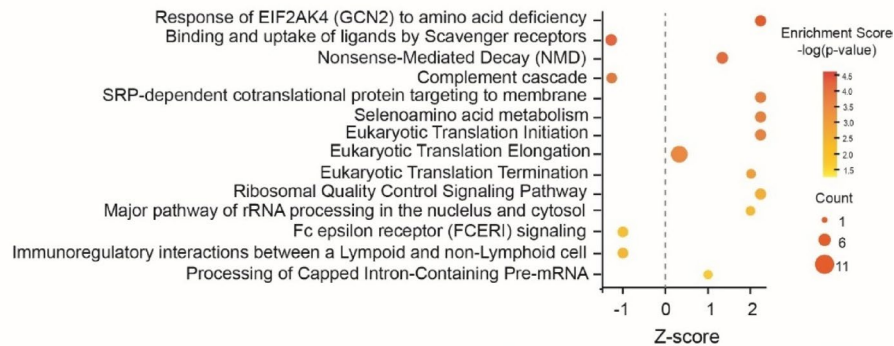
Human tear secretion is a body fluid that has not been widely documented for its use as a biomarker source, although its recollection is simple and non-invasive. Our results show that the presented pipeline for collection, protein extraction, proteomic analysis by LC-MS/MS and bioinformatics data processing allows the detection of a greater number of proteins than previously identified<sup>12,13,15–18</sup>. Moreover, it facilitated the identification of distinct proteomic profile that enable segregation of allergic from healthy subjects through unsupervised analysis.



**Fig. 2.** Proteomic quality analysis. **(A)** Principal components analysis. The lilac and light blue colors show the allergic and healthy subjects, respectively. **(B)** Hierarchy heatmap representing the clustering of proteins from tears, showing the 99 differentially expressed proteins clustered into healthy controls and allergic subjects, each group consisting of 4 subjects. The heatmap plot was generated using Pearson correlation and Euclidean distance between proteins. **(C)** Volcano plot showing the distribution of proteins from healthy controls and allergic subjects tears. The x-axis represents the  $\log_2$  of fold change (FC) and the y-axis the  $\log_{10}$  p value provided by EdgeR. The red dots represent 47 up-regulated proteins, and the light blue dots represent 52 down-regulated proteins (Pvalue < 0.05, and FC  $0.585 \leq x \leq -0.585$ ).

This study presents the development and optimization of a high-throughput, data-independent acquisition (DIA) methodology for efficient tear proteomic analysis. The optimized workflow achieves robust protein identification and quantification, characterized by a low incidence of missing values. Importantly, this method is scalable for the analysis of large cohorts, maintaining reasonable processing times.

We acknowledge that a limitation of our study is the small sample size, which restricts the ability to draw definitive conclusions or formulate hypotheses about the mechanisms associated with conjunctival allergy. The number of samples used in this study was limited, as it was part of an exploratory study aimed at observing the feasibility of isolating and characterizing proteins from human tear fluid and its potentiality. Besides, from an experimental and technical point of view, we have shown high reproducibility with our sample's replicas and how



**Fig. 3.** Enrichment analyses of pathways in tears proteome from allergic subjects. The bubble chart shows enriched proteins in several categories from 99 DEPs in allergic subjects using  $-\log(p\text{-value-adjust})$  with IPA analysis. The negative Z-score represents down-regulated pathways, and the positive Z-score represents up-regulated pathways. The up-regulated proteins were involved in 10 pathways categorized mainly in Translocation processes. The downregulated pathways with the most abundant proteins were related to immune responses. Count: represents the protein abundance in each pathway.

| Transcription factors | N° of genes in the dataset | N° of genes in the background dataset | % of genes | Fold enrichment | p-value | Target genes                                                                     |
|-----------------------|----------------------------|---------------------------------------|------------|-----------------|---------|----------------------------------------------------------------------------------|
| CREB1                 | 12                         | 973                                   | 14.45      | 2.26            | 0.0061  | KRT80, TRNT1, LP5, RAB25, MAGOH, NRNPU, NT5E, RPL24, OXCT1, STXBP3, PURB, TUBB2A |
| SPDEF                 | 4                          | 189                                   | 4.81       | 3.89            | 0.0196  | FCGBP, RPL26, TBC1D2, UBLCP1                                                     |

**Table 2.** Enrichment analysis for the targets of each transcription factor. Enrichment analysis of transcription factors from allergic subjects. The CREB1 and SPDEF were the most significantly enriched transcription factors in human tears samples from allergic subjects, by FunRich Tool. The table also shows the proteins encoded by each transcription factor target genes.

bioinformatic analysis can accurately and clearly separate each group. This work demonstrates the robustness and efficacy of our workflow, showcasing its capacity to detect significant variations in protein expression levels, sufficient to discriminate between study groups through unsupervised analysis. It is crucial to expand the sample size in future investigations to conclusively determine whether the observed changes are directly related to the pathology in question, or whether they could be influenced by injuries or inflammatory processes associated with other conditions.

Our analysis identified 99 DEPs. Among the top 5 upregulated we find ZFYVE19, HLA-DRB5, TRNT1, FCGBP, and RAB25. ZFYVE19 regulates cytokinesis checkpoint, allowing proper cell division. Loss of function mutations produces ciliopathies that compromises cell division<sup>20,21</sup>.

HLA-DRB5 is part of the major histocompatibility complex class II, which plays a crucial role in the presentation of antigens to CD4 T cells. Polymorphism associated with HLA-DRB5 has been linked to antibiotics allergy<sup>22</sup> and uveitis<sup>23</sup>. In addition, very recently, a study has described its upregulation can promote immune thrombocytopenia<sup>24</sup>. Interestingly, HLA-DRB5 expression has been detected before in the eye by RT-PCR from cytology samples, finding reduced levels in dry-eye patients<sup>25</sup>. TRNT1 is an enzyme that participates in tRNA processing, by adding CAA trinucleotide at its 3' end, which is required for aminoacylation and correct positioning on the ribosome. Some case studies have related TRNT1 mutations with B cell deficiency and type I interferonopathy, a condition characterized by overactivation of interferon pathways<sup>26,27</sup>. FCGBP is a mucin-like glycoprotein expressed in different mucous membranes of the organism, that plays a role in mucosal epithelial defense<sup>28</sup> fulfilling cytoprotective and anti-inflammatory roles<sup>29</sup>. It has been recently identified increased in the ocular surface of patients with dry eye, where it is assumed to exert a protective role<sup>30</sup>. RAB25 is a small GTPase that regulates intracellular trafficking pathways. Although the Rab family is known for controlling multiple key immunological processes, such as phagocytosis and phagosome maturation, neutrophil and macrophage degranulation<sup>31</sup> specifically for Rab25 there is only evidence linking decreased expression in skin lesions from atopic dermatitis patients, negatively correlating with its severity<sup>32</sup>.

Downregulated proteins, on the other hand, were KRT80, FLG, DCAF7, ITIH3 and ELP5. The KRT80 is a filament protein involved in the differentiation and structural arrangement of epithelial barrier. Although it has not a direct role in immune responses its expression has been related to higher infiltration of immune cells in various cancer types<sup>33,34</sup> and also in the context of tumor cells its inhibition has been shown to decrease the pro-tumoral and anti-inflammatory TGF- $\beta$ /SMAD signaling pathway<sup>35</sup>. FLG is an epidermal protein that interacts with keratin fibers to provide strength and elasticity to epithelial barriers. Loss of functions mutation for *flg* gene are related to impairment of barrier function and risk factors for atopic dermatitis, asthma, allergic rhinitis and

food allergies<sup>36,37</sup>. Interestingly, downregulation of FLG detected in tear samples from allergic individuals seems to reflect what happens with FLG systemically and not in the eye, as another study has been previously found FLG is upregulated in inflamed sclera tissue<sup>38</sup>. DCAF7, also known as WDR68/HAN11 is a scaffold protein that enables the assembly of multiprotein complexes involved in kinase signaling. It has been shown to participate in nucleotide excision repair<sup>39</sup> and ventral/dorsal patterning of craniofacial embryonic development<sup>40</sup>. We could not find evidence of immune related functions for DCAF7 in the literature. ITIH3 is part of a family of plasma protease inhibitors that interacts with hyaluronic acid acting as matrix stabilizers. No direct link has been established before with allergic responses, but it has been proposed that ITIH3 could be a mediator of thrombo-inflammation in gestational hypertension and preeclampsia<sup>41</sup>. Finally, ELP5 is part of a protein complex that regulates transcription by modifying tRNA and acetylating histones<sup>42</sup> which is a global process that can indirectly affect immune responses. However, so far there are no reports linking ELP5 directly with allergic responses or inflammatory pathways.

IPA analysis of 99 DEPs in samples from allergic individuals provided insight into potential molecular pathways that could be altered in this group. For **Translocation** pathway, which was found upregulated, we identified enrichment of ribosomal protein S19 (RPS19), shown to modulate the immune response during inflammatory processes by decreasing neutrophil activity and promoting monocyte activity<sup>43</sup>. Recently studies described its role in inhibiting the tumor-suppressor activity of the immune system through interaction with the complement receptor C5aR1<sup>44</sup>. Thus, the increase of RPS19 in the tears of allergic individuals may represent a counter-regulatory response to prevent neutrophils recruitment and avoid acute inflammation. In the skin, downregulation of EIF3F in atopic dermatitis has been shown to reduce inflammation and immune cell recruitment<sup>45</sup>.

IPA analysis also showed enrichment of General Control Non-Derepressible Protein 1 (GCN1), signal recognition particle 68 (SRP68), eukaryotic translation elongation factor 1 epsilon 1 (EEF1E1), and TRNT1. GCN1 is a key factor necessary for the activation of amino acid starvation sensing by GCN2, regulating co-translational protein quality control<sup>46</sup>. One study has shown that GCN1 is also necessary to mediate the anti-inflammatory responses elicited by amino acid restriction<sup>47</sup> proposing it could also participate in suppressing inflammation.

For the **Selenoamino acid metabolism** pathway, which was also upregulated we found eukaryotic translation elongation factor 1 epsilon 1 (EEF1E1), also known as AIMP3/p18, in addition to proteins enriched in the translocation pathway. EEF1E1 facilitates the assembly of Methionyl-tRNA synthetase (MARS) during the translation elongation step<sup>48</sup>. Immunologically, increased EEF1E1 expression correlates with immune cell infiltration in hepatocellular carcinoma<sup>49</sup>. Moreover, in ankylosing spondylitis, an inflammatory rheumatic disease, EEF1E1 upregulation suggests it may promote pro-inflammatory cytokine production, similar to other proteins of the EEF1E1 family, such as AIMP1, which promote the expression of pro-inflammatory genes in macrophages and dendritic cells<sup>50</sup>.

Interestingly, IPA analyses found four pathways directly related to immunological responses that were not identified by Reactome. These included pathways involved in the recognition and endocytosis of foreign molecules by macrophages (*binding and uptake of ligands by scavenger receptors*), complement cascade, mastocyte activation in response to antigen-IgE interactions (*Fc epsilon receptor (FCER1) signaling*), and immunoregulatory interactions between a lymphoid and a non-lymphoid cell. The downregulation in these pathways involves ferritin light chain protein (FTL), complement component 7 (C7) and immunoglobulins Heavy Variable 1–69 (IGHV1-69), Kappa Variable 2D-30 (IGKV2D-30), Lambda Variable 2–18 (IGLV2-18), and Lambda Variable 3–16 (IGLV3-16). FTL is a sub-unit of ferritin, the primary intracellular iron storage protein. It has been shown that FTL overexpression decreases the production of pro-inflammatory mediators such as TNF- $\alpha$ , IL-1 $\beta$ , nitric oxide and prostaglandin E2 in response to lipopolysaccharide (LPS)<sup>51</sup> while in the context of allergy, FTL downregulation may impair anti-inflammatory response, exacerbating the inflammatory storm. Although no specific literature on C7 was found regarding allergies, complement responses are tightly controlled in the eye to prevent continuous inflammatory processes which could lead to vision loss<sup>52,53</sup> so it makes sense that proteins participating in complement activation pathways are downregulated.

## Future perspectives

Tear proteomics holds immense promise as a minimally invasive strategy for advancing diagnostics and personalized medicine, particularly in allergy management. By identifying disease-specific biomarkers, this methodology enables the early detection of allergic conditions, paving the way for customized treatment strategies, including targeted anti-inflammatory therapies. This technique could become invaluable for monitoring immune status across various pathological conditions.

Understanding the molecular mechanisms of critical proteins such as FLG, and EIF3F could lead to the development of innovative therapeutic interventions. These therapies might focus on restoring barrier function or modulating inflammatory responses, benefiting not only individuals with allergies but also those with other inflammatory diseases.

Tear proteomics also offers a unique platform to investigate the influence of environmental and lifestyle factors, such as allergens and pollutants, on both ocular and systemic immune health. This application could drive preventive healthcare initiatives by identifying environmental triggers and their effects on the immune system. To further advance the field, integrating metabolomics and transcriptomic analyses with proteomic studies of tears could provide a more comprehensive understanding of the molecular pathways underlying allergies and related conditions. Such integrative approaches may unveil novel therapeutic targets and enhance our understanding of immune regulation.

In conclusion, the insights gained from tear proteomics emphasize its potential to revolutionize diagnostics, drive therapeutic innovations, and expand the scope of biomedical research. The translational opportunities

presented by this field underscore its significance in addressing both allergic diseases and broader health challenges.

## Materials and methods

### Study design and sample

The following study is exploratory in nature, with a cross-sectional design, aiming to standardize the isolation of tear secretion proteins from Schirmer Test strips, and to standardize a protein analysis method for discriminating differentially expressed proteins in healthy versus allergic persons.

Our population of interest corresponds to healthy men and women between 20 and 30 years old, and individuals suffering from seasonal allergies which affected their eyes, noted as irritation or watery eyes of the same age range, who can provide a Schirmer test > 10 mm in 5 min. The 'healthy' group reported that they did not have any allergies or other eye complications at the time of participating in the study. Exclusion criteria included any diagnosed ocular pathology, signs of ophthalmological inflammation or infectious disease in the last 6 months, ophthalmological surgical procedures in the last 12 months, pregnancy, and autoimmune diseases. The absence of these affections was self-reported through a short questionnaire, and the absence of eye alterations were also corroborated by the medical technologist of the research team.

The sample was taken by convenience and corresponds to a total of 8 fourth-year medical technology students with specialization in Ophthalmology and Optometry, who as part of their clinical training must apply the technique of Schirmer Test. All students gave their written consent to use their tear secretion before tear collection.

This study was approved by the Ethics Scientific Committee of Universidad San Sebastián (CEC-USS 140 – 23). All research was performed in accordance with the guidelines provided by the Declaration of Helsinki and the regulation of the CEC. Informed consent was obtained from all participants.

### Protein collection from tear secretion

Tear secretion was collected by the Schirmer Test, a routinely used test in ophthalmology to measure basal and reflex tear secretion without topical anesthesia. Subject were asked to look up and the lower eyelid of each eye was delicately moved downward to position the head of a graduated paper strip (Schirmer-Plus<sup>®</sup> Strip Biotech Vision Care PVT LTD, Gujarat, India) on the lower eyelid temporal zone. The subject was asked to maintain this position for 5 min. Once this time has elapsed, the test strips were removed from the patient's eyelids using tweezers and placed into sterile 1.7 ml tubes, free of DNase and RNase<sup>54</sup>. The length of the wet region of the Schirmer strip was recorded in mm. Immediately after collection, 400 µl of sterile 1X PBS (pH 7.4) with protease inhibitor cocktail (Roche, Basel, Switzerland) was added to each tube containing a test strip and stored at -80 °C until analysis.

### Protein extraction

We extracted proteins from the Schirmer strip according to the protocol described by Gijs and collaborators<sup>54</sup>. Briefly, the tubes containing the Schirmer strips at -80 °C were thawed on ice and the strips were cut into small 1 mm fragments with sterile scissors and placed in the same tube containing buffer. Then, strip pieces were incubated in the buffer for 90 min at 4 °C on a shaker at 900 rpm. With the help of a micropipette, the pieces of Schirmer strip were transferred to a new 0.5 ml tube with a hole at its end, made by a 25G needle. This 0.5 ml tube was positioned inside the initial 1.7 ml tube and centrifuged at 12,000 rpm for 10 min at 4 °C. At the end of the centrifugation, the 0.5 ml tube with the strip fragments was discarded and the liquid in the lower tube was stored at -80 °C until further analysis.

### Protein yield and quality assessment

Protein concentration for each eye tear of each patient was determined using the Qubit protein assay (ThermoFisher Scientific). Additionally, 20 µg of protein were seeded into an SDS-PAGE gel for Coomassie staining to reveal protein integrity.

### Protein digest to nLC-MS/MS

Proteins were denatured using 100 µl of lysis buffer (50 mM HEPES, pH 8, 1% (wt/vol) Triton X-100, 1% (vol/vol) NP-40, 1% (vol/vol) Tween 20, 1% (wt/vol) deoxycholate, 5 mM EDTA, 50 mM NaCl, 1% (vol/vol) glycerol, 1X Complete protease inhibitor, and 5 mM DTT), then they were incubated for half an hour at 60 °C, then the sample was homogenized by ultrasound for 2 min with cycles of 10 s at 40% intensity, subsequently it was alkylated using 20 mM of iodoacetamide in 25 mM of ammonium bicarbonate and incubate in darkness for 30 min. The protein extract was subjected to cleaning liquid/liquid extraction using the chloroform/methanol method. 1 vol of the protein extract was incorporated into 5 volumes of 100% methanol and a volume of 100% chloroform (vol/vol) was added then 3 volumes of milliQ water, centrifuged at 15,000 x g for 5 min, observing a protein disc, the soluble phase is eliminated and then the disc is washed with 400 µl of 100% methanol 4 times, and the protein pellets were dried in a rotary concentrator at 2000 rpm for 10 min at 40 °C.

The samples were resuspended in a maximum of 100 µL of 8 M Urea and 25 mM ammonium bicarbonate. The mixture was incubated for 5 min at 25 °C and vortexed several times. The samples were sonicated on ice with 10-second on/off pulses at 90% amplitude and incubated on ice for 5 min. Centrifugation was performed at 19,000 x g for 15 min at 4 °C, and the supernatant was transferred to a new tube. The proteins were immediately quantified using the Qubit Protein Assay reagent (#Q33212, Invitrogen). The samples were diluted 8X, and then, digestion was carried out with sequencing-grade trypsin (#V5071, Promega) at a 1:50 protease/protein (w/w) ratio and incubated for 16 h at 37 °C. Digestion was stopped by adding 10% formic acid. The samples were then cleaned using EVOTips following the supplier's instructions.

### Peptide fractionation and library construction

High pH reversed-phase fractionation was performed on an ÄKTA Avant25 (General Electric) coupled to a refrigerated fraction collection. Purified peptides were separated on a reversed-phase column BHE 2.1 cm x 5 cm (Waters) at a flow rate of 0.2 ml/min at pH 10. The binary gradient started from 3% buffer B (90% ACN in 5 mM Ammonium Formate pH 10), followed by linear increases to first 40% B within 30 min, to 60% B within 15 min, and finally to 85% B within 5 min. Each sample was fractionated into 24 fractions in 400 µl volume intervals. The fractions were dried in a vacuum-centrifuge and reconstituted in water with 2% ACN and 0.1% FA and concatenated in 8 fractions.

### nLC-MS/MS TimsTOF PRO 2 analysis

*Generation of spectral library:* Each fraction was injected into a nanoELUTE nano liquid chromatography system (Bruker Daltonics), Peptides (200 ng of digest) were separated within 60 min at a flow rate of 400 nL/min on a reversed-phase column Aurora Series CSI (25 cm x 75 µm i.d. C18 1.6 µm) (ionopticks Australia) with 50 °C. Mobile phases A and B were water and acetonitrile with 0.1 vol% FA, respectively. The % of B was linearly increased from 2 to 17% within 37 min, followed by an increase to 25% B within 15 min and further to 35% within 8 min, followed by a washing step at 85% B and re-equilibration. All fractions were analyzed on a hybrid trapped ion mobility spectrometry (TIMS) quadrupole time-of-flight mass spectrometer (TIMS-TOF Pro, Bruker Daltonics) via a CaptiveSpray nano-electrospray ion source. The MS was operated in data-dependent mode for the ion mobility-enhanced spectral library generation. We set the accumulation and ramp time was 100ms each and recorded mass spectra in the range from m/z 100–1700 in positive electrospray mode. The ion mobility was scanned from 0.8 to 1.3 Vs/cm<sup>2</sup>. The overall acquisition cycle of 0.5 s comprised one full TIMS-MS scan and 4 parallel accumulation-serial fragmentation (PASEF) MS/MS scans.

### DiaPASEF analysis

The peptides from all voluntaries were separated injected into a EVOSEP ONE nano liquid chromatography system (EVOSEP System), Peptides (200 ng of digest) were separated within 30SPD gradient on a reversed-phase column Bruker pepsep 15 (15 cm x 150 µm i.d. C18 1.6 µm) (Bruker, Daltonics) with 50 °C. Mobile phases A and B were water and acetonitrile with 0.1 vol% FA, respectively. When performing DIA, we define quadrupole isolation windows as a function of the TIMS scan time to achieve seamless and synchronous ramps for all applied voltages. We defined up to 16 windows for single 100 ms TIMS scans according to the m/z-ion mobility plane. During PASEF MSMS scanning, the collision energy was ramped linearly as a function of the mobility from 59 eV at 1/K0 = 1.6 Vs/cm<sup>2</sup> to 20 eV at 1/K0 = 0.6 Vs/cm<sup>2</sup>.

### Database searching

*For spectral library generation:* We used FragPipe computational platform (version 15) with MSFragger (version 3.2), Philosopher (version 3.4.13), and EasyPQP (<https://github.com/grosenberger/easypqp>; version 0.1.9) components to build spectral libraries. Peptide identification from tandem mass spectra (MS/MS) was done using MSFragger search engine, using raw (.d) files as input. Protein sequence databases H. sapiens (UP000005640) from UniProt (reviewed sequences only; downloaded on Feb. 15, 2021) and common contaminant proteins, containing 20,421 (H. sapiens) sequences were used. Reversed protein sequences were appended to the original databases as decoys. For the MSFragger analysis, both precursor and (initial) fragment mass tolerances were set to 20 ppm. Enzyme specificity was set to 'stricttrypsin', and fully enzymatic peptides were allowed. Up to two missed trypsin cleavages were allowed. Oxidation of methionine, acetylation of protein N-termini, -18.0106 Da on N-terminal Glutamic acid, and -17.0265 Da on N-terminal Glutamine and Cysteine were set as variable modifications. Carbamidomethylation of Cysteine was set as a fixed modification. The maximum number of variable modifications per peptide was set to 3. The final spectral library was filtered to 1% protein and 1% peptide-level FDR.

The processing of diaPASEF raw data was performed with DIA-NN v1.7.15 software<sup>55</sup>, which uses neural networks to perform library-based protein identification and intensity-based library previously generated by the MSFragger tool. The parameters of maximum mass accuracy tolerances were set to a default average of 10 ppm for both the MS1 and MS2 spectra. The samples were analyzed with match-between-runs (MBR) enabled; quantification mode was set to "any LC (high accuracy)". All other settings were left default. The DIA-NN output was filtered at a precursor q value < 1% and a global protein q value < 1%.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>19</sup> partner repository with the dataset identifier PXD067099.

### Bioinformatics analysis

Quantitative output .csv files generated by DIA-NN, containing protein identifiers and intensities per sample, were exported for downstream analysis in R, specifically for LFQ analysis. First, the dataset was split into samples groups (Healthy and Allergic). Missing values were imputed for each group using the missForest algorithm, requiring at least 60% presence within each group. Subsequently, intensity values for each run were adjusted to the median using quantile normalization to reduce technical variation. Significant differential protein expressions were determined using a Bayesian moderated t-test implemented in the limma package v.3.54.2. Any protein associated with a p-value < 0.05 was considered significant. Exploratory data analysis and visualization were created using R v.3.6.0 with EnhancedVolcano, ComplexHeatmap v.2.0.0, and base packages. Pathway enrichment analysis was conducted using ReactomePA v.1.42.0, querying the human database with logFC and p-value data for each statistical comparison. for transcriptional enrichment analysis we used FunRich Tool Vol 3.1.3<sup>56</sup>.

## Data availability

Data are available via ProteomeXchange with identifier PXD067099.

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## Author contributions

E.S, C.C and S.A participated in the recruitment of research subjects and the tear samples collection. C.C, S.A and A.V participated in tear sample extraction protocol. M. H processed tear samples for proteomic analysis. M. H, P. S and G. N participated in proteomic data processing and analysis. A.V, C. C, R. E and K. O participated in data interpretation and generation of final figures. K. O and C. V supervised the experimental work. K. O conceived the idea and generated the first draft, with inputs and revisions from A.V, C.C, R.E and M.H. E. K and C. V provided funding for proteomic analysis.

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## Declarations

## Competing interests

The authors declare no competing interests.

## Additional information

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