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Intraday and day-to-day variability of the tear proteome

E. PONZINI([1])([2])(*)

([1]) *Dipartimento di Scienza dei Materiali, Università degli Studi di Milano-Bicocca - Milano, Italy*

([2]) *Optics and Optometry Research Center, COMiB, Università degli Studi di Milano-Bicocca Milano, Italy*

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Summary. — Tear film is a dynamic biological fluid that is emerging as an attractive source of non-invasive biomarkers. Due to the minimal volume typically obtained during the collection, the in-depth study of the tear proteome presents significant challenges, particularly for personalized medical approaches. Despite the advances in high-resolution mass spectrometry, which have enabled more detailed profiling of the tear proteome, intraday and day-to-day variability of tear proteins, which could affect their utility as biomarkers, has not been well explored yet. This study aims to investigate the variations in the tear fluid proteome. Building on a foundational study that identified 932 proteins across 23 subjects, tear composition was monitored in two individuals over three weeks, collecting samples weekly both in the morning and in the afternoon. Differential analysis of ultrahigh-resolution shotgun proteomics data revealed a consistent abundance of tear proteins across the weeks, with only 27 proteins showing significant variation throughout the day. These findings enhance the understanding of tear fluid dynamics and support its potential in biomarker research.

1. – Introduction

Tear fluid is a dynamic biological fluid essential for ocular surface homeostasis and protection. In addition to its physiological roles, it has garnered increasing interest as a non-invasive biofluid for biomarker discovery in both ophthalmic and systemic diseases [1,2]. The tear proteome, which is composed by proteins involved in immune modulation, inflammation, and cellular communication, offers a unique source of biomarkers for disease pathophysiology and personalized medical applications. However, the limited volume available for collection presents substantial analytical challenges, particularly in the context of high-sensitivity proteomic investigations [1,2].

(*) E-mail: erika.ponzini@unimib.it

Recent advancements in high-resolution mass spectrometry have significantly enhanced the depth and accuracy of tear proteome characterization, enabling comprehensive protein profiling from minimal sample volumes. Previous studies have identified more than 1000 distinct tear proteins, establishing a robust reference dataset for biomarker research [3-8]. Despite these technological advancements, the intrinsic temporal variability of the tear proteome remains an understudied factor with potential implications for biomarker reliability and clinical translation.

Fluctuations in tear protein abundance may occur on both intraday and interday scales, influencing the reproducibility and diagnostic utility of tear-based biomarkers. While previous studies have reported variability in specific tear proteins [1, 2], a systematic investigation of these temporal dynamics is lacking. This study aims to assess temporal variations in the tear proteome by monitoring protein composition over a three-week period in two individuals. Tear samples were collected at weekly intervals, both in the morning and afternoon, and analyzed using ultrahigh-resolution shotgun proteomics. Differential abundance analysis was conducted to identify proteins exhibiting significant temporal fluctuations. By elucidating the extent of intra- and interday variability, this study provides a foundational understanding of tear fluid dynamics and contributes to the advancement of tear-based biomarker research.

2. – Materials and methods

Collection of tear fluid was performed using microcapillary tubes, ensuring minimal ocular irritation and contamination. Samples were immediately stored at -80 °C until further analysis. Proteomic characterization of tear fluid was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Prior to analysis, tear proteins were extracted and subjected to reduction, alkylation, and tryptic digestion. Peptides were then purified using C18 solid-phase extraction and dried under vacuum. Tryptic peptides were separated using an ultrahigh-performance liquid chromatography (UHPLC) system coupled to a high-resolution mass spectrometer. A reversed-phase C18 column was used for chromatographic separation under a gradient elution of acetonitrile and water containing 0.1% formic acid. Mass spectrometric data were acquired in a data-dependent acquisition (DDA) mode, ensuring comprehensive detection of both high- and low-abundance proteins. The MS1 scans were collected at high resolution, followed by MS2 fragmentation of the most abundant precursor ions using higher-energy collisional dissociation (HCD). Raw spectral data were processed using a proteomics software suite for peptide identification and protein quantification (Proteome Discoverer 2.3.0.523, ThermoFisher, San Jose, CA, USA). Peptide spectra were searched against a human protein database using a false discovery rate (FDR) threshold of 1% at both peptide and protein levels.

3. – Comparison with previous studies

With this protocol, it was possible to identify a total of 932 proteins in 23 subjects, with an average number of identifications of 579 ± 69 per run. This results represents a significant advancement in tear proteomics [8], particularly given the methodological constraints imposed to enhance its applicability to precision medicine. Compared to previous studies (fig. 1), Zhou *et al.* [5] reported a higher number of identified proteins (1543), but this was achieved by pooling samples from four individuals and applying

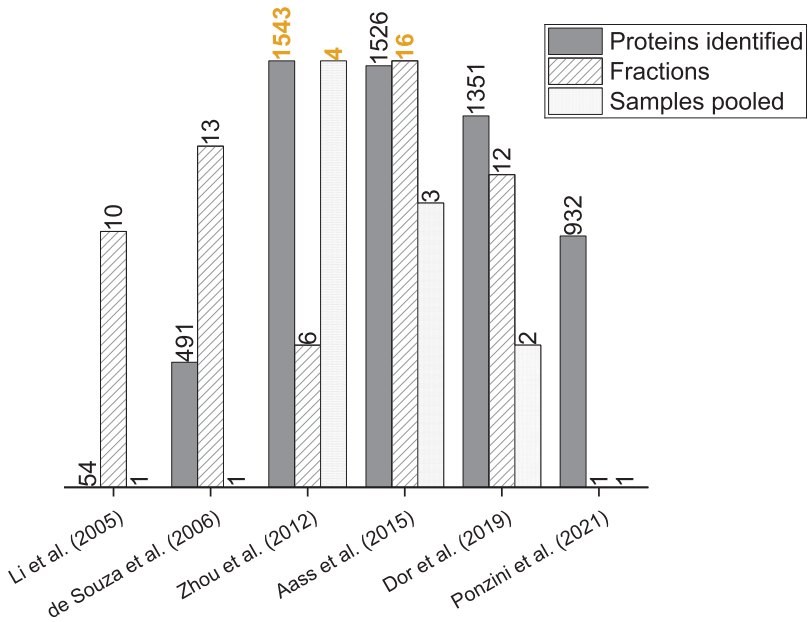

Fig. 1. – Number of protein identifications, fractions and samples pooled in this protocol and in previous studies on human tears from healthy subjects. Normalized data are shown.

extensive prefractionation (six fractions prior to LC-MS). Similarly, Aass *et al.* [6] identified 1526 proteins while using a lower number of pooled samples (three) compared to Zhou *et al.* [5] but the most extensive fractionation strategy among all previous studies (16 fractions). Despite the lower number of proteins identified with the present workflow [8], it is essential to highlight that this protocol uniquely avoids both sample pooling and prefractionation. These methodological choices are critical for applications in precision medicine, where inter-individual variability must be preserved, and minimal sample preparation is preferred for clinical feasibility. The ability to achieve a high depth of protein identification without these preprocessing steps underscores the robustness of this approach and its potential for personalized diagnostics and biomarker discovery.

4. – Intraday and day-to-day variability

A possible application of this protocol is the evaluation of intraday and day-to-day variability in the tear proteome. Understanding the temporal stability of tear proteins is crucial for assessing their reliability as biomarkers and optimizing sampling strategies in clinical and research settings. To investigate the temporal variability of the tear proteome, tear fluid was collected from two individuals over a three-week period. The two subjects were selected to minimize variability and ensure a controlled experimental setup. Both individuals were female, 20 years old, of Caucasian origin, and adhered to a Mediterranean diet. They reported no use of medications, no systemic or ocular diseases, and no use of contact lenses, which has recently been identified as a factor influencing tear composition [9]. The samples were taken once a week, in the morning and in the afternoon. The same standardized collection procedure was used throughout the study to minimize technical variability. All samples were analyzed using the LC-MS/MS

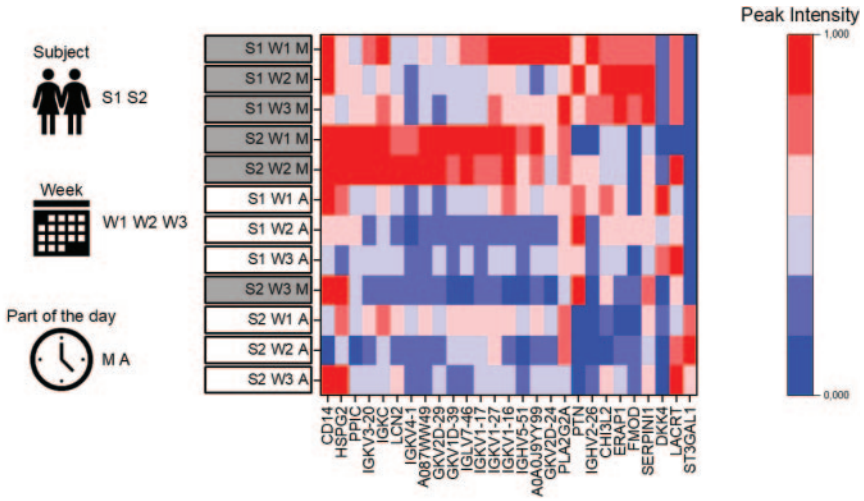

Fig. 2. – Double-hierarchical clustering analysis of 27 tear proteins that are differentially expressed during the day. Proteins were identified through LDA across 12 samples (gray: morning, M; white: afternoon, A). The abundance ratio of morning *versus* afternoon was determined by label-free quantification based on peak intensity and depicted using a color gradient from blue (low) to red (high). Samples were obtained weekly from two subjects (S1 and S2) over a span of three weeks (W1, W2, and W3).

workflow described above, and protein abundances were compared across different time points. Differential abundance analysis was performed to identify proteins exhibiting significant intraday and day-to-day variability. A pairwise comparison was performed and only proteins with F ratio $\geq$4 and uncorrected p-value $\leq$0.05 were retained. Finally, proteins selected by linear discriminant analysis (LDA) (here referred to as descriptors) were processed by hierarchical clustering applying the Ward's method and the Euclidean distance metric. Data processing was performed using JMP 15.1 SAS software. Analysis of the ultrahigh-resolution shotgun proteomics data revealed that the overall protein composition of tear fluid remained stable over the three-week period. While the majority of identified proteins exhibited consistent abundance levels, 27 proteins showed significant variation throughout the day (fig. 2).

These fluctuations suggest that certain tear proteins may be subject to diurnal regulation or influenced by external factors such as environmental exposure and physiological stress. Importantly, the observed variability underscores the necessity of standardizing sampling time in biomarker discovery studies to ensure reproducibility and clinical applicability. Among the proteins that exhibited significant intraday variability, most were upregulated in the morning compared to the afternoon. Notably, the list includes immune-related proteins such as CD14, immunoglobulin kappa and lambda variable chains (*e.g.*, IGKV3-20, IGKC, IGKV1-16), and inflammatory mediators such as LCN2 and PLA2G2A. The increased abundance of these proteins in the morning may reflect a higher basal immune readiness or an overnight accumulation of tear components due to reduced blinking and tear exchange during sleep. Additionally, proteins involved in extracellular matrix organization (HSPG2, FMOD) and enzymatic processing (ERAP1, ST3GAL1) also displayed diurnal variation, suggesting a complex regulatory mechanism underlying tear fluid composition. Interestingly, the most abundant proteins, such as

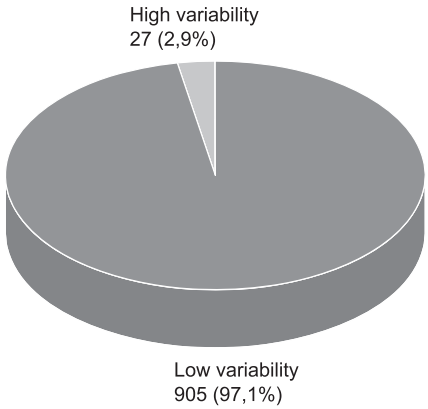

Fig. 3. – Percentage of the tear proteome with high (light grey) or low (dark grey) intraday variability.

lysozyme and lactoferrin, did not show significant intraday variability. This is particularly notable for lactoferrin, which has been reported as a reliable biomarker for several diseases and conditions [10-12]. Although this variability is important and should be acknowledged, we should take into account that only 27 proteins were found to have a remarkable variability over 905, which means that only 2.9% of the proteome displayed variability (fig. 3). This indicates that tear fluid, despite its complexity and direct interaction with the environment, is a robust and promising source of biomarkers.

5. – Conclusion

This study underscores the critical importance of considering individual variability when analyzing tear proteins. Sample pooling should be avoided, as it can mask unique biological differences that may provide valuable insights. Preserving individual variations is essential for obtaining accurate and meaningful data. The significance of diurnal changes in tear composition was also highlighted, with protein levels fluctuating throughout the day. To ensure accuracy and comparability in results, standardized collection times are necessary. This approach will help control for temporal variations and produce more reliable data for robust conclusions. Additionally, the potential of tear biomarkers for clinical applications was demonstrated. Although the tear proteome is complex, 2.9% of the proteins quantified in the study showed significant changes. This suggests that tear fluid holds great promise as a source for biomarkers, paving the way for non-invasive diagnostic techniques in various medical fields. In summary, addressing individual variability, diurnal fluctuations, and the biomarker potential of tears will contribute to enhancing the precision and applicability of tear-based biomarker research in future studies.

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