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# Single-tear proteomics for noninvasive biomarker discovery and precision medicine

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**Summary.** — In the last decades, the lacrimal fluid has gained increasing interest as a potential source of biomarkers, thanks to its accessibility, moderate complexity, and responsiveness to physiopathological conditions. Despite the still limited number of studies, tear investigation, in particular single-tear analysis, could offer unique contributions to the identification of noninvasive and easily accessible biomarkers, and to the development of a feasible approach to precision medicine. High-performance liquid chromatography-mass spectrometry (LC-MS) has led to promising approaches to tear proteomics, despite the intrinsic limitations in the sample amounts that can be collected. By ultrahigh-resolution, shotgun proteomics, we developed an effective analytical pipeline for single-tear analysis and we analyzed the tear fluid of 23 healthy volunteers, achieving high-confidence identification of 890 proteins. This study demonstrates the feasibility of single-tear quantitative proteomics, highlighting the unique contributions that this unconventional body fluid can offer to personalized approaches in biomedicine.

## 1. – Introduction

Thanks to the great improvements in mass spectrometry (MS)-based technologies, proteomics-based clinical investigation has rapidly moved from the identification of single biomarkers to the comprehensive profiling of protein expression. In the last decades, the field of biomarker discovery has been strongly influenced by MS-based proteomics of body fluids. Conventional sources of biomarkers are tissue biopses, blood and plasma, and cerebrospinal fluid, due to the in-depth characterization of their proteome. Nonetheless,

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these matrices present a broad dynamic range, and their collection is invasive. Therefore, the scientific community has been focusing on the analysis of peripheral body fluids for biomarker discovery, such as urine, saliva, sweat, fat aspirate and tears.

The tear film is structured in three main layers: a lipid layer containing lipids, an aqueous layer containing water, proteins and salts, and a mucous layer formed by mucins, which are high-molecular weight glycoproteins. Different types of tear can be collected: basal, reflex or emotional tears. Basal tear has the lowest sample volume (5–10 $\mu$L/eye) but the highest protein concentration.

In the literature, several methods have been reported to collect basal tear for proteomics, but the most widely employed are the Schirmer's test strip (STS) method and the microcapillary tube (MCT) method. The STS method is based on Schirmer's strips, which are thin paper strips that are placed in the conjunctival sac to absorb the tear fluid. Schirmer's strips are easy to handle and do not cause discomfort to the volunteers, but they cause reflex tearing and can injure the conjunctival surface and the microvasculature. In addition, tear proteins can bind and retain on the paper strip differently, according to their molecular weight (MW) and hydrophobic surface area. The MCT method is noninvasive, safe, and does not cause reflex tearing. Nonetheless, it must be taken into account that it requires a trained specialist and that sampling is interrupted by blinking.

Tear proteome investigation in healthy human subjects dates back to 2005, when Li *et al.* published the first research in this field, identifying 54 proteins [1]. After that first publication, other authors focused on this topic [2-6], increasing the knowledge on the tear proteome and reaching 1543 protein identifications [3].

Most of the published works rely on sample pooling, which consists in pooling collections from different subjects or on pooling sequential collections from the same subjects [2-6]. Sample pooling is based on the assumption that the main composition of body fluids is likely to have strong similarities among healthy donors and it provides several advantages, including increasing the sample quantity, reducing the inter-subject background noise and decreasing the number of analyses. However, it unavoidably loses the sample identity. Subject-to-subject variability is becoming particularly relevant because of the ambition and increasingly widespread practice of personalized medicine.

In this view, by ultrahigh-resolution, shotgun proteomics, we developed an effective analytical pipeline for single-tear analysis. We have analyzed the tear fluid of 23 healthy volunteers, achieving high-confidence identification of 890 proteins, with no sample pooling and no fractionation prior to MS-analysis.

## 2. – Materials and methods

For this study, 23 subjects (sex: 12 males, 11 females; average age: $23.7 \pm 2.6$ years) were selected. Volunteers were recruited according to the following criteria: volunteers aged between 18 and 35, of any gender and ethnicity, able to express their consent, with no systemic disease, ocular pathology, or cancer type. The recruitment and experimental procedures were approved by the local ethics committee for research on human beings (approbation No. 0055071/19, 11 July 2019).

Tears were collected by MCT. Proteins were purified from the tear samples by precipitation, then they were reduced, alkylated, and digested in solution by trypsin. Peptides were desalted and analyzed by LC-MS/MS.

The complete analytical pipeline is described in the work by Ponzini *et al.* [7].

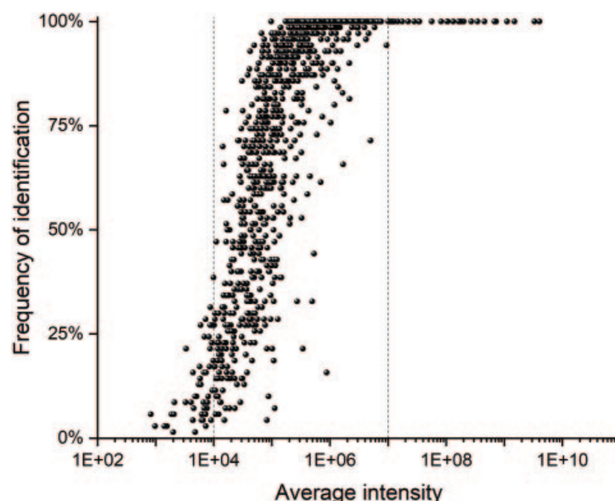


Fig. 1. – Average intensity and frequency of identification of each protein identified. The values are averaged over 70 runs. The range of intensity displayed by 87.5% of the proteins identified is highlighted by dashed lines.

## 3. – Results and discussion

The identified proteins are homogeneously distributed in a wide range of isoelectric points (pI) (from 4.5 to 10) and of molecular weight (MW) (from 50 to 500 kDa). The proteins with the highest MW are mucins, which are glycoproteins, and the proteins with the highest pI are histones. Label-free quantification was performed by peak intensity, peak area, and spectral counting, evidencing highly reproducible results and similar precision. Most of the proteins displayed a broad range of intensity, between $1 \times 10^4$ and $1 \times 10^7$ (87.5%), but no pronounced correlation was found between protein abundance and identification frequency (fig. 1).

The most abundant and frequent proteins were lysozyme C, lactotransferrin, and lipocalin-1, which are produced by the lacrimal gland.

A gene ontology analysis was performed by the Protein Analysis Through Evolutionary Relationships (PANTHER) program. The results were compared with the protein list published by Dor and coworkers [6], which represented the most recent study on human tear proteins of healthy subjects available in the literature. This comparison evidenced that translational and transcriptional proteins are less represented in our work, whereas extracellular proteins are more represented. This could be ascribed to the different collection method employed: indeed, our pipeline is based on MCT collection, whereas Dor and coworkers' method employed STS.

Hierarchical clustering led to the identification of 41 descriptors for females *vs.* males stratification that did not emerge from the aforementioned studies on pooled samples. In addition, two subjects were monitored weekly over 3 weeks. The samples presented 27 descriptors for the withdrawal time of day (morning *vs.* afternoon) but not by follow-up week, with a high predominance of immune system components in tears collected in the morning.

## 4. – Conclusion

This study demonstrates the feasibility of single-tear proteomics, which could be employed in different research fields, such as biomarker discovery [7, 8] or contact lens discomfort assessment [9]. Future perspectives include the application of complementary approaches for single-tear analysis, *e.g.*, FTIR and Raman spectroscopies [10], and MS-based proteomics.

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