

# Critical role of mass spectrometry proteomics in tear biomarker discovery for multifactorial ocular diseases (Review)

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**Abstract.** The tear film is a layer of body fluid that maintains the homeostasis of the ocular surface. The superior accessibility of tears and the presence of a high concentration of functional proteins make tears a potential medium for the discovery of non-invasive biomarkers in ocular diseases. Recent advances in mass spectrometry (MS) have enabled determination of an in-depth proteome profile, improved sensitivity, faster acquisition speed, proven variety of acquisition methods, and identification of disease biomarkers previously lacking in the field of ophthalmology. The use of MS allows efficient discovery of tear proteins, generation of reproducible results, and, more importantly, determines changes of protein quantity and post-translation modifications in microliter samples. The present review compared techniques for tear collection, sample preparation, and acquisition applied for the discovery of tear protein markers in normal subjects and multifactorial conditions, including dry eye syndrome, diabetic retinopathy, thyroid eye disease and primary open-angle glaucoma, which require an early diagnosis for treatment. It also summarized the contribution of MS to early discovery by means of disease-related protein markers in tear fluid and the potential for transformation of the tear MS-based proteome to antibody-based assay for future clinical application.

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## 1. Introduction

The tear film is a thin layer of body fluid secreted and produced by the meibomian gland, the main and accessory lacrimal glands, as well as the goblet cells in the conjunctiva (1). Tear fluid serves several functions: i) Lubricating the ocular surface; ii) providing nutrients to the corneal epithelium; iii) providing a moist and smooth surface for good vision; and iv) protecting the eyes against pathogens (2). The tear film is comprised of three layers: i) The lipid layer; ii) the aqueous layer; and ii) the innermost mucin layer with the aqueous and mucin layers forming a homogeneous layer (3,4). Altogether, it consists of proteins (including enzymes), metabolites, electrolytes, lipids and glucose, and serves a critical function in the ocular system (5). The total tear protein concentration ranges from 3.5 to 9.5 mg/ml in basal tears and reflex tears, but is higher in newborns, ranging from 11 to 13 mg/ml (6), and is increased in closed eye conditions (6 to 18 mg/ml) (7). It has been demonstrated that the proteins in the tear fluid play a significant role in regulating inflammatory responses (8), wound healing (9) and antibacterial protection (10). Mass spectrometry (MS) proteomic analysis not only provides a comprehensive characterization of tear fluid efficiently, but the multiplex nature of acquisition may also provide insights into the key mediators of biological responses and the status of the ocular surface (11). The high concentration of proteins and easy accessibility of tear fluid, compared with other ocular fluids, have made tears a sought-after target for proteomic studies in ophthalmology (12). However, the presence of abundant proteins, including lactotransferrin (LTF), secretory IgA, lipocalin-1 (LCN1) and lysozyme C (LYZ) detected using electrophoresis techniques (13,14) and the small volume of tears has hampered comprehensive protein analysis of low abundant proteins. The advancement in nano-scale liquid chromatography coupled MS (nanoLC-MS) that offers extended dynamic range and sensitivity to identify >1,000

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proteins, has opened up the possibility of tear biomarker research (15). With continued advances in techniques from sample preparation to MS acquisition, it is anticipated that the tear fluid will serve as an important matrix to develop molecular assays for ocular diseases and ophthalmology overall using the MS approach. Quantitative profiling and targeted MS methods have allowed multiplexed, reproducible screening of hundreds to thousands of proteins in a single, microliter volume (16) of tear fluid samples in early discovery, clinical trials and clinical proteomics application for the discovery of multifactorial ocular diseases.

## 2. Collection of tear fluid for clinical proteomics

Tear fluid can be obtained using several established collection methods, yet different sampling methods are known to affect the quality of tears samples and, consequently, the results of tear proteome analysis (17). The three most commonly 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.

*Cellulose sponges.* 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 (18). Additionally, the sponge sampling method enables the standardization of the tear collection volume (19). Nevertheless, a variety of sponges and extraction buffers have been used in different studies, thus making it difficult to directly compare their results (19,20). In addition, some cytokines, including interleukins and g-interferon, bind tightly to the sponge, making the recovery and extraction of these proteins more difficult (21).

*Schirmer's strips.* Schirmer's strips are used in the Schirmer's test for dry eye assessment (22). 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 (23) and improved protein identification than from tear fluid collected with a capillary tube (24). Although Schirmer's strips have been considered as a convenient and easy to perform method of tear collection, their use can cause strong irritation, leading to reflex tearing that results in unwanted dilution of tears (25). In addition, improper handling can also affect protein content (26). In particular, 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) (26).

Both the Schirmer's strip and cellulose sponge methods make use of absorptive materials that have contact with the conjunctiva, which can potentially damage the ocular surface. An increase in the number of certain proteins due to mechanical trauma of the conjunctiva has been reported (27,28). Hence, extra care should be taken to

minimize the trauma-induced stimulation of proteins during sample collection.

*Capillary tube.* To overcome the aforementioned 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 (29). Compared with the use of absorptive materials, this method is considered to be less invasive, to avoid reflex tearing, and to result in less protein disruption during the sample recovery process (30). However, it is time-consuming and requires precise handling, and may not be suitable for anxious or uncooperative patients and children (19). Improper handling of capillary tubes can induce reflex tears due to contact between the tube and the conjunctiva. In general, capillary tubes sampling is not always practicable and feasible in clinical studies that require reproducible data from large cohorts, particularly when children are involved (19). Furthermore, the collectible sample volume is limited. To overcome the limited tear volume of samples, pooling of tears from multiple subjects can be useful in research, but is undesirable in clinical studies as individual characteristics cannot be determined (31).

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 (32,33). 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.

## 3. MS proteomics approaches

Proteins are the key functional entities in the cell and arguably form the principle level of information required to understand any cellular function (34). Proteome refers to the entire protein complement expressed by the genome, while proteomics refers to the global analysis of protein mixtures (or their polypeptide components). Protein research and proteomics continue to develop and have become increasingly sophisticated. Proteomics research was initially qualitative, i.e., proteins were identified as being present in samples, providing the foundation for future research, but this alone was insufficient to characterize induced protein regulations and pathological conditions. For these purposes, proteomics analysis needed to be quantitative (35). Consequently, proteomic platforms with quantitative measurement of protein expression and protein post-translational modifications (PTMs) have been developed and become an integral and indispensable part of current proteomic studies (36). Discovery-based proteomics is typically conducted with a comparatively small set of samples to identify and quantify the differential expression of proteins. These proteins can then be verified and validated with a larger sample cohort to better account for biological variation, specificity and clinical longitudinal changes in expression. Data-dependent acquisition (DDA) was the first approach developed to survey abundant peptide masses in an unknown mixture, in which precursors were isolated and fragmented

to generate a unique fingerprint spectrum of amino acid sequences for peptide identification in a high-resolution mass spectrometer. However, DDA has poor quantitative performance, because of the bias towards redundant proteins and poor reproducibility of signals and coverage of low abundant proteins (37). Data-independent acquisition (DIA) utilizes software-controlled mass isolation windows across the chromatogram, providing superior reproducibility and consistent acquisition ideal for quantitative results (38). In particular, the Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) extended the data analysis concept of a targeted approach to achieve high-throughput DIA data extraction and statistical validation. SWATH-MS is one of the first methods to record all fragment ions of the detectable peptide precursors and highly multiplexed fragment ion maps included with low abundant peptides (39). DIA method, which relies on a high-quality mass spectral library generated from the DDA approach for peptide identification, remains the only label-free quantification method to survey and quantify the hundreds of thousands of proteins in complex biological samples without the prior knowledge of fragment mass transitions and peptide occurrences in the sample. Advances in proteomics were required and went hand in hand with intensive progress in computational interfaces, including databases, data processing algorithms, decoy peptides, accurate protein identification and data analysis of large proteome datasets (40). Isobaric tags for relative and absolute quantitation (iTRAQ) and Tandem mass tag (TMT) are isobaric labeling methods used in quantitative proteomics. These methods are based on the covalent labeling of the peptide with designated tags of reporter mass. Peptide samples are labeled and then pooled for preparation. This labeled approach allows multiplexed sampling and quantification of peptides that are ideal for pilot studies, as demonstrated in the analysis of tears in dry eye disease to quantify differential expressed proteins in a single MS acquisition (41).

Phosphorylation and glycosylation are common PTMs of proteins. A pilot study of phosphorylation enrichment using a titanium dioxide ( $\text{TiO}_2$ ) column identified a total of 13 phosphoproteins in tear fluid, including mamaglobin-B (SCGB2A1), clusterin and protein UNQ773. Of note, phosphoproteins LCN1, immunoglobulin k constant (IGKC), polymeric immunoglobulin receptor (PIGR), lacritin (LACRT), cystatin S (CST4), proline-rich protein 4 (PRR4), deleted in malignant brain tumors 1 protein (DMBT1), immunoglobulin heavy constant  $\alpha$  1 (IGHA1), LYZ and immunoglobulin J chain (IGJ) had differentially expressed protein levels in ocular diseases, as described in a previously mentioned study (42). The use of PTMs as a biomarker in ocular diseases is limited, despite the high abundance of phosphoproteins reported in tear fluid (43). For example, glycosylation in the proline-rich protein family, and LACRT can be identified without an enrichment procedure (37). Methylation and acylation were observed in LCN1 and LYZ, however there was statistically significant difference of PTMs in dry eye disease (44). A total of 50 N-linked glycans were identified in tears, five low abundance N-glycans ( $m/z$  864.4, 945.5, 994.9, 1039.0 and 1112.0) and one O-glycan ( $m/z$  665.2) were significantly different in patients with diabetes or diabetic retinopathy (45).

#### 4. Protein sample preparation for MS

One-dimensional and two-dimensional gel electrophoreses (1DGE and 2DGE, respectively) were early proteomic tools used to separate, visualize and determine the size of proteins (46). MS was used to identify spots of abundant proteins and relative quantification of proteins. With this technique, proteins are separated according to isoelectric points (pI) through isoelectric focusing (IEF). In 1974, LTF and LYZ were found to be the major protein constituents in tear fluid (47). Using 1DGE, scientists found serum albumin (ALB) and transferrin were significantly elevated in the tear fluid of eyes even in mild cases of acute catarrhal conjunctivitis (48). Using 2DGE, scientists found lower abundance of proteins in human reflex tears, including CST4, cystatin SN and  $\alpha$ -2-glycoprotein, in addition to the previously reported LTF and LYZ (49), in particular, high expression of PRR4 in reflex tears (50). The fluorescence visualization of proteins in GE improved sensitivity compared with traditional Coomassie brilliant blue staining (51). However, irrespective of the staining method used, GE has a limited resolution of complex protein mixtures, low abundance proteins and co-appearing protein isoforms. It is also incompatible with hydrophobic proteins, for which it has limited access to various protein classes (52). Additionally, its limited dynamic range has hampered proteomic analysis of biofluids, such as human plasma, in which protein concentrations can differ up to 12 orders of magnitude (53). Unicellular organisms were used to benchmark 2DGE. However, detection of low abundance proteins remained a problem in this paradigm, despite the use of extended separation range and increased sample load, only 193 proteins were identified. This has demonstrated the limitations of the technique in a relatively simple model, and led to the conclusion that GE is not suitable for comprehensive global protein detection and quantitative profiling of protein networks (54). One-dimensional-liquid chromatography (1D-LC) offered a solution to the limitations of GE and has become increasingly popular over the past decade. Compared with GE-based proteomics, LC-based proteomics have improved reproducibility, streamlined peptide separation, increased sample throughput and dynamic range, and reduced sample consumption (55). These advances enabled the characterization of proteins in tissues (56), cells (57), plasma (58) and tears (59). The separation efficiency of LC is dependent on peak capacity, i.e., the maximum number of proteins that can be resolved in each separation time in a single sample acquisition (60). In human plasma, which has a particularly complex protein content, the highest achievable peak capacity of LC was reported to be 1,500 (61). It was also reported that the theoretical peak capacity of GE is three times lower than LC methods, due to its confined and definite retention volume (62). Consequentially, MS-based targeted proteomics have been rapidly adopted for quantifying proteins in complex clinical samples (63,64). MS-based approaches perform particularly well with respect to assay sensitivity and specificity, when testing biomarker panels, rather than individual markers. Therefore, this technology has paved the way for multiparametric diagnostics that can significantly increase diagnostic accuracy (65). Currently, the only Food and Drug Administration (FDA)-approved, multi-parametric clinical

test is designed to aid in the diagnosis of ovarian cancer. This test, which uses five serum proteins [CA125, transthyretin, apolipoprotein A-I,  $\beta$ 2-microglobulin (B2M) and transferrin], correctly predicted ovarian cancer in 94% of cases, which was significantly improved compared with the 66% rate observed with a single-parametric assay based on CA125 alone (66,67). Therefore, MS-based proteomics is likely to become an important tool in the identification and application of multi-analyte biomarker panels, including the use of tear fluid samples for the diagnosis of ophthalmic diseases and conditions.

## 5. Normal tear proteome in healthy ocular condition

Under normal healthy ocular conditions, tear proteins are mainly released from the lacrimal gland (68), meibomian glands (69), goblet cells (70), and accessory lacrimal glands. Early discovery studies identified only 54 proteins in tears from subjects without eye diseases (37). The number of proteins identified increased notably to 491 in closed-eye tear fluid using hybrid linear ion trap-Fourier transform (LTQ-FT) and LTQ-Orbitrap mass spectrometers (71). The majority of the identified proteins were involved in the modulation of the immune system. They were responsible for carrying out immune, inflammatory responses as well as defense responses to pathogens. This study also focused on the identification of proteases and antioxidant enzymes. Among the 491 proteins identified, 64 were proteases or protease inhibitors, and 18 were antioxidant enzymes. These findings could explain the importance of tears in protecting the healthy ocular surface from noxious external stimulants and irritants. The results of studies of the proteome of human tears in healthy subjects are shown in Table I.

Tear proteome profiles of normal subjects have been the basic research standard for method assessment and development of the clinical use of tear proteomics. LTF, SCGB2A1, haptoglobin,  $\alpha$ -1-antitrypsin (SERPINA1), CST4, LCN1 and LACRT were found to be significantly upregulated in the tear fluid of female patients compared with male patients. A total of 253 proteins and 231 proteins were identified in the tears of male and female patients, respectively, using an electrophoresis method (72). The regulation of LCN1 in tear fluid was shown to be hormone-dependent in an experimental rabbit model, but the function of LCN1 in the tears of female patients remains unknown (73). Most upregulated proteins in the tear fluid of female patients were responsible for local immune defense responses. In-depth analysis with the use of fractionation, nanoscale reversed phase-liquid chromatography (nanoRP-LC), and TripleTOF 5600 MS resulted in the report of a comprehensive human tear proteome, comprising the discovery of 1,543 proteins in normal healthy subjects (15). This number of proteins was significantly higher than other reports, so it should be noted that isoforms of the same protein were counted as separate proteins and only 714 proteins of the reported proteins were repeatable and reproducible in the triplicate analysis. Another study reported a total of 747 proteins in human tears (74), of which 595 were also reported by Zhou *et al* (15). Using an optimized extraction method and two-dimensional strong cation exchange-reversed phase (SCX-RP) with greater orthogonality of separation, Aass *et al* (75) reported 1,526 proteins in tear-fluid. We have

converted the International Protein Index (IPI), GenInfo Identifier (GI) protein identification from the literature listed in Table I to a matched UniProt reviewed proteome for comparison. A total of 3,724 unique proteins (1% False Discovery Rate in each study) were identified in tear fluid, with 1,397 (60%) unique proteins only identified in Schirmer's strip samples, and 60 (3%) unique proteins in tear samples collected with a capillary tube (Fig. 1). These independent studies had reported the most tear protein analyzed with LTQ-Orbitrap (Thermo Fisher Scientific, Inc.) and TripleTOF 5600 mass spectrometers (SCIEX). The combination of these studies is likely to increase the confidence of such protein identification. We propose that 435 (19%) proteins commonly reported can be identified regardless of the tear fluid collection method and are the higher abundant, core protein in the composition of tear fluid. Comprehensive information of these 435 common proteins was derived from the UniProt database (<https://www.uniprot.org>) and is summarized in Table SI. Gene Ontology information on their biological processes and protein class was analyzed using the Omicsbean classification system (<http://www.omicsbean.cn>). The reported genes are categorized into 'Biological Process', 'Cell Component' and 'Molecular Function', and it was found that the enriched genes were involved in the top three pathways associated with 'vesicle-mediated transport', 'immune effector process' and 'exocytosis' (Fig. 2). These data were generated from a mixture of MS and vendors. Regardless of the MS system, common proteins are likely to be identified in tear fluid, but this will depend on the collection method. Several reports have stated that Orbitrap MS yields higher protein identifications compared with the TripleTOF MS (76-79).

## 6. Putative protein markers in tear fluid

*Dry eye disease.* Based on the definition and classification provided by the International Dry Eye Workshop in 2017 (80), dry eye disease is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film that leads to tear film instability and hyperosmolarity, ocular surface inflammation, and neurosensory abnormalities and associated ocular symptoms (81). The two common types of dry eyes are known as the aqueous-deficient and evaporative dry eyes (82). Clinical diagnosis of dry eye diseases is based on questionnaires, Schirmer test, phenol red thread test, tear breakup time, corneal staining and tear osmolarity (83,84). However, these assessments have shown poor reproducibility and large inter-test variability, as well as a poor correlation between the findings and subjective symptoms (85). Hence, an unmet need requires a reliable prognostic method when diagnosing dry eye diseases. Proteomic analysis of tear fluid has been increasingly used to identify biomarkers for ocular diseases.

Ocular surface inflammation is one of the major findings of patients with dry eye so several inflammatory proteins can act as possible biomarkers of dry eyes (86). It is reported that several inflammatory proteins are reported to be differentially expressed, including upregulated proteins of  $\alpha$ -enolase (ENO1),  $\alpha$ -1-acid glycoprotein 1 (ORM1), calgranulin A (S100A8), calgranulin B (S100A9), calvasculin (S100A4) and calgizzarin, and downregulated proteins of prolactin-inducible

Table I. Human tear proteome identification of healthy subjects using various proteomics approaches coupled with mass spectrometry.

A, Capillary tube					
First author, year	Sample preparation	Mass spectrometer(s)	Number of protein identification	Clinical condition(s)	(Refs.)
Li, 2005	SDS-PAGE; In-gel digestion; In-solution digestion	LXQ Deca (Thermo Fisher Scientific, Inc.); Reflex III (Bruker Corporation); QSTAR® Pulsar (SCIEX)	54	Open eye, normal subjects	(37)
de Souza, 2006	SDS-PAGE; In-solution digestion	LTQ-FT (Thermo Fisher Scientific, Inc.); LTQ-Orbitrap (Thermo Fisher Scientific, Inc.)	491	Closed eye, normal subjects	(71)
Ananthi, 2011	SDS-PAGE; In-solution digestion	MicroTOF-Q (Bruker Corporation)	54	Reflex tear fluid, normal subjects (n=40; F=20, M=20)	(72)
Shamsi, 2011	SDS-PAGE; In-gel digestion	Ultraflex III (Bruker Corporation)	182	Normal subjects (age=35±5; n=25; F=10, M=15)	(79)
Perumal, 2015	SDS-PAGE; In-gel digestion	LTQ Orbitrap XL™ (Thermo Fisher Scientific, Inc.)	78	Based and reflex tears (age between 20-33 years; n=20; F=10, M=10)	(50)
B, Schirmer's strip					
First author, year	Sample preparation	Mass spectrometer(s)	Number of protein identification	Clinical condition(s)	(Refs.)
Zhou, 2012	SCX-RPLC; In-solution digestion	TripleTOF 5600 System (SCIEX)	1,543	Normal subjects (age=36±14; n=4; F=3, M=1)	(15)
Aass, 2015	SCX-RPLC; In-solution digestion	LTQ Orbitrap XL™ (Thermo Fisher Scientific, Inc.)	1,526	Normal subjects (n=3)	(75)
Tong, 2015	RPLC; In-solution digestion	TripleTOF 5600 System (SCIEX)	747	Normal subjects (age=55.5±14.5; n=1,000; F=589; M=611)	(74)
Dor, 2019	RPLC; In-solution digestion	LTQ Orbitrap Velos Pro (Thermo Fisher Scientific, Inc.)	1,351	Normal subjects (age=37.6±18.6; n=8; F=4, M=4)	(78)
C, Capillary tube and Schirmer's strip					
First author, year	Sample preparation	Mass spectrometer(s)	Number of protein identification	Clinical condition(s)	(Refs.)
Green-Church, 2008	SDS-PAGE; SCX-RPLC; In-gel digestion	LTQ (Thermo Fisher Scientific, Inc.)	Total, 97; Common, 30; Schirmer's strip, 54; Capillary tube, 13	Closed eye, normal subjects (age=35±13; n=8; F=6, M=2)	(31)
Nättinen, 2020	RPLC; In-solution digestion	TripleTOF 5600+ System (SCIEX)	Total, 992; Common, 316; Schirmer's strip, 592; Capillary tube, 88	Normal subjects (n=31)	(24)



protein, LTF, LCN1 and LYZ (87). ORM1 protein promotes anti-inflammatory responses, whereas S100A8 and S100A9 proteins are pro-inflammatory proteins and are commonly found in the area of inflammation (88). Downregulated proteins LTF, LYZ and LCN1 are abundant proteins that protect against pathogens in tear fluid. The decreased expression of these proteins may explain why patients are prone to infectious ocular surface diseases (89). Notably, lipocalins promote the formation and maintenance of a compact and homogeneous outermost lipid layer of the tear film (90). Hence, decreased levels of lipocalins may lead to an unstable lipid layer, as well as an increased evaporation rate of the tear fluid. The levels of S100A8 and S100A9 are associated with the severity of meibomian gland dysfunction (MGD) and with symptoms of ocular redness and transient blurring in patients with dry eye (91). The upregulation of S100A8 and S100A9 occurs in response to the oxidative changes in redox regulation and inflammatory regulation (92). Significantly upregulated levels of ALB and downregulated lactase-phlorizin hydrolase, LCN1, SCGB2A1, and lipophilin A were reported in the evaporative dry eye disease (93). The increased level of ALB is an indication of passive exudation, i.e. a leaky blood-eye barrier in conjunctival vessels (94).

Another previous study reported the differential expression of PRR4, zymogen granule protein 16 homolog B (ZG16B), DMBT1, LACRT, opiorphin prepropeptide (PROL1), aldehyde dehydrogenase dimeric NADP-preferring (ALDH3A1), phosphatidylethanolamine-binding protein 1, serotransferrin (TF), together with S100A8, S100A9, SCGB2A1, ENO1 and ORM1 that were previously reported in the literature (95). The increased expression of PRR4 and ZG16B in dry eye disease may indicate an impaired neurological process of the lacrimal gland. Downregulation of DMBT1 impairs epithelial differentiation and cellular defense mechanisms, whereas the reduction of LACRT may account for the reduced tears secretion in patients with dry eye (96). The reduction of PROL1 affects the paracrine or autocrine pathway of the lacrimal system (97). The ALDH3A1 protein protects against the oxidative stress of toxic radicals on the corneal surface (98). The upregulation of TF protein was only identified in the aqueous deficient type of dry eye (95). A previous study demonstrated the complex molecular difference between dry eye disease and MGD. Thioredoxin, Ig  $\gamma$ -1, membrane-associated phospholipase A2, SERPINA1 and antileukoproteinase (SLPI) were found to be upregulated in patients with dry eye, yet these proteins were downregulated in MGD. In addition, lactoperoxidase (LPO) was significantly downregulated in dry eye disease and upregulated in MGD (99). The upregulation of these proteins suggests enhanced immune, host-defense and proteolytic responses in aqueous-deficient dry eye disease, whereas the contrary may be the case in the evaporative dry eye caused by MGD. Furthermore, the higher level of hyperosmolarity in patients with MGD may lead to an increased expression of oxidative stress-associated LPO protein in MGD when compared with patients with dry eye. The differential expression of proteins between MGD and dry eye suggest different regulatory processes. S100A8, S100A9 and ORM1 were identified as differentially expressed in all the dry eye studies reported in the present review (Table II). Hence, these proteins may

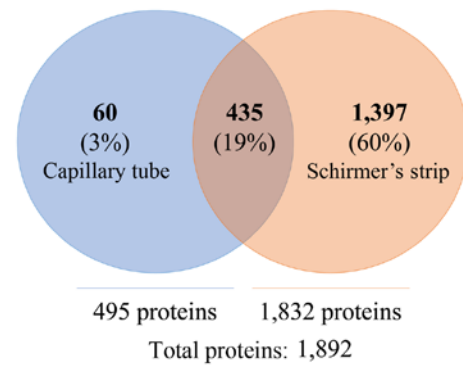


Figure 1. Venn diagram of the number of proteins (UniProt reviewed protein) collected with capillary tube or Schirmer strips in Table I. A total of 2,327 unique proteins (1% False Discovery Rate) were reported in tear fluid using mass spectrometry techniques. Of these, 495 and 1,832 unique proteins were identified in tears collected with a capillary tube and Schirmer's strip, respectively.

serve as biomarkers of dry eye disease, in addition to the established biomarker matrix metalloproteinase-9 (MMP-9) that is already being employed in the diagnosis of dry eye disease (100). Although anterior ocular inflammation is a typical feature of Sjögren syndrome, it is considered to be a systemic autoimmune disease with some distinct clinical presentation (101). Different from physiological dry eyes, aqueous-deficient and evaporative dry eyes, which could be clinically difficult to differentiate using routine clinical assessments. It was recently found that the elevated MMP-9 protein biomarker is non-specific and difficult to distinguish Sjögren syndrome from typical dry eye diseases (102). MS-based proteomics approaches enabled the discovery of the upregulation of other pro-inflammatory proteins, including LIM domain only protein 7, E3 ubiquitin-protein ligase and Copine-1, as well as in the involvement of TNF- $\alpha$  signaling (103,104), which suggested the possibility that specific molecular biomarkers may be developed for more specific clinical diagnosis.

These studies have provided preliminary data on protein biomarkers in tear fluid using MS techniques. However, there are several limitations of using tear proteomics to make a diagnosis of dry eyes. The tear sampling methods and ways of sample manipulation differ among the reported studies; hence, a direct comparison between these studies may not be appropriate. To achieve comparable results, standardization of sampling methods and sample manipulation protocols are required in the future. Additionally, S100A8 and S100A9, which were differentially expressed in all of the studies, were also reported in patients with glaucoma (Table II), indicating that these inflammatory proteins are not differentially expressed uniquely in patients with dry eye (105). In summary, several potential biomarkers have been identified in patients with dry eyes, but whether a diagnosis of dry eyes can be based on tear proteomics remains to be determined and defined. A signature panel of tear fluid biomarkers is needed to address overlap with other conditions to increase the specificity of tear fluid protein markers for the diagnosis of dry eye diseases.

**Diabetic retinopathy.** Diabetic retinopathy is a common complication of diabetes mellitus (DM). The condition is asymptomatic in the early stages of disease development, yet it

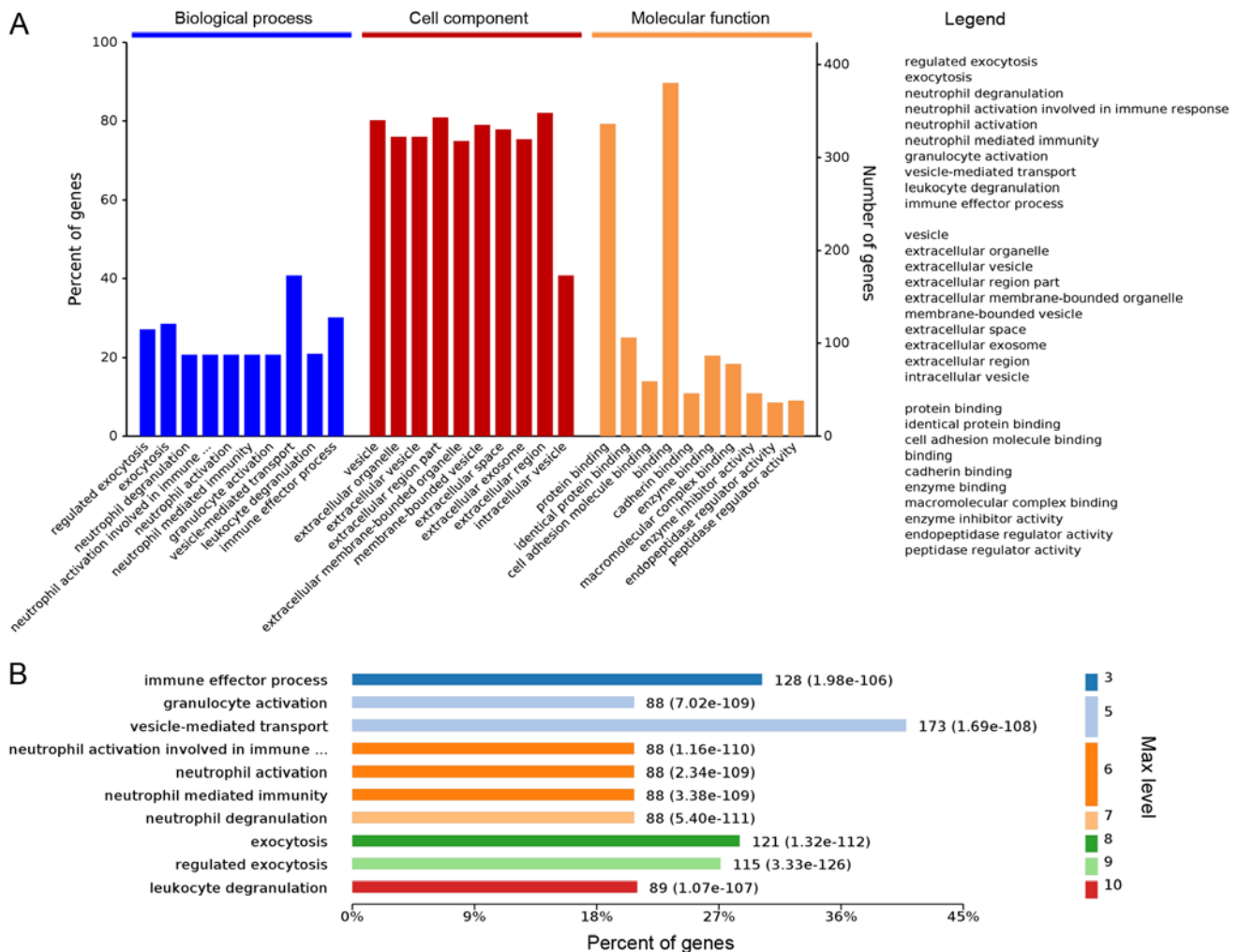


Figure 2. Omicsbean Gene Ontology analysis of the 435 common tear proteins. (A) The bars indicate the proportion of converted gene represented per 'Biological Process', 'Cell Component' and 'Molecular Function'. (B) Top 10 enriched significant pathways with the percentage of genes under each pathway of the 435 common proteins.

can cause irreversible blindness in its final stages. Tear composition can be affected by DM, although the tear film is not in direct contact with the retina (106). Hence, tear proteins may act as biomarkers for the screening of diabetic retinopathy. The relative abundance of LACRT, Ig lambda chain C region (LAC), LTF, LYZ, LCN1 and SCGB2A1 proteins were upregulated in patients with proliferative diabetic retinopathy (PDR) compared with non-PDR and healthy subjects (107). The upregulated expression levels of LTF, LAC and LACRT may reflect an increased inflammatory response, potentially caused by macular edema, vascular abnormalities, the proliferation of the ocular cells, and an indicator of the pro-proliferative environment that is essential for the progression of diabetic retinopathy (108).

**Thyroid eye disease.** Thyroid-associated orbitopathy (TAO) is an autoimmune disorder that affects the orbit. TAO is characterized by enlarged extraocular muscles, orbital tissue and inflammatory changes, including upper eyelid retraction, proptosis and erythema of the conjunctiva (109). There are two phases of TAO: The inflammatory phase, which requires anti-inflammatory treatment, and a later less active stage (110). The clinical diagnosis, assessment

and management of the disease are based on the Clinical Activity Score (CAS) (111). However, disease onset, prognosis and time course of TAO remain unclear. TAO mainly affects the extraocular muscles, eyelid and orbital tissue. These surrounding damaged tissues may release different proteins into tears or by passive transport from blood; therefore, tears may contain potential protein markers for the diagnosis of TAO (112). However, the composition of tears collected from patients with TAO need to be analyzed carefully as it may contain certain inflammatory proteins that are associated with exposure keratitis, which is a common complication of TAO (113).

In one previous study, the expression of three proteins was modulated in patients with TAO (114). LYZ was found to be upregulated, whereas PRP4 and B2M were downregulated in patients with TAO. LYZ is a proteolytic protein that is important in the immune response (115) and increased LYZ is found in patients with autoimmune diseases (116). The increase of LYZ may suggest increased inflammatory responses of the lacrimal gland. Lacrimal PRP4 can regulate the microflora of the eye to protect the ocular surface (117). The inflammatory processes of the orbit in TAO may decrease the lacrimal expression of PRP4. It has been demonstrated that increased

Table II. List of significantly differentiated tear protein abundance by MS-based proteomic analysis in ocular diseases.

A, Capillary tube					
First author, year	Separation	Mass spectrometry	Conditions	Differentially expressed protein markers, gene name	
				Upregulated	Downregulated (Refs.)
Versura, 2010	2D SDS-Page	Micromass® Q-ToF (Waters Corporation)	Evaporative dry eye (age=64.2±22.3; n=90; F=42, M=18)	ALB	LCN1, LTF, SCGB1D1, SCGB1D2, SCGB2A1 (93)
Csász, 2012	RPLC	ESI-MS/MS (QTRAP 4000, SCIEX)	General diabetic retinopathy (average age=61; n=145) Non-proliferative diabetic retinopathy (average age=56) Proliferative diabetic retinopathy (average age=64)	IGLC1, LACRT, LCN1, LFT, LYZ, SCGB1D1 CST4 APOA1, IGLC1, LACRT, LCN1, LFT, LYZ, PRB4, SCGB1D1, SCGB2A1	(107)
Soria, 2017	RPLC	SYNAPT G2-S HDMS System (Waters Corporation)	Aqueous deficient dry eye and control (age=54.58±21.55; n=24; F=14, M=10) Meibomian gland diseases and control (age=54.7±11.6; n=12; F=7, M=5)	LPO PLA2G2A, S100A6, S100A8, SERPINA1, SLPI, TXN ANXA1, CLU, LPO, ORM1	(99)
B, Schirmer's strip					
First author, year	Separation	Mass spectrometry	Conditions	Differentially expressed protein markers, gene name	
				Upregulated	Downregulated (Refs.)
Zhou, 2009	Online 2D SCX-RPLC	QSTAR-XL qTOF (SCIEX)	General dry eye and control (average age=60; n=56; F=43, M=23)	ENO1, ORM1, S100A4, S100A8, S100A9, S100A11	LCN1, LTF, LYZ, PIP (87)
Wong, 2011	Online 2D SCX-RPLC	QSTAR-XL qTOF (SCIEX)	Glaucoma and control (Topical antiglaucoma medications for >1 year; age=72±7; n=18; F=9, M=9) Glaucoma and control (Topical antiglaucoma medications for <1 year)	S100A8, S100A9, SCGB2A1, YWHAZ SCGB2A1, S100A8	PRR4 PRR4, S100A9, YWHAZ (105)
Matheis, 2012	1D-SDS-PAGE	Ultraflex MALDI-TOF/TOF (Bruker Corporation)	TAO and control [median age=45 (33-74); n=45]	CST4, LYZ	B2M, PRB4 (114)



B, Schirmer's strip

First author, year	Separation	Mass spectrometry	Conditions	Differentially expressed protein markers, gene name		(Refs.)
				Upregulated	Downregulated	
Pieragostino, 2012	1D-SDS-PAGE	Reflex IV MALDI-TOF (Bruker Corporation)	PXG and control (n=5)	TF, S100A4	AZGP1, CST1, CST2, CST4, IGHA1, KRT1, LACRT, LCN1, LYZ, OPRPN, PIP, PRR4, SCGB2A1, ZG16B	(136)
				IGHG1, IGHG2, IGHG4	ANXA1, AZGP1, CST1, CST2, CST4, IGHA1, IGHA2, IGKC, JCHAIN, KRT1, LCN1, LTF, LYZ, PIGR, PIP, PRR4, SCGB2A1	
					CST2, CST4, IGHG2, LACRT, PRR4, SCGB2A1	JCHAIN, KRT19, LGALS3, PIGR, PRDX1, S100A4, S100A8
Pieragostino, 2013	RPLC	LTQ Orbitrap (Thermo Fisher Scientific, Inc.)	POAG vs. PXG			
			Primary open angle glaucoma and control (age=55.4±14.5; n=9; F=4, M=5)	ACTB, ACTG1, ALB, AZGP1, B2M, CST4, HSPB1, IGHA1, IGHA2, IGKC, JCHAIN, LCN1, LTF, LYZ, OPRPN, PIGR, PIP, POTE, POTEF, POTEI, POTEJ, PRDX1, PRR4, TF, ZG16B		
			TAO and control [median age=45.5 (17-68); n=30; F=23, M=7]	MDN1, POTEI	PROL1, PRP4, S10A8, SMCA4	(117)
Matheis, 2015	1D-SDS-PAGE	Ultraflex MALDI-TOF/TOF (Bruker Corporation)	TAO with DE (TAO: DE) and control [median age=51 (31-70); n=30; F=25, M=5]	LEG3, SMCA4	UGDH	
			DE and control [median age=54.5 (32-80); n=30; F=23, M=7]	ANXA1, HSPB1, LEG3, S10A8, SMCA4		
			TAO and DE			
			TAO and TAO:DE	LYSC	ANXA1, CYTN, HSPB1, LEG3, PROL1, S10A8, SMCA4, UGDH	LEG3

Table II. Continued.

First author, year	Separation	Mass spectrometry	Conditions	Differentially expressed protein markers, gene name		(Refs.)	
				Upregulated	Downregulated		
Aass, 2016	RPLC	LTQ Orbitrap (Thermo Fisher Scientific, Inc.)	TAO:DE and DE		CYTN, PROL1, S10A8, SMCA4, UGDH	(122)	
			TAO and control [median age=57 (20-77); n=21; F=15, M=6]	APOD, AZGP1, CASP14, DCD, DMBT1, GPX3, LACRT, LYZ, MSLN, PLOD2, SLPI, ZG16B	CST5, PPL, SCGB2A2		
			Evaporative dry eye and control (F age=51.8±18.66; M age=52.9±20.45; n=20; F=10, M=10)	S100A8, S100A9	PROL1, PRR4, ZG16B		(95)
			Aqueous deficient dry eye (F age=49.6±14.74; M age=47.6±15.32; n=20; F=10, M=10)	ENO1, ORM1, PEBP1, S100A8, S100A9, TF	DMBT1, PROL1, PRR4, SCGB2A1, ZG16B		
Kishazi, 2018	1D-SDS-PAGE	LTQ Orbitrap (Thermo Fisher Scientific, Inc.)	Aqueous deficient and evaporative dry eye (F age=58.78±17.42; M age=57.73±19.38; n=20; F=10, M=10)	ALDH3A1, ENO1, ORM1, PEBP1, S100A8, S100A9, TF	DMBT1, LACRT, PROL1, PRR4, SCGB2A1, ZG16B	(125)	
			TAO and control (age=46.92±11.25; n=28; F=21, M=7)	CST3, HP, NQO1, SERPINA3, TXNDC5	ABHD14B, ADH5, ALDH1A1, PLA2G2A, STAT1		
TAO, thyroid-associated orbitopathy; PXG, pseudoexfoliative glaucoma; POAG, primary open angle glaucoma; DE, dry eye.							

levels of inflammation and higher CAS values are associated with lower levels of PRP4, indicating the progressive nature of the inflammatory lacrimal gland dysfunction in patients with TAO (114). B2M belongs to the major histocompatibility complex class I molecules and also plays an important role in immune responses (118). The downregulation of B2M may reflect altered immune function in this auto-immune disease.

Patients with TAO can have signs and symptoms similar to dry eye syndrome, which can result in delayed diagnosis of TAO (117). In comparison to normal subjects, transcription activator BRG1 (SMCA4), PROL1, PRR4 and S100A8 proteins were downregulated, whereas midasin, POTE ankyrin domain family member I and LYZ proteins were upregulated in patients with TAO (117). In comparison to patients with dry eye, UDP-glucose 6-dehydrogenase (UGDH), annexin A1, cystatin-C (CST3), heat shock protein  $\beta$ 1 (HSPB1), galectin-1, PROL1, S100A8 and SMCA4 proteins were downregulated in patients with TAO (117). The apoptosis of lacrimal cells can cause the downregulation of PROL1 and PRR4 proteins, the protective enzymes secreted by the lacrimal acinar cells in TAO (119). The damage to the lacrimal cells can reduce the number of cystatin proteins, which perform protective function in the tears (120). UGDH protein is responsible for the indirect production of the glycosaminoglycans that are expressed in fibroblasts in the active phase of TAO (121). The downregulation of UGDH protein can be explained by the fact that the majority of the patients involved in this study were in advanced and inactive stages of TAO. A similar study reported 12 upregulated proteins in patients with TAO, including caspase-14, SLPI, dermcidin (DCD), procollagen-lysine 2-oxoglutarate 5-dioxygenase 2, mesothelin, apolipoprotein D, glutathione peroxidase 3, zinc- $\alpha$ -2-glycoprotein 1, DMBT1, ZG16B and LACRT (122). The overexpression of CASP14, SLPI and LYZ proteins may represent the inflammatory responses of the ocular surface, orbital tissue or lacrimal gland. However, the exact function of CASP14 in tear fluid remains unclear. DCD protein has anti-microbial properties and has been detected in conjunctival cells (123). Increased amounts of DCD protein suggest more bulbar conjunctiva inflammation in patients with TAO (124). In a more recent study, retinal dehydrogenase 1, SERPINA3 and CST3 proteins were found to be upregulated in tear fluid obtained from patients with TAO (125). CST3 protein is a cysteine protease inhibitor that is concentrated and expressed in the retinal pigment epithelium (126). The concentration of CST3 protein in the blood is associated with thyroid functioning (127). The downregulation of retinol dehydrogenase 11 protein may result in reduced synthesis of retinoic acid, hence, affecting the visual pigment and leading to vision loss (128). Increased expression of SERPINA3, a protein responsible for mediating inflammatory responses, may reflect the increased level of eye inflammation in TAO, which is an autoimmune disease with orbital inflammatory responses. Different biomarkers for TAO have been identified across different studies (Table II), further validation should be carried out to confirm potential biomarkers and these biomarkers should be analyzed according to the severity or different stages of TAO.

**Primary open-angle glaucoma (POAG).** Glaucoma is a progressive neurodegenerative disease that causes optic nerve head damage, retinal nerve fiber layer defects, and is associated with the loss of the visual field (129). It is one of

the main causes of blindness worldwide (130). The underlying mechanism of glaucoma remains unclear, and the clinical diagnosis of glaucoma relies on several assessments, including tonometry, dilated fundus image examination, visual field test, gonioscopy and pachymetry (129). Visual field impairment is a cause of irreversible damage to retinal ganglion cells (131). Tear fluid proteomic profiling may provide novel insights into the understanding and diagnosis of glaucoma and may serve to monitor therapy, including the side effects of medication. POAG is the most common subtype of open-angle glaucoma in the European population (132). The damaged trabecular meshwork and modification of the aqueous humor leads to an impaired drainage system. The accumulation of fluid increases the intraocular pressure (IOP) of the eye (133). Pseudoexfoliative glaucoma (PXG) is another subtype of POAG and is characterized by the production and accumulation of abnormally high concentration of fibrillar and proteinaceous substances in the anterior segment of the eyes (134). These substances can block the ocular drainage system and thus increase the IOP of the eye, one of the risk factors of glaucoma (135). A total of 23 differentially expressed proteins have been reported in POAG and PXG. Cystatin-SA, CST4, SCGB2A1, Ig  $\gamma$ -2 chain C region and PRR4 proteins were found to be upregulated in POAG, but not PXG. Peroxiredoxin-1, IGJ, galectin-3, PIGR, keratin type I cytoskeletal 19, S100A4, S100A8 and LACRT were found to be downregulated in POAG compared with PXG samples. More importantly, keratin type I cytoskeletal 10 and apolipoprotein A-II proteins are unique to POAG tear fluid (136). B2M, HSPB1, IGHA1, immunoglobulin heavy constant  $\alpha$ 2, IGJ, IGKC, LTF, LYZ, PIGR, TF and ALB proteins were also upregulated in patients with POAG (136). The modulation of these proteins between treated and untreated POAG groups indicated that PGA works effectively via the anti-inflammatory mechanism. Proteomics was applied to monitor patients chronically treated with topical antiglaucoma medications, finding that SCGB2A1, S100A8, S100A9 and 14-3-3  $\zeta/\delta$  proteins were upregulated, whereas PRR4 was downregulated in patients with glaucoma treated with IOP lowering medication (105). The results indicated that the use of topical antiglaucoma medications for >1 year affects the ocular surface by inducing inflammatory responses. The tear fluid proteome of the medically treated patients with glaucoma and patients with dry eyes compared with normal control subjects have shown upregulation of S100A8 and S100A9 proteins in both glaucoma and dry eye patients. Proteins expressed in medically treated glaucoma eyes (SCGB2A1, 14-3-3  $\zeta/\delta$ , PRR4) or dry eyes (ENO1, S100A4) did not exhibit a common expression pattern between conditions (137). These results suggested that distinct, yet complex mechanisms lead to different inflammatory responses in ocular diseases that can be distinguished using MS-based proteomic techniques.

## 7. Conclusions

The present review provided a brief introduction to the development of proteomics platforms for tear proteome studies. The proteome identified in normal tear fluid and its expression in dry eye syndrome, diabetic retinopathy, thyroid eye disease

and POAG were summarized. MS-based methods have evolved rapidly with technological advances in high-resolution mass spectrometers and data analysis tools for a variety of discovery-based experiments, resulting in ever-larger proteomic datasets in tear fluid. With respect to accurate quantitative proteomics, DIA and labeled tags offer consistent quantification of proteins in disease conditions for both pilot and large cohort studies. MS technology continues to improve and has enabled in-depth protein profiling, reliable quantification with superior flexibility for assay development, and remains the only antibody-free approach for protein analysis in biological samples. The consistent results of analyzing the microliter volumes of tear fluid or differentiated proteins has demonstrated the potential development of assays for ocular diseases and ophthalmology overall using a variety of MS approaches. For future approved molecular diagnostics, a custom-made antibody-based assay or point of care diagnostic molecular kit could be developed to target specific proteins, taking full advantage of established, lower-cost, and ease of use into clinical use.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

JYWM and YHS drafted the manuscript. JFB edited and formatted the manuscript. TCL conceived the idea, proofread the manuscript, and provided financial support. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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