Progress in Retinal and Eye Research 106 (2025) 101338

Contents lists available at ScienceDirect

Progress in Retinal and Eye Research

journal homepage: www.elsevier.com/locate/preteyeres

A comprehensive scoping review of methodological approaches and clinical applications of tear fluid biomarkers

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ARTICLE INFO

Keywords:
Tear fluid
Tear fluid collection
Biomarkers
Tear fluid analysis
Schirmer's strip
Glass capillaries
Tear fluid applications

ABSTRACT

Tear fluid is an emerging source of disease biomarkers, drawing attention due to its quick, inexpensive, and non-invasive collection. The advancements in detection techniques enable the measurement of ultra-low biomarker levels from small sample volumes typical of tear fluid. The lack of standardized protocols for collection, processing, and analysis of tear fluid remains a significant challenge. To address this, we convened the Tear Research Network Review Taskforce in 2022 to review protocols from the past three decades, providing a comprehensive overview of the methodologies used in tear fluid biomarker research.

A total of 1484 articles published from January 1974 to May 2024 from two electronic databases, Embase and Ovid MEDLINE, were reviewed. An exponential increase in the number of articles on tear fluid biomarkers was observed from 2015 onwards. The two most commonly reported collection methods were; glass capillaries (45.2%), and Schirmer's strips (25%), with glass capillary tube collection remaining the most frequent method until 2019, when Schirmer's strips became the leading method. Most articles analyzed tear fluid proteins (65%) and focused on a single analyte (32.3%). In recent years, an increase was observed in the type and number of examined analytes.

The differences in the reported methodologies and protocols underscore the need for standardization and harmonization within the field of tear fluid biomarkers to minimize methodological differences and reduce variability in clinical outcomes. Consistent and detailed reporting is essential for improving the reproducibility and validity of tear fluid studies, in order to advance their potential clinical applications.

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https://doi.org/10.1016/j.preteyeres.2025.101338
Received 20 September 2024; Received in revised form 4 February 2025; Accepted 4 February 2025
Available online 13 February 2025
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1. Introduction

Tear fluid is a highly dynamic and complex biological fluid, playing a crucial role in maintaining ocular surface health (Bron et al., 2004; Rolando and Zierhut, 2001). Although often perceived as merely a lubricant to maintain the primary optical surface of the eye, its functions extend far beyond this role (Tutt et al., 2000). Tear fluid consists of a mixture of proteins (more than 1500), lipids, mucins, water, ions, and many other ingredients (Aass et al., 2015; Akkurt Arslan et al., 2021). Tear fluid protects the cornea and conjunctiva against environmental pathogens, accelerates wound healing after injury, and ensures sustained eye comfort and optimal vision quality (Zhou et al., 2004, 2012). Recent improvements in the sensitivity and accessibility of immunoassays, omics techniques (e.g., proteomics, lipidomics, metabolomics, genomics), and high-resolution tear film imaging (Herbaut et al., 2019; Jones et al., 2022; Ponzini et al., 2022), have led to a rapidly growing interest to study tear fluid characteristics for measurable indicators of health or disease. Tear fluid biomarkers are being used to assess risk or presence of disease and the response to the treatment of disease (Gutman and Kessler, 2006; Hagan et al., 2016). Additionally, tear fluid analysis can now serve as a valuable tool to better understand the physiological and pathological processes of both ocular and systemic health (Barmada and Shippy, 2020; Hagan et al., 2016; Tseng and Tsubota, 1997).

Despite the identification of hundreds of potential tear fluid biomarkers (Altman et al., 2023; Khanna et al., 2022; Ma et al., 2021; Ponzini et al., 2022), translating these discoveries into clinical diagnostic and prognostic tools remains difficult. Significant challenges hinder progress in this area, most notably the variability in tear fluid analysis. This variability stems from the absence of standardized methods for tear fluid collection, analysis, interpretation, and reporting. As a result, this variability creates substantial limitations to the widespread use of tear fluid as a source of biomarkers among researchers and clinicians worldwide. The lack of universally accepted standardization amongst investigators has led to discrepancies among published studies using different tear fluid collection and analysis methodologies contributing to inconsistent research findings. This inconsistency, coupled with frequent small sample sizes in clinical trials, decreases the reliability and reproducibility, complicates the interpretation of data, and delays the translation of tear fluid analyses from bench to bedside. Without a unified framework for studying tear fluid, the scientific community struggles to draw meaningful conclusions, hindering the development of tear fluid-biomarker-based tools. Taken together, these issues highlight the untapped potential of tear fluid analysis as a non-invasive tool for predicting and monitoring the ocular surface, systemic diseases, environmental influences, and various other conditions.

To unlock the full potential of tear fluid as a routine diagnostic body fluid, the critical steps in tear fluid collection, storage, extraction, and preparation, and their impact on analysis should be standardized. This scoping review presents the past and current techniques for tear fluid collection, explores the analytical considerations necessary for optimizing data extraction, and discusses the applications of tear fluid biomarkers in both health and disease. Additionally, the current challenges and future directions necessary for establishing tear fluid as a routine point-of-care (POC) test are also discussed.

The Tear Research Network (TRN) (www.tearresearchnetwork.com) is a newly developed international network bringing together clinicians, researchers, and industry professionals, with over 200 members from five different continents, dedicated to advance the field of tear fluid research. Members meet several times a year during international meetings or by videoconferences to discuss challenges and future directions of tear fluid research. The first highlighted challenge was the lack of consensus regarding methods for tear fluid collection and analysis. The goal of the TRN is to provide evidence-based data to fill the current challenges in tear fluid research. Prior to developing international guidelines filling these gaps, the TRN thought it necessary to provide a comprehensive overview of the current literature on tear fluid collection and analysis, presented in the current study.

2. Tear fluid physiology

Tear fluid forms the outermost component of the ocular surface and covers the corneal and conjunctival epithelial cells (Gipson, 2007). The tear fluid provides a smooth surface required to maintain the optical properties of the cornea, and plays the same role as blood throughout the body for the avascular and transparent cornea. This involves delivering oxygen and nutrients, clearing waste, defending against pathogens, and repairing damage to the ocular surface (Schaumberg et al., 2003).

Other tear fluid functions include lubrication, prevention of dehydration of the cell membranes, maintenance of osmosis of the ocular surface, protection against pathogens, and nutrition of underlying cells (Ohashi et al., 2006). The tear fluid contains a variety of proteins, lipids, mucins, electrolytes, nucleic acids, and small metabolites, which are in continuous interaction with the ocular surface and epithelial cells, reflecting the biochemical and physiological state of the cornea and conjunctiva (Fig. 1). Tear fluid analysis is therefore of importance for monitoring ocular health and disease (Barmada and Shippy, 2020).

Traditionally, the tear film was described as composed of three layers: an inner mucin layer, a middle aqueous layer, and an outer lipid layer (Wolff, 1946). However, with a continuous improvement in understanding the complex, dynamic nature of tear fluid and facilitated by technological advancements, this model has evolved to a two-layer structure (Segev et al., 2020). In this updated model the tear film is divided into two distinct layers: the muco-aqueous layer and the lipid layer (Willcox et al., 2017) (Fig. 1). The muco-aqueous layer, a gel-like composite of aqueous fluid and mucin, features a concentration gradient of mucins that decreases from the apical epithelial cells toward the aqueous component (Willcox et al., 2017). The outer lipid layer is composed of polar lipids adjacent to the muco-aqueous layer, and nonpolar lipids. The surface lipid layer of the tear film is secreted mainly by the Meibomian glands and acts as an interface between the muco-aqueous layer and the air, reducing evaporation and lowering the surface tension (Fig. 1) (Pflugfelder and Stern, 2020). Under normal conditions, the tear fluid thickness is 3 μm (King-Smith et al., 2000) and the tear fluid volume is 5–10 μL, with a basal secretion rate of 1.2 μL/min (Tiffany, 2008), and a turnover rate of 16% per minute (Maitchouk et al., 2000b).

The lacrimal functional unit (lacrimal glands, ocular surface – cornea, conjunctiva and Meibomian glands -, lids and the sensory and motor nerves) regulates tear fluid production, delivery and clearance to maintain ocular surface homeostasis (Pflugfelder and Stern, 2020; Stern et al., 1998a, 1998b). The activity of the lacrimal gland can be divided into basal, reflex and emotional secretions (Murube, 2009). A neural reflex loop, involving trigeminal fibers, transmits signals from the cornea to the central nervous system, driving tear production and blinking, with sympathetic and parasympathetic efferent pathways terminating in the secretory glands (Cox and Nichols, 2014; Parra et al., 2010). While traditionally, accessory lacrimal glands were thought to handle basal secretion and the main lacrimal gland reflex secretion, studies also emphasize the role of corneal innervation in tear fluid regulation (King-Smith et al., 2000; Maitchouk et al., 2000a). Dysfunction of the lacrimal functional unit can result in dry eye disease (DED) or other ocular surface conditions, altering tear fluid composition and activating innate and acquired immunity and inflammatory processes (Behrens et al., 2006; Pflugfelder and de Paiva, 2017).

2.1. Proteins

The muco-aqueous tear fluid layer is rich in proteins that support homeostasis, wound healing, and defense (Pflugfelder and Stern, 2020). Proteinaceous growth factors such as epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β), regulate inflammation and

promote epithelial and stromal healing (Yoshino et al., 1996). Common blood-derived proteins in tears include albumin, immunoglobulins, and transferrin.

Tear fluid also contains antimicrobial proteins like lysozyme, lipocalin, and lactoferrin, that inhibit bacterial growth and infections (Dartt, 2011; Flanagan and Willcox, 2009; Pflugfelder and Stern, 2020). Moreover, secretory immunoglobulin A, β-defensins and S100 proteins prevent pathogens from adhering to epithelial cells, enhancing protection against infections like *Acanthamoeba* and *Staphylococcus aureus* (Jäger et al., 2010; Lan et al., 1997; McDermott, 2004; Zhou et al., 2012).

Additionally, tear fluid contains a variety of anti-inflammatory proteins and antioxidant factors, such as interleukin-1 receptor antagonist, TGF-β2, antioxidants, and vitamin A, to protect the ocular surface by regulating inflammation and reducing oxidative stress. (Contreras-Ruiz and Masli, 2015; Pflugfelder et al., 2015). Protease inhibitors, including secretory leukocyte protease inhibitor and tissue inhibitors of matrix metalloproteinases (TIPs), regulate protease activity, providing further protection of the ocular surface (Ohashi et al., 2006).

2.2. Nucleic acids

Various nucleic acids are present in tear fluid, including RNA, DNA, and microRNA (miRNA). In biomarker research, most emphasis has been on miRNAs because they are crucial for regulating gene expression and intercellular communication. Weber et al. previously emphasized high levels of miRNAs in tear fluid (Weber et al., 2010a). Research on miRNAs in tear fluid has identified specific miRNAs associated with DED and other ocular diseases including Sjögren's disease, infectious keratitis, vernal keratoconjunctivitis, glaucoma, diabetic macular edema, and diabetic retinopathy, and Alzheimer's disease (Altman et al., 2023; Hindle et al., 2019; Hirota et al., 2015; Inubushi et al., 2020b; Kenny et al., 2019; Kim et al., 2019, 2022; Saxena et al., 2015; Wang et al., 2020; Wong et al., 2023). Their stability, straightforward detection, and accessibility make miRNAs promising for clinical applications, though this research field is still in its early stages (Rassi et al., 2017).

2.3. Lipids

The lipid layer is composed of a complex mixture of polar and nonpolar lipids, including wax esters, cholesterol esters, sphingolipids and phospholipids, and forms a structured interface with the aqueous layer below (Bron et al., 2004). Polar lipids enhance tear film cohesion and spread, while the outer nonpolar lipids create a hydrophobic barrier that minimizes water loss. Key functions of the lipid layer include reducing evaporation, stabilizing the tear film, and providing lubrication during blinking to protect epithelial surfaces from mechanical stress. It also supports antimicrobial defense and helps maintain the ocular surface temperature by limiting evaporative cooling (Bron et al., 2004). Lam et al. reported in 2014 the most comprehensive human tear lipidome to date, with more than 600 individual lipid species from 17 distinct lipid classes (Lam et al., 2014). Rantamäki et al. demonstrated that polar lipids are the most prevalent lipid species (Rantamäki et al., 2011). In Meibomian gland dysfuncation, impaired lipid spreading and lipid layer thinning contribute to tear fluid instability, increased evaporation, and dysregulation of ocular surface homeostasis biomarkers (Braun et al., 2015).

2.4. Mucins

Tear fluid mucus, composed of water and mucin glycoproteins, plays an important role in maintaining epithelial hydration, barrier function, and wettability, while reducing friction during blinking. Key mucins include membrane-associated types like MUC1, MUC4, and MUC16, which form part of the glycocalyx, as well as gel-forming mucins like MUC5AC, MUC5B, MUC2 that are secreted by goblet cells (Gipson and Argüeso, 2003; Pflugfelder et al., 2000, 2015; Pflugfelder and Stern, 2020).

Glycosylation is crucial to allow mucins to effectively perform their protective role on the ocular surface (Argüeso, 2022). Glycosylation

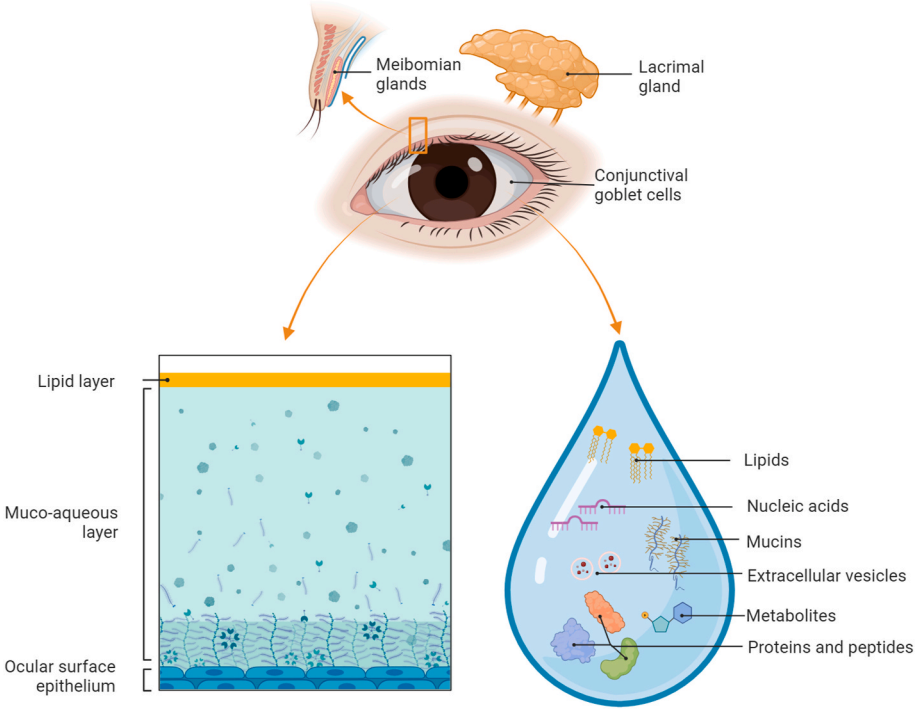


Fig. 1. Tear fluid physiology, structure and components. Tear fluid is produced and secreted by the lacrimal glands, Meibomian glands, and conjunctival goblet cells. It consists of two distinct layers: the lipid layer and the muco-aqueous layer. Tear fluid is rich in a variety of analytes, including lipids, nucleic acids, mucins, extracellular vesicles, metabolites, proteins, and peptides. Created in BioRender. Gijs et al. (2024) BioRender.com/r84k399.

provides the hydrophilic properties necessary for lubrication, maintaining a stable tear film, protecting the ocular surface from desiccation, and acting as a barrier against pathogens by preventing their adhesion to the epithelial cells (Brockhausen et al., 2018; Uchino, 2018).

Disruption of mucin disulfide linkages or goblet cell function can impair tear fluid stability and ocular surface health, contributing to DED and inflammatory conditions such as Sjögren disease and Stevens-Johnson syndrome (Pflugfelder et al., 1990, 1997, 2015; Pflugfelder and Stern, 2020).

2.5. Metabolites

Metabolites are small molecules involved in various biological functions, including energy provision, structural formation, signaling, enzyme modulation, catalysis, defense, and molecular interactions. Unlike the proteome and lipidome, the human tear fluid metabolome is less studied (Brown et al., 2024; Brunmair et al., 2022; Chen et al., 2011; Dammeier et al., 2018; Khanna et al., 2022; Kropp et al., 2023). In tear fluid high concentrations of taurine, L-glutamic acid, and L-glutamine can be detected, while L-valine, L-isoleucine, and others were found in lower concentrations (Nakatsukasa et al., 2011). Despite these findings, more research is needed to fully understand the tear fluid metabolome.

2.6. Extracellular vesicles

Extracellular vesicles (EVs) are a diverse group of lipid bilayer-enclosed membrane structures, including apoptotic bodies, microvesicles, exosomes, and other subtypes. These vesicles are either shed from the plasma membrane or arise from the endosomal system (Welsh et al., 2024). The composition of EVs varies depending on the cell of origin, the process of their formation, and the functional state of the cell (Shiju and Yuan, 2024b). This variability makes EVs ideal carriers of potential disease markers (Amorim et al., 2022; Chatterjee and Singh, 2023; Cross et al., 2023; Hefley et al., 2022; Martins et al., 2024; Shiju and Yuan, 2024b; van der Merwe and Steketee, 2017; Yeung et al., 2022). To date, as reviewed in Shiju and Yuan, some EV cargo molecules have been identified in tear fluid as potential biomarkers for DED, Sjögren's disease, primary open angle glaucoma, diabetic retinopathy, and thyroid disease (Shiju and Yuan, 2024a).

3. Evaluation of tear fluid measures and composition

3.1. Tear fluid volume

Tear fluid volume is important for ocular surface health and its reduction is a key pathogenic progress in aqueous deficiency DED. Tear fluid volume can be measured using various methods. Slit-lamp techniques, which assess tear meniscus height, curvature, and cross-sectional area, are commonly used in clinical settings. These techniques demonstrate good diagnostic accuracy and correlate with the severity of DED. It has also shown good correlation with spectral-domain optical coherence tomography (OCT) meniscometry (Bartuzel et al., 2014). The Schirmer's test estimates tear fluid volume by placing a paper strip on the lower lid for 5 min and measuring wetting length. The Schirmer's test is used to diagnose aqueous deficiency DED (e.g., Sjögren disease), but is less effective for detecting changes in tear fluid quality, such as evaporative DED from Meibomian gland dysfunction. (Cuevas et al., 2012; Willcox et al., 2017). Several diagnostic cut-off values have been suggested, ranging from 5 mm/5 min (Bron et al., 2007) to 10 mm/5 min (de Monchy et al., 2011; Wolffsohn et al., 2017).

3.2. Tear film stability

Tear film stability can be evaluated by the measurement of the tear film breakup time (TBUT) (Sweeney et al., 2013). This is the time interval between a complete blink and the onset of the first tear film break. Normal fluorescein-based TBUT values vary between 10 and 35 s (Singh et al., 2022), while reference values for non-invasive TBUT (NIBUT), measured by videokeratoscopy or interferometry (Sweeney et al., 2013), range from 8 to 12 s. A shorter TBUT is indicative of DED.

3.3. Tear fluid lipid layer thickness

The lipid layer can be routinely assessed using interferometry techniques. With an estimated thickness of around 40–100 nm in normal eyes (Bai et al., 2022), the tear film can be assessed quantitativelyor qualitatively. Interferometry is a non-invasive technique which allows visualization of the lipid layer by means of specular reflection (Purkinje I image). The lipid layer thickness can be quantified using the modified Guillon grading scale when assessing the tear film reflection on the slit lamp, or automatically graded in nm in certain devices (Bai et al., 2022; Guillon, 1998). The interferometric patterns are able to reflect clinical tear fluid dynamics, thereby distinguishing clinical subtypes of DED (Arita et al., 2016).

3.4. Tear fluid osmolarity

Tear fluid osmolarity refers to the concentration of dissolved salts and electrolytes like sodium, potassium, and chloride into the tear fluid. Hyperosmolarity in DED is typical and has the highest positive correlation with disease severity, classified as normal (302.2 ± 8.3 mOsm/L), mild-moderate (315.0 ± 11.4 mOsm/L) and severe (336.4 ± 22.3 mOsm/L). 316 mOsm/L is a widely accepted specific threshold to differentiate moderate from severe DED (Greiner et al., 2023; Jacobi et al., 2011). Severely affected subjects show higher average and variability in tear fluid osmolarity, highlighting its instability and the challenges of using it as a consistent ocular surface health biomarker (Keech et al., 2013). Prior the current point-of-care osmometers (TearLab and i-Pen), osmolarity was a technique mostly used in research settings, due to the high cost and complexity, either with a freezing-point depression osmometry (Pena-Verdeal et al., 2019) or, vapor pression osmometry method (Gokhale et al., 2013).

3.5. Tear fluid components

Altered tear fluid composition, as seen in DED and other inflammatory ocular surface conditions, can lead to conjunctival epitheliopathy, corneal epithelial disease, nociceptor stimulation, and pain (Korb et al., 2005; Mehra et al., 2020; Pflugfelder and Stern, 2020; Roy et al., 2023). Total tear fluid protein concentrations usually range from 6 to 10 mg/mL, but rises during pathological conditions (Boehm et al., 2011; de Souza et al., 2006; Ng et al., 2000; Zhou and Beuerman, 2012; Zhou et al., 2012). For example, the level of MMPs in tear fluid increases with the loss of ocular surface barrier function in DED. The *InflammaDry*® POC device (Rapid Pathogen Screening, Inc/Quidel) measures tear fluid MMP-9 levels in 10 min, with levels above 40 ng/mL indicating a positive result. However, this test is not specific to the exact source of ocular surface inflammation (Kaufman, 2013).

The level of tear fluid cytokines and chemokines reflect the level of epithelial disease. Several cytokines have been quantified by proteomics analysis and shown to be dysregulated in DED and other ocular surface disease, including Th1, Th17, tumor necrosis factor alpha, interferon gamma, IL-1 beta and IL-6 (reviewed in Di Zazzo et al., 2019; Jackson et al., 2022; Ponzini, 2024; Zhou and Beuerman, 2012).

Tear fluid composition can be modified by several circumstances and conditions including open- or closed-eye environments, DED, ocular allergy, keratoconus, infectious keratitis, conjunctivochalasis, ocular rosacea, contact lens wear, blepharitis and glaucoma. These compositional changes have been reviewed extensively in the scientific literature (Nättinen et al., 2022; Zhou and Beuerman, 2012). Recently, changes in the tear fluid protein composition of eyes with limbal stem cell deficiency treated with autologous cultivated limbal epithelial

transplantation, has also been reported (Figueiredo et al., 2023).

Changes in tear fluid composition extend beyond ocular health, providing insights into systemic health. Tear fluid biomarkers have been reported in various systemic diseases with and without ocular complications, such as diabetes, breast cancer, cystic fibrosis and neurodegenerative disorders, reviewed in (Hagan et al., 2016; Ponzini, 2024). As an example, tear fluid proteomics have demonstrated the ability to discriminate healthy women from women with breast cancer, highlighting differential expression of proteins involved in systemic immune pathways and metabolic cascades (Inubushi et al., 2020a; Lebrecht et al., 2009). Another example is the detection of SARS-CoV-2 mRNA in tear fluid of patients affected with coronavirus disease 2019 (Gijs et al., 2021b; Mastropasqua et al., 2021). Tear fluid protein analysis of numerous neurodegenerative disorders such as Multiple sclerosis, Amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, and Parkinson's disease have yielded specific biomarkers for each disease (Ami et al., 2021b; Gijs et al., 2021a, 2024; Hamm-Alvarez et al., 2019; Maass et al., 2020; Tomečková et al., 2023a). Finally, tear fluid is directly exposed to the environment, making its composition sensitive to external changes. This sensitivity can be utilized to assess exposure to environmental pollutants (Calonge et al., 2017; Miglio et al., 2021).

4. Tear fluid collection

In order to investigate the tear fluid composition in health or disease, tear fluid biomarker research begins with the critical step of tear fluid collection. This initial phase is fundamental, as the quality and accuracy of subsequent analyses depend significantly on the effectiveness of the collection process. Proper collection techniques ensure that the samples are representative and reliable for studying tear fluid biomarkers. The most commonly used methods for tear fluid collection in biomarker research are described below, along with their advantages and disadvantages, and are illustrated in Fig. 2.

4.1. Glass capillaries

Tear fluid can be collected by fire polished glass capillaries. Tear fluid (5–10 μL) from the forniceal reservoir within the conjunctival sac is drawn into the tube through capillary action. This technique has minimal contact with the ocular surface, thereby avoiding irritation of the conjunctiva and collection of epithelial cells (Pieczyński et al., 2021). However, the collection process can be slow, especially in patients with DED, or can be difficult to perform in certain patient groups like young children or patients with Parkinsonian disorders.

4.2. Schirmer's strips

Tear fluid can also be collected using filter paper strips, called Schirmer's strips, a technique routinely employed in clinical practice to assess tear fluid volume (Bachhuber et al., 2021). Schirmer's strips are inexpensive and the collection method is easy to learn, making it advantageous for clinical applications in analyzing tear fluid biomarkers. Yet, limitations of Schirmer's strips include irritation of the ocular surface after removal of the strip, particularly in DED patients, as well as collection of conjunctival epithelial cells (Akkurt Arslan et al., 2021; Bachhuber et al., 2021; Gijs et al., 2023b; Koduri et al., 2023). In addition, the wetting length of Schirmer's strips can vary depending on the manufacturer due to differences in pore size, cellulose fiber density, and strip size (Gijs et al., 2023b).

4.3. Sponges

Other absorbent-based methods, including, cellulose or polyurethane mini sponges, porous polyester rods or cellulose acetate filter rods, can also be used to collect tear fluid (López-Cisternas et al., 2006). Typically, the absorbent material is placed in the lower tear meniscus and held in position for a set period of time to collect tear fluid. Generally, this way of collecting tear fluid is easy and quick to perform (López-Cisternas et al., 2006; Soria et al., 2013). In order to estimate the collected volume, sponges can be weighed before and after collection (Inic-Kanada et al., 2012). A drawback is the retention of fluid within the absorbent material, which can significantly reduce the amount of recoverable tear fluid components (Inic-Kanada et al., 2012). Additionally, mechanical contact between the sponge and the ocular surface may induce reflex tearing, which can alter the natural tear fluid composition.

4.4. Ocular surface wash

Flushing the ocular surface with a fixed volume of saline solution allows for a gentle way of tear fluid collection (Nair et al., 2021). After application of the solution using a micropipette on the ocular surface, the patient is asked to close their eye and roll their eyeball in all directions several times before collection by a glass capillaries or micropipette. This technique is particular useful in the case of DED, where the tear fluid volume is low. However, it dilutes the collected tear fluid, preventing accurate measurement of the original volume and making it more challenging to detect low-abundance biomarkers.

4.5. Swabs

An ocular or eye swab is conducted by swabbing the conjunctiva

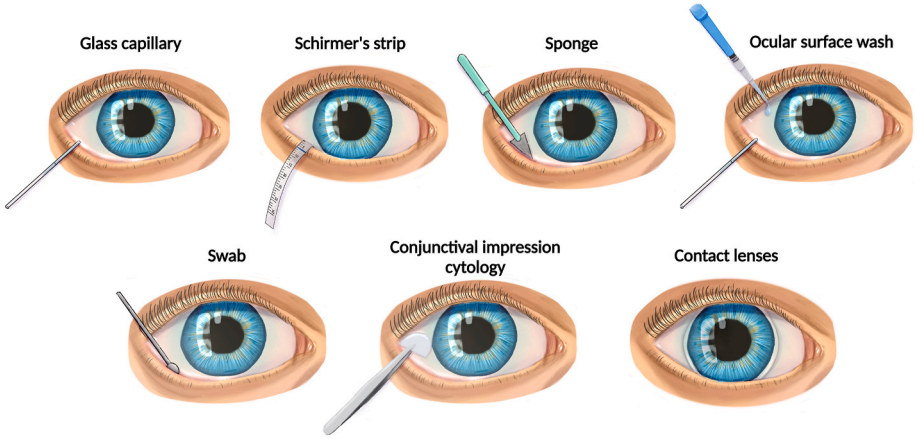


Fig. 2. The most commonly used methods for tear fluid collection in biomarker research.

(Sabage et al., 2022). This simple and inexpensive process collects tear fluid along with conjunctival cells, making it advantageous for studies analyzing both cellular and fluid components. In clinical routine, eye swabs are already used for the investigation of superficial bacterial eye infections, such as conjunctivitis (Drew et al., 2015). However, swabs collect an undetermined volume of tear fluid and may retain analytes within their absorbent material, complicating the precise quantification of analytes. The mechanical swabbing action may also stimulate reflex tearing.

4.6. Conjunctival impression cytology

Conjunctival impression cytology, although not specifically designed for the purpose of tear fluid collection, is sometimes used to investigate tear fluid biomarkers (Corrales et al., 2003). This technique collects the outermost layers of the ocular surface by applying a membrane or filter paper to it. Ocular surface cells will adhere to the paper, along with some tear fluid (Calonge et al., 2004b). Conjunctival impression cytology in clinical routine allows for (immune)histological or molecular assessment for several disorders of the ocular surface, such as DED, viral infections, or vitamin A deficiency (Calonge et al., 2004a).

4.7. Contact lenses

The literature has predominantly reported the use of contact lenses in the field of tear fluid biomarker research as sensors for detecting and monitoring physical or chemical changes through colorimetric, optical, or electrochemical techniques (Bai et al., 2024; Jones et al., 2021; Mann and Tighe, 2013; Moreddu et al., 2020; Shetty et al., 2024; Yang et al., 2020). Newer studies are exploring the use of contact lenses as collection tools to investigate molecular changes in tear fluid. Tear fluid can be collected using contact lenses simply by removing the lens from the ocular surface (Boychev et al., 2024a, 2024b; Roden et al., 2024). The process of collecting tear fluid using contact lenses may result in protein deposition on the lens and a reduction or depletion of specific tear fluid components due to absorption (Mann and Tighe, 2013) This may provide insight into tear fluid dynamics, lens interaction with the ocular surface and molecular changes induced by lens wear (Willcox, 2019). Contact lenses provide a patient-friendly approach to collect tear fluid since many individuals are already used to its wear, it is associated with low irritation, and can be worn for a long time in a user-independent way (Yang et al., 2020). Nonetheless, when collecting tear fluid with contact lenses, it is important to recognize that even normal, uncomplicated lens wear can inherently trigger inflammation, as evidenced by clinical symptoms and biochemical changes (Efron, 2017; Insua Pereira et al., 2022).

5. Methods

This scoping review was conducted to explore the past and current tear fluid collection methods, extraction, preparation, pre-analytical steps, and analysis methods used in tear fluid biomarker research. The literature search followed the PRISMA Extension for Scoping Reviews (Tricco et al., 2018) and was conducted on May 21, 2024 using two electronic databases, Embase (January 1974–May 2024) and Ovid MEDLINE (January 1946–May 2024). The full search strategy is detailed in Appendix A. The inclusion and exclusion criteria for the included articles are presented in Fig. 3A.

Covidence web-based collaboration software platform was used to streamline the data extraction. Titles and abstracts of identified records were screened to assess eligibility based on the inclusion and exclusion criteria. Next, data were extracted from the remaining articles using a standardized data extraction form (Appendix B). All steps were performed by two independent reviewers and a third reviewer was solicited in case of disagreement. The initial literature search yielded a total of 10,555 articles (Fig. 3B). Of these, 8292 articles did not meet the inclusion criteria. 688 articles were then excluded during the full-text screening when these were conference abstracts, letters to the editor, animal studies, or focused only on the development or validation of a new method. Ultimately, 1484 full-text journal articles were reviewed. All 1484 included articles are listed in Appendix C, along with the most relevant extracted data, while only the references cited in the text appear in the reference list at the end of the manuscript. Data were analyzed to provide insight into the past and current practices of tear fluid methods used in tear fluid biomarker research. Descriptive statistics (e.g. frequencies, percentages) were analyzed in Microsoft Excel (2016) and figures were created with GraphPad Prism 10 and RStudio (www.r-project.org).

6. Global trends in tear fluid research

The leading countries that contributed to tear fluid biomarker research (based on the first author's institution) were the USA (15%), China (10%), and Japan (9%), followed by India (6%), Italy (6%), and South Korea (5%) (Fig. 4A). A steady increase, followed by an exponential rise starting from 2015, can be observed in the number of publications on tear fluid (Fig. 4B). This growth is likely driven by recent advances in high throughput protein and proteomic approaches that allowed for the quantification and identification of multiple proteins from a small volume with high sensitivity (Zhou and Beuerman, 2012).

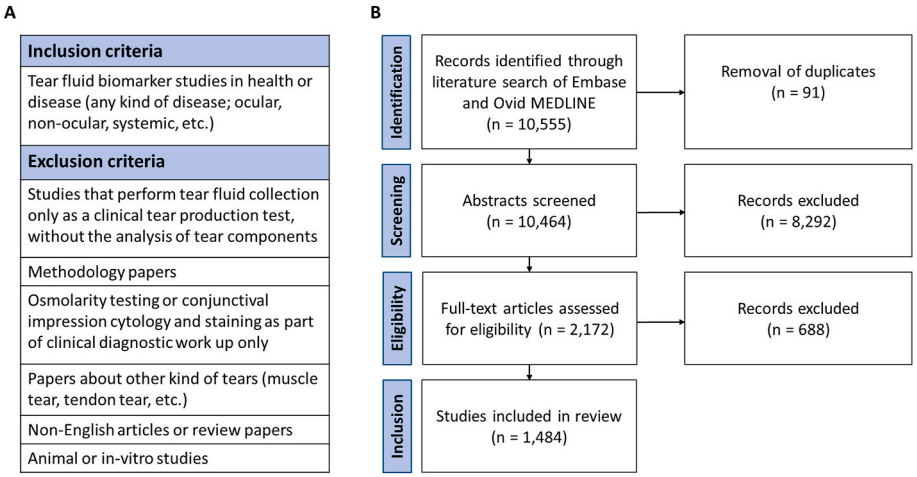


Fig. 3. (A) Inclusion and exclusion criteria of the included articles in this scoping review. **(B)** Visual representation of the literature search methodology and results from January 1974–May 2024.

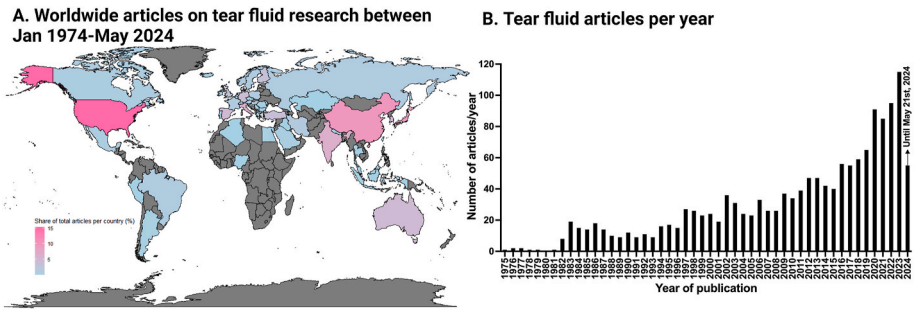


Fig. 4. Global trends in tear fluid articles (N=1484). (A) Percentage of tear fluid articles per country over the review period. **(B)** Cumulative number of tear fluid articles published per year from January 1974 to until May 21, 2024.

7. Overview of practices for tear fluid collection

Across 1484 included articles, glass capillary tube was the most commonly reported method for tear fluid collection (45%), followed by Schirmer's strip (25%) (Fig. 5A). Over time, glass capillary tube collection remained the most frequent method reported until 2019, when Schirmer's became the leading collection method (Fig. 5B). The increased use of Schirmer's strips may be explained by their easy and established use in clinical practice (Posa et al., 2013; Wolffsohn et al., 2017). In addition, Schirmer's strips that are collected during clinical practice can, with patient permission, be easily stored in a biobank for use in future research. Another distinction between the two methods is that research has shown that the level of proteins that can be extracted from Schirmer's strips is higher and differs in content compared to capillary glass tubes (Green-Church et al., 2008; Stuchell et al., 1984).

Less frequently used collection methods included sponges (2.6%), swabs (1.5%), ocular surface wash/flush (3.7%), conjunctival impression cytology (1.1%), and contact lenses (0.3%) (Fig. 5A). Rarely reported methods were grouped under ''other collection methods'' (8%), including micropipettes, filter paper discs, polyester wicks, and polyester rods. These results indicate that tear fluid biomarker research employs a variety of collection methods. While each method offers distinct advantages tailored to different research needs, the lack of a single standardized collection method complicates research comparability.

In most studies (67%), tear fluid was analyzed from a single sampling

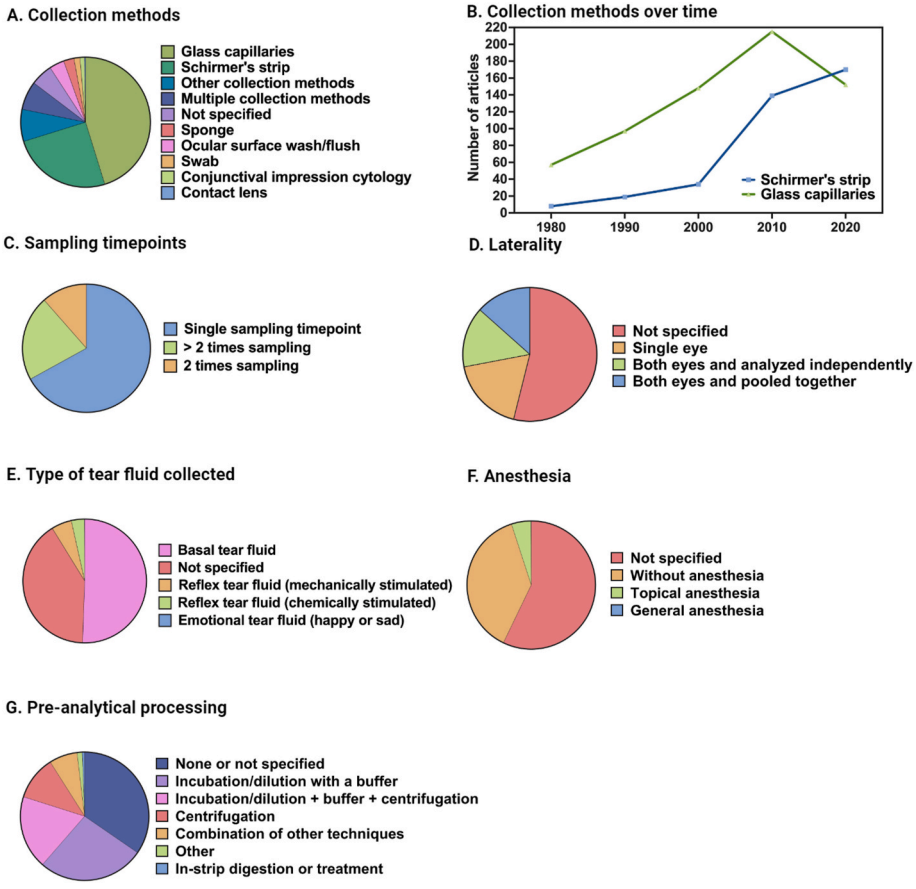


Fig. 5. Use of tear fluid collection methods in tear fluid biomarker research. This figure presents an overview of the tear fluid collection procedure described in the 1484 included articles. **(A)** Types of collection methods utilized. **(B)** Trends in collection methods. **(C)** Frequency of tear fluid sampling per study. **(D)** Collection from either a single eye or both eyes. **(E)** Type of tear fluid collected: basal, emotional, or reflex tear fluid. **(F)** Usage of anesthetics during collection. **(G)** Pre-analytical processing steps.

timepoint, whereas sampling twice (for example before and after an intervention) or more than two times, was less frequent (Fig. 5C). When collecting tear fluid, another consideration is whether to collect it unilaterally or bilaterally. Bilateral samples can be pooled or analyzed separately. The decision to collect tear fluid monocularly, binocularly, or by pooling depends on the study's objectives, hypothesis, biomarker behavior, disease condition (unilateral/bilateral, symmetric/asymmetric), and required sample volume for the detection method. Studies have shown no significant differences in protein concentration between bilateral samples in the absence of unilateral ocular conditions (Guntermann et al., 2023). For conditions affecting both eyes symmetrically, for example with systemic diseases, analyzing a single eye may be sufficient (Guntermann et al., 2023). However, for asymmetric conditions or when inter-eye variability is important, sampling both eyes separately (without pooling) is likely necessary. Although the majority of articles did not specify it, there is an almost evenly distributed number of articles that collected tear fluid unilaterally, bilaterally as a pooled sample, and bilaterally with independent analysis (Fig. 5D).

Another consideration is what type of tear fluid to collect. In our scoping review, basal tear fluid accounted for the majority of collected tear fluid (51%) (Fig. 5E). Basal tear fluid forms the tear film on the ocular surface and is produced continuously (Bachhuber et al., 2021). In contrast, reflex tear fluid is triggered by mechanical or chemically stimulation and thereby increase the tear fluid flow rate, leading to the dilution of tear fluid components (Fullard and Snyder, 1990). Reflex tear fluid was less commonly collected in tear fluid biomarker research, with mechanically stimulated reflex tear fluid making up 5.3% and chemically stimulated tear fluid 3.4% of the reviewed articles (Fig. 5E). If the tear biomarker of interest is expressed at low levels, dilution from reflex tear fluid could make detection more challenging. Therefore, reflex tear fluid is not always ideal for tear fluid biomarker analysis which is in contrast with their clinical value.

For tear fluid collection, topical anesthetics can be used to ensure that only basal tear fluid is collected. However, it is important to consider the potential impact of anesthetic eye drops on tear fluid composition, a topic that requires further research. Tear fluid collection using glass capillaries is typically done without anesthesia, with care taken to avoid touching the ocular surface epithelium to prevent reflex tearing (Pieczyński et al., 2021). While the majority of articles did not specify the use of anesthetics, our scoping review showed that no anesthesia was used in 37% of articles, and only 5% described the use of topical anesthesia (Fig. 5F). In the 1484 reviewed articles, each of the tear fluid collection methods has been conducted both with and without the use of anesthesia, except for methods involving contact lenses. It thus appears that the use of anesthesia is not inherently part of any specific tear fluid collection methodology but depends on the aims and design of the study.

Finally, the different pre-analytical processing steps were reviewed. Pre-analytical steps are essential for ensuring accurate interpretation of tear fluid analysis These steps are crucial for processes such as eluting tear fluid from the collection method or adding a buffer to the tear fluid to protect against protein degradation. Additionally, given that analytes can degrade at room temperature over time, proper storage is essential. Tear fluid samples can be stored short-term (up to 24 h) at 4 °C, while storage at −80 °C is recommended for preserving analytes over extended periods (Chiang et al., 2022; Gijs et al., 2023b). Additionally, in Schirmer's strips addition of protease inhibitors before storage results in higher protein content compared to dry storage (Gijs et al., 2023b).

The majority of reviewed papers (65%) use a pre-analytical processing step. The most frequently used pre-analytical processing step was incubating or diluting the collected tear fluid with a buffer, reported in 27% of all articles (Fig. 5G). This was often combined with centrifugation (19%). The use of centrifugation alone was less frequent, occurring in 11% of all articles, and in-strip digestion was rarely reported. Furthermore, multiple combinations of pre-analytical processing techniques (7%), and other less common pre-analytical processing techniques such as drying of Schirmer's strips were also used. Nonetheless, in 35% of all articles there was no use of a pre-analytical processing step or it was not specified (Fig. 5G).

8. Overview of approaches in tear fluid analysis

Subsequent to its collection, tear fluid can be analyzed in the lab. In this scoping review, we observed a wide variety in the number and type of investigated analytes. Most articles investigated up to five analytes simultaneously (Fig. 6A). Specifically, 32.3% of articles evaluated a single analyte, and 31.9% of articles explored 2–5 analytes. Up to the year 2000, the majority of studies analyzed 1 to 5 analytes, but after that, there has been a sustained increase in the number of studies examining more than 5 analytes (Fig. 6B). Additionally, there has been a growth in the number of studies evaluating an unlimited number of analytes with omics approaches, which accounted for 13% of the articles (Fig. 6A and B). These numbers highlight the development of novel high-precision and more sensitive detection methods, the improvement of analysis techniques which have progressively moved from gel-based to gel-free strategies and the use of protein panels instead of individual proteins, seeking to overcome the wide dynamic ranges and slow processes to be able to put them into clinical practice and precision medicine (Ponzini et al., 2022).

For the type of analytes investigated in tear fluid, proteins were shown to be the principal analyte of interest (65.1%), followed by antibodies (6.2%), nucleic acids (4.8%), peptides (4.0%), and drugs (3.7%) (Fig. 6C). When examining the top 10 most commonly investigated tear fluid analytes, the dominance of cytokines, chemokines and enzymes such as IL-6 and MMP-9 is evident (Table 1). Proteins were primarily analyzed in tear fluid samples obtained through glass capillaries (69.6%), followed by Schirmer's strips (68%), ocular surface wash/flush (61.3%), sponges (60%), and contact lenses (50%) Fig. 6D). While some articles mentioned the use of conjunctival impression cytology for protein analysis, this collection method was primarily used for the analysis of nucleic acids (28.6%) and mucins (28.6%). Additionally, swabs were predominantly used for the analysis of nucleic acids (45.8%) and microbes (41.6%) (Fig. 6D).

A large variety of techniques were used for the analysis of tear fluid analytes, with a majority (50.8%) being protein (immuno)assays (Fig. 6E). Liquid chromatography–mass spectrometry (LC-MS) and LC-MS/MS were shown to be the second most commonly used technique (12.5%). In addition, the use of this technique showed a steep increase between 1990 and 2010 (Fig. 6F), which aligns with the increase that was observed in the number of articles that analyzed an unlimited number of analytes (Fig. 6B). Of the 1484 articles selected for detailed analysis, a majority of 1251 articles used a single technique, while 233 articles utilized two or more techniques to analyze their tear fluid samples. In the coming years, the use of multiple analytical techniques per tear fluid sample is likely to grow, driven by the enhanced sensitivity of assays. For example, recent research has shown that a Schirmer's strip can be cut in half, with each half reserved for different purposes, further maximizing sample use (Jones et al., 2022). Traditional techniques, such as Raman and infrared spectroscopy, are witnessing a revival in their application to tear fluid (Ami et al., 2021a; Capaccio et al., 2019; Thomas et al., 2024; Tomečková et al., 2023b). Yet, comparisons of the sensitivities of these techniques with currently available omics methods remain to be validated at large scale.

After analysis, the raw tear fluid data needs to be converted into meaningful results. A variety of normalization methods have been used in the literature so far. The most commonly used techniques involve normalization based on tear fluid total protein content (6.7%) or tear fluid volume (wetting length) (6.6%) (Fig. 6G). Less common, normalization techniques were based on the level of an endogenous protein (2%) or tear fluid weight (1.5%). Methods based on tear fluid volume or tear fluid weight are used to correct dilution errors while normalizing based on the total protein content corrects potential reflex tearing.

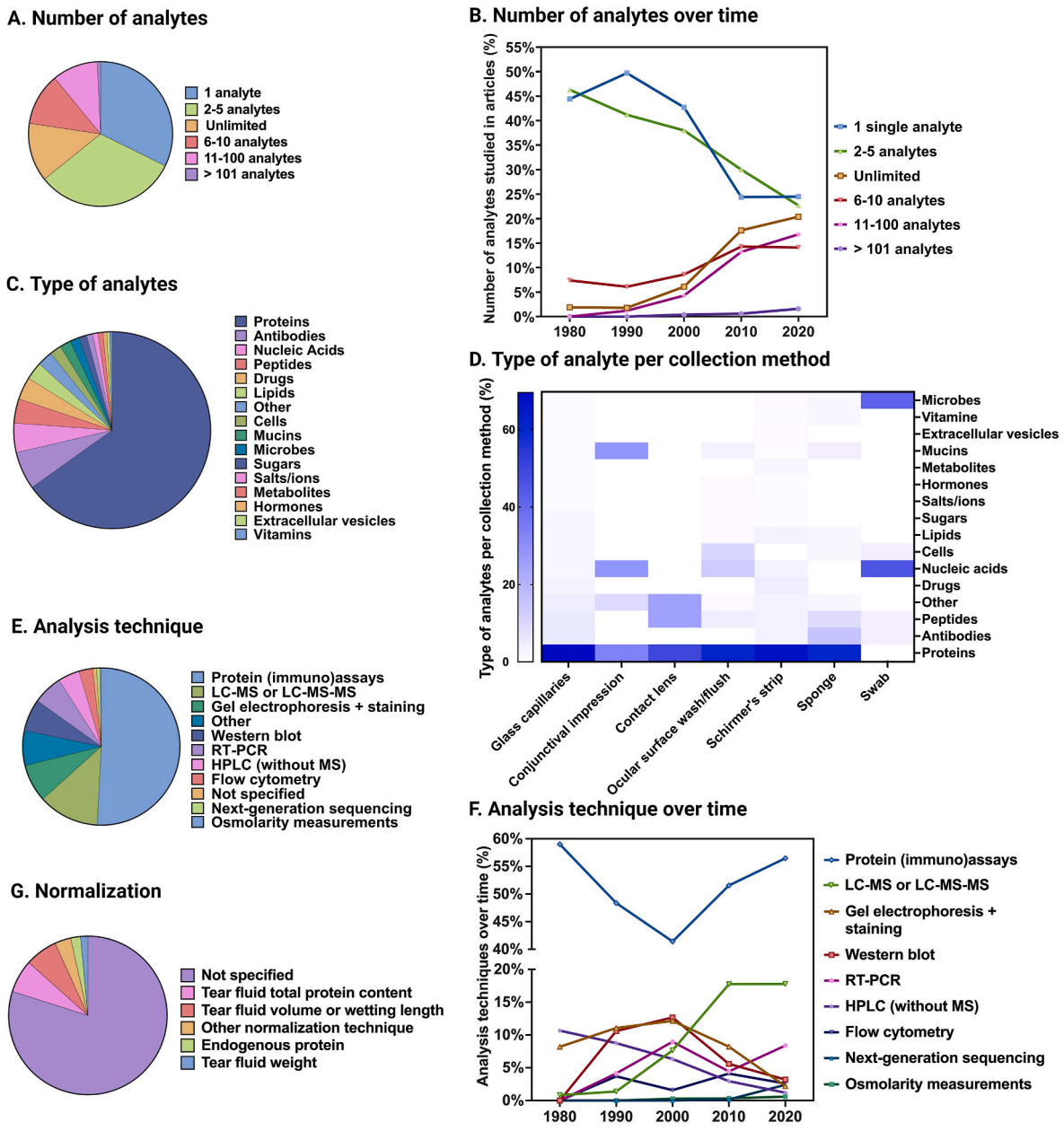


Fig. 6. Tear fluid analysis. (A) Number of analytes (N = 1484). **(B)** Number of analytes over time (N = 1484). **(C)** Type of analytes (N = 1698). **(D)** Type of analytes investigated with different collection methods (N = 1332). **(E)** Analysis techniques (N = 1835). **(F)** Analysis techniques over time (N = 1835). **(G)** Normalization (N = 1484).

However, these methods are not ideal, for example the migration length of the Schirmer test does not directly correspond to a specific volume (Gijs et al., 2023b). Normalization using an endogenous protein, such as lactoferrin or lysozyme, or total protein content allows for comparison between different body fluids (Gijs et al., 2023b). However, these two approaches have disadvantages, including the need to use part of the already limited tear fluid volume, the costs for additional assays, and potential variability in endogenous protein levels due to disease. For example, the levels of lactoferrin and lysozyme can be altered in DED (Jia et al., 2023; Ponzini et al., 2020). Despite these challenges, normalization using an endogenous protein appears to be a promising method, as it is well-established in other body fluid analyses, such as using creatinine levels to adjust for urine flow rate (Gijs et al., 2023b). A small number of studies (3.3%) used alternative normalization techniques, like Z-scores or analyte levels detected in healthy controls. However, most reviewed articles (79.9%) did not specify whether raw data were normalized (Fig. 6G). Further evidence is needed to standardize the normalization method for future tear fluid research and to improve the comparability and reproducibility of tear fluid studies.

9. Overview of tear fluid applications in research

Tear fluid can be used across medical disciplines and for various biomarkers indications (Evans et al., 2001; Grus et al., 2002; Hagan et al., 2016). Thanks to the non-invasive nature of tear fluid collection, tear fluid studies can range from pilot studies with small size population to large multicenter validation studies with large patient groups, or population studies. In this review, most studies included between 1 and 100 participants (85%) (Fig. 7A). A remaining 11.2% of articles included between 100 and 300 participants, and 1.6% had over 300 participants with a maximum of 2510 participants observed for one study. Most articles included a control group and their patient groups of interest

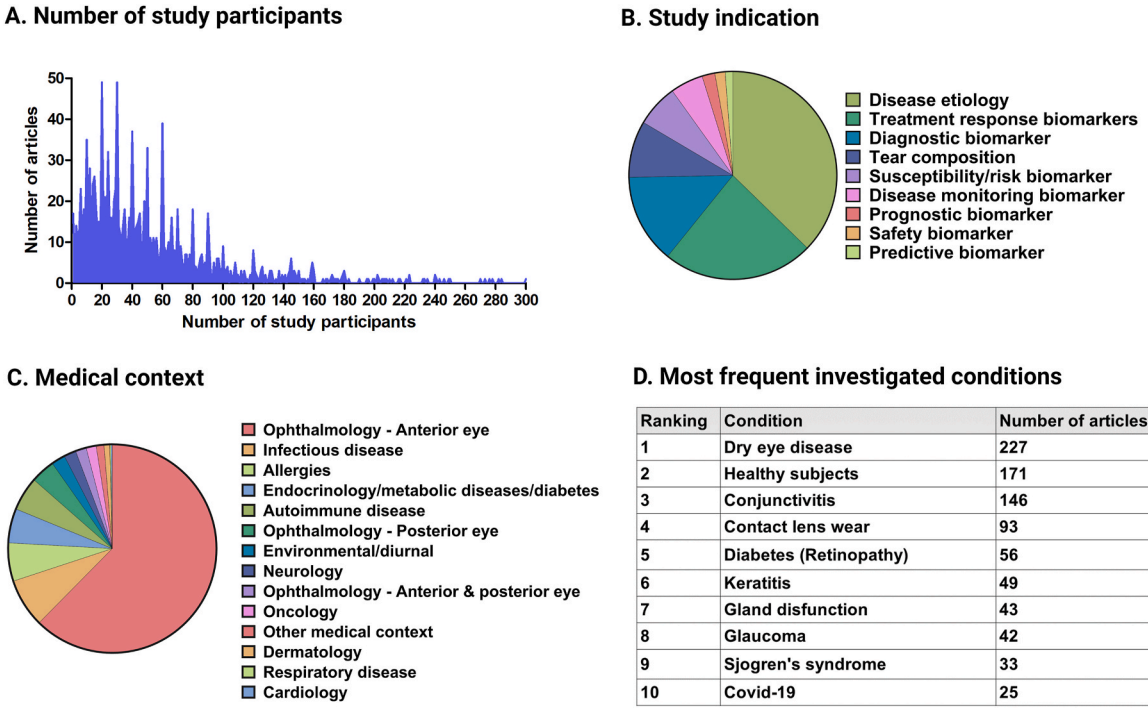
Table 1
Top 10 of most commonly investigated analytes in tear fluid research (N=1484). Note that the percentages do not app up to 100% because several articles investigated more than one of these analytes.

Ranking	Abbreviation	Full name	Number of articles	% of total articles
1	IL-6	Interleukin-6	267	18
2	IL-8	Interleukin-8	248	16.7
3	TNF-α	Tumor necrosis factor alpha	227	15.3
4	IL-1β	Interleukin-1 beta	203	13.7
5	IL-10	Interleukin-10	181	12.1
6	MMP-9	Matrix metalloproteinase-9	147	9.9
7	CCL2	Chemokine ligand 2	144	9.7
8	IFN-γ	Interferon gamma	137	9.2
9	IL-2	Interleukin-2	133	9
10	IL-4	Interleukin-4	129	8.7

(54.5%), while others included patients (29.2%) or healthy controls (13.6%) only. Studies that only included healthy controls often aimed to detect new analytes, set reference values or monitor biomarkers in a healthy condition that may be altered due to diurnal effects or environmental exposure.

Biomarkers are primarily used for diagnostic purposes, helping to distinguish between patients and healthy controls by highlighting specific differences in biological markers (Group, 2016). In the reviewed literature, the majority of articles focused on tear fluid biomarkers in the context of disease etiology (37.3%) and diagnostic biomarkers (13.9%), which both compare biomarker levels between patients and healthy controls (Fig. 7B). In studying disease etiology, the main focus is often on understanding the underlying physiology of the disease, while identifying potential diagnostic biomarkers may be a secondary goal. Treatment response biomarkers (23.5%) are the second most often investigated indication (Fig. 7B). Assessing the treatment response, allows for future personalized adjustments to therapeutic strategies (Group, 2016). 6.6% of the articles investigated susceptibility or risk biomarkers in tear fluid, to see if biomarkers in tear fluid can indicate the potential of somebody developing a disease or condition who does not currently have the disease (Fig. 7B). One of the main advantages of tear fluid is that it is possible to sample multiple times a day, week or month, making it an excellent candidate fluid for disease monitoring biomarkers and was studied for this indication by 5.1% of all articles. Least frequently investigated were prognostic (2%), safety (1.6%) and predictive biomarkers (1.2%) in tear fluid (Fig. 7B). Lastly, 8.8% of the articles focused on tear fluid composition in healthy subjects. This indicates that the composition of tear fluid is not yet fully discovered, but is likely to happen in the near future especially with the advent of more sensitive detection techniques.

The main medical context in which tear fluid biomarkers are studied is ophthalmology, particularly the anterior segment of the eye (62.4%) (Fig. 7C). Reflecting this focus, some of the most frequently investigated conditions in tear fluid include DED and contact lens wear, both of which are among the top 10 studied conditions (Fig. 7D). Tear fluid biomarkers were also investigated in the context of infectious diseases (7.5%), allergies (5.9%), and autoimmune diseases (5.3%) (Fig. 7C). This includes diseases such as herpes simplex virus, allergic conjunctivitis (Neil et al., 2020), and Sjögren's disease. Another medical context studied is endocrinology, which includes metabolic diseases and diabetes (5.3%) (Fig. 8C), with diabetic retinopathy as one of the most frequently investigated conditions within this medical context (Fig. 7D). The first papers investigating tear fluid for the posterior eye came in the 1990's, and exponentially increased from 2010 onwards, comprising 7.3% of all papers published after 2020. Due to this growing interest in recent years, glaucoma was the 8th most frequently investigated disease (Fig. 7D). Additionally, papers investigating neurological diseases have exponentially increased since the 2000's, with 3.2% of all papers published after 2020. In the coming years, tear fluid biomarker research is likely to continue to increase and expand into other medical fields. Particularly in neurology, where cerebrospinal fluid (CSF), which is invasive to access, remains the only body fluid of focus (Gijs et al., 2021a, 2024; Hamm-Alvarez et al., 2019; Maass et al., 2020; Tomečková



Ranking	Condition	Number of articles
1	Dry eye disease	227
2	Healthy subjects	171
3	Conjunctivitis	146
4	Contact lens wear	93
5	Diabetes (Retinopathy)	56
6	Keratitis	49
7	Gland disfunction	43
8	Glaucoma	42
9	Sjogren's syndrome	33
10	Covid-19	25

Fig. 7. Applications of tear fluid research. (A) Number of study participants included in tear fluid studies (N = 1427) (N = 24 studies with >300 participants excluded, N = 33 articles excluded due to not specified). **(B)** Study indications of tear fluid research (N = 1484). **(C)** Medical context in which tear fluid is studied (N = 1484). **(D)** Ranking of the top 10 conditions most frequently investigated in tear fluid biomarker research.

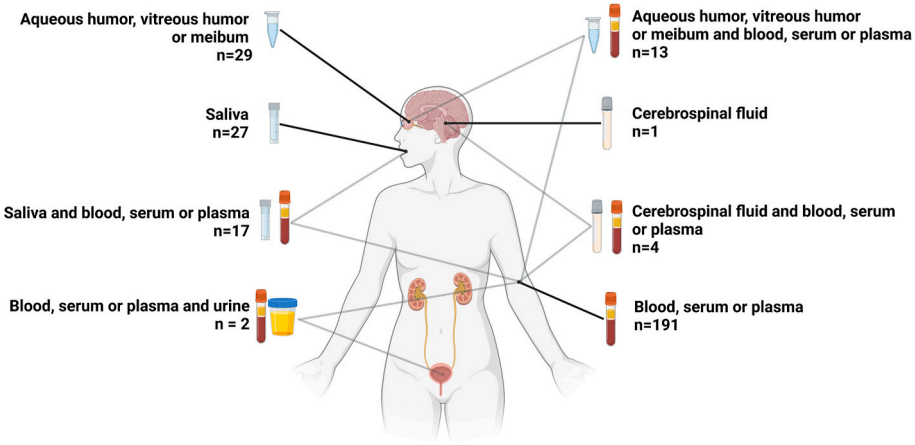


Fig. 8. Overview of other well-known body fluids that have been studied in combination with tear fluid. Created in BioRender. Gijs et al. (2024) BioRender.com/b31z453.

et al., 2023a).

10. Tear fluid and its relationship with other body fluids

In tear fluid biomarker research, tear fluid is sometimes investigated in conjunction with other body fluids. Our results show that 332 articles (22.4%) included another body fluid (Fig. 8). Most articles compared biomarkers in tear fluid with a single body fluid (n = 263), whereas a number of articles collected two (n = 36) or more (n = 33) other body fluids from the same study subject. Blood, serum or plasma were most often investigated together with tear fluid. Body fluids that are categorized in the 'other' section include nasal secretions, sweat, or gingival crevicular fluid.

There are several reasons to investigate multiple body fluids. Firstly, examining multiple body fluids for a certain biomarker may offer a more comprehensive understanding of a disease or condition. Secondly, studying how biomarkers behave across different body fluids can provide insights into the pathophysiology of diseases, helping to understand how a disease affects the body as a whole. Thirdly, this approach can identify the best source of a biomarker for clinical applications, focusing on the fluid with the highest concentration and the most convenient, non-invasive collection method. Below, tear fluid and other body fluids are described, highlighting its distinct properties but also the emerging clinical applications of tear fluid in diagnostics.

10.1. Tear fluid and blood, serum, and plasma

In this review, 252 articles co-investigated tear fluid with blood, serum, or plasma. When comparing tear fluid with blood/plasma/serum for biomarker identification, several key differences emerge, presenting unique advantages and challenges for each fluid type. Blood contains approximately ten times more proteins than tear fluid, with plasma featuring around 3000 to 5000 proteins, including enzymes, hormones, clotting factors, and transport proteins. Serum, which is plasma minus clotting factors, has a similar protein content but lacks certain components such as fibrinogen. In contrast, tear fluid contains fewer proteins, and the total protein concentration is lower (6–11 μg/μL) (Zhou and Beuerman, 2017), compared to plasma and serum (60–80 μg/μL) (Leeman et al., 2018), yet it is sufficiently informative for diagnostic purposes. This is supported by research on total RNA and miRNA content across human body fluids (Weber et al., 2010b; Hulstaert et al., 2020). Using the Human miScript Assay panel from Qiagen, which included qPCR assays for 714 different human miRNA species, 320 miRNAs were detectable in tear fluid, while 349 miRNAs were detectable in plasma. The top 10 miRNAs present in tear fluid were miR-335, miR −325, miR −377, miR −586, miR −518e, let-7i, miR-539, miR −616, miR −302d, and miR-589. Although RNA concentration was found to be 1.83 times higher in tear fluid, plasma contained a greater diversity of unique miRNA species overall, with miR-637 being the only miRNA uniquely detected in tear fluid (Weber et al., 2010b). Interestingly, a report using a dedicated mRNA enrichment-sequencing method found that mRNA concentration in tear fluid is approximately 318 times higher than in plasma. The study also revealed that tear fluid contains a significantly more diverse repertoire of mRNA, with 13,366 distinct mRNA compared to 4152 mRNA in serum. Additionally, tear fluid contains about 2.2 times more miRNA than serum (Hulstaert et al., 2020).

A significant advantage of tear fluid over blood is the absence of blood cells, which may mask or degrade biomarkers of interest. Moreover, tear fluid collection is less invasive and more patient-friendly compared to blood collection. This preference is supported by a study in which 74% of participants favored tear fluid collection over blood collection (Quah et al., 2014). In addition, collecting blood samples can be challenging, particularly in vulnerable populations such as the elderly or (premature) infants.

10.2. Tear fluid and saliva

Similar to tear fluid, saliva is collected easily, non-invasively and cost-effective. Saliva contains two major digestive enzymes, salivary amylase and lingual lipase, that are essential in digestion of dietary starches and fats. However, these enzymes are likely to breakdown biomarkers of interest, particularly carbohydrates and lipid biomarkers.

Saliva has been investigated in combination with tear fluid in 76 articles. Several of these articles measured viral particles and antibodies against viruses. For example, antibodies against herpes simplex type I and Epstein Barr were found in tear fluid, whereas antibodies to mumps were found in parotid saliva (Coyle and Sibony, 1988). Detection of varicella zoster virus DNA in tear fluid and saliva allowed tracking the dynamics of disease in patients with Ramsay Hunt syndrome (herpes zoster oticus) (Hiroshige et al., 2002). In addition, SARS-CoV-2 was found in these fluids in symptomatic and asymptomatic patients (Karia et al., 2020)). Other applications that investigated tear fluid together with saliva include to diagnose Toxoplasma gondii (Nayeri et al., 2022), to assess the clinical symptoms of radiated neck and head cancer patients (Aqrawi et al., 2020; Hynne et al., 2022), in females with evaporative DED (Fineide et al., 2023), in patients with Parkinson's disease (Jiménez-Jiménez et al., 2023), ocular rosacea (Vieira et al., 2012) and keratoconus (Sharif et al., 2019).

10.3. Tear fluid and aqueous humor, vitreous, and meibum

The other ocular fluids, aqueous humor and vitreous, as well as meibum are increasingly being recognized as valuable sources of disease biomarkers. Each of these fluids contains unique molecular signatures that can provide insights into various physiological and pathological conditions (Magni et al., 2010). In our findings, 44 articles co-investigated tear fluid with aqueous humor, vitreous, and/or meibum.

Unlike tear fluid, the collection of aqueous humor is highly invasive. Aqueous humor can be obtained during cataract surgery, but yields only small volumes, typically less than 50 μL, and can only be performed once per eye, limiting its potential for repeated analysis. Furthermore, aqueous humor has a very low endogenous RNA content (0.002 ng/mL), and does not contain a rich mRNA repertoire, with only 107 mRNAs and 20 miRNAs detected (Hulstaert et al., 2020).

Vitreous humor collection, performed through a procedure known as vitreous tap or vitrectomy, involves surgical intervention and carries significant risks. This procedure is generally reserved for specific medical indications, such as diabetic retinopathy, age related macular degeneration, and retinal detachment. Although vitreous humor allows for the collection of larger volumes (0.5–2 mL) compared to tear fluid, its invasive nature and the associated risks make it less suitable for routine or repeated sampling (Mishra et al., 2023; Sampani et al., 2024).

Meibum, a lipid-rich secretion from the Meibomian glands, requires specialized techniques for collection, yielding very small volumes, often similar to or smaller than those obtained from tear fluid. While it is less invasive than aqueous humor and vitreous collection, it is more complex and less comfortable than tear fluid collection. Consequently, meibum is primarily used in specific conditions, such as DED and Meibomian gland dysfunction (Asiedu, 2022).

10.4. Tear fluid and urine

Similar to tear fluid and other body fluids, urine is a rich source of biomarkers. Urine collection is non-invasive and can be done easily, making it convenient for both patients and researchers. In addition, urine is produced in large volumes, providing ample sample material for analysis. However, urine composition can vary significantly due to factors such as hydration levels, diet, and time of day, potentially affecting biomarker consistency. In addition, not all diseases or conditions produce detectable changes in urine biomarkers, limiting its utility for certain types of diagnostics.

Urine biomarkers were investigated alongside tear fluid in 16 articles. Applications include the diagnosis of Alzheimer's disease and Parkinson's disease (Bălașa et al., 2020; Dutta et al., 2023), detection of SARS-CoV-2 (Kwon et al., 2021), detection of specific biomarkers, such as alanine and lactate in patients with diabetic retinopathy (Du et al., 2022) and to assess the hydration status in diseases and in healthy people, including athletes (Barley et al., 2020; Bennet et al., 2021; Villiger et al., 2018).

10.5. Tear fluid and cerebrospinal fluid

To date, only 14 articles have investigated tear fluid in conjunction with CSF. CSF and tear fluid can both reflect systemic and localized health conditions, but they differ significantly in their composition and collection methods. CSF, produced by the choroid plexus in the brain, circulates within the brain's ventricles and around the spinal cord, playing a crucial role in protecting the central nervous system (CNS). While tear fluid has a protein content approximately ten times higher than CSF, both fluids contain proteins, RNA, and miRNA that serve as valuable biomarkers for disease diagnosis (Gijs et al., 2024). However, CSF collection is invasive, risky, and can cause significant patient anxiety, particularly limiting its routine clinical use. The procedure, requiring a lumbar puncture, is typically performed only once during the course of a disease, restricting information on disease progression, treatment response, and overall management. It also demands a specialized hospital setting and a neurologist's expertise, takes up to 30 min, and often causes patient stress before and during the procedure. Additionally, lumbar punctures can occasionally fail due to movement of the patient, or difficulty to locate the correct space in the spine.

CSF is traditionally used to identify markers of neurological diseases. Several articles investigated these neurological markers in tear fluid, including biomarkers for Alzheimer's disease (Gijs et al., 2021b), Prion disease (Schmitz et al., 2023), Parkinson's disease (Hamm-Alvarez et al., 2019; Maass et al., 2020) and Multiple sclerosis (Hümmert et al., 2019; Salvisberg et al., 2014). Interestingly, a recent study suggested that mutant huntingtin, the biomarker for Huntington's disease, was present in much higher levels in tear fluid than in CSF (Gijs et al., 2024).

10.6. Tear fluid advantages and challenges

Tear fluid offers several compelling advantages over other body fluids for diagnostic purposes. First and foremost, tear fluid collection is non-invasive, eliminating the discomfort and risks associated with needles or surgical procedures. This makes it a safe and low-risk option, with the only potential side effect being a temporary dry feeling in the eyes (Tham et al., 2023). The collection process is inexpensive and does not require specialized training or equipment, making it feasible to perform in a variety of settings, including at home or in non-medical environments. Additionally, tear fluid sampling is well-suited for translation into POC or home testing, significantly increasing patient compliance. Tear fluid is readily available at any time of day, further enhancing its utility as a diagnostic fluid.

However, some challenges are associated with tear fluid collection and analysis. The small volume of tear fluid can limit the number of tests that can be performed from a single sample, and the collection process may be challenging for individuals who are uncomfortable with eye contact, such as young children or those with mental disabilities. In such cases, tear fluid sampling can be performed after administering local anesthesia to minimize discomfort. Additionally, environmental factors, such as humidity and temperature, and diurnal variations can affect tear fluid samples, introducing variability (Calonge et al., 2017; Jones et al., 2024). Another drawback is that most commercially available tests, such as ELISA assays, have been validated for other body fluids, but not for tear fluid.

Furthermore, both systemic and topical antibiotics can alter the natural tear fluid composition and microbial flora of the ocular surface, impacting the levels of proteins, lipids, and metabolites in tear fluid (Doan et al., 2020). This alteration can complicate the interpretation of tear fluid data, potentially leading to inaccurate or misleading diagnostic results. Additionally, other medications, such as those affecting blood pressure or tear fluid production, can also modify tear fluid composition, making it essential to consider a patient's medication history during analysis. Despite these challenges, the overall benefits of tear fluid make a promising medium for non-invasive diagnostics.

11. Future use of tear fluid as point-of-care tests

Tear fluid is highly responsive to physiological changes, such as stress, infection, and inflammation, making it a valuable medium for real-time health monitoring. In addition, tear fluid is easy to collect, making it ideal for POC testing. Translating tear fluid into a POC test is obvious for two reasons. Firstly, the most common POC tests are strip-based (similar to pregnancy tests) or microfluidic devices using glass capillaries, both of which are widely used in ophthalmology and require minimal development time (Jia et al., 2023; Wang et al., 2024). Additionally, because the field of ophthalmology is already familiar with these methods, implementing a POC test in the clinic is likely to be easier compared to other medical disciplines. POC tests allow clinicians to obtain immediate results without sending samples to a lab, waiting for

analysis, or delaying treatment decisions. This rapid, on-site testing is particularly beneficial in ophthalmology, where early diagnosis and timely treatment can significantly impact patient outcomes and streamline the clinical workflow.

Extensive research on DED has driven the development of POC tests to assist clinicians in accurately diagnosing the type and severity of DED. These techniques measure either osmolarity or specific tear fluid proteins.

Tear fluid hyperosmolarity arises from either reduced aqueous tear fluid secretion (aqueous-deficient DED) or enhanced evaporation of the tear film's aqueous layer from the exposed ocular surface (evaporative DED) (Baudouin et al., 2013; Willcox et al., 2017). In 2008, the *TearLab Osmolarity System*, developed by Tear Lab Group, received US Food and Drug Administration (FDA) approval. This device measures the electric impedance of a 50 nL tear fluid sample collected from the tear fluid meniscus, offering precise osmolarity readings (Benelli et al., 2010). Another portable device, the *I-Pen Osmolarity System* by I-MED Pharma, provides rapid measurements of tear film osmolarity. With a cut-off value of 318 mOsm/L, the *I-Pen* system demonstrated a sensitivity of 90.9% and specificity of 90.6% for diagnosing DED, making it suitable for clinical use (Park et al., 2021). However, the *TearLab* has shown superior accuracy, precision, and alignment with clinical interpretations, whereas the *I-Pen* system has exhibited insufficient performance in differentiating between DED and non-DED subjects (Nolfi and Caffery, 2017). Recent studies have further indicated that the *I-Pen* system's accuracy declines outside of the mild DED range, whereas the *TearLab* system maintains consistency across various osmolarity levels. Both devices, however, exhibit low reproducibility *in vivo*, raising concerns about the reliability of single-eye measurements in DED diagnosis (De Luca et al., 2023; Tashbayev et al., 2020; Tavakoli et al., 2022). Introduced in 2022 by Trukera Medical, *ScoutPro* uses temperature-corrected impedance measurements to indirectly gauge osmolarity. Evaluations by Trukera Medical have reported a sensitivity of 64% and specificity of 71% for a reference value of 316 mOsm/L (TrukeraMedical, 2022).

The pathogenesis of DED is often linked to intrinsic immunoregulatory pathways dysfunction, leading to the release of pro-inflammatory mediators and contributing to ocular surface inflammation. Elevated levels of MMP-9 in tear fluid are associated with ocular surface barrier dysfunction in DED (Wolffsohn et al., 2017). To address this, Rapid Pathogen Screening Inc./Quidel has developed the *InflammaDry* POC test, which detects MMP-9 levels above 40 ng/mL, aiding in the identification of inflammatory causes and guiding anti-inflammatory treatment initiation (Choi et al., 2023). Accurate tear fluid sample volume is crucial for the test's reliability, as deviations can result in false-positive outcomes (Bang et al., 2020). The *T-POC* Quantitative Testing Platform by Verséa Ophthalmics offers a comprehensive approach to analyzing potential allergic, aqueous-deficient, or evaporative causes of DED through the measurement of lactoferrin and immunoglobulin E (IgE) in tear fluid. Lactoferrin, a multifunctional glycoprotein with antimicrobial and anti-inflammatory properties, is essential for maintaining ocular surface health (Vagge et al., 2020), and its low levels are indicative of aqueous-deficient DED (Da Dalt et al., 1996; Danjo et al., 1994). IgE testing may be used to distinguish allergic conjunctivitis from DED by detecting allergen-induced IgE binding. The T-POC platform utilizes gold nanoparticle conjugation for precise detection, providing a reliable colorimetric readout. The *TearScan 270* microarray system from Beye provides real-time, quantitative results for lactoferrin and IgE levels in tear fluid using a 500 nL sample, analyzed within the TearScan unit (Chao and Tong, 2018). Another platform that was recently introduced is the *i-ImmunDx*™ Platform from Seinda Pharmaceuticals. They developed a series of tear fluid biomarker POC test cassettes (including Lymphotoxin-α (LTα3, LTA), IgE, MMP-9, IL-6, and IL-8) that can be read by their *i-ImmunDx*™ Analyzer.

In addition to the currently available commercial options for investigating tear film biomarkers, several emerging tear fluid-based POC tests are in development. Innovations like smart contact lenses, microfluidic devices (Li et al., 2022), and integrated (immuno)sensor systems are being developed to enable continuous, non-invasive and real-time health monitoring solution (Liu et al., 2024). For instance, glucose biosensors embedded in smart contact lenses offer a promising alternative to traditional blood glucose monitoring, showing a strong correlation between tear fluid glucose and blood glucose levels across various species (Park et al., 2024). However, factors such as "personalized lag time", which account for variations in tear composition due to the contribution of reflex tear fluid caused by mechanical stimulation of the conjunctiva while wearing smart contact lenses, must be contemplated to improve accuracy (Park et al., 2024). Immunosensor technology capable of quantifying pro-inflammatory mediators like TNF at the picogram level has been reported to be more rapid and sensitive than conventional ELISAs. This technology requires a smaller sample volume while maintaining high specificity (Cruz et al., 2019). Graphene-based sensors are also being explored for monitoring stress hormones like cortisol in tear fluid, with potential applications in managing conditions such as Cushing's disease, autoimmune diseases, cardiovascular complications, type 2 diabetes and neurological disorders (reviewed elsewhere). These sensors offer the ability to measure cortisol levels in human tear fluid with a detection limit as low as 10 pg/mL (Ku et al., 2020), and can transmit real-time data wirelessly to smartphones, making them a convenient option for continuous health monitoring. Together, these emerging technologies are paving the way for more accessible, continuous, and non-invasive health monitoring, holding significant promise for early diagnosis, disease management, and personalized medicine.

12. Conclusions and recommendations

This review provides a comprehensive global overview of the current methods used in tear fluid research, emphasizing a critical challenge: the lack of standardization across methodologies. While each technique offers its own advantages and disadvantages, the absence of standardized protocols makes it difficult to compare results and draw consistent conclusions, hindering progress in the field. Our findings reveal that over eight different collection methods are in use, each of which can be performed under varying conditions leading to variability in research outcomes. While this scoping review is valuable for understanding current practices and guiding future harmonization efforts, it is important to note that the most commonly used methods are not necessarily the most effective ones. Therefore, the next step for the field, and specifically for the Tear Research Network (TRN), is to gather and evaluate the evidence that is needed to establish a consensus. This process will involve drafting standardized operating procedures (SOPs) to ensure consistent and reliable tear fluid analysis across the field.

This review also highlights critical knowledge gaps within the field. Firstly, a significant limitation identified in the reviewed studies is the lack of detailed information necessary for reproducing experiments. In 57.1% of the articles, critical aspects such as the use of anesthesia were not specified, yet these details are crucial for understanding and replicating the studies accurately. To address this issue, the Tear Research Network's next task is to develop comprehensive methodology and reporting guidelines for tear fluid collection and analysis to ensure that all essential steps (e.g. time of collection, pre-analytical processing, normalization) and conditions are clearly documented in research articles. Secondly, the initial step of this review, i.e. the systematic search, was complicated by the fact that 165,805 articles referenced "tear" in their titles or content, but the majority were not related to tear fluid. Instead, these articles often discussed muscle tears, tendon tears, and similar topics, which required extensive and time-consuming filtering to identify relevant studies. Additionally, tear fluid has several synonyms, such as tear film, lacrimal fluid, tear drop and tears, which complicates the process of identifying relevant literature for researchers in this field. This challenge emphasizes the need for a standardized terminology.

While lacrimal fluid refers to the overall liquid produced and secreted by the lacrimal glands, tear fluid is a broader term that encompasses all the liquid covering the ocular surface, including contributions from both the lacrimal glands, Meibomian glands, and other structures. In contrast, the tear film specifically describes the structured, multi-layered coating that covers the ocular surface. We recommend consistently using the term "tear fluid" in future tear fluid biomarker-related research and reviews.

The articles included in this review were limited to those that collected and analyzed tear fluid, but it did not address tear fluid biobanking. Tear fluid biobanking represents a valuable strategy for advancing future research, as it allows for the long-term preservation of tear fluid samples for subsequent analysis. This approach can facilitate the discovery of new biomarkers, enable longitudinal studies, and support the reproducibility of findings. Incorporating tear fluid biobanking into research workflows could enhance the potential for collaboration and innovation in tear fluid-based diagnostics. Given the anticipated improvements in detection techniques, which are likely to enhance both sensitivity and specificity, having a well-maintained repository of tear fluid samples will be crucial. By preserving these samples, researchers can take advantage of future technological advancements to re-evaluate and refine their findings, potentially uncovering new biomarkers or refining diagnostic methods. However, the field still lack SOPs for biobanking, which would be highly beneficial to harmonize practices and ensure consistency in sample handling, storage and analysis.

Finally, several exciting developments and innovations in the field are worth noting. Collection methods are being adapted into POC tests for immediate results, and digital (immuno)sensors are being introduced for continuous monitoring (Cruz et al., 2019; Ku et al., 2020). Detection techniques are increasingly capable of handling small volumes with reduced void volumes, enabling the analysis of undiluted tear fluid. Additionally, the latest immunoassays, such as those from Olink, NULISA, and Affymetrix, now allow multiplexing of up to 200 analytes in a single sample, minimizing the need for large sample volumes (Cross et al., 2023; Feng et al., 2023; Gijs et al., 2023a). Another promising advancement is the investigation of tear fluid for the diagnosis and monitoring of systemic and neurological diseases (Gijs et al., 2021a, 2024; Hamm-Alvarez et al., 2019; Maass et al., 2020).

While this review provides a thorough analysis of tear fluid research methodologies, several limitations in our approach should be considered. First, the exclusion of inaccessible articles, non-English articles, grey literature, and studies that did not specifically focus on tear fluid biomarkers may have introduced selection bias, limiting the comprehensiveness of the review. Additionally, although our search strategy was extensive, there is a possibility that some relevant studies were missed due to variations in terminology and inconsistent reporting in the field. Furthermore, the use of two databases (Embase and Ovid MEDLINE) could have excluded research indexed in other relevant databases, potentially narrowing the scope of our findings.

In conclusion, tear fluid collection is non-invasive, relatively inexpensive, requires no specialized training, and can be performed in various settings, including at home. Despite these advantages, it is crucial to accurately identify the levels of biomarkers in tear fluid and correlate them with specific stages of diseases or conditions. Making routine tear fluid testing a reality will require combined efforts from analytical scientists and clinical ophthalmologists to advance analytical technologies and integrate them into clinical practice. Sustained collaboration, particularly with physician-scientists who can identify clinical limitations as opportunities for scientific discovery, will be essential. Given the significant clinical advantages of tear fluid and the rapid pace of ophthalmic discovery, tear fluid analysis holds promise to become a routine test for health monitoring health in both clinical and non-clinical environments.

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Declaration of competing interest

No conflicts of interest.

Acknowledgements

We would like to thank to following people for their work and support in the making of this scoping review: Ward van Deurse, Sobia Hashmee, Nicholas Wang, Swaminathan Sethu, Abdullah Mohamed, Lasse Jørgensen Cehofski, Debasmita Kanungo, Romy op de Laak, Maria Markoulli, Amy E. Ross, Nikhil Ashok, Arka Subhra, Robert Graham, and Shiju Thomas Michael.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preteyeres.2025.101338.

Data availability

The data is available in appendix 3.

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