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The Growing Need for Validated Biomarkers and Endpoints for Dry Eye Clinical Research

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Purpose. Biomarkers with minimally invasive and reproducible objective metrics provide the key to future paradigm shifts in understanding of the underlying causes of dry eye disease (DED) and approaches to treatment of DED. We review biomarkers and their validity in providing objective metrics for DED clinical research and patient care.

Methods. The English-language literature in PubMed primarily over the last decade was surveyed for studies related to identification of biomarkers of DED: (1) inflammation, (2) point-of-care, (3) ocular imaging, and (4) genetics. Relevant studies in each group were individually evaluated for (1) methodological and analytical details, (2) data and concordance with other similar studies, and (3) potential to serve as validated biomarkers with objective metrics.

Results. Significant work has been done to identify biomarkers for DED clinical trials and for patient care. Interstudy variation among studies dealing with the same biomarker type was high. This could be attributed to biologic variations and/or differences in processing, and data analysis. Correlation with other signs and symptoms of DED was not always clear or present.

Conclusions. Many of the biomarkers reviewed show the potential to serve as validated and objective metrics for clinical research and patient care in DED. Interstudy variation for a given biomarker emphasizes the need for detailed reporting of study methodology, including information on subject characteristics, quality control, processing, and analysis methods to optimize development of nonsubjective metrics. Biomarker development offers a rich opportunity to significantly move forward clinical research and patient care in DED.

Overview. DED is an unmet medical need — a chronic pain syndrome associated with variable vision that affects quality of life, is common with advancing age, interferes with the comfortable use of contact lenses, and can diminish results of eye surgeries, such as cataract extraction, LASIK, and glaucoma procedures. It is a worldwide medical challenge with a prevalence rate ranging from 8% to 50%. Many clinicians and researchers across the globe are searching for better answers to understand the mechanisms related to the development and chronicity of DED. Though there have been many clinical trials for DED, few new treatments have emerged over the last decade. Biomarkers may provide the needed breakthrough to propel our understanding of DED to the next level and the potential to realize our goal of truly personalized medicine based on scientific evidence. Clinical trials and research on DED have suffered from the lack of validated biomarkers and less than objective and reproducible endpoints. Current work on biomarkers has provided the groundwork to move forward. This review highlights primarily ocular biomarkers that have been investigated for use in DED, discusses the methodologic outcomes in providing objective metrics for clinical research, and suggests recommendations for further work.

Keywords: biomarker, dry eye, clinical research, inflammation

Dry eye disease (DED) is a multifactorial condition difficult to categorize given the less than precise definitions currently used. One of the most often quoted definitions was developed by over 60 worldwide experts and published as part of the dry eye workshop report (DEWS): Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability, with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.[1] As more research and information becomes available, the definition will no doubt be modified,[2] but it is unlikely to be significantly simplified in the near future given that there is no universally accepted "gold standard" to diagnose DED. Despite the common occurrence of DED, routine diagnosis and clinical evaluation often are subjective and typically based on patient symptom reporting with poor correlation between signs and symptoms.[3–7] While multiple clinical assessments do exist to examine qualitative and quantitative facets of the ocular surface and tear functional unit,[8,9] no universal consensus exists as to which of the specific assessments should be included in the diagnostic workup.[10] Moreover, established threshold values for defining the distinction between normal and pathologic states on each assessment often are chosen semiarbitrarily, especially as the

disease manifests in a spectrum of severity. Additionally, in many of the assessments the measure may be affected by use of drops, touching the eye, and so forth. For example, Schirmer's test, which has been used routinely to determine the amount of tear secretion, is performed by applying a standardized filter paper to the eye for 5 minutes and then measuring the length of wetness on the paper to correlate with tear production; however, its physical presence on the eyelid often stimulates reflex tear secretion, which is distinct from the basal tear production intended to be measured and, thus, can affect the measured levels.[10] Other tests, though called objective, require the clinician to score the change on the ocular surface, such as vital dye staining of the cornea, and, therefore, are open to significant observer bias. As a result, poor correlations often are demonstrated between typically used assessment findings (signs) and subjective symptoms where the patient's general pain sensitivity threshold also may be a crucial factor.[3,5,11–13]

Nonetheless, most clinicians would say, "we know it when we see it and even with current methods we can make the diagnosis of DED during a standard ocular examination." However, more definitive diagnostic tests, improved ability to determine severity of disease, and methods to determine efficacy of treatment, in the clinical setting and in clinical trials are much needed.

Experience over the last decade with clinical trials in DED has demonstrated the low success rate with few new treatments reaching regulatory approval.[14,15] There are likely several contributing factors to the poor success rates, including ineffective treatments, trial length, environmental influences, heterogeneity of the population and disease, and that signs do not always correlate with symptoms. In addition, the chosen primary endpoints may be less than objective or repeatable; of 57 DED clinical trials listed on clinicaltrials.gov in 2010, 33% used symptoms (dryness, grittiness, redness, and so forth) as primary endpoints, and 40% used signs (corneal staining, Schirmer's test, tear breakup time, and so forth).[14]

To address the needs of clinical trials and to expand our understanding of DED, there is an acute need for the identification and validation of biomarkers using minimally invasive methods that will lead to objective metrics to help us create a roadmap for improved understanding of the mechanisms at work in DED, provide better endpoints for clinical trials, and superior patient care.

Biomarkers as Objective Tools to Support Diagnosis, Treatment Options, and Therapeutic Drug Development

A biomarker is defined as a characteristic that is measured objectively and evaluated as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention.[16,17] Further, biomarkers do not come in "one size fits all." They can be classified as diagnostic biomarkers, monitoring biomarkers, predictive biomarkers, and so forth.[17–20] As stated by BEST Resource FDA-NIH Biomarker Working Group, "biomarkers should be objective — free of biases by either the patient or observer, reproducible, and provide a metric." Finally, key characteristics of a usable biomarker include specificity, sensitivity, simplicity, reliability, reproducibility, multiplexing capability, and cost and time needed for the methodology used.[21]

Overall, not all biomarkers, as in other fields, will be validated as surrogate endpoints for clinical research involved in testing efficacy and safety of new treatments for DED.[22] A surrogate endpoint, in brief, is "expected to predict clinical benefit or harm,"[20] and so needs clear evidence of its rationale and its ability to predict clinical benefit. Some biomarkers may best serve clinical trials by enhancing patient selection to provide more uniform subject groups and provide easier comparability between clinical trial results.[23]

As we search for biomarkers to better define DED, we are struck again with the definition and the oft-repeated line that DED has a "multifactorial" pathogenesis. Our current knowledge may be more comparable to calling all joint pain "arthritis" with no separation of osteoarthritis and rheumatoid arthritis; obviously, we do not treat all joint pain the same way and instead direct treatment to the specific mechanisms at work. Though patient-reported outcomes are key to understanding and treating symptomatic diseases, such as DED, they have not provided objective repeatable metrics that are needed for clinical trials. Biomarker data will likely lead to better categorization and more effective treatment of DED and maybe even development of companion diagnostics that will associate biomarker status with specific treatments. As such, the scientific, economic and regulatory impact of validated biomarkers and surrogate endpoints have the potential to revolutionize the approach to DED.

The following sections review studies on biomarkers from human subjects with DED that have the potential to provide minimally invasive objective metrics that could be useful for clinical trials and patient care.

Biomarkers of Inflammation

Even though the pathogenesis of DED is not fully understood, it is recognized that immune-mediated inflammation has prominent roles in its development and progression.[24–28] Ocular inflammation, of course, can be part of many diseases and, therefore, is not diagnostic of DED, but it may be useful to determine severity, and has been used in clinical trials and other studies to evaluate efficacy of treatment (listed in Tables 1–6). For inflammatory biomarker studies on DED patients, two approaches have primarily been used: impression cytology (IC) and tear sampling.

Impression Cytology (IC)

This technique, which involves briefly touching the conjunctival surface to remove cells, has been a key minimally invasive means of sampling cells from the ocular surface. The technique, which initially was used by investigators to examine the cytologic and morphologic characteristics of the ocular surface,[29] is now coupled to an array of analytical processing techniques to probe the cellular and molecular expression patterns of inflammatory biomarkers on the ocular surface in DED[30,31] (summarized in Table 1). Though the recovered cells have been analyzed by light microscopy, immunocytochemistry, and mRNA polymerase chain reaction, flow cytometry is the most commonly used method as it lends itself to objective measures of multiple inflammatory biomarkers in each sample.[30,32] HLA-DR is one of the most common biomarkers of inflammation in DED that has been studied using IC, while little has been done to look at other markers of inflammation (summarized in Table 1). Currently, the Dry Eye Assessment and Management (DREAM) randomized clinical trial of Omega-3 supplements is investigating a series of markers, using IC sampling, to determine common effector cells and their level of activation (Hom MM. *IOVS*. 2016;57:ARVO E-Abstract 2844).[33]

Sampling. The general protocol for IC specimen collection for flow cytometry is based upon a method introduced by Baudouin et al.[34] In brief, a porous membrane, following a single anesthetic drop, is applied to the corneal surface. It is

Table 1. Markers Studied in Cells Obtained From Conjunctival Impression Cytology Samples of DED Patients

Markers Studied	Method of Assessment
HLA-DR	Flow cytometer[173]
86 genes including *IL-6, IL-9, CCL24, CCL18, IL-10, IFN-γ, CCL2* and *EGRR*	mRNA[174]
ICAM-1 and HLA-DR	Flow cytometer[175]
CD45, CD3 and HLA-DR	Flow cytometer[176]
NLRP3, caspase-1, IL-1β, and IL-18	mRNA[177]
HLA-DR	mRNA[178]
HLA-DR	Microscopic evaluation[43]
HLA-DR	Flow cytometer[41]
HLA-DR and ICAM-1	mRNA[179]
CCL20, IL-8, and eotaxin-2	mRNA[180]
PAX6, IL-1β, and SPRR1B	mRNA[181]
TNF-α	mRNA[182]
96 genes including *HLA-DRB5, PSCA, FOS, lysozyme, TSC22D1, CAPN13* and *CXCL6*	mRNA[183]
HLA-DR and CD11c	Microscopic evaluation[184]
HLA-DR	Microscopic evaluation[185]
HLA-DR	Flow cytometer[32]
HLA-DR	Flow cytometer[36]
HLA-DR	Flow cytometer[186]
HLA-DR	Flow cytometer[187]
MUC1, MUC2, MUC4, MUC5AC, and MUC7	mRNA[188]
HLA-DR	Flow cytometer[189]
MUC1	mRNA[190]
CD3, CD11a and HLA-DR	Flow cytometer[191]
CD3, CD11a and HLA-DR	Flow cytometer[192]
CK19, CD45, CD3, CD4, CD14, CD56 and HLA-DR	Flow cytometer[38]
IL-1β, IL-6, IL-8, and TNF-α	mRNA[193]
MUC16	mRNA[194]
CCR4, CCR5, and HLA-DR	Flow cytometer[44]
HLA-DR	Flow cytometer[195]
ICAM-1	Flow cytometer[196]
CCR5 and CD45	Flow cytometer and mRNA[37]
HLA-DR	Microscopic evaluation[197]
HLA-DR	Flow cytometer[198]
ICAM-1, M1/MUC5AC, and HLA-DR	Flow cytometer[42]
CD40, CD40ligand, APO2, Fas and HLA_DR	Flow cytometer[199]
EGFR, ErbB2, and ErbB3	Microscopic evaluation[200]
CD23 and HLA-DR	Flow cytometer[34]
CD23 and HLA-DR	Microscopic evaluation[201]

important to point out that while most studies have reported the use of either polyether sulfone filters or Eyeprim (Tomlins P, et al. *IOVS*. 2013;54:ARVO E-Abstract 5430),[35] no consensus exists on the size of membrane to be used, which may impact the number of cells recovered for analysis. Once removed from the eye, the samples are placed immediately in physiologic solution, which typically contains 0.05% paraformaldehyde and allows for gentle fixation to maintain the morphologic and antigenic integrity of the cells. This is of particular importance in multicenter trials where samples are stored before being shipped to a central reading center that is masked to subject treatment and to clinical signs or symptoms.[32]

Processing. The processing of IC samples for flow cytometry involves mechanical separation of the cells from the membrane by vortex or gentle agitation, followed by recovery using centrifugation. Two important components of this step are the time lapse between collection and processing and the number of cells recovered. While some studies report the time lapse between collection and processing of cells, most studies fail to mention this important detail. As indicated in a study by Epstein et al,[32] antigenic integrity of the cells and, thus, the flow cytometer data output of HLA-DR expression, appeared stable up to 4 weeks of storage at 4°C. Another study has reported an even shorter time period of 10 days for obtaining reproducible expression of HLA-DR.[36] There also is a lack of information in most studies on the number of cells recovered. In the few studies that have included this information,[34,36–38] the cell numbers have ranged from 2000 to 200,000 per eye. Unlike blood, where analysis is not limited by cell number, information on cell numbers obtainable from IC samples will be critical in deciding the number of antigens that can be targeted for a study as lower cell numbers may result in statistically insignificant data output for cell subgroup analysis. The cell samples then are processed for immunostaining with conjugated monoclonal antibodies to the intended targets. Antibody dilutions depend on the specific antibody used, the manufacturers' recommendation or, for more stringency, titration for best signal-to-noise ratio. In case of multiple antigen analysis, careful selection of fluorochromes is needed to enhance the ability to differentiate markers.

Analysis. Analysis of samples involves data acquisition by a flow cytometer and postacquisition analysis. For this, a variety of machines and analysis software are used by different investigators. While this variation cannot be overcome, it is important to recognize that this can be the biggest contributor to variability that may be observed in data reported by various groups. Added to this is the subjective nature of analysis, specifically gating; when multiple parameters are involved this adds to the less than consistent nature of data generated by multiple labs studying the same parameter(s) by flow cytometry. Flow cytometry is a common way to identify biomarkers in many human diseases, such as oncology.[39] The processing and analyzing issues that contribute to variability of the results are issues for all studies involving flow cytometry and efforts are being made to address this very issue by "harmonization of flow cytometers" and protocols.[39,40]

HLA-DR Expression Variability in DED. Despite the use and adoption of IC for flow cytometry, variability in sampling, processing and analysis, as detailed above, has hampered intergroup reproducibility of results and their comparison. Table 2 lists studies that have looked at HLA-DR expression in IC samples from DED patients and normal individuals. A wide variability in percentage of HLA-DR expression in DED patients is seen among studies, with values ranging from 1.2 to 64.2. For example, a study by Fernandez et al.[41] reports the percentage of HLA-DR–positive cells in IC samples from DED patients to be 7.17 ± 6.10, while another study[42] shows it to be 56.9 ± 24.6. While the wide range of percentages in DED samples could be attributed to differences in the patient groups (age, proportion of male to female, severity, and so forth), more striking is the variation in percentage of HLA-DR expressed in IC samples from normal subjects (with no history of ocular disease or clinical ophthalmic abnormality), a group where interstudy variation should be minimal. In this group, percentage of HLA-DR–positive cells has ranged from 1.95 ± 1.46[43] to 22.1 ± 19.1.[44] All of the above once again emphasizes the need for stricter quality control with collective standardization of procedures as well as demonstration of reliability and repeatability of each step, and a need for optimization of nonsubjective data analysis tools.

Table 2. Reported Levels of HLA-DR Expression in Cells Obtained From Conjunctival Impression Cytology Samples From DED Patients

Study Description	Groups and Interventions	Dry Eye		Normal	
		n	Mean ± SD	*n*	Mean ± SD
Comparison between patients treated with cyclosporine A cationic emulsion (CsA CE) or Vehicle (V) for 3 and 6 mos.[173]	DED + CsA CE baseline	154	64471AFU		
	DED + CsA CE mo 1	154	52306AFU		
	DED + CsA CE mo 6	154	49917AFU		
	DED + V baseline	91	67663AFU		
	DED + V mo. 1	91	66825AFU		
	DED + V mo. 6	91	70062AFU		
Comparison between 10-wk treatment with topical tacrolimus (TT) or methylprednisolone (TM) in patients with ocular graft-versus-host disease (oGVHD)[175]	oGVHD TT baseline	24	8.7%		
	oGVHD TT 10 wks	24	4.7%		
	oGVHD TM baseline	16	9.5%		
	oGVHD TM 10 wks	16	7.2%		
Comparison between glaucoma patients treated with one (group 1), two (group 2), or three and more (group 3) anti-glaucoma medication, normal individuals, and DED patients.[43]	DED	20	37.10% ± 13.56%	20	1.95% ± 1.46%
	Group 1	40	24.25% ± 7.13%		
	Group 2	20	35.05% ± 8.14%		
	Group 3	20	42.00% ± 5.83%		
Comparison between pre- and post-30–d treatment with eye drops containing polyethylene glycol and propylene glycol plus gelling agent hydroxypropyl guar[41]	DED baseline	19	7.17% ± 6.10%		
	DED 30 d	19	3.77% ± 2.12%		
The correlation between HLA-DR expression and corneal fluorescein staining in patients with moderate to severe DED participating in a randomized clinical trial with cyclosporine treatment[202]	DED + cyclosporine baseline	154	64471AFU		
	DED + V baseline	91	67663AFU		
	DED + cyclosporine mo 1	154	52306AFU		
	DED + V mo 1	91	66825AFU		
	DED + cyclosporine mo 6	154	49917AFU		
	DED + V mo 6	91	76062AFU		
Effect of oral supplementation of omega-3 and omega-6 fatty acids on a conjunctival inflammatory marker in dry eye patients[203]	DED + fatty acid baseline	58	53438AFU		
	DED + placebo baseline	63	62249AFU		
	DED + fatty acid mo 3	58	38553AFU		
	DED + placebo mo 3	63	59159AFU		
Comparison between oGVHD patients and normal individuals[185]	oGVHD	27	30.1%	19	7.65%
Validity of HLA-DR as an inflammatory biomarker by comparing levels between normal individuals, patients with mild, moderate, or severe KCS[36]	KCS mild	12	25.7%	12	23.3%
	KCS moderate	12	30.8%		
	KCS severe	12	41%		
Comparison of 15- and 30-d Pranoprofen 0.1% plus sodium hyaluronate 0.1% (PFSH) orsodium hyaluronate 0.1% (SH) treatment in DED[186]	DED PFSH baseline	30	44.2% ± 11.4%		
	DED PFSH 15 d	30	33.4% ± 8.0%		
	DED PFSH 30 d	30	30.7% ± 5.6%		
	DED SH baseline	30	43.6% ± 8.6%		
	DED SH 15 d	30	42.0% ± 7.4%		
	DED SH 30 d	30	42.3% ± 9.9%		
Comparison of HLA-DR in response to hyperosmolar stress in DED patients and normal individuals[189]	DED	25	46.2% ± 7.2%	15	7.2% ± 1.1%
Comparison of cataract patients treated with tobramycin and dexamethasone drops (UT) or with UT plus hydroxypropyl-Guar inclusion (HP-Gaur) post-cataract surgery[191]	UT pre-surgery	21	4.7% ± 2.8%		
	UT post-surgery	21	6.8% ± 4.5%		
	HP-Guar pre-surgery	27	5.3% ± 3.0%		
	HP-Guar post-surgery	27	1.8% ± 1.7%		
Compare the effect of 30 d of treatment with Viscofresh 0.5% (carmellose sodium 0. 5%, CS) versus Lubristil (sodium hyaluronate 0.15%, SH) in DED[192]	DED CS baseline	7	67.1% ± 18.4%		
	DED CS 30 d	7	8.9% ± 9.9%		
	DED SH baseline	8	64.2% ± 31.4%		
	DED SH 30 d	8	36.7% ± 29.3%		
Immune response in the conjunctival epithelium of patients with DED[38]	DED	15	≈40%	15	≈5%
Th1 and Th2 responses on the ocular surface[44]	KCS	17	52.4% ± 12.1%	17	22.1% ± 19.1%
	Uveitis	26	57.4% ± 21.1%		
	VKC	24	23.9% ± 26.8%		
HLA-DR expression on conjunctival epithelial cells from patients with cystic fibrosis (CF) and mild KCS[195]	CF KCS	25	16.9% ± 10.3%	25	8.1% ± 1.9%
Expression of HLA-DR in the early stages of DED[197]	Mild DED	16	1.3% ± 0.2%	16	1.2% ± 0.2%
	Moderate DED	16	1.8% ± 0.2%		

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Table 2. Continued

Study Description	Groups and Interventions	Dry Eye		Normal	
		n	Mean ± SD	*n*	Mean ± SD
Monitor the effects of 3- and 6-mo 0.05% cyclosporin A (Group 1) and 0.1% cyclosporine A (Group 2) treatment vs vehicle (Control) on the expression of HLA-DR in KCS patients[198]	Group 1 baseline	51	61.7% ± 29.5%		
	Group 1 3 mo	36	39.0% ± 31.4%		
	Group 1 6 mo	44	39.5% ± 33.1%		
	Group 2 baseline	53	57.5% ± 31.7%		
	Group 2 3 mo	30	41.7% ± 33.6%		
	Group 2 6 mo	39	38.6% ± 33.0%		
	Control baseline	51	≈55%		
	Control 3 mo	32	≈45%		
	Control 6 mo	41	≈46%		
Investigate the inflammatory status of conjunctival epithelium in Ocular Rosacea and KCS[42]	KCS	13	56.9% ± 24.6%	12	9.9% ± 5.9%
	Ocular rosacea	13	46.6% ± 23.7%		
Inflammatory Markers in conjunctival epithelial cells of patients with DED[199]	KCS	169	57.0% ± 3.2%	50	≈7% ± 3.0%
Immunopathologic findings in conjunctival cells using impression cytology specimens[201]	DED	24	59.0% ± 27.0%	17	2.0% ± 3.6%

Table 3. Observed Differences of IL-6 Levels in Tears From DED Patients and Normals

Study Description	Groups or Interventions	Dry Eye		Normal	
		n	Mean pg/mL ± SD	*n*	Mean pg/mL ± SD
To determine tear cytokine profiling data in a prospective case-control study in DED patients with or w/o human immunodeficiency virus (HIV) infection[66]	DE with HIV	34	174.7 ± 127.5		
	DE w/o HIV	32	119.5 ± 86.7		
To develop a tear molecule level-based predictive model based on a panel of tear cytokines and their correlation with clinical features. in ocular chronic graft versus host disease (cGVHD) in a controlled environmental research lab[174]		22	119.5 ± 117.4	21	51.4 ± 48.5
To investigate changes in signs, symptoms, and tear cytokines following punctal plug occlusion in patients with dry eye[65]	Baseline	29	6.1 ± 6.7		
	Wk 1 after punctal occlusion	29	5.8 ± 5.7		
	Wk 3 after punctal occlusion	29	4.3 ± 3.7		
To explore a method for measuring tear cytokines with 5 μL tear sample volume and 80% reduced Luminex reagents compared to previous protocols[59]	MilliPlex			1000	12.9 ± 1.4
	DA bead plate			1000	9.2 ± 0.9
To determine if staying in controlled environmental conditions (CEC) for 2 h can induce acute exacerbations of signs and symptoms in dry eye and asymptomatic subjects[204]	Before CEC	19	81.4 ± 33.6†	20	29.6 ± 5.8†
	After CEC	19	69.7 ± 12.4†	20	54.3 ± 8.3†
To compare serum and tear inflammatory and anti-inflammatory cytokine levels of rosacea patients with the healthy controls and evaluate the correlation of tear cytokine levels with tear function parameters[205]	Rocacea w/o ocular findings	12	12.7 ± 19.1	22	24.2 ± 25.9
	Rocacea w ocular findings	20	13.7 ± 27.4		
To explore changes in lacrimal gland and tear inflammatory cytokines in thyroid associated ophthalmopathy (TAO) patients[206]	Active TAO	27	107.3 ± NA	32	8 ± NA
	Inactive TAO	21	21.8 ± NA		
To provide standard operating procedures (SOPs) for measuring tear inflammatory cytokine concentrations Randomized DE patients were treated with omega-3 or placebo for 3 mo[8]	DE w Ω3-baseline	7	53.2 ± 65.8	20	7.4 ± 5.6
	Placebo-baseline	7	151.8 ± 254.8		
	DE w Ω3-mo 3	7	181.1 ± 257.6		
	Placebo-mo 3	10	144.5 ± 314.4		

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Table 3. Continued

Study Description	Groups or Interventions	Dry Eye		Normal	
		n	Mean pg/mL ± SD	*n*	Mean pg/mL ± SD
To determine cytokine and chemokine concentrations in the tears of patients with DED[207]	DES1	130	22.5 ± 10.5*	70	10 ± 10.5*
	DES2	130	35.5 ± 10.5*		
	DES3	130	27.5 ± 10.5*		
To assess clinical outcomes and tear cytokine levels in patients with moderate and severe MGD after treatment with oral minocycline and artificial tears versus artificial tears only[57]	Baseline	30	14.8 ± 13.2		
	2M artificial tear	30	9.1 ± 11		
	Baseline	28	15.7 ± 20.64		
	2M oral minocycline w artificial tear	28	3.9 ± 4.9		
This report describes a procedure that can be used to recover tears from the Schirmer strip for the measurement of multiple tear cytokines as well as MMPs by Luminex technology[68]				5	600 ± 200†
This study analyzes tear cytokine levels and their clinical correlations in patients with moderate evaporative-type DED due to MGD[208,209]		46	200.0 ± NA*†		
				18	130.4 ± 12.3†
To compare tear cytokine and chemokine concentrations in asymptomatic control and dysfunctional tear syndrome (DTS) patients and determine the correlations between tear inflammatory mediators and clinical severity[67]	DTS	30	238.0 ± 278.2	14	26.5 ± 21.8
	DTS w/MGD	9	289.0 ± 272.2		
	DTS w/o MGD	21	210.0 ± 282.9		
To determine the levels of 8 important cytokines and 1 chemokine in tears of patients with dry eye disease[193]		7	1625.7 ± 430.9	7	632.3 ± 167.9
To determine the concentration of interleukins (IL-1β and -6) and MMP-9 (pro-MMP-9) in the tears of patients with different ocular surface diseases and to examine the possible relationship between the disorders and molecular inflammation[210]		20	16.5 ± 10.6	36	8.2 ± 2.7
To determine the levels of IL-6 and TNF-α in tears of patients with DES[211]		36	18.6 ± 8.9	14	3.6 ± 3.4

NA, not available; DE, dry eye; DES, dry eye syndrome; ADDE, adaptive immune in patients with aqueous-deficient DED; LDDE, lipid-deficient dry eye.

* Data estimated from Figure.

† Standard error.

Tears

Tears are an accessible source of biological material that can be obtained minimally invasively, and they have been analyzed extensively for a number of biomarkers in DED.[45–47] Inflammatory mediators released in tears have been recognized as one of the key components in ocular surface inflammation that have prominent roles in the pathophysiology of DED.[48] Of the multiple inflammatory biomarkers under investigation, cytokines and chemokines are the most frequently reported and studied in DED.[45–47] Components of lipid metabolism also have been reported to be correlated with clinical measures of DED, and include secretory phospholipase A2 (sPLA2),[49] prostaglandin E2 (PGE2),[50] arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and leukotriene B4 (LTB4).[51,52] Using a relatively new technology, isobaric tag for relative and absolute quantitation technology (iTRAQ) proteomics, tear proteins like α-enolase, α-1 acid glycoprotein 1, S100 A8/calgranulin A, S100 A9/calgranulin B, S100 A9/calgranulin B; S100 A4, and S100 A11 have been shown to be upregulated in DED tears.[53,54]

Sampling. A number of techniques, including, microcapillary tubes, minisponges, Schirmer's test strips, and tear wash, have been used to collect tears.[47] Microcapillary tubes and Schirmer strips are the most frequently used and show comparable outcomes by Western blot analysis,[55] whereas different ophthalmic sponges with various extraction buffers have yielded diverse results.[56] Tear volumes obtained with the tear wash method vary from patient to patient,[57] and there is no evidence supporting its comparability to other methods.

Processing/Analysis. Luminex, a cytometric bead–based multiplex technology developed by Luminex Corporation (Austin, TX, USA), allows for the simultaneous analysis of multiple cytokines in each sample and processing multiple samples at one time.[58] A recent advancement of miniaturized, wall-less multiplex cytokine assay, named DropArray, allows for the relative and absolute quantification of tear cytokines with 1/5 of volume and reagents normally needed for routine Luminex assay,[59] possibly allowing for analysis of small tear volumes. In brief, the Luminex method involves loading a fixed volume of diluted tears onto assay plates according to

Table 4. Observed Differences of TNF-α Levels in Tears From DED Patients and Normals

Study Description	Groups and Interventions	Dry Eye		Normal	
		n	Mean pg/mL ± SD	*n*	Mean pg/mL ± SD
To determine tear cytokine profiling data in a prospective case-control study in DED patients with or w/o HIV infection[66]	DE with HIV	34	21.7 ± 90.9		
	DE w/o HIV	32	35.1 ± 30.6		
To develop a tear molecule level-based predictive model based on a panel of tear cytokines and their correlation with clinical features in cGVHD in a controlled environmental research lab[174]		22	36.4 ± 74.9	21	20.2 ± 17.2
To investigate changes in signs, symptoms, and tear cytokines following punctal plug occlusion in patients with dry eye[65]	Baseline	29	1.5 ± 1.5		
	Wk 1 after punctal occlusion	29	1.9 ± 1.9		
	Wk 3 after punctal occlusion	29	1.4 ± 1.2		
To explore a method for measuring tear cytokines with 5 μL tear sample volume and 80% reduced Luminex reagents compared to previous protocols[59]	MilliPlex			1000	1 ± 0.1
	DA bead plate			1000	1.5 ± 0.3
To explore changes in lacrimal gland and tear inflammatory cytokines in TAO patients[206]	Active TAO	27	5.8 ± NA	32	3.3 ± NA
	Inactive TAO	21	6 ± NA		
To provide standard operating procedures (SOPs) for measuring tear inflammatory cytokine concentrations. Randomized DE patients were treated with omega-3 or placebo for 3 mo[58]	DE w Ω3-baseline	7	23.1 ± 49.1	20	7.5 ± 8.7
	Placebo-baseline	7	6 ± 10.2		
	DE w Ω3-mo 3	7	38.9 ± 75.9		
	Placebo-mo 3	10	38.8 ± 55.8		
To assess clinical outcomes and tear cytokine levels in patients with moderate and severe MGD after treatment with oral minocycline and artificial tears vs. artificial tears only[57]	Baseline	30	5.4 ± 8.9		
	2M artificial tear	30	5.9 ± 7		
	Baseline	28	4.7 ± 6.3		
	2M oral minocycline w artificial tear	28	2.1 ± 2.5		
This report describes a procedure that can be used to recover tears from the Schirmer strip for the measurement of multiple tear cytokines as well as MMPs by Luminex technology[68]				5	35 ± 10†
This study analyzes tear cytokine levels and their clinical correlations in patients with moderate evaporative-type DED due to MGD[208,209]		46	100.0 ± NA*†	18	47.5 ± 3.3†
To compare tear cytokine and chemokine concentrations in asymptomatic control and DTS patients and determine the correlations between tear inflammatory mediators and clinical severity[67]	DTS	30	464.4 ± 392	14	126.8 ± 44.5
	DTS w/MGD	9	323.2 ± 251.9	14	126.8 ± 44.5
	DTS w/o MGD	21	542.9 ± 445.6	14	126.8 ± 44.5
To determine the levels of 8 important cytokines and 1 chemokine in tears of patients with DED[193]		7	435.7 ± 145.6	7	250.6 ± 63.2
To determine the levels of IL-6 and TNF-α in tears of patients with DES.[211]		36	3.7 ± 3.45	14	<0.5 ± NA

* Data estimated from Figure.
† Standard error.

manufacturer's instruction. To ensure consistent results between plates and batches, a serially diluted mixture of cytokine standards with known concentrations, to obtain a standard curve, suitable internal control (pooled tear samples with known concentrations), and external controls (provided in kit) also are loaded onto the assay plate. Following incubation to allow binding of analyte to capture antibodies coated to the beads, a biotinylated detection antibody and its reporter molecule, Streptavidin-PE conjugate, are introduced to complete the reaction on the surface of each microsphere. The plates are read on a laser flow-based detection instrument. The fluorescent intensity and bead counts are measured, and data output is reported as median fluorescent intensity (MFI) and can be translated to concentration based on standard curves for known cytokines/chemokines. Using this technology, Huang et al.[60] have shown good measuring repeatability of many immune mediators in tears of DED patients. While intrastudy repeatability appears to be possible with this technology, observed interstudy variation is a serious concern and discussed in the subsequent section.

Table 5. Observed Differences of IL-8 Levels in Tears From DED Patients and Normals

Study Description	Groups and Interventions	Dry Eye		Normal	
		n	Mean pg/mL ± SD	*n*	Mean pg/mL ± SD
To determine tear cytokine profiling data in a prospective case-control study in DED patients with or w/o HIV infection[66]	DE with HIV	34	6518.3 ± 4509.7		
	DE w/o HIV	32	3917.4 ± 4006		
To develop a tear molecule level-based predictive model based on a panel of tear cytokines and their correlation with clinical features in cGVHD in a controlled environmental research lab[174]		22	7131.2 ± 15956.8	21	385.2 ± 401.7
To investigate changes in signs, symptoms, and tear cytokines following punctal plug occlusion in patients with dry eye[65]	Baseline	29	74 ± 55		
	Wk 1 after punctal occlusion	29	78.6 ± 67.2		
	Wk 3 after punctal occlusion	29	61.1 ± 57.2		
To determine if staying in CEC for 2 h can induce acute exacerbations of signs and symptoms in dry eye and asymptomatic subjects[204]	Before CEC	19	999.4 ± 424.2†	20	690.8 ± 146.7†
	After CEC	19	901.8 ± 211.6†	20	789.4 ± 145†
To compare serum and tear inflammatory and anti-inflammatory cytokine levels of rosacea patients with the healthy controls and evaluate the correlation of tear cytokine levels with tear function parameters[205]	Rocacea w/o ocular findings	12	426.6 ± 508.3	22	275.5 ± 296.2
	Rocacea w ocular findings	20	277.8 ± 301.9		
To provide SOPs for measuring tear inflammatory cytokine concentrations. Randomized DE patients were treated with omega-3 or placebo for 3 mo[58]	DE w Ω3-baseline	7	53.2 ± 65.8	20	NA ± NA
	Placebo-baseline	7	151.8 ± 254.8		
	DE w Ω3-mo 3	7	181.1 ± 257.6		
	Placebo-mo 3	10	144.5 ± 314.4		
To characterize tear protein markers in DED. Sampling at d 0 and 7, no treatment involved[60]	DE1-d 0	30	3310.0 ± NA	22	4600.0 ± NA
	DE2-d 0	29	5380.0 ± NA		
	DE3-d 0	21	9730.0 ± NA		
	DE1-d 7	30	3890.0 ± NA	22	3800.0 ± NA
	DE2-d 7	29	6070.0 ± NA		
	DE3-d 7	21	8190.0 ± NA		
To assess clinical outcomes and tear cytokine levels in patients with moderate and severe MGD after treatment with oral minocycline and artificial tears versus artificial tears only[57]	Baseline	30	86.6 ± 53.8		
	2M artificial tear	30	101.5 ± 81.4		
	Baseline	28	88.3 ± 168.3		
	2M oral minocycline w artificial tear	28	72.5 ± 161		
This report describes a procedure that can be used to recover tears from the Schirmer strip for the measurement of multiple tear cytokines as well as MMPs by Luminex technology[68]				5	1150 ± 50†
This study analyzes tear cytokine levels and their clinical correlations in patients with moderate evaporative-type DED due to MGD.[208,209]		46	2000.0 ± NA*†		
				18	322.7 ± 33.5†
To compare tear cytokine and chemokine concentrations in asymptomatic control and DTS patients and determine the correlations between tear inflammatory mediators and clinical severity[67]	DTS	30	1510.0 ± 1671	14	176 ± 72
	DTS w/MGD	9	1303.0 ± 661	14	176 ± 72
	DTS w/o MGD	21	1657.0 ± 2393	14	176 ± 72
To determine the levels of 8 important cytokines and 1 chemokine in tears of patients with DED[193]		7	48508.6 ± 9397.3	7	16791.4 ± 2841.2

* Data estimated from Figure.
† Standard error.

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Table 6. Observed Differences of IL-17A Levels in Tears From DED Patients and Normals

Study Description	Groups and Interventions	Dry Eye *n*	Dry Eye Mean pg/mL ± SD	Normal *n*	Normal Mean pg/mL ± SD
To determine tear cytokine profiling data in a prospective case-control study in DED patients with or w/o HIV infection[66]	DE with HIV	34	20.1 ± 72.4		
	DE w/o HIV	32	215.9 ± 145.1		
To explore adaptive immune in patients with ADDE and LDDE[212]	ADDE	2	1.8 ± 1		
	LDDE	11	1.3 ± 0.5		
	Combined	7	1.4 ± 0.4		
	Generic			9	1.1 ± 0.2
To develop a tear molecule level-based predictive model based on a panel of tear cytokines and their correlation with clinical features in cGVHD in a controlled environmental research lab[174]		22	12.2 ± 12.4	21	20.9 ± 17.7
To investigate changes in signs, symptoms, and tear cytokines following punctal plug occlusion in patients with dry eye[65]	Baseline	29	0.6 ± 0.5		
	Wk 1 after punctal occlusion	29	1 ± 1.8		
	Wk 3 after punctal occlusion	29	0.6 ± 0.4		
To explore changes in lacrimal gland and tear inflammatory cytokines in TAO patients[206]	Active TAO	27	17.7 ± NA	32	9 ± NA
	Inactive TAO	21	11.8 ± NA		
To provide SOPs for measuring tear inflammatory cytokine concentrations. Randomized DE patients were treated with omega-3 or placebo for 3 mo[58]	DE w Ω3-baseline	7	53.2 ± 65.8	20	NA ± NA
	Placebo-baseline	7	151.8 ± 254.8		
	DE w Ω3-mo 3	7	181.1 ± 257.6		
	Placebo-mo 3	10	144.5 ± 314.4		
To determine cytokine and chemokine concentrations in the tears of patients with DED[207]	DES1	130	1.8 ± 1.5*	70	5 ± 1.2*
	DES2	130	1.1 ± 1.2*		
	DES3	130	1.8 ± 1.8*		
To assess clinical outcomes and tear cytokine levels in patients with moderate and severe MGD after treatment with oral minocycline and artificial tears versus artificial tears only[57]	Baseline	30	5.1 ± 4.9		
	2M artificial tear	30	4.8 ± 6.6		
	Baseline	28	4.5 ± 7.9		
	2M oral minocycline w artificial tear	28	1.8 ± 1.7		
This study analyzes tear cytokine levels and their clinical correlations in patients with moderate evaporative-type DED due to MGD[208]		46	40.0 ± NA*†		

* Data estimated from Figure.
† Standard error.

TNF-α, IL-6, IL-17a, and IL-8 in DED. Though a number of different cytokines/chemokines have been analyzed in tears of DED patients,[45–47] this review will focus on 4 cytokines that have shown to be consistently elevated in DED tears compared to non-DED controls, and thought to have a mechanistic role in DED: (1) TNF-α for general inflammatory status of the ocular surface[61] (Table 3), (2) IL-6, which has pro- and anti-inflammatory roles, may provide important information on ocular immune status and on treatment effect[62] (Table 4), (3) IL-17a, which is secreted by specialized T helper 17 (Th17) subpopulation[63] (Table 5), and (4) IL-8, which is important in chemotaxis to mediate macrophage and epithelial innate immunity[64] (Table 6). As can be observed in the Tables, a wide range of concentrations has been observed for these cytokines. For example, while tears from DED patients tested for IL-8 showed higher mean values for IL-8 compared to normals (Table 6), the reported concentrations ranged from 74 +_55 pg/mL[65] to 6518.3 +_4509.7 pg/mL[66] for DED and from 176 +_72 pg/mL[67] to 1150 +_50 pg/mL in normals.[68] While some of the variability, as with IC studies (Table 2), may be due to biologic variations, the technique used is likely an issue as well. However, the variation in concentrations observed with "normal" subjects is remarkable. It must be noted that under the umbrella of Luminex technology, studies have used assay kits from different manufacturers, different instruments, or even the same instrument with different panels or settings, which may contribute significantly to the wide range of concentrations observed for the same cytokine. Even after the sample processing is completed, data analyzed and reported with different stringent curve fitting models vary significantly, for example, best curve fitting, five-parameter curve fitting, four-parameter curve fitting, cubic spline fitting, or linear polation fitting (Milliplex Analyste User Guide). Few reports describe the analysis algorithm used. Therefore absolute concentrations are likely not comparable between studies and reporting percent change or ratios may be a more useful metric for reporting analyte levels in tears at baseline and with treatment in clinical trials.

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Table 7. Tear Osmolarity in Clinical Trials involving DED Patients

Study Description	Groups and Interventions	Dry Eye *n*	Dry Eye Mean mOsm/L ± SD	Normal *n*	Normal Mean mOsm/L ± SD
Multicenter prospective interventional placebo controlled double masked study. Treatment = 1680 mg EPA/560 mg DHA (2240 mg omega-3 total)[213]	Omega-3, baseline	54	326.2 ± 15.8		
	Omega-3, 12 wks	54	306.9 ± 12.1		
	Omega-3, 6 wks	54	309.4 ± 13.4		
	Placebo, baseline	51	326.0 ± 15.4		
	Placebo, 6 wks	51	317.0 ± 20.5		
	Placebo,12 wks	51	317.7 ± 19.7		
Cross-sectional association study investigating predictors of discordance between signs and symptoms of DED[3]	Min	648	275.0		
	Median	648	314.0		
	Max	648	390.0		
Cross-sectional study looking at clinical characteristics of DED with chronic pain syndromes[214]	DED + chronic pain	74	309.4 ± 1.9*		
	DED + no chronic pain	351	311.6 ± 0.9*		
Cross-sectional study 3 consecutive osmolarity measurements taken at 1-min intervals in a session[79]	Sjögren's syndrome (SS)	18	307.0 ± 15.8	8	301.0 ± 10.5
	Blepharitis	11	304.0 ± 14.6		
Study to compare tear osmolarity measurements between two different methods (TearLab, Vapro5520)[215]	TearLab normal			52	299.2 ± 10.3
	Vapro 5520 normal			48	298.4 mmol/kg ± 10.0
Study to evaluate tear osmolarity in Non-SS and SS DED patients and compare to normals[80]	Non-SS DED	39	296.8 ± 46.5	44	303.5 ± 12.9
	SS DED	39	303.36 ± 17.2		
Cross-sectional study evaluating tear osmolarity in DED patients with SS vs. normals[77]	SS DED	20	301.9 ± 11.4	20	294.9 ± 8.3
Observational study looking at TO and other clinical findings in DED and normals[81]	DED	129	308.9 ± 14.0	71	307.1 ± 11.3
Study assessing the diagnostic performance of tear osmolarity[78]	Mild DED	55	298.1 ± 10.6	25	295.5 ± 9.8
	Moderate DED	57	306.7 ± 9.5		
	Severe DED	29	314.4 ± 10.1		
Study comparing two different techniques of testing tear osmolarity (TearLab and Clifton) with tests done in DED and normals[216]	DED Tearlab	15	321.0 ± 16.5	21	308.0 ± 6.2
	DED Clifton	15	323.0 ± 14.7	21	310.0 ± 7.2

* Standard error of the mean (SEM).

Point of Care Biomarkers

In contrast to the above discussed biomarkers, tear osmolarity and matrix metalloproteinase-9 (MMP-9) are the only biomarkers that are approved by the United States Food and Drug Administration (FDA), have commercially available point-of-care measurement devices, and have the potential to provide objective metrics in DED patient care as well as for clinical research.[8,69–71]

Tear Osmolarity

High tear osmolarity is considered as one of the "core mechanisms" of DED, and can lead to an increase in inflammation and further damage to the ocular surface.[1,72,73] Measurement of tear osmolarity recently has become attractive because of the availability of commercial machines that are FDA approved for point-of-care use.[74] These machines can measure osmolarity with very small tear volumes (nanoliters) and are easy to use.[72,75] Studies have shown that increased osmolarity is potentially diagnostic of DED (Table 7),[72,76–78] but there is wide variation from study to study and, in some cases, the DED readings are similar to normal.[79–81] Furthermore, the cutoff value for DED is not clear[82,83] (Table 7). For instance, one study suggested the cutoff value was 308 mOsm/L,[80] while another reported 316 mOsm/L.[84] Other studies have not shown correlation with clinical signs and symptoms.[81,85] Though studies have shown the reliability of some of the available devices using standardized solutions,[74,86,87] this does not necessarily indicate reliability when tears are sampled from the ocular surface. Rather than determining one value, it may be best to repeat measurements for each eye to demonstrate stability or lack of stability, indicative of DED.[88,89] However, variation in measurements taken within a single session can be high, even among normal subjects.[79]

In summary, it remains to be seen if tear osmolarity has the potential to be an objective biomarker for use in clinical trials; further studies will need to specifically elucidate how it can be used, including number of tests per subject, cutoff point, and correlation with clinically relevant findings.

Matrix Metalloproteinases-9 (MMP-9)

MMP-9 is an enzyme important for tissue remodeling and has important roles in the inflammatory process of DED.[90,91] A number of studies show high levels of MMP-9 in tears in DED (Table 8). The FDA-approved office test, called InflammaDry, provides results as positive or negative using a cut-off level of 40 ng/mL in tears. One study has suggested this test provides 85% sensitivity and 94% specificity in diagnosing DED.[92] However, the value of MMP-9 as a biomarker for DED is challenged by the

Table 8. MMP-9 in Tears of DED Patients

Study Description	Sample Size	Assessment Method	Baseline	Intervention
Retrospective single center chart review of DE pts. If MMP-9+ then treated with cyclosporine 0.05%, omega-3, and artificial tears[217]	100 DED	Inflammatory	MMP-9+: 60% (60/100)	MMP-9+:54% (26/48) became MMP9- with treatment MMP-9-: 6% (2/30) became MMP9+ (only 78% of total returned for follow-up visit)
Study correlating MMP-9 test with clinical findings in DED[218]	47 DED, 54 normals	Inflammatory	40% DED MMP-9+ 5% of normals MMP-9+. MMP-9 positivity correlated well with signs and symptoms of DED	n/a
DED post-LASIK patients vs. normals for a point-of-care test for MMP-9 and ELISA test for MMP-9 concentration[219]	14 DED post LASIK, 34 normals	Inflammatory + ELISA	57% post-LASIK were MMP-9+ with Inflammatory, 0% normals MMP-9+ with Inflammatory DED: 52.7 ± 32.5 ng/mL MMP-9 (50% >40 ng/mL) Normal: 4.1 ± 2.1 ng/mL MMP-9	n/a
Multicenter placebo controlled double masked study on the effect of Omega-3 on MMP-9 and other clinical findings in DED[213]	105 DED (54 Omega-3, 51 Placebo)	Inflammatory	48/105 (46%) MMP-9+ Omega-3 = 28 MMP-9+Placebo = 20 MMP-9+	Omega-3 = 68% **(19/28)** became MMP-9-Placebo = 35% **(7/20)** became MMP-9-
Study comparing signs and symptoms of MMP-9+ vs. MMP-9- DED patients[93]	128 DED	Inflammatory	39% MMP-9+ no statistically significant difference of signs and symptoms between MMP-9+ and MMP-9-	n/a
Determine the negative and positive agreement of a point-of-care MMP-9 test in confirming the diagnosis of DE[220]	146 DED, 91 normal	Inflammatory	Total positive agreement of 86% (126/146), negative agreement of 97% (88/91)	n/a
Determine the negative and positive agreement of a point-of-care MMP-9 test in confirming the diagnosis of DE[92]	143 DED, 63 normal	Inflammatory	Sensitivity of 85% (in 121 of 143 patients), specificity of 94% (59 of 63)	n/a
MMP-9 levels in tears of patients with conjunctivochalasis (CCh; DED or nonDED) before and after surgery vs. normals[221]	12 CCh, (4 CCh + DED), (8 CCh + non-DED), 5 normals	ELISA	CCh (DED) = 254.55 ± 73.70 ng/mL, CCh (non-DED) = 207.74 ± 74.89 ng/mL, normal = 20.32 ± 5.21 ng/mL	CCh (DED) = 109.05 ± 5.27, CCh (non-DED) = 39.1 ± 20.6
Study looking at MMP-9 levels in different ocular surface diseases[210]	77 Ocular surface disease (13 blepharitis, 19 allergic eye disease, 20 DED, 25 CCh) 18 normal	ELISA	Blepharitis = 58.56 ± 30.1 ng/mL, allergic eye disease = 132.33 ± 77.99 pg/mL, DED = 97.25 ± 49.5 ng/mL, CCh = 126.40 ± 101.97 ng/mL, normal = 23.61 ± 17.4 ng/mL	n/a

observations that there is lack of correlation among MMP-9, other standard tests, and disease symptoms.[93] In addition, elevated MMP-9 can be associated with other ocular surface diseases involving inflammation, such as ocular allergy.[69]

MMP-9 testing may not be diagnostic for DED but it has the potential to enhance patient selection in clinical trials, especially those evaluating anti-inflammatory treatments. Further research evaluating repeatability in DED and normals without change in intervention, as well as studies correlating clinical tests with DED signs and symptoms, will contribute to understanding its usefulness.

Ocular Imaging

Imaging may provide minimally invasive metrics about the ocular surface and meibomian glands (MG), and possibly a

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better differentiation between aqueous deficiency and evaporative dry eye.[1,94]

Tear Film Stability and Tear Meniscus. Two features of tears, tear film stability and tear meniscometry, have been shown to be affected in DED.[1] The traditional method to assess tear breakup time is through slit-lamp examination using fluorescein dye (FBUT).[1] FBUT is measured by observing for dark spots on the ocular surface through a slit-lamp with incident cobalt blue filtered light. The fluorescein illuminates the tear film and the dark spots indicate that the tear film has begun to break up.[95] However, this traditional test, though considered objective, likely is impacted by placement of the drops, concentration, volume of fluorescein used, and low test repeatability and reproducibility.[94] Newer imaging technologies offer minimally invasive testing methods since, typically, no eye drops or contact with the eye are involved, and analysis provides automated numeric output.[8] Noninvasive tear breakup time (NITBUT) is measured through the use of computer software that analyzes reflections of placido rings on the ocular surface. Rapid distortion of the rings is indicative of tear film instability and high NITBUT.[96]

NITBUT, such as that obtained using the Oculus Keratograph, has been shown to correlate with DED and provide statistically significant differences between DED and normals.[97–100] Recent studies have shown good intraexaminer repeatability and interexaminer reproducibility of NITBUT in normals and DED patients.[99,101] However, there are varying results comparing NITBUT with the traditional FBUT.[99,102–106] There also may be variability depending on the machine used.[107] More research is needed to determine which method is the better diagnostic tool for DED.

Tear meniscus height (TMH) is a common aspect of tear meniscometry that can be measured noninvasively using infrared light to capture an image and then measuring the height of the tear meniscus with an electronic ruler,[108] or with optical coherence tomography (OCT), which uses high wavelength light waves to take images of the anterior segment that then are analyzed,[109] and also by briefly touching the edge of the lower tear meniscus using a specially designed meniscometry strip.[110] TMH has been shown to correlate with DED and the traditional measurements for DED.[97,110–120] Some studies suggest there is good repeatability and reproducibility of TMH measurements in DED and normals.[101,114,115,118,120–122] However, TMH taken with different machines may not be comparable.[111,115,121]

In addition to TMH, tear meniscus cross-sectional area (TMA) and tear meniscus depth (TMD) also are common parameters used to observe the menisci of DED patients.[120] TMA and TMD are measured using OCT, similarly to TMH. Since some user input is needed to designate the borders of the tear film to measure, there is some subjectivity to the measurements. However, some studies have shown repeatability and reproducibility in using OCT to measure TMA and TMD.[120,122] They also have been shown to correlate with DED and traditional measurements for DED.[116,119,120] However, TMA and TMD measurements using OCT have not yet been incorporated in any multicenter clinical trial.

Overall, there is no consensus on parameters to be evaluated for tear meniscometry, and it is not clear which would be the most clinically significant.

Meibomian Gland. MGs have an important role in providing lipids to the tear film, which helps to retard the evaporation of tears from the ocular surface.[123] MG dysfunction (MGD), defined by the International Workshop on MGD as a "chronic, diffuse abnormality of the MGs, commonly characterized by terminal duct obstruction and/or qualitative/quantitative changes in the glandular secretion," is a leading cause of evaporative DED.[1,123–125] Recently, imaging of the MGs under infrared illumination has become easier to perform and can be included in clinical trial testing,[126–129] but may not be sufficient for diagnosis of MGD or DED. Some studies suggest MG dropout, using direct- or transillumination of the lid, shows a strong relationship between MGD and DED.[130–134] However, interpretation of meibography is just beginning to be developed and further work will be needed to validate mechanisms of analysis, including development of automated systems and/or reading centers, and correlation of MG changes with clinical findings.[135,136]

Lipid Layer. The lipid layer has a critical role in tear film stability and the maintenance of ocular surface health.[123,137,138] Alterations in the lipid layer thickness (LLT) have been shown to be a possible good indicator of DED,[97,98,139,140] and specifically of MGD.[141,142] The lipid layer of the tear film can be assessed using simple, noninvasive imaging technology, such as an interferometer.[97] Different studies have found variation in LLT measurements between DED patients and normals; we might expect DED patients to have thinner LLT than normals,[142,143] but this is not always observed.[144] Further research is needed to demonstrate reliability, repeatability, reproducibility, and validity of LLT in DED.

Bulbar Redness. Bulbar redness is a nonspecific ocular response due to vasodilation of the conjunctival and/or anterior scleral blood vessels[145] and is observed in DED and often is a complaint of patients with DED.[146] New imaging technology has been developed that automatically provides a bulbar redness score, and some research suggests that it correlates with patient and observer grading (Gadaria-Rathod N, et al. *IOVS*. 2013;54:ARVO E-Abstract 527).[147] Whether this can serve as a validated biomarker for DED remains to be seen.

Corneal Surface Anatomy. Morphologic changes of the corneal epithelium and sub-basal nerves have been shown to occur in DED.[148–155] This has been studied using in vivo confocal microscopy, which allows visualization of the layers of the cornea.[156] Some effort has been made to identify potential predictive anatomic biomarkers on the corneal surface and the MGs, primarily using confocal microscopy.[149,151] Several groups also are investigating the potential applications of MultiPhoton microscopy for studying ocular surface anatomy.[157]

The feasibility of using confocal microscopy for assessing acinar density and diameter, secretion reflectivity, and periglandular inflammation for diagnosis of MGD has been explored by some groups.[158–160] A comparison between normal and MGD patients regarding the aforementioned morphologic structures and cells showed the potential to diagnose MGD with high sensitivity and specificity.[158] Confocal microscopy showed morphologic abnormalities and inflammatory changes in MGs of patients with Sjögren's syndrome and MGD that was not easily distinguishable by the usual clinical exams.[160]

Visualization of corneal nerve density may be potentially important where signs do not match symptoms (i.e., patients with dry eye symptoms but with a normal standard clinical examination),[149,161] and may be a sign of neuropathic pain.[148,155,161–163] However, the relationship between DED and corneal nerve changes is not clear. One study has reported that improvement in corneal fluorescein staining score and symptomatology following treatment was evident only in the patient group showing near normal corneal sub-basal nerve fiber length.[149] In contrast another study showed that corneal sub-basal nerve fiber length was similar in the patient group that showed clinical improvement and the patient group with no clinical improvement following treatment.[151] Notably, though nerve length was similar, sub-basal dendritic cell density was decreased in the patient group that responded positively to treatment.[151] Nonetheless,

including corneal nerve changes in DED clinical trials, would require extensive instruction for each site and support of automated analysis. Corneal sensitivity testing, an alternate means of measuring corneal innervation, also would be helpful.[152,164,165] However, there are only limited data on the repeatability and reliability of available esthesiometers, such as the Cochet-Bennet[166] and Belmonte[167] esthesiometers. Measuring peripheral cutaneous sensitivity also may be helpful in understanding the pain of DED and provide a more uniform rating of ocular symptoms, but has not been evaluated extensively in humans as yet.[168]

Overall, data suggest that many of the ocular imaging measurements are repeatable; however, further investigation on the correlation with other signs and symptoms of DED will likely better elucidate the role of these methods for clinical trial use as objective metrics.

Genetics

Genetics of human disease is a rapidly growing area for developing biomarkers to identify risk of disease, response to treatment, and so forth. However, little has been done to date on DED,[169–171] primarily because DED still is considered a multifactorial disease. Genetic studies likely will be more useful when we can better characterize the DED patient population and identify subgroups.

Considerations for Developing Validated Biomarkers With Objective Endpoints for DED

Considerable efforts already have been initiated, but additional work is needed before we have validated biomarkers with minimally invasive objective metrics that will be generally acceptable in clinical research and for patient management in DED. Results must be reproducible and comparable with multiple studies on the same biomarker. For instance, how useful would testing for cholesterol be if the same subject got significantly different results depending on what laboratory was used? More studies to validate standard operating procedures would add to the acceptance of biomarker usage. Research publications must provide greater detail to allow easier review and comparison with other research on the same biomarkers. Some recommendations include:

- Subjects: Studies often described subjects as "healthy controls" and "dry eye patients." The listing of inclusion and exclusion criteria will help with interstudy comparison of data among subcategories of patients along the DED spectrum.
- Methodology: Details of techniques used, including quality control, will allow other researchers to repeat the experiment/study which would add validity to using the particular biomarker.
- Result reporting: Focusing on statistical differences, rate of change rather than absolute values of the reported data, may be more useful. For example for flow cytometry, since gating and analysis still are not automated, it probably is best to look at trends (percent change or ratios) rather than absolute percentage or AUF or any other metric. Similarly for tears, where concentrations appear to vary significantly, between studies, reporting of trends along with concentration will be more useful.
- Clinical relevance: Does this biomarker provide important information to the clinical setting?
- Reproducibility: Including repeatability of test, interobserver agreement.
- Purpose of biomarker: Need to state the use of the biomarker, for example, for diagnosis, establishing severity, assessing change, clinical trial surrogate endpoint, and so forth.
- Clearly stating sources of potential bias: This would include whether biomarker processing and analysis were masked to clinical attributes and/or treatment of the subjects, funding sources, and all potential sources of conflict of interest.
- Ease of use: For research setting, multisite clinical trials, and/or in-office patient care.
- Reading centers: Provide information on standard operating procedures.
- Reporting: Consider making available information on biomarkers that were not correlated with the disease as well as those that were; development of standardized reporting methodologies.
- Systemic biomarkers: Not discussed in this review, but a potential source of biomarkers to identify underlying pathogenesis and patients at risk for DED, such as markers for Sjögren's Syndrome.[172]
- Statistical Analysis: Methodology should be clearly stated.

Summary/Conclusion

There is a growing body of research and interest in developing biomarkers for DED, for understanding underlying pathophysiology of DED, diagnosis, classification, and treatment efficacy, and for endpoints in clinical trials. Basic research in DED with tissue/organ cultures and animal models will continue to provide direction for potential biomarkers that then will need to be evaluated in patients to validate their role in human disease. All studies, including those from single centers, using small sample size, and so forth, in humans with DED have taken our understanding further and point to areas that would benefit from larger masked studies to validate a biomarker. Multisite clinical trials to date have incorporated biomarkers to monitor inflammatory changes and support drug mechanisms of action. In-office testing now is available for some biomarkers with growing information on their potential usefulness for improved clinical care. Going forward, composite "scores" incorporating several biomarker measurements may be most useful. Our collective efforts have been successful in providing a roadmap for future work in biomarkers in DED. Biomarkers with minimally invasive and reproducible objective metrics will provide the key to future paradigm shifts in understanding of the underlying causes of DED and approaches to treatment of DED.

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