

Prospective, Multicenter, Clinical Evaluation of Point-of-Care Matrix Metalloproteinase-9 Test for Confirming Dry Eye Disease

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Purpose: The aim of this study was to determine the negative and positive agreement of a point-of-care matrix metalloproteinase-9 test in confirming the diagnosis of dry eye and to evaluate the ease of use by untrained ophthalmic technicians.

Methods: The study was a prospective, sequential, masked, clinical trial with 4 clinical trial sites. The InflammaDry test was compared with the clinical assessment of tear break-up time, Schirmer tear testing, and corneal staining for the confirmation of dry eye, both with and without the inclusion of the Ocular Surface Disease Index (OSDI), as a confirmatory test.

Results: The study enrolled 237 patients. If the OSDI is included in the definition for mild dry eye, the InflammaDry test was shown to have a total positive agreement of 81% (127/157) and a negative agreement of 98% (78/80). The removal of the OSDI shifted the categorization of 11 patients previously considered positive for dry eye to become categorized as negative for dry eye. If the OSDI is excluded from the definition of dry eye, the InflammaDry test demonstrates a positive agreement of 86% (126/146) and a negative agreement of 97% (88/91) against the clinical assessment.

Conclusions: The InflammaDry test demonstrates a high positive and negative agreement for confirming suspected dry eye disease. In addition, the test was safely and effectively performed by untrained operators. These findings support the intended use of the InflammaDry test as an aid in the diagnosis of dry eye.

Key Words: dry eye, matrix metalloproteinase, MMP-9, inflammation, ocular surface, clinical study

(*Cornea* 2014;0:1–7)

According to the Dry Eye Workshop (DEWS) report, dry eye is a multifactorial disease of tears and the ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface.¹ It is also accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.¹ Dry eye is affected by the relationship between the amount of tears produced, rate of tear evaporation, and the presence or absence of inflammation.

Symptoms alone are inadequate for the diagnosis of dry eye, because the same symptoms can be experienced with a range of ocular surface conditions and tear film disorders. Additionally, both symptoms and signs can vary greatly depending on the environmental conditions to which patients are exposed in their daily lives. Only 57% of symptomatic patients have been shown to have objective signs of dry eye.^{2–6} This finding has been attributed to the symptoms preceding the signs, or the differing etiology and pathophysiology of dry eye.⁷

The most common objective diagnostic test for dry eye, the Schirmer tear test, has been in use for >100 years.⁸ The Schirmer tear test lacks standardization⁹ and is inaccurate and unrepeatable because of the reflex secretion produced by its irritating nature.¹⁰ This test is limited to the measurement of tear production,¹¹ while overlooking the evaporative aspects of dry eye.⁹ The discomfort, time inefficiency, and lack of sensitivity associated with Schirmer tear testing further limit its use. However, the low cost of strips and ease of application has led the Schirmer tear test to become the most common clinical test for lacrimal secretory function in dry eye.

Tear break-up time (TBUT) measurement with fluorescein is another widely used technique for the clinical diagnosis of dry eye. TBUT is considered to be more reliable than the Schirmer test, because it is repeatable and minimally invasive; however, the instillation of a topical anesthetic can destabilize the tear film and lead to an artificially accelerated TBUT.^{12–14} Further, all forms of tear break-up measurement fail to give direct information on tear production.⁹ Because support staff assess most patients before the clinician's evaluation, the ability to accurately perform TBUT or corneal staining before the application of a topical anesthetic is limited by patient flow.

Received for publication January 8, 2014; revision received April 12, 2014; accepted May 3, 2014.

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The design of the study and collection of data were sponsored by Rapid Pathogen Screening Inc (RPS). No sponsorship was received for the analysis and interpretation of study data. R. Sambursky has an affiliation with RPS but was not an investigator. The remaining authors have no funding or conflicts of interest to disclose.

Clinical Trial Registration—URL: <http://www.clinicaltrials.gov>. Unique identifier: NCT01692964.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.corneajrnl.com).

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Ocular surface staining with vital dyes such as Rose Bengal, lissamine green, and fluorescein has also been used to diagnose dry eye disease.^{15–17} The disadvantage of staining is that dry eye cannot be clinically differentiated from other conditions that lead to ocular surface staining such as medication toxicity (including topical anesthetic), poor lid apposition, underlying infection, or trauma.¹⁸ Additionally, these staining techniques are not likely to be used in early dry eye or mild dry eye.¹⁸

Outside the research setting, the majority of dry eye-affected patients encounter an ophthalmic technician as part of the initial patient work-up. Typically, patients report on the presence of symptoms. The Ocular Surface Disease Index (OSDI) is a questionnaire that was developed to identify and quantify the common symptoms associated with dry eye as a means to measure the therapeutic effect of a dry eye medication.¹⁹ The OSDI consists of 12 questions, each scored by the patient. This assessment has been found to be subjective, to lack specificity, and to be prone to operator-dependent analytical errors, preventing it from routine clinical use.^{1,20–22}

Increased osmolarity has been described in patients having dry eye,²³ because reduced tear secretion and/or increased evaporation results in the loss of fluid that isotonic tears cannot overcome.²⁴ Elevated tear osmolarity is considered an important indicator of dry eye. Specifically, normal tear osmolarity is understood to be reflected by tears in the range of 275 to 307 mOsm/L, whereas hyperosmolarity is indicated by a tear fluid osmolarity ≥ 308 mOsm/L in 1 or both eyes, or a >8 mOsm/L difference in tear osmolarity between the eyes.²⁵

Versura et al²⁶ measured tear osmolarity in 25 normal subjects and in 105 dry eye-affected patients and found that the normal values were 296.5 ± 9.8 mOsm/L. Values were shown to increase as dry eye severity increases (mild to moderate to severe dry eye, respectively: 298.1 ± 10.6 vs. 306.7 ± 9.5 vs. 314.4 ± 10.1 , $P < 0.05$).²⁶ In a study performed by Lemp et al,²⁵ osmolarity was determined to be more sensitive than other clinical signs. In this study, the most sensitive threshold between normal and mild or moderate subjects was found to be 308 mOsm/L, whereas the most specific threshold was found to be at 315 mOsm/L.²⁵ At a cutoff of 312 mOsm/L, tear hyperosmolarity exhibited 73% sensitivity and 92% specificity.²⁵ In another study performed by Tomlinson et al²⁷, a cutoff value of >316 mOsm/L, derived from the distribution of osmolarity values, was used to diagnose dry eye disease with an effectiveness of 73% sensitivity, 90% specificity, and 85% positive predictive value. Osmolarity measurements have been shown to vary between sample measurements, and reflect the inherent tear film instability of dry eye disease.^{28,29}

Matrix metalloproteinases are proteolytic enzymes that are produced by stressed epithelial cells on the ocular surface. Specifically, matrix metalloproteinase-9 (MMP-9) is an inflammatory marker that has consistently been shown to be elevated in the tears of patients with dry eyes. Elevated MMP-9 levels in patients with moderate to severe dry eye disease correlate with clinical examination findings.³⁰ Altered corneal epithelial barrier function is the cause for ocular irritation and visual morbidity in dry eye disease. MMP-9 seems to play a physiological role in corneal epithelial desquama-

tion. Although MMP-9 does not provide information about tear production, increased MMP-9 activity in dry eye may contribute to deranged corneal epithelial barrier function, increased corneal epithelial desquamation, and corneal surface irregularity.³⁰

MMP-9 activity is also elevated in ocular surface conditions such as blepharitis and Sjögren syndrome; however, these underlying conditions lead to the development of inflammatory dry eye disease that would be accurately detected using the InflammDry test.³¹ Other conditions such as infection,³² allergy,³³ pterygium,³⁴ and conjunctivalchalasis,³³ have been associated with an elevated level of MMP-9, but are readily clinically differentiated from dry eye disease. According to an analysis performed by Sambursky and O'Brien,³⁵ normal levels of MMP-9 (nanograms per milliliter) in human tears range from 3 to 40 ng/mL.

The aforementioned characteristics and limitations of dry eye diagnostic tools suggest that diagnosis of this multifactorial disease may be improved upon with a protocol inclusive of multiple diagnostic tools (Fig. 1). A new single use, noninvasive, inexpensive, disposable test that can accurately aid in the confirmation of the diagnosis of dry eye, such as InflammDry, provides valuable information without imposing infrastructure challenges. InflammDry will cost less than the test's anticipated reimbursement. Using direct sampling microfiltration technology, the InflammDry immunoassay detects elevated levels of MMP-9 (≥ 40 ng/mL) in tears to confirm the diagnosis of dry eye disease.

The InflammDry test was evaluated in a prospective, multicenter, masked clinical trial to determine the negative and positive agreement of the test in confirming the diagnosis of dry eye disease. The term positive agreement is used in lieu of sensitivity when reporting the performance data of a diagnostic test against which there is no single diagnostic gold standard to compare. Similarly, the term negative agreement is used in lieu of specificity when reporting the performance data of a diagnostic test against which there is no single diagnostic gold standard to compare. The clinical trial took place over a 7-month period and used untrained ophthalmic technicians (operators) at 4 clinical sites representing a combination of academic and private practices.

MATERIALS AND METHODS

Study Design

The study design was a prospective, sequential, masked, clinical trial. Those patients who were clinically determined by an ophthalmic clinician to meet enrollment criteria were included in the study (see Table, Supplemental Digital Content 1, <http://links.lww.com/ICO/A230>).

Institutional review board approval was first obtained. A subject's participation was limited to a 1-time event that occurred at the time of specimen collection. There were no follow-up visits necessary for this study. The subjects did not incur any costs associated with the study procedures. Before starting the clinical trial, each site conducted positive and negative external controls on the InflammDry test to confirm the functionality of the test reagents.

DRY EYE DIAGNOSTIC PROTOCOL

Initial Visit

NOTE: The InflammDry test may be performed independent of other testing. If TearLab osmolarity testing is performed, it must be performed before InflammDry or any other testing. TearLab test results may be negatively impacted by reflex tearing. Reflex tearing does not affect InflammDry test results. However, InflammDry must be performed before the installation of any drops.

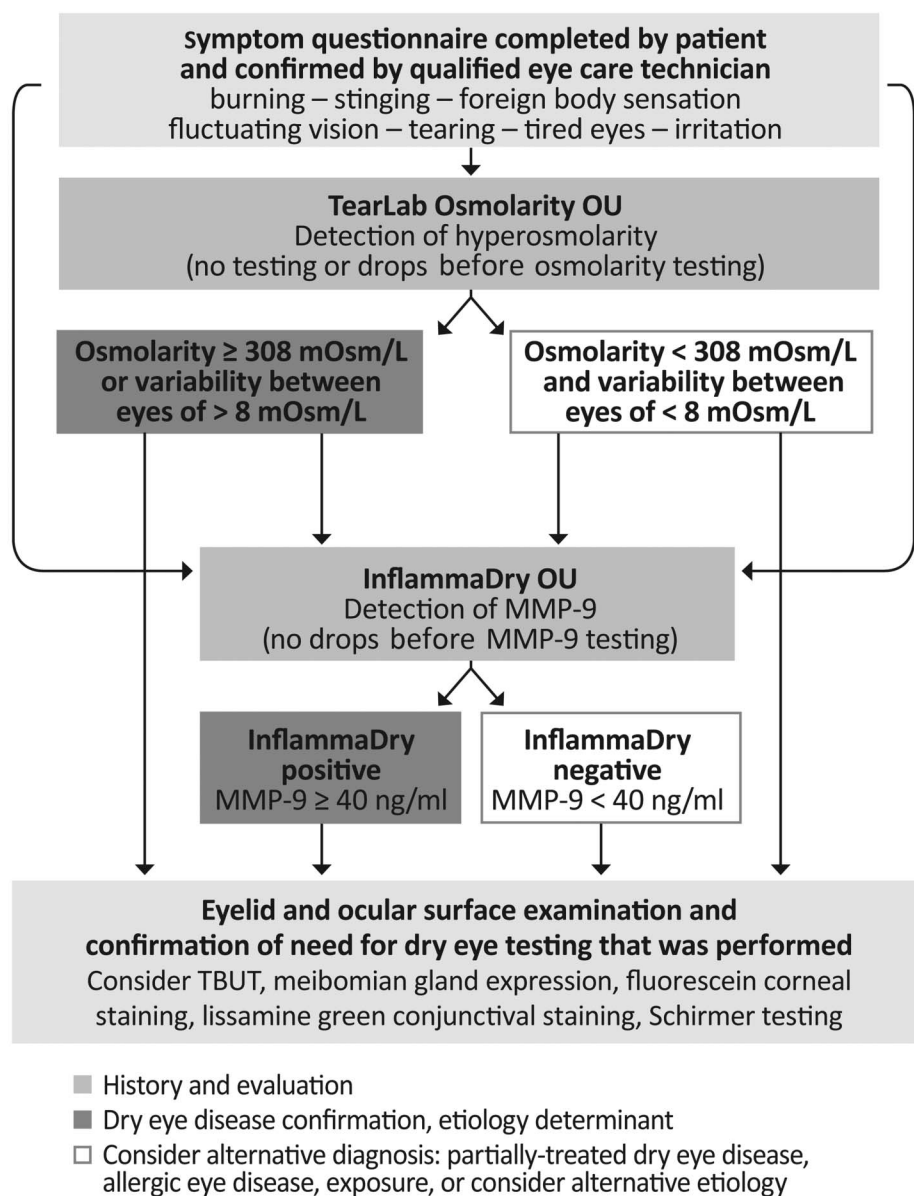


FIGURE 1. Dry eye diagnostic protocol, initial visit.

At the office visit, before any study-related procedures, the subjects were screened through a standard of care history and a slit-lamp examination. After determining that the patient qualified for enrollment into the study, an investigator or delegated study personnel obtained an informed consent. Upon obtaining the subject's consent, study personnel interviewed the subject and documented data, including the subject's age, gender, race, and patient history, on a sponsor-provided Case Report Form.

Study Visit Testing

Study testing was done on the subject's more symptomatic eye. If no difference existed, the right eye was tested.

InflammaDry

The ophthalmic technician (operator) performing the InflammaDry test was limited to the manufacturer's instructions for use as their only resource. In addition, the untrained operator was unaware of the patient's clinical history and did not perform or learn of any subsequent test results including the OSDI survey, TBUT, corneal fluorescein staining, or the Schirmer tear test.

First, to perform the InflammaDry test, the untrained operator collected a tear sample from the patient's palpebral conjunctiva. The operator then gently dabbed the provided sample collector in multiple locations along the palpebral conjunctiva; they released the lid after every 2 to 3 dabs to allow the patient to blink. This was repeated 6 to 8 times, and then the sampling fleece was allowed to rest against the conjunctiva for at least 5 seconds or until the sampling fleece was saturated with tears (5–10 μ L). Adequate saturation of the sampling fleece was indicated by a pink color or glistening appearance. Next, the test was assembled by snapping the sample collector onto the provided test cassette. The assembled test was then dipped into the provided test buffer solution for 20 seconds for activation. Last, after 10 minutes had elapsed, the test values were read. The presence of 1 blue line and 1 red line in the test result window indicate a positive test result ($\text{MMP-9} \geq 40 \text{ ng/mL}$). The intensity of the red line is directly related to the amount of MMP-9 present; thus, mild dry eye is associated with fainter lines than more severe dry eye is. The presence of a red line of any intensity confirms the presence of elevated MMP-9. One blue line indicates a negative test result ($\text{MMP-9} < 40 \text{ ng/mL}$). All InflammaDry tests were analyzed within 24 hours of activation.

Per the trial protocol, invalid test results were to be documented and reported to show usability, but these results were not to be included in any positive or negative agreement calculations. There were no invalid test results in this study. The InflammaDry test has built-in procedural controls, including a blue control line. In the unlikely event that the test is not run properly or the reagents do not work, the blue control line will not appear, indicating an invalid test result.

Ocular Surface Disease Index

To evaluate patient-reported symptoms associated with dry eyes, the OSDI survey was completed. OSDI scores range

from 0 to 100, where 0 indicates no disability and 100 indicates complete disability.

Fluorescein Tear Break-up Time

The TBUT was evaluated 2 minutes after the inferotemporal bulbar conjunctiva was touched with a 1-mg sodium fluorescein strip (wet with preservative-free saline). Subjects were instructed to blink, and the precorneal tear film was examined under blue-light illumination with a biomicroscope and 10 \times objective. The interval between the blink and the appearance of the first dark spot or discontinuity in the precorneal fluorescein-stained tear layer was then recorded. Three separate readings were taken for each eye, and the results were averaged.

Corneal Fluorescein Staining

The ocular surface was also examined 2 minutes after fluorescein instillation into the tear film as described above. The Oxford grading scheme was used to grade the intensity of corneal fluorescein staining in 5 different zones of the conjunctiva and cornea (central, superior, temporal, inferior, and nasal). The result was based on the number of dots on a 5-point scale: no dot = 0; 1 to 5 dots = 1; 6 to 15 dots = 2; 16 to 30 dots = 3; and >30 dots = 4. Additionally, if there was 1 area of confluence, 1 point was added. Two points were added if there were ≥ 2 areas of confluence or if filamentary keratitis was present.

Schirmer Tear Test

Topical anesthetic was then introduced into the inferior fornix. The Schirmer tear test was performed by placing Schirmer test strips (Tear Flo, Alta Loma, CA) over the lower lid margin, at the junction of the lateral and middle thirds, for 5 minutes. The strip wetting was measured and recorded in millimeters. If complete wetting of the strips occurred before 5 minutes, and if the person administering the Schirmer tear test felt that an initial response occurred because of reflex tearing, then it was documented and the test was repeated after measures had been taken to prevent reflex tearing (ie, reanesthetizing the eye, removing any potential irritants, and waiting a few minutes). If, after every effort to prevent reflex tearing, a similar complete wetting of the strips occurred before 5 minutes, then the result was documented and accepted.

Clinical Assessment and Dry Eye Disease Severity

The InflammaDry test was compared with the clinical assessment as specified in Table 1. Derived from the DEWS criteria, the clinical assessment was developed to represent a combination of symptoms and signs. The clinical trial used the same metrics for TBUT, Schirmer tear testing, and corneal staining as described in the DEWS criteria. However, conjunctival injection, conjunctival staining, and the presence of meibomian disease were not tested or used to characterize the severity of dry eye disease. In general, the worst severity for

TABLE 1. Dry Eye Disease Severity Grading

Clinical Testing	Negative Control	Mild Grade 1	Moderate Grade 2	Moderately Severe Grade 3	Severe Grade 4
OSDI score*	<13	≥13	≥13	≥13	≥13
TBUT, s	>10	≤10	≤10	≤5	0 (Immediate)
Staining (0–5)	0 (None)	0 (None)	1–2	3	≥4
Schirmer, mm/5 min	>10	≤10	≤10	≤5	≤2

*Study data were analyzed with and without the inclusion of the OSDI as a confirmatory test for dry eye.

any sign tested determined the overall severity. Patients were categorized to the highest severity level at which all required criteria were satisfied. Patients who did not meet all the required clinical criteria for a given severity grade were considered to be at the next lower grade.

Sample Size Justification and Statistical Significance

The study concluded with a total of 237 patients, 146 in the dry eye group and 91 in the control group. Using a binomial 1-sided test against 75% that was significant at the 0.05 alpha level, the sample size of 146 patients provided >90% power to test against a null hypothesis of 75% positive agreement. In the control group, the sample size of 91 patients provides 83% power to detect a negative agreement of at least 75%. The InflammaDry test demonstrated a positive agreement of 86% (126/146) with a *P* value of <0.0001 and 95% confidence interval of 0.80 to 0.91 and a negative agreement of 97% (88/91) with a *P* value of <0.0001 and 95% confidence intervals of 0.91 to 0.99.

RESULTS

The study enrolled 237 patients consisting of 164 women and 73 men between the ages of 18 and 94 years with a mean age of 53 years. The categorization of dry eye severity was analyzed with and without the inclusion of the OSDI as a confirmatory criterion for the presence of dry eye disease. Eleven patients were found to have an elevated OSDI without any objective confirmatory testing as shown in Table 2.

Table 3 demonstrates the categorization of dry eye-affected patients enrolled based on the signs they had presented, with and without the inclusion of the OSDI as a confirmatory criterion for the presence of dry eye. The removal of the OSDI shifted the categorization of 11 patients previously considered positive for dry eye to become categorized as negative for dry eye.

Table 4 demonstrates the performance of the InflammaDry test against the clinical assessment that both includes and excludes the OSDI as a confirmatory test for the presence of

dry eye. If the OSDI is included in the definition for mild dry eye, the InflammaDry test was shown to have a total positive agreement of 81% (127/157) and a negative agreement of 98% (78/80). If the OSDI is excluded from the definition of dry eye, the InflammaDry test demonstrates an 86% (126/146) positive agreement and a 97% (88/91) negative agreement against clinical assessment as an objective confirmatory criterion for the presence of dry eye.

DISCUSSION

According to the American Academy of Ophthalmology's Preferred Practice Pattern for dry eye disease, many ocular surface diseases produce symptoms that are similar to those associated with dry eye. Although it is useful to identify characteristics of the symptom causative factors, such as adverse environments, prolonged visual efforts, or ameliorating circumstances, tests are required to confirm the diagnosis of dry eye disease. The 2 major factors that contribute to dry eye independently, deficient aqueous tear production and increased evaporative loss, may also be present together.¹

The preferred practice pattern states that no single test is adequate for establishing the diagnosis of dry eye and recommends a combination of TBUT, Schirmer tear testing, and staining as the current gold standard. For mild disease, a rapid TBUT may indicate an unstable tear film, whereas aqueous tear deficiency may be diagnosed with the Schirmer tear test. Minimal or no dye staining of the ocular surface may exist with mild dry eye disease.

The InflammaDry test was compared with clinical assessment, defined as the presence of subjective symptoms of suspected dry eye accompanied by at least one of the following objective confirmatory clinical signs: reduced Schirmer tear test, reduced TBUT, or the presence of corneal staining. Each of these objective measurements provides distinct information about the condition of the ocular surface. The trial was performed at 4 clinical sites. Similar to the data by Chotikavanich et al,³⁰ MMP-9 was found to be elevated over the entire range of dry eye severity (Table 3). Moreover, MMP-9 was consistently elevated in patients with mild dry eye despite the lack of corneal staining in study participants with mild dry eye (Table 1).

Three out of the 4 clinical sites had 4 different untrained ophthalmic technicians perform the testing, whereas 1 site used only 1 untrained ophthalmic technician to enroll all their patients. Two clinical sites, using a total of 8 different untrained technicians, enrolled >74% of the patients. These sites demonstrated the best performance despite having the

TABLE 2. OSDI Discordance from Dry Eye Confirmatory Testing

No. Subjects	OSDI	TBUT	Schirmer Tear Test	Staining
11	≥13	>10	>10	0

TABLE 3. Patient Dry Eye Severity Grading With and Without OSDI

Confirmatory Testing With and Without OSDI Inclusion*	No Dry Eye Grade 0	Mild Grade 1	Moderate Grade 2	Moderately Severe Grade 3	Severe Grade 4
Without OSDI	91	49	76	20	1
InflammaDry positive	3	47	60	18	1
With OSDI	80	60	76	20	1
InflammaDry positive	2	48	60	18	1

*Study data were analyzed with and without the inclusion of the OSDI as a confirmatory test for dry eye.

greatest number of untrained technicians participating. Of the 2 remaining sites, only 1 site enrolled a significant number of patients (21%), but this clinical site used only a single untrained technician to perform all the testing, and this site accounted for the majority of the reported false negatives observed in the study. The remaining clinical site enrolled <5% (12 patients). Taken together, the analysis showed that nearly 90% of the untrained technicians were effective at running and interpreting the test, whereas 1 operator accounted for most of the false negative results.

There is a general trend for patients to report more severe dry eye symptoms relative to the clinical signs observed by their clinician.³⁶ Symptoms have been shown to be insufficient for the diagnosis and management of dry eye; thus, a consensus of clinical signs is recommended for the diagnosis of dry eye.² A study conducted by Amparo et al³⁷ showed no correlation between changes in patient-reported symptoms using OSDI and changes in tear osmolarity or corneal fluorescein staining. A comparable study conducted by Caffery et al³⁸ showed no significant correlation between tear osmolarity and the self-assessment of dry eye in a nonclinical population of 249 convention attendees.

Similarly, if the OSDI is included as a confirmatory test, the positive and negative percent agreements of the InflammaDry test changes. In this trial, inclusion of the OSDI results lead to 11 patients being potentially falsely characterized as having dry eye disease despite the lack of any demonstrable objective signs. These results suggest that the OSDI may be a good screening tool for identifying patients with symptoms consistent with dry eye but is not suitable as a confirmatory diagnostic test.

The InflammaDry clinical trial was designed as an all comers trial where all patients enrolled reported at least 1 symptom consistent with dry eye over the preceding month.

Only approximately two-thirds of patients with symptoms consistent with dry eye disease tested positive with any objective confirmatory test, including TBUT, Schirmer tear testing, or corneal staining. Of those who tested positive with any objective confirmatory test, approximately 85% tested positive with InflammaDry. These results suggest that 50% of all symptomatic patients and nearly all of those confirmed as dry eye have significant ongoing inflammation. Similarly, a study reported by McDonald shows that less than half of all symptomatic subjects (42.8%), symptomatic cataract-affected patients (48.9%), and symptomatic laser-assisted in situ keratomileusis-operated subjects (42.7%) had actual dry eye disease as tested by tear osmolarity (unpublished data submitted for presentation at the American Society of Cataract and Refractive Surgeons 2014 Symposium).

Dry eye is a multifactorial, chronic disease and inflammation occurs in most, but not in all, patients with dry eye. Another possible explanation for the discordance between dry eye symptoms and both hyperosmolarity and elevated MMP-9 may be the intermittent nature of mild dry eye disease, which leads to symptoms only at the time of an environmental stress. These patients would most likely demonstrate a higher rate of signs if tested at that time. Thus, mild dry eye could be thought of as intermittent moderate disease, differentiated primarily by the temporal frequency of symptoms.

Because inflammation is found throughout the lacrimal unit, MMP-9 levels are unlikely to be affected by reflex tearing.³¹ A study on relative humidity by Tesón et al³⁹ demonstrated that MMP-9 levels increase in the presence of low relative humidity. However, additional studies are needed to assess the variability of MMP-9 levels in dry eye-affected patients.

The reported clinical study supports the use of MMP-9 as a marker for dry eye and the InflammaDry test as a clinical aid in the diagnosis of dry eye disease. Additionally, the

TABLE 4. Performance Results of MMP-9 Test Compared with Confirmatory Testing

N = 237		Clinical Assessment + OSDI + TBUT + Schirmer + Staining		Positive % Agreement 95% CI	Negative % Agreement 95% CI
InflammaDry	Positive	127	2	81% (127/157) (74%, 87%)	98% (78/80) (91%, 100%)
	Negative	30	78		
		Clinical Assessment + TBUT + Schirmer + Staining			
InflammaDry	Positive	126	3	86% (126/146) (80%, 91%)	97% (88/91) (91%, 99%)
	Negative	20	88		

clinical performance demonstrated by untrained ophthalmic technicians in this trial correlates with that reported in a previous prospective clinical trial by Sambursky et al⁴⁰ in which investigators at 7 clinical sites determined an InflammDry test sensitivity of 85% (121/143) and specificity of 94% (59/63).

Because InflammDry is a qualitative test, it is not designed or intended to monitor the disease state after the initiation of treatment. However, several investigators have suggested that a combination of clinical variables, including the measurement of surface epitheliopathy/staining, along with various biomarkers such as MMP-9, may be the most reliable prognosticator for response to therapy.³⁷ Therefore, identifying symptomatic dry eye-affected patients with underlying inflammation may guide patient management and therapeutic recommendations, including artificial tear replacement, punctal occlusion, or antiinflammatory therapeutics such as a short course of corticosteroids, oral doxycycline, or long-term maintenance treatment with cyclosporine.

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JOURNAL CLUB

Sensitivity and Specificity of a Point-of-Care Matrix Metalloproteinase 9 Immunoassay for Diagnosing Inflammation Related to Dry Eye

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Objectives: To determine the clinical sensitivity, specificity, negative predictive value, and positive predictive value of a rapid point-of-care diagnostic test to detect elevated matrix metalloproteinase 9 levels (InflammaDry).

Methods: In a prospective, sequential, masked, multicenter clinical trial, InflammaDry was performed on 206 patients: 143 patients with clinical signs and symptoms of dysfunctional tear syndrome (dry eyes) and 63 healthy individuals serving as controls. Participants were assessed as healthy controls or for a clinical diagnosis of dry eye using the Ocular Surface Disease Index, Schirmer tear test, tear breakup time, and keratoconjunctival staining.

Main Outcome Measures: The sensitivity and specificity of InflammaDry were compared with clinical assessment.

Results: InflammaDry showed sensitivity of 85% (in 121 of 143 patients), specificity of 94% (59 of 63), negative predictive value of 73% (59 of 81), and positive predictive value of 97% (121 of 125).

Conclusion: Compared with clinical assessment, InflammaDry is sensitive and specific in diagnosing dry eye.

Application to Clinical Practice: Dry eye is often underdiagnosed resulting from poor communication between the clinical assessment of dry eye severity between clinicians and patients. This often leads to a lack of effective treatment. Matrix metalloproteinase 9 is an inflammatory biomarker that has been shown to be elevated in the tears of patients with dry eyes. The ability to accurately detect elevated matrix metalloproteinase 9 levels may lead to earlier diagnosis, more appropriate treatment, and better management of ocular surface disease. Preoperative and perioperative management of inflammation related to dry eyes may reduce dry eyes that develop after laser in situ keratomileusis, improve wound healing, and reduce flap complications. Recognition of inflammation may allow for targeted perioperative therapeutic management of care for patients who undergo cataract and refractive surgery and improve outcomes.

Trial Registration: clinicaltrials.gov Identifier: NCT01313351

JAMA Ophthalmol. 2013;131(1):24-28

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DYSFUNCTIONAL TEAR SYNDROME, more commonly referred to as *dry eyes*, is a common condition that is often underdiagnosed. Dry eyes range in severity from asymptomatic or episodic symptoms of ocular discomfort to a chronic condition requiring therapeutic intervention. Inflammatory mechanisms have been shown to be an integral underlying cause of chronic dry eye.¹

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Dry eyes result from the complex interrelationship between the amount of tears produced and rate of tear evaporation that leads to hyperosmolarity and ocular surface inflammation.¹ Tear hyperosmolarity is one com-

ponent of the inflammatory cascade that results in elevated levels of matrix metalloproteinase 9 (MMP-9), which is a proteolytic enzyme that is produced by stressed epithe-

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lial cells and is elevated in patients with dry eye and ocular surface disease.² Matrix metalloproteinase 9 affects the corneal epithelial cells to produce inflammatory cytokines and other mediators that further augment the inflammatory cycle. Inflammation may destabilize the tear film and cause ocular surface epithelial disease.³⁻⁸ Increased activity of MMPs, such as MMP-9, influence wound healing^{9,10} and contribute to the pathologic alterations to the ocular surface that lead to a dry eye state.⁷⁻⁹ Ocular irritation and visual morbidity in dry eye disease result from an

altered corneal epithelial barrier function. Matrix metalloproteinase 9 levels are elevated in the tear fluid of patients with dry eyes^{11,12} above the level of expression of MMP-9 in healthy eyes, which ranges from 3 to 40 ng/mL.^{2,13-16} Higher levels of MMP-9 are present in patients with more severe dry eyes and correlate with clinical examination findings.² Abnormally elevated MMP-9 levels (>40 ng/mL) have been shown² to correspond with moderate to severe dry eye disease, as defined by dysfunctional tear syndrome severity levels of 2 to 4 identified in the Dry Eye Workshop report.¹

Currently, the diagnosis of dry eye is based on a clinical examination, which is occasionally accompanied by ancillary testing. Symptoms of dry eyes as described by patients include burning, dryness, foreign-body sensation, ocular pain, blurred vision, photophobia, and visual fatigue. Clinical signs of dry eye include positive vital staining of the ocular surface, decreased tear breakup time (TBUT), reduced corneal sensitivity, and decreased functional visual acuity.¹⁷

The inconsistency and lack of correlation between clinical symptoms and signs of dry eyes and diagnostic test results make the diagnosis and treatment of this condition challenging.¹⁸ In addition to being uncomfortable for the patient, Schirmer tear testing produces inconsistent results. Theoretically, hyperosmolarity testing may be an option for identifying patients with dry eye, but current techniques lack reproducibility and are more expensive than other methods of testing. Most important, neither TBUT nor Schirmer tear testing provides direct evidence of ocular surface inflammation or detects elevated levels of MMP-9.

InflammaDry (Rapid Pathogen Screening, Inc) is a 10-minute in-office immunoassay designed to detect abnormally elevated MMP-9 levels (>40 ng/mL). Any positive test result suggests ocular surface disease consistent with moderate to severe dry eye. The presence of elevated levels of MMP-9 may identify patients who otherwise would not receive the diagnosis clinically. This information may help lead to successful therapy for these patients to relieve symptoms and optimize the ocular surface. The purpose of this clinical trial was to determine the sensitivity and specificity of InflammaDry compared with the clinical assessment of dry eyes.

METHODS

A prospective, sequential, masked multicenter clinical trial was performed to evaluate InflammaDry at 7 clinical trial sites from December 1, 2010, through March 14, 2011. The sites consisted of academic and private ophthalmology practices in various regions across the United States. The test was successfully performed on 206 patients: 143 patients with clinical signs and symptoms of dry eyes and 63 healthy individuals serving as controls. Institutional review board approval was received and informed consent was obtained by an investigator at each site.

The sample size calculation was based on enrolling a minimum of 160 participants to test the hypothesis that there was no significant difference in the percentage of patients detected to have MMP-9 levels greater than 40 ng/mL between InflammaDry and the clinical assessment. The method is based on testing a hypothesis that the sensitivity and specificity of InflammaDry is at least 85%. Rejection of the null hypothesis signals that the sensitivity and specificity of InflammaDry in detecting clinically dry eyes is at least 85%. This hypothesis was used twice, first for sensitivity and second for specificity. A sample of at least 100 case patients

for the sensitivity hypothesis provides more than 90% power to detect a sensitivity of at least 85%, using a 1-sided exact binomial test with $\alpha = .05$ and an effect size of 10%. A sample of at least 60 controls for the specificity hypothesis provides 86% power to detect a specificity of at least 85%, using a 1-sided exact binomial test with $\alpha = .05$ and an effect size of 10%. Power and sample size calculations were performed using commercial software (nQuery Advisor, version 6.1; Statistical Solutions).

At the office visit, patients were screened using clinical history and signs. First, a clinical history was performed using the Ocular Surface Disease Index (OSDI).¹⁹ Patients were then assessed for clinical signs. Each patient had fluorescein dye introduced into the inferior cul-de-sac and was tested for TBUT. Additionally, any corneal staining was noted. After the ocular surface was analyzed for TBUT and staining, anesthetic was inserted and a 5-minute Schirmer test was performed. Last, an independent health care professional masked to the clinical evaluation was asked to analyze each InflammaDry test result, independently confirming each result.

OCULAR SURFACE DISEASE INDEX

To evaluate the symptoms associated with dry eyes, the OSDI survey was first completed. The OSDI is a global assessment measure consisting of 12 questions, each scored by the patient. It has been used to evaluate the severity of symptoms and response to previous treatment in patients with dry eye. The OSDI scores range from 0 (no disability) to 100 (complete disability).¹⁹

FLUORESCEIN TEAR BREAK-UP TIME

The TBUT was evaluated 2 minutes after the inferotemporal bulbar conjunctiva was touched with a sodium fluorescein strip (wet with preservative-free saline). Participants were instructed to blink, and the precorneal tear film was examined under blue-light illumination with a biomicroscope and 10× magnification. The interval between the blink and the appearance of the first dark spot or discontinuity in the precorneal fluorescein-stained tear layer was then recorded.

CORNEAL FLUORESCEIN STAINING

The ocular surface was examined 2 minutes after fluorescein instillation into the tear film. The Oxford grading scheme was used to grade the intensity of corneal fluorescein staining in 5 zones of the conjunctiva and cornea (central, superior, temporal, inferior, and nasal) based on the number of dots on a 5-point scale (no dot, 0; 1-5 dots, 1; 6-15 dots, 2; 16-30 dots, 3; and >30 dots, 4; if there was 1 area of confluence, 1 point was added; 2 or more areas of confluence, 2 points were added; and if there was filamentary keratitis, 2 points were added).

SCHIRMER TEAR TEST

Topical anesthetic was introduced into the inferior fornix. A Schirmer test was performed by placing Schirmer test strips (Alcon Laboratories) over the lower eyelid margin, at the junction of the lateral and middle thirds, for 5 minutes. The strip wetting was measured and recorded in millimeters. If complete wetting of the strips occurred before 5 minutes, the Schirmer score was extrapolated linearly to the 5-minute end point and considered a response to reflex tearing.

Patients were required to be aged 18 years or older for enrollment. The clinical diagnosis of dry eyes consisted of meeting all the following criteria: an OSDI increased to 13 or more, Schirmer test results of less than 10 mm in 5 minutes, a reduced TBUT in less than 10 seconds, and the presence of keratoconjunctival stain-

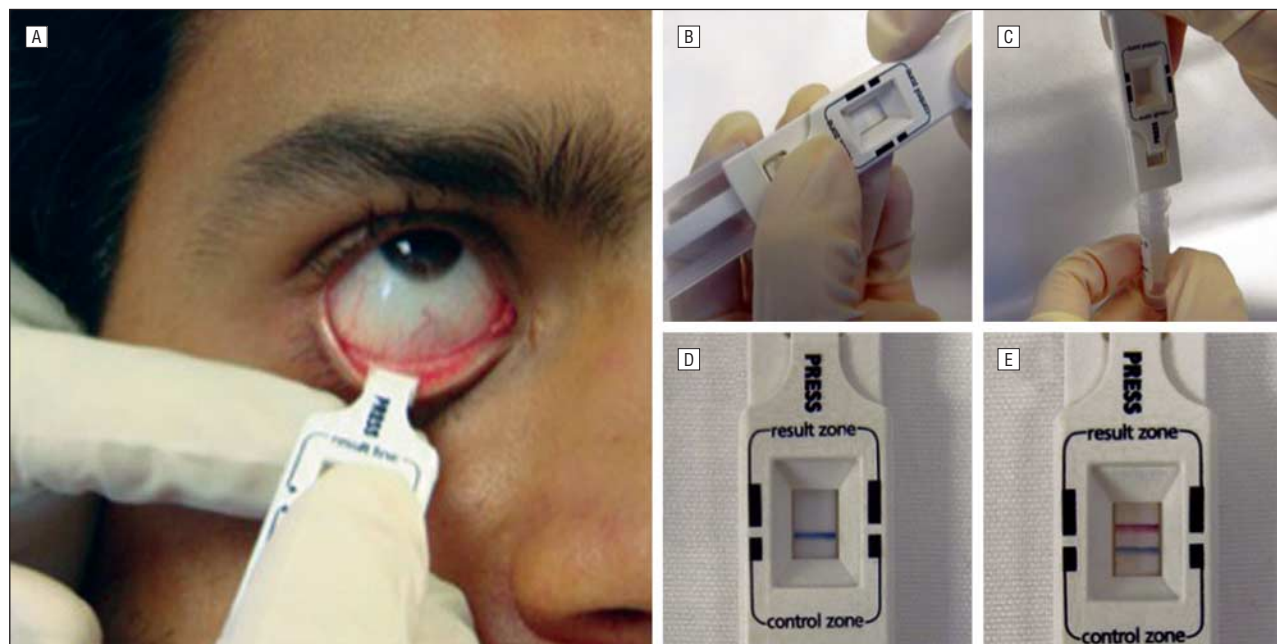


Figure. InflammDry 4-step procedure. A, Sample collector dabs the palpebral conjunctiva. B, Sample collector is assembled to the test cassette. C, Assembled test is dipped into a buffer solution for 20 seconds. D, At 10 minutes, 1 blue line is negative. E, At 10 minutes, 2 lines (1 blue and 1 red) are positive.

ing. Healthy controls required an OSDI score of 7 or less, Schirmer test results of 10 mm or more in 5 minutes, TBUT of 10 seconds or more, and no keratoconjunctival staining.

Patients with allergies or a recent history of ocular injury, trauma, contact lens use, surgery, or pregnancy and those with chronic inflammatory or infectious processes were excluded. In addition, patients using topical or systemic medications known to suppress MMP-9 were excluded.

After a minimum of 30 minutes following the conclusion of the Schirmer test and the introduction of any topical medications, patients who met the inclusion criteria were enrolled. The eye with the greatest corneal staining or, if the eyes had the same amount of staining, the lowest Schirmer test value, had 10 μ L of tears collected and analyzed with the InflammDry test. If the patients were tested on a subsequent office visit, they returned within 72 hours of the initial screening to have the InflammDry test performed. During any interim period between the initial screening and collecting the InflammDry sample at a follow-up visit, patients were instructed to not initiate any oral or topical medications (including nutritional supplements that were not being used before the initial screening), to not have any new punctal occlusion, and to not change their artificial tear use or frequency of use.

InflammDry

Before the clinical trial, each study site conducted a positive and negative external control on InflammDry to ensure that the test reagents were working properly. InflammDry was used according to the manufacturer's instructions.²⁰

InflammDry is a patented and proprietary modification of a traditional lateral flow device and uses direct sampling microfiltration technology. Two antigen-specific antibodies capture MMP-9 antigens in the sample, and this complex is captured in a proprietary mode at the test result line, giving rise to a visually observable signal. The intensity of the visual test result line correlates with the amount of MMP-9 in the sample.

The test is rapid, requiring only 10 minutes for a result. InflammDry consists of 2 parts: a sterile sample collector and an immunoassay test strip in a plastic test cassette housing. After the

sample collector is used to collect the tear fluid, it is assembled to the test cassette. Sample transfer to the test strip happens automatically without any pretreatment or dilution of the sample.

The test is initiated when the absorbent pad of the test strip is dipped into a buffer solution. After 10 minutes, the result is visible in a readout window. The presence of 1 blue line (control line) indicates a negative (MMP-9, <40 ng/mL) result, whereas 2 lines (blue control line and red test line) indicate a positive (MMP-9, \geq 40 ng/mL) result (**Figure**).

RESULTS

The study enrolled 206 patients: 143 patients with dry eyes confirmed by clinical examination and 63 healthy controls. Only patients found to meet the inclusion criteria participated in this investigational device testing. Participants ranged in age from 18 to 88 years; 152 (73.8%) were women and 54 (26.2%) were men; 136 (66.0%) were white, 11 (5.3%) were black, 49 (23.8%) were Hispanic, 9 (4.4%) were Asian, and 1 (0.5%) was of other ethnicity.

InflammDry results were compared with clinical assessment using detailed criteria discussed in the "Methods" section. If the clinical examination criteria were positive, the patient was deemed to have dry eyes. If the clinical examination criteria were negative, the patient was deemed not to have dry eyes. The percentage of sensitivity of the InflammDry test was calculated by comparing the InflammDry positive results with all clinically defined true-positives. To determine specificity, all negative InflammDry samples were compared with samples deemed to be clinically defined true-negatives.

When compared with the clinical assessment, InflammDry showed a sensitivity of 85% (in 121 of 143 patients) and specificity of 94% (59 of 63) (**Table**). Both findings were statistically significantly higher than 70% using a binomial 1-sided test against 70% ($P < .001$ for both comparisons). Additionally, InflammDry demonstrated a nega-

tive predictive value of 73% (in 59 of 81 patients) and a positive predictive value of 97% (121 of 125) (Table).

COMMENT

Dry eye is a condition that is often chronic and prevalent, affecting 5% to 30% of the population aged 50 years or older.¹ Classically, dry eyes referred to tear volume deficiency, typically associated with Sjögren syndrome²¹⁻²³; however, most patients with dry eye do not have associated systemic conditions and many do not have low aqueous tear production.³ Quality of life can be affected by dry eye symptoms.²⁴ As with other chronic conditions, the psychological impact is significant and, through utility assessments, patients have reported willingness to trade years at the end of life for an opportunity to be free of dry eye, which found that the utility of moderate dry eye was similar to that of moderate angina.²⁵

A study by Chalmers et al²⁶ indicated a low level of agreement between the assessment of the severity of dry eye disease between clinicians and patients, confirming the widely suspected underdiagnosis of dry eye in the clinical setting, particularly in female and elderly populations. Similar to how the treatment of headache is correlated with the patients' assessment of severity, clinicians' underestimation of the severity of dry eye among the elderly may leave hundreds of thousands of patients, especially women, without proper treatment of this chronic symptomatic condition.^{27,28} The discrepancy between the assessment of dry eye severity by clinicians and patients often results in patients receiving treatment of artificial tears without consideration of the cause of the symptoms or the potential effectiveness of other treatments.²⁶ Confirmation of the presence of dry eye will drive the initiation of appropriate therapy. Although artificial tears have been reported²⁹ to improve symptoms of irritation and decrease ocular surface dye staining in dry eye, their use has not been found to improve conjunctival squamous metaplasia. Anti-inflammatory therapy, however, has been reported to improve signs and symptoms of ocular surface disease and therefore is a more targeted and effective therapeutic option.³⁰

Chronic dry eye syndrome was characterized in the 2007 Report of the Dry Eye Workshop into levels of severity based on the presence or absence of a combination of symptoms and signs.¹ Chotikavanich et al² showed that MMP-9 activity increases proportionally along with increasing severity of dry eye syndrome, elevated levels of MMP-9 correlate with clinical examination findings, and MMP-9 may be a more sensitive marker than clinical signs. Patients without dry eye syndrome showed average MMP-9 levels of 8 ng/mL, whereas those with dry eye syndrome showed the following progressive increase in the detectable levels of MMP-9 in tears: level 1, 36 ng/mL; level 2, 66 ng/mL; level 3, 101 ng/mL; and level 4, 381 ng/mL.² The progressive increase of MMP-9 in tears as dry eye syndrome severity increases further supports the positive result shown by the InflammDry test when levels of MMP-9 in tears are above 40 ng/mL. The InflammDry limit of detection was confirmed by serial dilutions of native MMP-9 protein. The purity of the native MMP-9 protein was first established using gel electrophoresis and the concentration confirmed by an MMP-9 enzyme-linked immunosorbent assay kit (Micro

Table. Sensitivity, Specificity, and Positive and Negative Predictive Values

Characteristic	Results, % (No./No.) (N = 206)
InflammDry test result, positive/negative, No.	
Dry eye	121/22
Control	4/59
Sensitivity ^a	85 (121/143)
95% CI	78.7-90.5
Specificity ^a	94 (59/63)
95% CI	7.6-99.7
Positive predictive value	97 (121/125)
95% CI	93.7-99.9
Negative predictive value	73 (59/81)
95% CI	63.2-82.5

^aP < .001.

Bicinchoninic Acid Protein Assay; Pierce Protein Research [a division of Thermo Fisher Scientific, Inc]). InflammDry false-negatives occurred uniformly across all levels of dry eye severity, which suggests that these false-negatives may have been the result of inadequate collection and transfer of tear samples.

InflammDry is designed to detect abnormally elevated MMP-9 present in the late phase of the inflammation cycle and therefore may be more clinically relevant than causal mechanisms or acute symptoms. Although MMP-9 activity is elevated in symptomatic dry eyes as well as in other clinically easily identifiable ocular surface conditions, InflammDry also reveals asymptomatic ocular surface disease and the hidden inflammation of dry eyes. One limitation of the present study is that participants with recent surgery, trauma, or other inflammatory conditions that may lead to elevated MMP-9 levels were excluded to prevent false-positive results. Patients taking anti-inflammatory medications that may decrease MMP-9 levels and lead to false-negative results were also excluded.

Tear MMP-9 activity shows a strong correlation with the results of routine dry eye diagnostic tests²; however, currently, there is a lack of consensus on the diagnostic standards for dry eye disease. The reported sensitivity and specificity of routine dry eye diagnostic methods, such as the Schirmer test, TBUT, corneal staining, and OSDI, show a variable sensitivity, ranging from 42% to 90%, and specificity, ranging from 17% to 89%,³¹ compared with InflammDry's sensitivity of 85% and specificity of 94%.

Tear osmolarity has been shown to be a useful dry eye diagnostic method, but currently available tear osmolarity test results have been variable³² and show a sensitivity range of 64% to 73% and a specificity range of 71% to 92%.^{33,34} Although tear osmolarity helps to confirm the presence or absence of dry eye, it provides limited information to guide therapeutic management. Identifying elevated levels of MMP-9 to confirm the presence of inflammation related to dry eye disease may lead to more targeted therapeutic interventions and management of the condition.

Reducing ocular inflammation is the target for many dry eye therapeutics. Topical cyclosporine treatment inhibits T-lymphocyte proliferation^{35,36} and decreases the levels of MMP-9 expression in the conjunctival epithel-

lial cells.³⁷ Corticosteroids inhibit inflammation and decrease the production of inflammatory cytokines and MMP-9 by the corneal epithelium.^{30,38} Doxycycline preserves the tight junction network, increases corneal smoothness, preserves corneal barrier function, and leads to a reduction in the production and activity of MMP-9.^{12,30,39,40} In animal models, topical azithromycin reduces MMP-9 on the ocular surface, especially in inflammatory conditions such as rosacea.⁴¹ It is postulated that patients with positive test results with InflammDry may benefit from initial intervention with anti-inflammatory therapy. Use of topical medications with anti-inflammatory properties, such as cyclosporine, corticosteroids, doxycycline, and azithromycin, may suppress MMP-9 levels in the tears, thus leading to a false-negative InflammDry test result.

Furthermore, patients who test negative with InflammDry and positive with a clinical examination may have dry eye without significant accompanying inflammation and may benefit from artificial tear replacement or punctal occlusion. If artificial tears have been ineffective in relieving these patients' symptoms, a therapeutic trial of anti-inflammatory therapy to target subclinical inflammation may be necessary. Thus, evaluating levels of MMP-9 may be clinically helpful for diagnosing, classifying, and monitoring inflammatory dry eyes.

Submitted for Publication: January 26, 2012; accepted June 18, 2012.

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Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest Disclosures: Dr Sambursky has an affiliation with Rapid Pathogen Screening, Inc (RPS); however, none of the principal investigators have any financial interest in InflammDry or in RPS.

Funding/Support: The design and conduct of the study and the collection of data described in the article were sponsored by Rapid Pathogen Screening, Inc.

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MMP-9 and the perioperative management of LASIK surgery

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Current Opinion in Ophthalmology 2011, 22:294–303

Purpose of review

Hyperosmolarity is a central mechanism causing ocular surface inflammation and eye irritation in typical patients suffering from tear dysfunction. Tear composition in dry eyes, or dysfunctional tear syndrome, may destabilize the tear film and cause ocular surface epithelial disease. Increased activity of matrix metalloproteinases (MMPs), especially MMP-9, plays a critical role in wound healing and inflammation and is primarily responsible for the pathologic alterations to the ocular surface that leads to a dysfunctional tear state.

Recent findings

Altered corneal epithelial barrier function is the cause for ocular irritation and visual morbidity in dry eye disease. The increased MMP-9 activity in dry eyes may contribute to deranged corneal epithelial barrier function, increased corneal epithelial desquamation, and corneal surface irregularity.

Summary

Dry eye is one of the most common complications of photorefractive keratectomy and laser in-situ keratomileusis (LASIK). LASIK has both a neurotrophic effect on the cornea and leads to a physical change in corneal shape that results in a change in tear dynamics, leading to ocular surface desiccation. The reduction in tear function after LASIK may induce an increase in osmolarity and consequently raise the concentration of proinflammatory cytokines and MMP-9 in the tear film, which results in dry eyes and insufficient attachment between the corneal flap and the corneal bed. Appropriate diagnosis and management of dysfunctional tear syndrome may lead to less postoperative LASIK complications.

Keywords

dry eyes, dysfunctional tear syndrome, keratoconjunctivitis, laser in-situ keratomileusis, metalloproteinase 9, MMP-9

Curr Opin Ophthalmol 22:294–303
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1040-8738

Introduction

Tear composition in dry eyes, or dysfunctional tear syndrome, may destabilize the tear film and cause ocular surface epithelial disease [1–6]. Increased activity of matrix metalloproteinases (MMPs), especially MMP-9, plays a critical role in wound healing and inflammation [7,8] and is primarily responsible for the pathologic alterations to the ocular surface that leads to a dysfunctional tear state [5,7].

MMPs are a family of 23 zinc and calcium ion-dependent proteolytic enzymes produced by stressed ocular surface and glandular epithelial cells, as well as by the immune cells that infiltrate these tissues. The MMPs are integrally involved in angiogenesis, inflammation, wound repair, and tissue remodeling through their ability to degrade extracellular matrix components [9–11]. These enzymes are subclassified according to their substrates: the collagenases (MMP-1, MMP-8, and MMP-13) degrade fibrillar collagen types I, II, and III; the gelati-

nases (MMP-2 and MMP-9) degrade types IV and VII collagen that are found in basement membranes; the stromelysins (MMP-3 and MMP-10) and the matrilysins (MMP-7 and MMP-26) degrade proteoglycans, laminin, and glycoproteins; and the membrane-type (MTMMP-1, MTMMP-2, and MTMMP-3), which are bound to epithelial cell membranes, can activate other MMPs [12].

MMP-9, or gelatinase b, plays a vital role in several physiological and pathological processes, and pathologically can disrupt or disintegrate extracellular matrices [13–15]. Secreted from cells as a 92-kDa proenzyme [10], MMP-9 is the primary matrix-degrading enzyme produced by the corneal epithelium [16] and is instrumental in the pathogenesis of sterile corneal ulceration [5] and dry eye [17], as levels of MMP-9 increase as tear clearance decreases. Preformed MMP-9 may also be released from the secretory granules of neutrophils recruited by any associated inflammation [18] and subepithelial expression of MMP-9 parallels basement membrane degradation from human neutrophils [19,20].

MMP-9 tissue inhibitors of metalloproteinases (TIMPs) bind and inactivate the proenzyme [10].

After injury, and in response to the release of cytokines, several MMPs in the cornea are upregulated by transcription or activation [21]. MMP-9 has been found to be of central importance in catalyzing the cleavage of epithelial basement membrane components [19,22]. Additionally, hyperosmolarity stimulates the production of inflammatory factors such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and MMP-9 and activates the mitogen-activated protein kinase (MAPK) signaling pathways in the ocular surface epithelial cells [23]. The activation of MAPK signaling pathways is known to stimulate the expression of MMP-9, and the production of inflammatory cytokines, actuation of matrix degrading enzymes, and recruitment of T lymphocytes [24], which leads to further ocular surface instability, damage, and dry eye.

Pathophysiology

The proinflammatory cytokine IL-1 is an important mediator of inflammation and immunity and is implicated in corneal and ocular surface diseases rosacea, bullous keratopathy, keratoconus, and sterile corneal ulceration [5,25,26]. The precursor and the mature 17-kDa forms of IL-1 α are both biologically active, whereas the precursor form of IL-1 β possesses minimal biological activity and requires cleavage to the 17-kDa mature form to become active [27]. MMP-9 protein and mRNA was shown to upregulate synergistically in rabbit and human dermal fibroblasts when exposed to a combination of growth factors, IL-1, and TNF- α [28]. MMP-9 is induced by IL-1 in human corneal stromal cells [29]. Tear fluid growth factor and cytokines are secreted by the lacrimal glands and are produced by the epithelial and inflammatory cells that reside on the ocular surface. As tear clearance decreases in dry eye conditions, the concentration of pro-inflammatory cytokines, such as IL-1 increases [5,30].

Solomon *et al.* [4] demonstrated that dry eye disease is accompanied by an increase in the proinflammatory forms of IL-1 (IL-1 α and mature IL-1 β). IL-1 is a potent inducer of other inflammatory cytokines such as IL-6, IL-8, and TNF- α and can stimulate the production of MMP-9 by epithelial and inflammatory cells [4,10,31,32]. Previous studies have demonstrated that IL-6 can also induce the expression of MMPs [33,34]. IL-1 β is activated in the extracellular environment by a number of proteases, including leukocyte elastase, granzyme A, MMP-2, and MMP-9 [27,35,36]. Further, MMP-9 was found to be the most efficient activator of precursor IL-1 β [36]. The stable concentrations of IL-1 α and precursor IL-1 β in tear fluid after induction of reflex tearing suggests that the lacrimal glands secrete these cytokines

Key points

- Metalloproteinase 9 (MMP-9) is an inflammatory marker that has consistently been shown to be elevated in the tears of patients with dry eyes.
- MMP-9 activity may be a more sensitive diagnostic marker for dry eyes than clinical signs.
- Preoperative dry eye condition is a major risk factor for more severe dry eye after surgery and should be identified prior to surgery.
- Preoperative and perioperative management of inflammation related to dry eyes may reduce dry eyes after laser in-situ keratomileusis, improve wound healing, and reduce flap complications.

[4]. Hyperosmolar stress and the elevated inflammatory cytokines present in dry eye such as IL-1, TNF- α , and TGF- β increase expression of MMP-9 by the corneal epithelium.

Dry eyes: definition and quality of life

Historically, the term dry eye implied tear volume deficiency, mainly associated with Sjögren's syndrome [37–39], but it is now widely appreciated that the majority of dry eye patients do not have associated systemic conditions and many do not have low aqueous tear production [1]. According to the Dry Eye Work Shop (DEWS) definition, dry eyes 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.

Risk factors for dry eyes include patients taking other medications (e.g., antihypertensives, antidepressants, or hormone replacement therapies) [40], patients with autoimmune inflammatory diseases [41], contact lens wearers [42], laser in-situ keratomileusis (LASIK) and refractive surgery patients [43], and postmenopausal women [44].

Symptoms of dry eyes as described by patients include burning, dryness, foreign-body sensation, ocular pain, blurred vision, photophobia, and visual fatigue. Clinical signs of dry eye include positive vital staining of ocular surface, decreased tear film breakup time and Schirmer's tests, reduced corneal sensitivity, and decreased functional visual acuity [45]. The discordance between symptoms, clinical signs, and diagnostic test results are inconsistent, making the diagnosis and treatment of this condition challenging [46].

Quality of life can be significantly affected by dry eye symptoms, as documented by several validated survey

instruments [47]. The psychological impact of this chronic condition is suggested by a utility assessment of patients' willingness to trade years at the end of life for an opportunity to be free of dry eye [48].

Mechanism of action

Hyperosmolarity is a central mechanism causing ocular surface inflammation and eye irritation in typical patients suffering from tear dysfunction [49••]. Dry eye induces inflammation on the ocular surface, evidenced by elevated levels of the inflammatory cytokines, chemokines, adhesion molecules, and MMPs in the tear film and on the ocular surface [3,6,23,50–52]. A significant increase in the concentration and activity of MMP-9 has been reported in the tear fluid of human dry eye patients [4,5,53] as well as in the corneal epithelium and tear fluid of mice with experimental dry eye [54]. MMP-9, in particular, is a nonspecific inflammatory marker that has consistently been shown to be elevated in the tears of patients with dry eyes.

Decreased tear production and tear clearance lead to chronic inflammation of the ocular surface. This inflammatory response consists of cellular infiltration of the ocular surface by activated T lymphocytes, with increased expression of adhesion molecules and inflammatory cytokines, increased concentrations of inflammatory cytokines in the tear fluid, and increased activity of MMP-9 in the tear fluid. Increased MMP-9 expression and activity of MMP-9 causes aggravation of symptoms because they cleave epithelial basement membrane components and tight junction proteins that maintain corneal epithelial barrier function [55–57]. Epithelial damage, along with the progressive lymphocytic infiltration and the released cytokines, induces inflammation and apoptosis. Expression of the proapoptotic markers by the conjunctival epithelium has been found to be high in dry eye [58]. Significant positive correlation has been observed between the levels of inflammatory cytokines in the conjunctival epithelium and the severity of symptoms of ocular irritation, including corneal fluorescein staining, and the severity of conjunctival squamous metaplasia in

patients with Sjögren's syndrome keratoconjunctivitis [59].

Desiccating stress had no effect on levels of TIMP transcripts in the corneal epithelium, suggesting that MMPs are more inducible by dry eye stress than are TIMPs [12]. There are three levels of MMP regulation: transcriptional control, activation of the secreted pro-enzyme, and inhibition of the enzyme by the TIMPs [60,61]. Each MMP is inhibited by at least one of four recognized TIMPs. It has been proposed that MMP activity is related to the ratio of the concentration of an MMP to its TIMP. An altered balance of MMPs and TIMPs has been implicated in the pathogenesis of corneal ulceration and wound healing [62,63]. Although both latent-MMP-9 and TIMP-1 concentrations were elevated in tear fluid obtained from patients with rosacea compared with normal controls, no significant difference in the TIMP-1-to-pro-MMP-9 ratio between the two groups was seen [53].

Experimentally induced dry eye is associated with acutely increased markers of ocular surface inflammation and with epithelial cell apoptosis [51]. MMP-9 produced by the corneal epithelium has been found to impede re-epithelialization of the cornea after experimental thermal injury in animal models [20], and increased MMP-9 activity disrupts corneal epithelial barrier function, produces corneal surface irregularity in an experimental murine model of dry eye [54], and increases production of inflammatory mediators on the ocular surface that is similar to human dry eyes [23,64]. MMP-9 contributed significantly to the altered corneal epithelial permeability in dry eyes, because corneal epithelial barrier function was preserved in experimental dry eye MMP-9 knockout mice [54].

Several human studies demonstrate elevated MMP-9 levels in the tear fluid of patients with dry eyes [53,65]. Expression of MMP-9 by the ocular surface epithelia in normal healthy eyes is low [66]. The normal levels of MMP-9 (ng/ml) in human tears range from 3 to 41 ng/ml (see Table 1). [4,52,67–69,70•]. Increased production of MMP-9 by the corneal epithelium has been

Table 1 Levels of matrix metalloproteinase 9 in healthy normal controls

Study	Normal controls	Average MMP-9 levels (ng/ml)	Standard deviation (ng/ml)	Upper range (ng/ml)
Acera <i>et al.</i> [52]	18	23.61	17.4	41
Chotikavanich <i>et al.</i> [67]	16	8.39	4.70	13
Solomon <i>et al.</i> [4]	17	7.2	2.1	9
Leonardi <i>et al.</i> [68]	10	10.5	0.2	11
Lema <i>et al.</i> [69]	20	6.9	1.4	8
Honda <i>et al.</i> [70•]	28	22.7	14	37
Total/average/range	109	13.2		3.9–41

MMP, matrix metalloproteinase.

found in the eyes of human patients suffering from sterile corneal ulceration [71].

In addition, MMP-9 activity was found to be greater in the tear fluid of patients with ocular rosacea than in normal controls [5]. Ocular rosacea and other dry eye conditions are associated with an increased incidence of recurrent corneal epithelial erosion (RCEE) and sterile corneal stromal ulceration. RCEE has been reported to occur in up to 12% of patients with ocular rosacea [72,73]. Solomon *et al.* [4] found increased activity of MMP-9 in the tear fluid of patients with meibomian gland disease and Sjögren's syndrome. MMP-9 concentration and activity is significantly higher in the tear fluid of patients with delayed tear clearance, especially in patients with recurrent corneal epithelial erosion [5].

Chotikavanich *et al.* [67] showed that levels of MMP-9 in patients with dysfunctional tear syndrome (DTS) were not significantly different between male and female participants and there was no significant difference in the tear MMP-9 activity between each decade of age between 20 and 80 years in both normal controls and dry eye patients. Each DTS group had significantly higher mean levels of tear MMP-9 activity than the normal individuals, and the most severe DTS group (DTS4) was found to have the highest mean MMP-9 activities that were significantly higher than the other DTS groups. These findings indicate that tear MMP-9 activity is significantly elevated, even in mild DTS, and that this may be a more sensitive diagnostic marker than clinical signs. Also, MMP-9 activities strongly correlate with clinical parameters [67]. Additionally, using gelatin zymography of tear fluid samples, Solomon *et al.* [4] demonstrated minimal or no 92-kDa pro-MMP-9 was observed in normal controls; however, greater levels of pro-MMP-9 were found in tear fluid samples taken from patients with dry eye who had meibomian gland dysfunction (MGD), patients with non-Sjögren's aqueous tear deficiency, and those with Sjögren's disease. Further, a quantitative MMP activity assay showed a 66-fold increase in patients with MGD and a 90-fold increase in patients with Sjögren's disease compared with normal individuals.

Diagnosis

It also is widely appreciated that there is little correlation between the symptoms of patients and the clinical test results in dry eye patients [24,38,74].

Tear stability can be evaluated with noninvasive diagnostic technologies [noninvasive tear breakup time (TBUT)] or with application of fluorescein to the tear film to measure tear film breakup time. Schirmer's tests are used to gauge the volume of tears. There are other

methods for analyzing tear interference images [75,76], and technologies such as corneal topography have been used to provide noninvasive tear stability information [77–79]. Even the technique for performing TBUT measurements with fluorescein varies significantly among clinicians depending on whether dye-impregnated strips or commercially available drops are used to perform the test.

A relatively new diagnostic device from TearLab (OcuSense, Inc., San Diego, California, USA) allows for the quantitative measurement of tear osmolarity. Studies of dry eye patients provide well validated cutoff values of 316 mOsm/l [80] or 317 mOsm/l [81] for dry eye disease. Tear osmolarity correlates with increasing severity of dry eye. Also a high correlation is found between tear hyperosmolarity and clinical score.

A rapid, 10-min point-of-care immunoassay that utilizes direct sampling microfiltration technology and detects the presence of elevated MMP-9 in 10 µl of tears, called the RPS InflammDry Detector (Rapid Pathogen Screening Inc., Sarasota, Florida, USA), is also available. Two antigen-specific antibodies capture MMP-9 antigens in the sample and this complex is trapped at the test line giving rise to a visually observable signal. The intensity of the visual test line correlates with the amount of MMP-9 present in the sample. Since the detection limit of this qualitative test is set at 40 ng/ml, any positive test is indicative of an abnormal level of MMP-9 [82]. Elevated MMP-9 levels in patients with moderate to severe dry eye correlate with clinical examination findings and have even been shown to be a more sensitive diagnostic marker than clinical signs [67].

Management

Treatment of tear dysfunction and lid margin disease may return the ocular surface to health. Artificial tears, especially preservative-free formulations, are useful. Punctal occlusion and nutritional supplements containing omega-3 fatty acids may be appropriate. Some suggest the use of short courses of topical steroids or cyclosporine 0.05% drops to optimize the ocular surface, as it has been found to increase goblet cell density [83] and to accelerate the return of corneal sensitivity postoperatively [84].

Laser in-situ keratomileusis: laser in-situ keratomileusis-associated dry eyes

Dry eye is one of the most common complications of photorefractive keratectomy (PRK) and LASIK [85,86]. LASIK surgery is the most commonly performed vision correction surgery in the United States [87]. Signs and symptoms of tear dysfunction occur early in the

postoperative period and resolve in nearly all patients by 6–9 months. In a recent review article, Toda [88] reported that signs or symptoms of dry eye after LASIK were found in 50% of patients at 1 week postoperatively, 40% at 1 month, and 20–40% at 6 months.

LASIK-associated dry eye is a major cause of patient dissatisfaction [43,89,90]. Although post-LASIK dry eye is usually short-lived, some patients complain of severe symptoms [1,91]. Other complications, such as fluctuating vision, decreased best spectacle corrected visual acuity, and severe discomfort occurs in approximately 10% of patients [92].

Clinical signs of post-LASIK dry eye include positive vital staining of the ocular surface, decreased TBUT, reduced corneal sensitivity, and the presence of a reduced Schirmer's tear test score [93–95]. The post-LASIK decrease in TBUT, basal tear secretion, and Schirmer's scores may persist for months [96,97]. Thus, LASIK has both a neurotrophic effect on the cornea and leads to a physical change in corneal shape that results in a change in tear dynamics, leading to ocular surface desiccation [92].

Preoperative risk factors

Although most patients have reduced basal tear production in the first few months following LASIK, only about half of patients are symptomatic [77,78,93,95–102]. The impact of using traditional methods of dry eye evaluation in the preoperative management of LASIK is variable. Studies have shown that preoperative Schirmer's scores below 10 mm are associated with postoperative tear dysfunction [95] and Ambrosio [92] showed that preoperative dry eye condition is a major risk factor for more severe dry eye after surgery and should be identified prior to surgery.

Among patients seeking refractive surgery, Toda *et al.* [103] found that more than 75% of patients interested in refractive surgery had preoperative symptoms or signs of dry eye. Despite having preexisting mild-to-moderate dry eye, a patient can have LASIK performed safely if care is taken to optimize the ocular surface prior to LASIK, and in some cases, if care of the ocular surface is continued after surgery [103–105]. Patients with preexisting tear dysfunction have poorer postoperative ocular surface health and more severe symptoms of tear dysfunction after LASIK and their corneal sensitivities take longer to recover compared to patients without dry eye [95,101,103].

Long-term contact lens wear may also predispose patients to tear film instability before and after surgery [97,101]. Long-term contact lens wearers demonstrate

significantly reduced tear secretion and corneal sensitivity before and after LASIK [96,97,106].

Gender has not been shown to be a risk factor for post-LASIK tear dysfunction. Two retrospective studies found significant associations between female gender and chronic post-LASIK tear dysfunction [99,107], yet a prospective study found no association [85]. Age also has not been shown to be an important risk factor for post-LASIK tear dysfunction [85,107].

Although objective clinical signs of tear insufficiency were not demonstrable, patients undergoing LASIK for high myopia reported ongoing dry eye symptoms 2–5 years after surgery [108]. In addition, Asian patients show a higher prevalence of chronic post-LASIK tear dysfunction [109].

Surgical considerations

LASIK-associated dry eye is one of the most frequent LASIK complications, which is believed to be attributable to the transection of large numbers of the afferent sensory nerve fibers in the cornea during the lamellar cut. This disturbs the lacrimal gland-ocular surface functional unit and promotes the development of LASIK-associated dry eye [77,93,95–101,110]. Corneal sensitivity decreases after LASIK because of surgical amputation and laser ablation of the nerve fibers innervating the central cornea [93,96–98,110–115].

Ablation depth and higher myopic refractive corrections also positively correlate with decreased corneal sensitivity [116,117]. Studies have reported conflicting results regarding the effects of hinge location on development of post-LASIK corneal hypoesthesia and dry eye, suggesting that further research is needed to determine the role hinge location plays in post-LASIK tear dysfunction [85,112,118–120].

Suction related to microkeratome use in LASIK leads to altered central corneal shape [121] and loss of goblet cells [99,122], the density of which has been found to decrease immediately postoperatively. Loss of goblet cells results in a subsequent reduction in goblet cell mucin, a stabilizing molecule in the tear film, which, if reduced, may lead to tear film instability [122]. These changes are present in all post-LASIK dry eye patients but are more significant in patients with chronic dry eye symptoms [99]. Goblet cell density may take 6 months to return to baseline values after LASIK [122].

In addition, tear fluorescein clearance time is increased post-LASIK, which may be due to less frequent blinking [96]. Toda *et al.* [93] found that the blink rate of LASIK patients was decreased by up to 40%, and the difference

in mean blink rate before and after LASIK remained statistically significant at postoperative months 3, 6, and 12.

The possible mechanisms for post-LASIK dry eye may be related to a neurotrophic effect, damage of goblet cells, and altered corneal shape [89]. Previous studies suggest that intraoperative risk factors for developing post-LASIK dry eye include higher refractive correction [116], deeper ablation depth [117], thicker flap [123], superior flap hinge [118], and narrow flap hinge [124].

Laser in-situ keratomileusis flap-related complications

LASIK flap analysis indicates that corneal wound healing does not terminate at 3 months after LASIK, and that corneal wound healing likely lasts for a long period following LASIK. This implies that insufficient attachment between the corneal flap and the corneal bed lasts for a prolonged period [125[•]]. Patients have been identified in whom the dislocation of corneal flaps [126[•],127] or ectasia consisted of a progressive deformation and thinning of the cornea [128] after LASIK, and this appears to be related to corneal wound healing.

In a rabbit model, MMP-2 and MMP-9 were predominantly localized behind the leading edge of migrating epithelium, which may indicate a role for these enzymes in stromal remodeling or early basement membrane assembly [129]. Azar *et al.* [130] used zymography to compare MMP expression after PRK or LASIK in the rabbit cornea and found that stromal levels of MMP-2 and MMP-9 increased after both procedures, with high MMP-9 expression also localized in the epithelium during re-epithelialization.

Significant expression of MMP-9 is observed in the peripheral LASIK wound margin scar in all eyes analyzed. The presence of MMP-9 may represent a marker of ectasia after LASIK and can lead to ongoing basement membrane remodeling with consequences in the stroma such as prolonged keratocyte apoptosis, which results in decreased synthesis of extracellular components, corneal thinning, and ectasia.

Post-LASIK epithelial ingrowth is associated with elevated MMP-9 [131^{••}]. The incidence of epithelial ingrowth is about 1% after LASIK [132] and develops in the interface through one of two known mechanisms for epithelial ingrowth: epithelial invasion and epithelial implantation. Lower endothelial counts, thinner flap thickness, and enhancement are risk factors for the development of epithelial ingrowth [89] through delayed sealing of the flap edge and/or poor adhesion of the flap interface. Epithelial cells under the flap gradually lose

their viability over time and finally undergo apoptosis with or without fibrosis. If the sealing of the flap edge is not tight or adhesion of interface is not firm, epithelial cells may survive and proliferate [89].

Epithelial ingrowth develops slowly in the beginning from week 1 to several weeks after LASIK and appears as variously shaped areas of transparent sheets with milky or whitish islands [132]. Although most cases heal spontaneously, some require surgical removal. Epithelial invasion grows in two distinct ways: outside invasion and flap epithelial invasion. The latter type is often seen after enhancement and may be resistant to treatment. Patients with compromised attachment of corneal epithelium before LASIK may develop recurrent corneal erosion, which sometimes requires phototherapeutic keratectomy [89].

Perioperative ocular surface management

The reduction in tear function after LASIK may induce an increase in osmolarity and consequently raise the concentration of proinflammatory cytokines and MMP-9 in the tear film [133]. In a dry eye characterized by a reduced tear production and a decreased tear clearance, pro-MMP-9 enzyme is accumulated and activated subsequently in the tears [92]. The importance of inflammation in the pathogenesis of dry eye is underscored by reports that the signs and symptoms of dry eye markedly improve with anti-inflammatory therapies such as glucocorticosteroids and cyclosporine [134,135]. Smith *et al.* observed a correlation between tear MMP-9 levels and clinical evidence of disease progression [66]. Additionally, cyclosporine was found to significantly decrease the number of meibomian gland inclusions in patients with MGD [136], leading to further tear stabilization.

Many patients who develop LASIK-associated dry eye without prior symptoms or signs of dry eye have a marked response to topical cyclosporine treatment, which treats the underlying inflammation and may benefit nerve regeneration [92]. Cyclosporine inhibits T-lymphocyte proliferation on the ocular surface cells [137,138]. Further, topical cyclosporine treatment significantly decreases the levels of apoptosis and MMP-9 expression in the conjunctival epithelial cells of thyroid patients with dry eye [139^{••}]. Patients should be routinely re-examined 4–6 weeks after beginning treatment with cyclosporine. After initiating cyclosporine, symptoms and signs of dry eye will improve in 50–60% of patients within 1 month; however, a significant proportion of patients with chronic dry eye take several months to respond to topical cyclosporine [7,140].

Treatment with anti-inflammatory medications such as topical corticosteroids [141] and topical cyclosporine

[142] also has been shown to increase goblet cell density in tear dysfunction associated with non-Sjögren's and Sjögren's diseases. Furthermore, ocular surface inflammation has been shown to correlate strongly with signs and symptoms of tear dysfunction in patients with Sjögren's disease [59].

Therapy of dry eye, with methylprednisolone and doxycycline was shown to preserve the tight junction network, increase corneal smoothness, preserve corneal barrier function, and lead to a reduction in the production and activity of MMP-9 [143]. Corticosteroids are global inhibitors of inflammation and have been reported to decrease the production of a number of inflammatory cytokines (IL-1, IL-6, IL-8, TNF- α , GM-CSF) and MMP-9 by the corneal epithelium [144].

Doxycycline is well known for its therapeutic efficacy in treating MMP-mediated ocular surface diseases, such as rosacea, recurrent epithelial erosion, and sterile corneal ulceration [73,145]. Doxycycline has been found to inhibit MMP-9 activity in human corneal epithelial cells [65]. Additionally, topical azithromycin was recently shown to reduce MMP-9 levels.

Management of the ocular surface during LASIK, as well as long-term management of the tear film and ocular surface, can minimize ocular surface damage and the risk of adverse outcomes, leading to a reduction in the severity and duration of dry eye symptoms and signs [101]. Optimization of the preoperative ocular surface with artificial tears, nutrition supplementation, punctal occlusion, and topical cyclosporine in patients with symptoms or signs of dry eye prior to LASIK decreases the incidence of postoperative symptoms [92]. Konomi *et al.* [107] suggest that topical anti-inflammatory therapeutics could normalize the ocular surface and improve the quality of the tear film after LASIK. Although topical steroids may have the most potent and rapid anti-inflammatory action, long-term treatment is not advisable because of the side-effects of corticosteroids, especially cataract formation and glaucoma [146]. Cyclosporine, however, has minimal side-effects compared with steroids and may be used for long periods without deleterious effects in the eye [135,146,147].

For patients who develop persistent neurotrophic keratopathy after LASIK, traditional treatment modalities should be implemented including preservative-free artificial tears, punctal occlusion, cyclosporine and azithromycin therapy, bandage contact lenses, autologous serum, and tarsorrhaphy. Autologous serum drops can dramatically increase corneal sensitivity to near normal levels and improve clinical parameters of ocular surface health [49•].

Conclusion

Preoperative dry eye or tear film instability is a major risk factor for increased dry eye and should be identified prior to LASIK surgery [92]. MMP-9 is an inflammatory marker that has consistently been shown to be elevated in the tears of patients with dry eyes and may be a more sensitive diagnostic marker than clinical signs [67]. Perioperative management of inflammation related to dry eyes may reduce post-LASIK dry eyes, improve wound healing, and reduce flap complications [92].

Acknowledgement

Financial Disclosure: Robert Sambursky, MD is the Chief Medical Officer and an equity shareholder of Rapid Pathogen Screening, Inc. (RPS).

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 308).

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The Practical Detection of MMP-9 Diagnoses Ocular Surface Disease and May Help Prevent Its Complications

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Purpose: To evaluate the importance and practicality of testing for matrix metalloproteinase 9 (MMP-9) in dry eye and ocular surface disease. This enzyme, which can cause tissue damage, seems also to be the most reliable diagnostic indicator of ocular surface disease.

Methods: Enzyme-linked immunosorbent assay, polymerase chain reaction, diffusion, and InflammDry, a new rapid immunoassay by RPS (Rapid Pathogen Screening Inc).

Results: MMP-9 measurement is sensitive and accurate for diagnosing dry eye and ocular surface disease and compares favorably in both sensitivity and specificity against the existing methods of dry eye diagnosis. Abnormal elevations of MMP-9 may predict post-laser in situ keratomileusis complications and refractive complications such as epithelial ingrowth and corneal ulceration. The presence of elevated MMP-9 on the ocular surface will identify those patients who should receive antiinflammatory therapy, such as cyclosporine, and may predict those patients who will respond to this therapy.

Conclusions: A rapid in-office test that is sensitive for identifying inflammatory dry eye and ocular surface disease may facilitate better preoperative management of the ocular surface. Optimization of the ocular surface perioperatively would be expected to reduce complications from laser in situ keratomileusis and other surgeries that often make the underlying disease worse. This test may also indicate the need for antiinflammatory therapies, such as cyclosporine or steroids, and also may predict those patients who are more likely to respond.

Key Words: matrix metalloproteinases, keratitis sicca, ocular surgery, inflammation, nano-detection, LASIK

(*Cornea* 2013;32:211–216)

In 1969, Dohlman and his co-workers identified metalloproteinases as substances produced in ocular surface diseases that were important contributors to corneal ulceration, destruction, and perforation.^{1–3} Although there are more than a dozen matrix metalloproteinases (MMPs) that can dissolve collagen and other tissues (Table 1), this review is primarily concerned with MMP-9 (also called gelatinase-B). Although numerous important articles have documented the role of these metalloproteinases

in ocular surface disease, both as a marker of the disease and as an agent of their pathology, they are being reviewed now because, for the first time, a new in-office immunoassay for the detection of MMP-9 is available. This can be of great value for both the detection of ocular surface disease and the prevention of complications after laser in situ keratomileusis (LASIK) and other ophthalmic surgeries.⁴

KERATITIS SICCA

Keratitis sicca is a complex disease with various components of aqueous deficiency from the lacrimal and accessory lacrimal glands, meibomian gland deficiency, and abnormal lipids stimulating evaporation and inflammation of the eyelids. It is usually referred to as dysfunctional tear syndrome (DTS), dry eye, and keratitis sicca. It occurs, in part, as a function of aging and atrophy of the accessory and lacrimal glands; as Sjogren syndrome, an immunomodulated disease; as a function of other autoimmune diseases (virtually all autoimmune diseases, such as lupus, graft-versus-host disease, rheumatoid arthritis, Stevens–Johnson syndrome, toxic epidermal necrolysis, and other skin diseases are associated with keratitis sicca); and can be exacerbated by a variety of common drugs, such as antihistamines, antihypertensives, and antidepressants. In its severe form, DTS can lead not only to disruptions of vision and corneal ulceration but also to corneal perforation and blindness. For clinicians, two potentially separate aspects of ocular surface disease are of primary concern, first, making the diagnosis of ocular surface disease,⁵ and second, determining the optimal therapy and monitoring its efficacy.⁶ In ocular surface disease associated with inflammation, where the degree of inflammation may be demonstrated by elevated tear levels of MMP-9, clinicians can better predict those patients who will respond to antiinflammatory therapy such as cyclosporine.⁷

The precise diagnosis of dry eyes has been clinically problematic. The Schirmer tear test, although extremely useful, sometimes correlates poorly with the patient's symptoms and the other clinical appearances of dry eyes. Tear break-up time, similarly, is useful, but often correlates poorly with the clinically observable phenomena and patient signs and symptoms.^{8–11} It is thought that hyperosmolarity may be the gold standard for the diagnosis of dry eyes, but current test results have been variable¹² and show sensitivity and specificity that range from 64% and 71%,¹³ respectively, to 73% and 92%.¹⁴ Moreover, tests are expensive and it has been difficult to collect tears without some stimulation. As we consider this as clinicians and consider the possibility of different therapies, it is possible that hyperosmolarity may be less important

Received for publication August 5, 2011; revision received February 8, 2012; accepted March 2, 2012.

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TABLE 1. Classification of MMPs, Their Inhibitors, and Potential Inducers of Transcription

Collagenases	Gelatinases	Stromelysins	Membrane-type MMPs	Matrilysin	Enamelysin	Metalloelastase	Others	Inhibitors	Potential Inducers of Transcription
MMP-1	MMP-2	MMP-3	MMP-14	MMP-7	MMP-20	MMP-12	MMP-19	TIMP-1	BSG
MMP-8	MMP-9	MMP-10	MMP-15	MMP-26			MMP-21	TIMP-2	TCF-20
MMP-13		MMP-11	MMP-16				MMP-23A	TIMP-3	TNF
			MMP-17				MMP-23B	TIMP-4	
			MMP-24				MMP-27		
			MMP-25				MMP-28		

BSG, basigin; TCF, transcription factor; TIMP, tissue inhibitors of matrix metalloproteinases; TNF, tumor necrosis factor.

than the presence or absence of ocular inflammation in the causation of signs and symptoms. Chotikavanich et al¹⁵ showed that the MMP-9 level correlates with symptom severity scores in dry eye patients as well as with their decreased low-contrast visual acuity and tear break-up time. In fact, elevated levels of MMP-9 may be a more sensitive diagnostic marker than clinical signs.¹⁶

Early studies of MMP-9 involved relatively complex assays using the dissolution of gelatin or radiolabeled gelatin by the MMP.¹⁵ These and similar assays, which will be discussed later, were complex and suitable only for laboratory investigation as opposed to routine clinical diagnosis. Despite this, much very important work was done. For example, tears and saliva were initially tested in patients with Sjogren syndrome.¹⁷ In 1998, Konttinen et al¹⁸ showed that a collagenase is present in the saliva of Sjogren patients and raised the question whether this gelatinase activity, which can degrade basement membrane collagens, was present and changed in the saliva of Sjogren patients. Subsequently, it was shown that when the tears and saliva of patients with Sjogren disease were compared with those of healthy controls, MMP-9 (as well as some other active MMPs and inflammatory inhibitors) was significantly elevated.¹⁹ It was suggested that this gelatinase enzyme could not only be an accurate marker for the disease but a factor in producing the inflammatory disease itself.^{19–21} More recently, the presence of MMP-9 in patients with pure keratitis sicca,^{15,22} or DTS was studied by Pflugfelder and his colleagues. They found that in patients with DTS, the MMP-9 activity in unstimulated tear fluid was much higher than that in healthy controls and that the mRNA transcripts for MMP-9 were higher.

Nichols et al⁸ showed that “although patient-reported symptoms are moderately repeatable from visit to visit, many of the procedures clinically used to diagnose and monitor dry eye syndromes are largely unrepeatable.” The clinical study by Chotikavanich et al¹⁵ working with Pflugfelder concludes, “MMP-9 appears to be a potentially useful biomarker for diagnosing, classifying, and monitoring dysfunctional tear syndrome” (Fig. 1).²²

The Pflugfelder group has done considerable laboratory work with mice using an evaporation-induced keratitis sicca.^{23,24} They found that this desiccating stress stimulates the production of MMP by the corneal epithelium.^{25,26}

Corrales et al²⁵ working with Pflugfelder, showed that desiccating stress stimulates the expression of MMPs by the

corneal epithelium in mice and that in a strain-dependent fashion, MMPs cause the disruption of the corneal barrier, thus increasing permeability (staining) and corneal irregularity.

CORNEAL ULCERATION AND MMP-9

In experimental dry eyes, the production of MMP-9 is involved in the disruption of the corneal epithelial barrier function. In fact, knockout mice without MMP-9 are resistant to corneal epithelial barrier disruption.²³ After the disruption of the epithelial barrier, Smith et al²⁷ originally postulated and Matsubara et al²⁸ later showed that the basement membrane can be dissolved by MMP-9, facilitating corneal ulceration. Fini et al²⁹ showed that the metalloproteinases are produced by the surrounding corneal epithelium and facilitate corneal ulceration and disrupt healing.

The mechanisms of MMP-9 production will be discussed below, but ulceration seems to result from an imbalance between the production of MMP-9 and its ability to overwhelm naturally secreted tissue inhibitory metalloproteinase proteins. In fact, MMP-9 has been found to be produced by the conjunctiva at the head of active pterygia and is thought to be a primarily responsible factor in dissolving the basement membrane as pterygium advances. It may well be that the presence or absence of MMP-9 will indicate which pterygia are active and need to be excised,^{30,31} but further study is required for this.

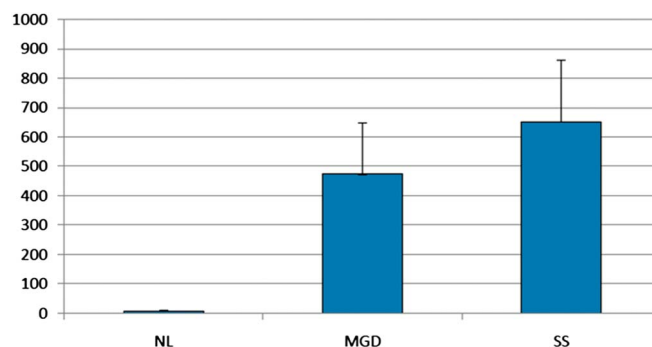


FIGURE 1. MMP-9 activity in tear samples of normal subjects (NL), patients with meibomian gland disease (MGD), and Sjogren syndrome (SS).²² Reprinted with permission from Association for Research in Vision and Ophthalmology, 2001.

TREATMENT OF DRY EYES AND OTHER OCULAR SURFACE DISEASE

In the past, effective MMP inhibitors have been synthesized. One of them, Galardin, was tested clinically. It was found to delay corneal destruction after pseudomonas infection. It was then tested on 556 patients with a variety of corneal ulcers, and it significantly reduced corneal perforations in clinical trials. It is not totally clear why Galardin was never approved and commercialized, but at the time it was tested, it was not feasible to determine those patients who had elevated metalloproteinase levels and those who did not, nor was it possible to measure those patients who actually responded to the treatment in terms of proteinase inhibition. It seems likely that the results would have been far more dramatic had a convenient clinical assay been possible for patient selection and evaluation of therapy because some of the ulcers may have been chronic and no longer subjected to proteinase activity.

In a rat thermal injury model,^{23,29} it has been shown that MMP-9 delays corneal epithelial healing and reepithelialization is impeded by these products of the resident corneal epithelial cells, which destroy adhesive structures at the basement membrane zone. Failure to reepithelialize was found to correlate with an increase in the amounts of gelatinolytic MMPs present in the rat cornea; inhibition of that synthesis correlated with inhibition of basement membrane dissolution.

PRODUCTION OF MMP-9 AND INFLAMMATORY CYTOKINE EXPRESSION AND INHIBITION

Brignole et al³² showed that 6 months of treatment with topical cyclosporine A can reduce inflammatory markers in patients with keratitis sicca. Turner et al³³ found a similar reduction in inflammatory markers after 6 months of treatment of patients with moderate to severe dry eyes with cyclosporine ophthalmic emulsion. In culture and in experimental desiccation keratitis, both cyclosporine^{7,34} and corticosteroids³⁵ can decrease the production of inflammatory markers such as MMP-9. Similarly, doxycycline, which has been found especially useful in the treatment of meibomian gland disease,³⁶ also decreases the production and effect of MMP-9.³⁷ It has been postulated that inflammation activates a mitogen-activated protein kinase, and this in turn activates the production of the MMP-9. This pathway seems especially susceptible to doxycycline inhibition.³⁷

From the point of view of an ophthalmologist, the presence of elevated levels of MMP-9 indicates an active ocular surface inflammation as part of dry eye or other ocular surface disease. Because cyclosporine^{7,34} and corticosteroids³⁵ have been shown to decrease production of inflammatory markers such as MMP-9 in human clinical trials, elevated levels of this marker seem to be a specific indication of patients who might be particularly susceptible to antiinflammatory treatment. Similarly, it seems likely that a decrease in MMP-9 may herald an improvement in the health of the ocular surface and patient symptoms.

MMP-9 IN LASIK AND OTHER OCULAR SURGERY

In any ocular surgery, the presence of undiagnosed ocular surface disease can be a hazard. In surgeries such as

LASIK or photorefractive keratectomy (PRK), any ocular surface disease can be made worse by the procedure and can result in serious complications.³³ Because dry eye is one of the most common complications of LASIK and PRK,^{37,38} diagnosis and management of ocular surface disease before surgery may lead to less postoperative complications.¹⁶ MMP-9 has been implicated in poor epithelial healing, and epithelial ingrowth after LASIK surgery.³⁹ Additionally, the reduction in tear function after LASIK may lead to an increase in the concentration of proinflammatory cytokines and MMP-9 in the tear film, which results in dry eyes and insufficient attachment between the corneal flap and the corneal bed.¹⁶ The failure to detect this inflammatory cytokine, which can signal potential complications, might be considered in the future to be below the standard of care.

Not only in LASIK surgery but also in cataract patients and patients undergoing other ocular surgery, corneal ulceration has been reported in patients who have dry eyes and ocular surface disease before the surgery. It is likely that testing for MMP-9 will be the simplest and most reliable way to rule out ocular surface disease and avoid problems such as this. In a practical sense, because of the frequency of ocular surface disease and the unreliability or irreproducibility of many of the tests used to detect ocular surface disease, the detection of elevated levels of MMP-9 might be even more important than the topographical testing for keratoconus and its forme fruste in terms of the frequency of avoiding serious complications.

PRK AND PTK

MMPs play an important role in epithelial regeneration and healing.⁴⁰ Coming from the healing epithelial cells or leucocytes that infiltrate, MMPs may cleave Bowman layer, the site of the hemidesmosomes that anchor the epithelial cells. After epithelial closure, MMPs become undetectable. They may well play a role in persistent or recurrent epithelial defects as well as persistent haze, which has been treated with Bowman membrane transplantation; this might be effective for recurrent erosions. Clinical studies indicate that inhibition of MMPs may be as effective as stromal puncture in treating recurrent erosions and preventing epithelial defects either alone (eg, with doxycycline or corticosteroids) or in combination with other therapies.^{41–43}

VERNAL CONJUNCTIVITIS

Just as other ocular surface disease that can damage the surface and the cornea can produce MMP-9, vernal conjunctivitis has been especially correlated with MMP-9 production.^{44–47} It has been suggested that eosinophils and mast cells may be involved in its production, but high levels of MMP-9 have been associated with vernal keratoconjunctivitis. The corneal damage and the ulceration seen in cases of vernal conjunctivitis may be because of the secretion of proteinases and gelatinases, often associated with the eosinophilic basic protein, and a reduction in these mediators may be an early indication of an adequate response to therapy.

OTHER CONDITIONS

Other ocular conditions, once they reach a point of significant surface inflammation, can also be associated with MMP-9 production. These include fungal keratitis,⁴⁸ burns, very advanced keratoconus with irregular surface,⁴⁹ and other factors that can seriously damage the surface. In these cases,⁵⁰ as in others, MMP-9 is a built-in indicator of significant ocular surface disease and also a mediator that can prolong and be responsible for the pathogenesis of the disease. Although these types of ocular conditions may be easily detectable with history or clinical presentation, many more patients have elevated levels of MMP-9 that may cause damage to the ocular surface and may not be detectable without the use of a testing device.

NONOCULAR DISEASE

The measurement of the elevation of MMP-9 has been found to be important in a variety of nonocular diseases.⁵¹ Just as it may be associated with pterygium and basement membrane dissolution, it is apparently involved in joint destruction with rheumatoid arthritis, but more recently, and importantly, it has been found to be a significant factor produced by cancers, especially those that are prone to metastasize. MMP-9 levels have been found to be elevated in breast cancer, aggressive prostate cancer, and bladder cancer. It may play a vital role in cancer metastasis and its measurement may be an important clinical indicator of this. The idea that a simple test could be done on urine, or a fingerstick drop of blood, to gauge the presence and potential metastatic predilection of a tumor, makes this whole field particularly exciting, especially because this testing may now be able to be made available as a simple and practical in-office screening test.

MECHANISMS OF MMP-9 PRODUCTION AND ACTIVITY

A variety of inflammatory mediators seem to have the potential of inducing and being correlated with MMP-9 production.^{52–55} Briefly, the MMP-9 seems to be produced in part through a mitogen-activated protein kinase pathway,⁵⁰ but other kinases also seem able to stimulate it. It seems to activate interleukin 1 and is, in fact, correlated with interleukin 1 production.^{56,57} MMP-9 is secreted as a zymogen, a proenzyme, that is physiologically activated by other proteases. Active MMP-9 may be bound and inactivated by tissue inhibitors of matrix metalloproteinases.^{15,52} It is one of a family of MMPs, but for ophthalmology seems to be the most important.

PRACTICAL MEASUREMENT OF MMP-9 AND ITS AFFECT ON TREATMENT DECISIONS

The earliest work with MMP-9 (gelatinase-B) was done by measuring its ability to dissolve gelatin. This was a relatively complex test that required hours and was difficult to quantitate, but it is still used. It has been improved with a variety of electrophoresis and other techniques (zymography). Western blotting techniques have been used to

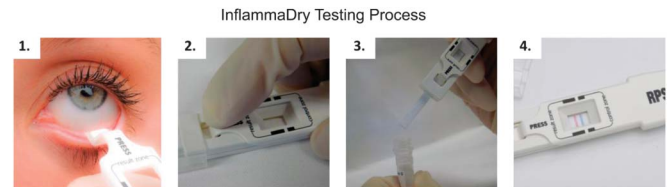


FIGURE 2. InflammDry testing process: 1, collect a tear sample from the lower conjunctiva; 2, snap the sample into the test; 3, dip test into buffer to activate; 4, read the results—2 lines = positive, 1 line = negative.

purify and identify it, as has immunohistochemistry, enzyme leak immunoassays, and MMP-9 capture activity measurements.^{21,32,54} InflammDry, a new, inexpensive, disposable single-use assay that provides a result in 10 minutes and can easily be done in the office, changes the utility of MMP-9 from a primarily research phenomenon to one that can and should be used in regular clinical diagnosis as a measure of the health of the ocular surface (Fig. 2). This new assay measures both active and latent MMP-9 (total MMP-9). No direct comparison has been made between elevated MMP-9 levels as determined by the new assay and MMP-9 activity assays that measure enzyme activity. According to the international InflammDry package insert, clinical study data demonstrate high sensitivity and specificity.⁵⁸

SUMMARY AND CONCLUSIONS

MMP-9 (gelatinase B) is produced in ocular surface disease and is important because it is a reliable indicator of the presence of this disease. In itself, it can cause corneal ulceration and complications after eye surgery such as LASIK, which may make ocular surface disease worse. MMP-9 is elevated in dry eye syndromes and its detection and measurement are not only a reliable indicator of the disease but also may be a reliable indicator of those patients who will respond to antiinflammatory therapy with agents such as cyclosporine or corticosteroids.

The development of an inexpensive and rapid test that can be easily done in the office is vital to the diagnosis of ocular surface disease, and probably the test best correlated with patient symptoms. In fact, elevated levels of MMP-9 may be a more sensitive diagnostic marker than clinical signs. This new test is indicated to detect and avoid complications of ocular surface disease before LASIK and other ocular surgeries as well as to detect hidden cases of dry eye disease that may not be easily identified through the clinical examination, particularly because inflammation is often present long before clinical signs appear. Additionally, the availability of this practical method to detect elevated levels of MMP-9 may facilitate the targeted therapeutic management of symptomatic patients.

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Production and Activity of Matrix Metalloproteinase-9 on the Ocular Surface Increase in Dysfunctional Tear Syndrome

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PURPOSE. To evaluate production and activity of metalloproteinase (MMP)-9 on the ocular surface of patients with dysfunctional tear syndrome (DTS) and determine any correlation between MMP-9 activity and clinical parameters.

METHODS. Forty-six patients with newly diagnosed DTS and 18 control subjects were recruited. Complete ocular surface examinations were performed. Tear MMP-9 activity was assessed with an MMP-9 activity assay in 1 μ L of unstimulated tear fluid. Using conjunctival epithelial cells from 19 patients with DTS and 16 controls, levels of MMP-9 and its regulating cytokine mRNA transcripts were evaluated by semiquantitative real-time PCR.

RESULTS. Each of four DTS severity-based groups had significantly higher mean MMP-9 activities than did the control group, which was 8.39 ± 4.70 ng/mL. The DTS4 group had the highest MMP-9 activity (381.24 ± 142.83 ng/mL), for which the mean was significantly higher than that of other DTS groups. In addition, patients with DTS had significantly higher levels of IL-1 β , IL-6, TNF- α , and TGF- β 1 mRNA transcripts in their conjunctival epithelia than did the control subjects. Tear MMP-9 activities showed significant correlation with symptom severity scores, decreased low-contrast visual acuity, fluorescein tear break-up time, corneal and conjunctival fluorescein staining, topographic surface regularity index (SRI), and percentage area of abnormal superficial corneal epithelia by confocal microscopy.

CONCLUSIONS. Tear MMP-9 activity was significantly higher in patients with DTS. This activity was associated with increased mRNA expression of MMP-9 and its regulating genes and correlated strongly with clinical parameters. MMP-9 appears to be a potentially useful biomarker for diagnosing, classifying, and

monitoring DTS. (*Invest Ophthalmol Vis Sci.* 2009;50:3203–3209) DOI:10.1167/iovs.08-2476

It has been proposed that inflammatory mechanisms are involved in the pathophysiology of dysfunctional tear syndrome (DTS), the more encompassing term for dry eye disease, proposed by the Delphi Dry Eye Panel Report in 2006.¹ It is now recognized that changes in tear composition in DTS may destabilize the tear film and cause ocular surface epithelial disease.^{1,2–6} Matrix metalloproteinases (MMPs) are proteolytic enzymes produced by stressed ocular surface and glandular epithelial cells, as well as by the inflammatory/immune cells that infiltrate these tissues. Increased activity of MMPs has been implicated in these pathologic ocular surface changes.^{5,7}

MMPs play a vital role in wound healing and inflammation.^{8,9} Increased levels of MMP-3 and -9 have been detected in the tear fluid of patients with keratoconjunctivitis sicca (KCS).^{10,11} Among the MMPs, MMP-9 has been found to be of central importance in cleaving epithelial basement membrane components and tight junction proteins (such as ZO-1 and occludin) that maintain corneal epithelial barrier function.^{12–14} MMP-9 belongs to the gelatinase group of metalloproteinases that degrade denatured collagen; native collagens type IV, V, and VII; and elastin. Expression of MMP-9 by the ocular surface epithelia in normal healthy eyes is low.¹⁵ Increased production of MMP-9 by the corneal epithelium has been found in the eyes of individuals with sterile corneal ulceration.¹⁶ Increased MMP-9 activity has been associated with disruption of corneal epithelial barrier function and corneal surface irregularity in an experimental murine model of dry eye.¹⁷ MMP-9 knockout mice showed significantly less alteration of epithelial barrier function in response to experimental desiccating stress than did wild-type mice.¹⁷ This protective effect was abrogated by topical application of MMP-9 to the ocular surface.¹⁷

Previous human studies have found an increased concentration of pro-MMP-9 measured by enzyme-linked immunosorbent assay (ELISA) in tear fluid of patients with ocular rosacea.^{5,10} Solomon et al.⁴ found increased activity of MMP-9 in the tear fluid of patients with meibomian gland disease and Sjögren's syndrome.⁴ Most MMPs are secreted as inactive zymogens (proMMPs) that require extracellular activation before they are able to cleave extracellular matrix components. This study was designed to measure the activity of the total active form of MMP-9 in normal eyes and in those in the various DTS groups classified by the Delphi panel and the Dry Eye Workshop (DEWS). The correlation between tear MMP-9 activity and clinical parameters of DTS was determined. Finally, mRNA transcript levels of MMP-9 and its regulating cytokines in the conjunctival epithelium were measured.

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Supported by NIH Grant EY11915 (SCP), an unrestricted grant from Research to Prevent Blindness, The Oshman Foundation, The William Stamps Farish Fund, and The Hamill Foundation.

Submitted for publication June 23, 2008; revised November 18, 2008, and January 16, 2009; accepted May 18, 2009.

Disclosure: S. Chotikavanich, None; C.S. de Paiva, None; D. Li, None; J.J. Chen, None; F. Bian, None; W.J. Farley, None; S.C. Pflugfelder, None

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TABLE 1. DTS Severity-Based Classification

Group	Symptom Severity Score	TBUT	Conjunctival Staining Signs*	Corneal Staining Signs†
Normal	≤20	>7 s	0	0
DTS1	>20	≤7 s	≥1 <3	<2
DTS2	>20	≤7 s	≥3	<6
DTS3	>20	≤7 s	≥3, mild conj. injection	6–11, filamentary keratitis
DTS4	>20	≤7 s	≥3, marked conj. injection; scarring	≥12, epithelial defect, ulceration

* Obtained from the van Bijsterveld grading scheme.²⁵

† Obtained from the Baylor grading scheme.²⁶

METHODS

The clinical protocol was approved by the Baylor College of Medicine Institutional Review Board and was in accordance with the tenets of the Declaration of Helsinki. After providing informed consent, 46 patients with DTS, newly diagnosed by a single investigator (SCP), and 18 asymptomatic control subjects were recruited for measurement of tear MMP-9 activity. A subset of 19 patients with newly diagnosed DTS and 16 asymptomatic control subjects were also enrolled for evaluating gene expression in the conjunctival epithelium. The following ocular surface and tear examinations were sequentially performed on all study participants: completion of the Ocular Surface Disease Index (OSDI) symptom questionnaire,^{18,19} collection of unstimulated tear samples, fluorescein tear break-up time (TBUT), corneal and conjunctival fluorescein staining, and Schirmer I test. Finally, impression cytology to obtain cells for evaluating gene expression was performed.

Criteria for diagnosis of DTS included an OSDI score >20^{1,18–20} with one or more of the following signs: TBUT ≤ 7 seconds,^{19,21} punctate corneal fluorescein staining, or Schirmer I score <10 mm. Subjects were excluded if they were using any topical medications or if they had another ocular surface disease.

The patients were classified into four levels of severity according to reported criteria of the DEWS²² report, as shown in Table 1. Lid margin disease and tear signs could be present at any severity level. When not all criteria of a severity group were met, severity grading was based on the worst parameter. Asymptomatic control subjects were recruited from employees of Baylor College of Medicine presenting for routine eye examinations.

Participants completed the OSDI questionnaire containing 12 items measuring visual function, ocular irritation symptoms, and effects of stressful environmental conditions.¹⁸ The score ranged from 12 (no symptoms) to a maximum of 64.

Measurement of High- and 10% Low-Contrast Visual Acuity

High (100%)- and low (10%)-contrast best corrected visual acuity was measured with a standard EDTRS chart mounted on a stand 4 m from the spectacle plane of the subject. The background luminance of the chart was set at 85 cd/m², and ambient luminance was adjusted to 3 cd/m².^{23,24} The difference between high- and low-contrast acuity was recorded.

Computerized Videokeratoscopy

Videokeratoscopic examination (TMS-2 Corneal Topography System; Tomey, Waltham, MA) was performed as previously reported.²⁰ The SRI was calculated by the instrument's software and recorded.

Tear Fluid Collection

Tear fluid (0.5 μL) was atraumatically collected with a 0.5-μL glass capillary tube (Drummond Scientific, Broomall, PA) by capillary action from the inferior tear meniscus of each eye. Tear samples from both eyes (1 μL total) were eluted into a sterile tube containing 9 μL of PBS and 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO).

The tubes were sealed with a cap containing a rubber O-ring to prevent evaporation and immediately stored at –80°C until activity assays were performed.

Fluorescein TBUT

After the inferior tarsal conjunctiva was touched with a sodium fluorescein strip (Fluor-I-Strip; Bausch & Lomb Pharmaceuticals Inc., Tampa, FL) wet with preservative-free saline (Unisol; Alcon, Fort Worth, TX), the precorneal tear film was examined under blue-light illumination, as previously reported.²⁰

Conjunctival and Corneal Fluorescein Staining

The ocular surface was examined 2 minutes after fluorescein instillation into the tear film, as described earlier. The intensity of conjunctival fluorescein staining was recorded with a modified van Bijsterveld grading scheme on a scale of 0 (none) to 3 (confluent) in the nasal conjunctiva and temporal conjunctiva.²⁵ The Baylor grading scheme was used to grade the intensity of corneal fluorescein staining²⁶ in five different zones of the cornea (central, superior, temporal, inferior, and nasal) based on the number of dots on a 5-point scale: no dots, 0; 1 to 5 dots, 1; 6 to 15 dots, 2; 16 to 30 dots, 3; >30 dots, 4. If there is one area of confluence add 1; two or more areas of confluence, add 2; filamentary keratitis, add 2.

Schirmer I Test

A Schirmer I test was performed by placing Schirmer strips (Alcon) over the lower lid margin, at the junction of the lateral and middle thirds, for 5 minutes. The length of strip wetting was recorded in millimeters.

Confocal Microscopy

Confocal microscopy was performed on the cornea of a subset of seven patients with DTS and three normal control subjects (Retina Tomograph II in combination with the Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany). After topical application of anesthesia, a drop of optical coupling medium gel (GenTeal, 0.3% hypromellose; Novartis Ophthalmics, Basel, Switzerland) was applied to the inferior conjunctival fornix. Images were sequentially captured from the air superficial corneal epithelial interface posterior to the subepithelial nerve plexus. For analysis, the area of abnormal superficial epithelia, defined by singular or multiple hyperreflective opaque cells or cells with pyknotic or snake like nuclei was measured in digital images with NIHJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) by two masked observers. The abnormal area was calculated as the average value of the percentage over the total (400 × 400 μm) field area of four randomly selected areas in the central cornea.

Collection of Conjunctival Epithelial cells

Conjunctival epithelial cells were obtained by an impression cytology technique. One drop of anesthetic was instilled into both eyes, and

TABLE 2. Clinical Features of DTS Patients and Controls

Group	Subjects (n)	Symptom Scores	SRI Score	TBUT (s)	Corneal Staining*	Conjunctival Staining	Schirmer I (min)
Control	18	16.59 ± 1.79	0.54 ± 0.10	9.56 ± 1.34	0	0	12.61 ± 7.62
DTS	46	35.70 ± 10.51	1.61 ± 0.82	5.55 ± 3.03	4.78 ± 5.22	1.43 ± 2.56	13.63 ± 9.69
DTS1	15	34.67 ± 12.67	1.48 ± 0.56	7.20 ± 3.00	0.73 ± 0.96	0	19.60 ± 9.22
DTS2	11	34.64 ± 10.34	1.25 ± 0.43	5.00 ± 2.19	3.00 ± 2.00	1.36 ± 2.25	16.64 ± 11.45
DTS3	9	33.63 ± 9.58	1.03 ± 0.70	5.56 ± 3.13	4.89 ± 4.43	1.22 ± 2.11	10.50 ± 6.57
DTS4	11	40.22 ± 8.41	2.54 ± 0.82	3.44 ± 2.70	12.00 ± 4.02	3.64 ± 3.53	6.11 ± 2.52

Data shown are mean ± SD.

* Measured by Baylor grading scheme.¹⁹

excess fluid was dried with a tissue. Sterile nitrocellulose filter papers (0.45 μ m HA, 45 mm circles, Cat No. HAWP04700; Millipore, Bedford, MA) with a tapered rectangular cut of 5 × 8 × 6 × 10 mm were applied to the temporal and inferior bulbar conjunctiva of each eye. The papers were gently peeled off the surface of the conjunctiva with a pair of forceps and placed in a tube containing RNA lysis buffer.

Tear MMP-9 Activity

Total MMP-9 enzyme activity was measured with an MMP activity assay kit (Biotrak; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. In brief, 100 μ L of each pro-MMP standard (0.125–4 ng/mL), diluted tears (1 μ L of tears in 9 μ L PBS and 0.1% BSA diluted in 90 μ L assay buffer), and assay buffer (for blanks) were incubated at 4°C overnight in wells of a microtiter plate precoated with anti-MMP-9 mouse monoclonal capture antibody. Plates were washed four times with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.05% Tween-20. Total MMP-9 activity was measured by activating bound pro-MMP-9 with 50 μ L of 1 mM *p*-amino phenylmercuric acetate (APMA) in assay buffer at 37°C for 1.5 hours. Detection reagent (50 μ L) was added to each well, and samples were incubated at 37°C for six hours. Active MMP-9 was detected through its ability to activate a modified prodetection enzyme that subsequently cleaved its chromogenic peptide substrate. Absorbance was read at 405 nm in a microplate reader (Versamax; Molecular Devices, Sunnyvale, CA). The activity of MMP-9 in a sample was determined by interpolation from a standard curve. Tear sample absorbance readings were multiplied by a dilution factor of 100.

RNA Isolation and Real-Time PCR

Gene expression in the conjunctival epithelium was evaluated in a subset of 19 DTS and 16 normal subjects. Conjunctival epithelial RNA, enriched for mRNA, was isolated from impression cytology samples by using guanidine-isothiocyanate-containing lysis buffer followed by selective binding of RNA to the silica-gel-based membrane (RNeasy Micro kit; Qiagen, Gaithersburg, MD). The RNA concentration was measured by its absorption at 260 nm, and the samples were stored at –80°C until used for polymerase chain reaction (PCR). First-strand cDNA was synthesized from 0.2 μ g of total RNA with random hexamers by M-MuLV reverse transcription (Ready-To-Go You-Prime First-Strand Beads; GE Health Care, Inc., Arlington Heights, IL). Real-time PCR was performed with specific probes (TaqMan MGB; Applied Biosystems, Inc. [ABI], Foster City, CA) for GAPDH, MMP-9, MMP-3, IL-1 β , IL-6, TNF- α , and TGF- β 1 (Assay IDs: Hs 99999905_m1, Hs 00234579_m1, Hs 00233962_m1, Hs 00174097_m1, Hs 00174131_m1, Hs 00174128_m1, and Hs 99999918_m1, respectively), with PCR master mix (TaqMan Gene Expression Master Mix; ABI), in a commercial thermocycling system (Mx3005P QPCR System; Stratagene, La Jolla, CA), according to the manufacturer's recommendations. Assays were performed in duplicate in each experiment. The results of quantitative PCR were analyzed by the comparative CT method,²⁷ where the target change was $2^{-\Delta\Delta C_t}$. The cycle threshold (C_t) was determined using the primary (fluorescent) signal as the cycle at which the signal crosses a user-defined threshold. The results were normalized by the C_t value of

GAPDH, and the relative mRNA level in the normal control group was used as the calibrator.

Statistical Analysis

Statistical analyses were performed with commercial software (Prism; GraphPad, La Jolla, CA, and Excel; Microsoft, Redmond, WA). Data are expressed as the mean ± SD. The normality of data was checked with the Kolmogorov-Smirnov test using the Dallal and Wilkinson approximation. One-way analysis of variance (ANOVA) or the Kruskal-Wallis test was used to detect statistical differences among multiple groups with normal or non-normal distribution, respectively. Statistical comparisons of tear MMP-9 activity levels between groups were performed by two-sample *t*-test. Inflammatory cytokine mRNA transcript levels were compared with the unpaired two-tailed *t*-test. Correlations between tear MMP-9 activities and clinical parameters, including symptom severity scores, decrease of low-contrast visual acuity, TBUT, and percentage area of abnormal superficial corneal epithelia by confocal microscopy were determined by logarithmic regression. Correlations between tear MMP-9 activities and SRI scores, corneal fluorescein staining scores, and conjunctival fluorescein staining scores were determined by polynomial regression. Statistical significance was calculated by Spearman correlation, which makes no assumption about the normality of the data. *P* < 0.05 was considered to be statistically significant.

RESULTS

Features of Study Groups

Forty-six patients with DTS with a mean age of 54.7 ± 14.7 (80% female, 20% male) and 18 controls with a mean age of 41.7 ± 13.0 (72% female, 28% male) were included for tear MMP-9 activity measurement.

The clinical features of control subjects and DTS subjects stratified by severity level are provided in Table 2. Associated ocular surface diseases found in each DTS severity level are noted in Table 3.

Clinical Parameters

Patients with DTS had a significantly greater mean decrease in 10% low-contrast visual acuity (0.24 ± 0.06 logMAR) than did control subjects (0.17 ± 0.04 logMAR) (*P* = 0.001). The decrease in low-contrast acuity showed significant and positive correlation with responses to two questions about blurred symptoms on the OSDI questionnaire (r^2 = 0.39, *P* < 0.001), but this correlation was not found for high-contrast acuity (r^2 = 0.07, *P* = 0.16).

In the subset of subjects evaluated by confocal microscopy, the percentage area of abnormal superficial corneal epithelial cells measured over the total image field area was 17.35% ± 15.41% in subjects with DTS (DTS2 [*n* = 3], DTS3 [*n* = 3], and DTS4 [*n* = 1]), compared with 0.34% ± 0.38% in control subjects (*n* = 3; *P* < 0.03). Some eyes with DTS had abnormal

TABLE 3. Ocular Surface Conditions in DTS Severity Groups

DTS Severity Level	Condition
DTS1	Anterior blepharitis (1) MGD (3), MGD with rosacea (1) Prior LASIK (1) Conjunctivochalasis (5) Salzmann's nodular degeneration (1) Epithelial basement membrane dystrophy (2) Thyroid eye disease with lagophthalmos and decreased blink frequency (1)
DTS2	MGD (9) ATD without systemic disease (2)
DTS3	MGD (4), MGD with rosacea (1) Sjögren syndrome (SS) (1) Prior LASIK (1) Hypoesthesia following acoustic neuroma tumor excision (1)
DTS4	Blepharoconjunctivitis with punctal swelling (1) ATD without systemic disease (1) Stevens-Johnson Syndrome (SJS) (2) Sjögren Syndrome (SS) (6) Graft versus host disease (GVHD) (1) Radiation induced punctal stenosis (1)

Numbers in parentheses are the number of subjects with each condition. MGD, meibomian gland disease; ATD, aqueous tear deficiency.

superficial corneal epithelia with a polygonal shape and highly reflective cytoplasm, with bright-appearing (pyknotic) nuclei surrounded by a dark perinuclear space. Representative confocal images are shown in Figure 1.

Tear MMP-9 Activity

The tear MMP-9 activities in all groups of patients with DTS are presented in Table 4. There was no significant difference in tear MMP-9 activity between male and female participants in the normal and DTS groups. Although the mean age of patients with DTS was higher than that of the normal control subject (54.7 vs. 41.7 years, $P = 0.01$), there was no significant difference in tear MMP-9 activity in each decade of age between 20 and 80 years in the normal and DTS groups.

Expression of MMP-9 and Regulating Cytokine Genes

Levels of mRNA transcripts encoding MMP-9 and its regulating inflammatory cytokines in conjunctival epithelia obtained by impression cytology from 19 patients with DTS, recruited from DTS1 ($n = 3$), DTS2 ($n = 2$), DTS3 ($n = 4$), and DTS4 ($n = 10$) and 16 normal subjects, evaluated by real-time PCR, are shown in Figure 2. Significantly higher levels of MMP-9, IL-1 β , IL-6, TNF- α , and TGF- β 1 transcripts were observed in patients with DTS than in normal subjects ($P < 0.05$). MMP-3 transcripts were undetectable in samples obtained from normal subjects and those with DTS.

TABLE 4. Tear MMP-9 Activity in Normal Control and DTS Groups

Group	MMP-9 Activity (ng/mL)
Normal ($n = 18$)	8.39 \pm 4.70
DTS1 ($n = 15$)	35.57 \pm 17.04*
DTS2 ($n = 11$)	66.17 \pm 57.02*
DTS3 ($n = 9$)	101.42 \pm 70.58*†
DTS4 ($n = 11$)	381.24 \pm 42.83*‡

Data shown are the mean \pm SD.

* $P < 0.008$ Compared with normal.

† $P < 0.003$ Compared with normal and DTS1.

‡ $P < 0.001$ Compared with normal and the other DTS severity groups.

Correlation of Tear MMP-9 Activities with Clinical Parameters

The correlations between tear MMP-9 activity and all clinical parameters were evaluated (Fig. 3). Tear MMP activities showed significant and positive correlation with symptom severity scores, SRI scores, corneal fluorescein staining scores, conjunctival fluorescein staining scores ($P < 0.001$), decreased low-contrast visual acuity ($P = 0.002$), and showed significant and negative correlation with fluorescein TBUT ($P < 0.001$). They also showed significant and positive correlation with percentage area of abnormal superficial corneal epithelia in confocal images ($r^2 = 0.64$, adjusted $r^2 = 0.57$, $P = 0.005$) in the subset of patients subjected to confocal microscopy.

DISCUSSION

DTS is a common ocular surface disease that can affect productivity and quality of life. Diagnostic criteria and management guidelines for DTS have been proposed by the Delphi panel and DEWS workshop. In this study, tear MMP-9 activity was evaluated in groups of patients with DTS classified by severity.

Each of the DTS groups had significantly higher mean levels of tear MMP-9 activity than the normal subjects. Moreover, the most severe DTS group (DTS4) was found to have the highest mean MMP-9 activity, significantly higher than the other DTS groups. The mean MMP-9 activities in the DTS1 and -2 groups were not significantly different from each other; however, the DTS3 group had significantly higher mean MMP-9 activity than the DTS1 group. These findings indicate that tear MMP-9 activity is significantly elevated, even in mild DTS and that this may be a more sensitive diagnostic marker than clinical signs. This elevation appears to be clinically significant, and tear MMP-9 activity may prove to be a better marker of disease severity than traditional clinical signs.

Tear MMP-9 activity showed strong correlation with the results of conventional diagnostic tests of DTS (symptom severity scores, SRI scores, fluorescein TBUT, and corneal and conjunctival fluorescein staining scores). Those clinical find-

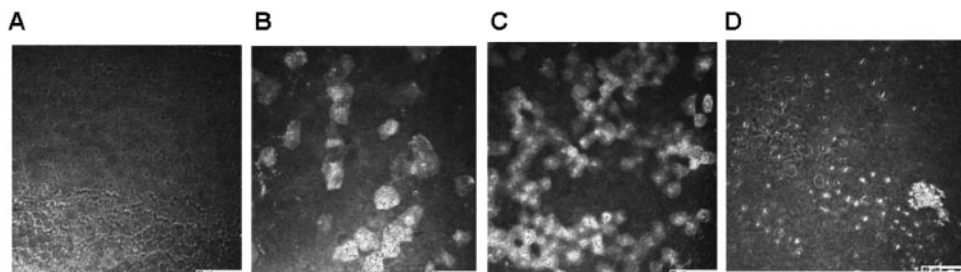
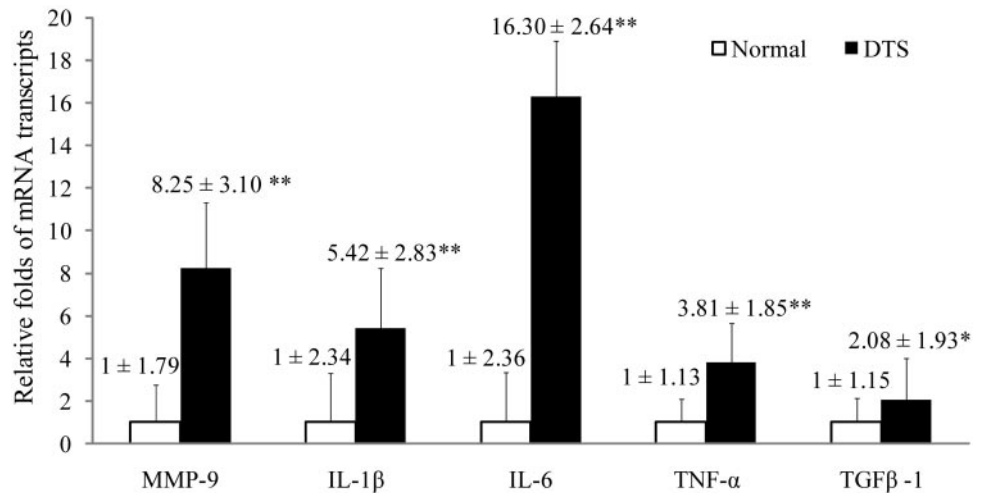


FIGURE 1. Representative confocal images of superficial corneal epithelium of (A) normal control, (B) DTS2, (C) DTS3, and (D) DTS4 subjects. Scale bar, 50 μ m.

FIGURE 2. Relative levels of MMP-9, IL-1 β , IL-6, TNF- α , and TGF- β 1 mRNA transcripts in conjunctival epithelia obtained from normal subjects ($n = 16$) and patients with DTS ($n = 19$). All data (mean \pm SD) were compared with the normal control: * $P < 0.05$; ** $P < 0.002$.



ings certainly can be attributed to the reported ability of MMP-9 to degrade epithelial tight junction and basement membrane proteins, leading to altered epithelial permeability and poor epithelial adherence.¹⁷

In the present study, we also measured 10% low-contrast visual acuity. Although the impact of DTS on visual function can be assessed by 100% high-contrast visual acuity, low-contrast vision may be a more functional and sensitive measure of the impact of this condition on visual quality. Previous studies in normal subjects have found that mesopic low-contrast acuity

shows a stronger correlation with retinal image quality than does mesopic or photopic high-contrast acuity.^{28,29} Moreover, this technique enabled detection of loss of vision in keratoconus that was not detected with high-contrast testing.³⁰ In addition, a previous study found that there was greater impact on restoration of low-contrast visual acuity when performing a Zernike-based optical correction.³¹ In our study, besides finding a significant correlation between the decrease of 10% low-contrast visual acuity and tear MMP-9 activities, we also found that the decrease in low-contrast acuity correlated more

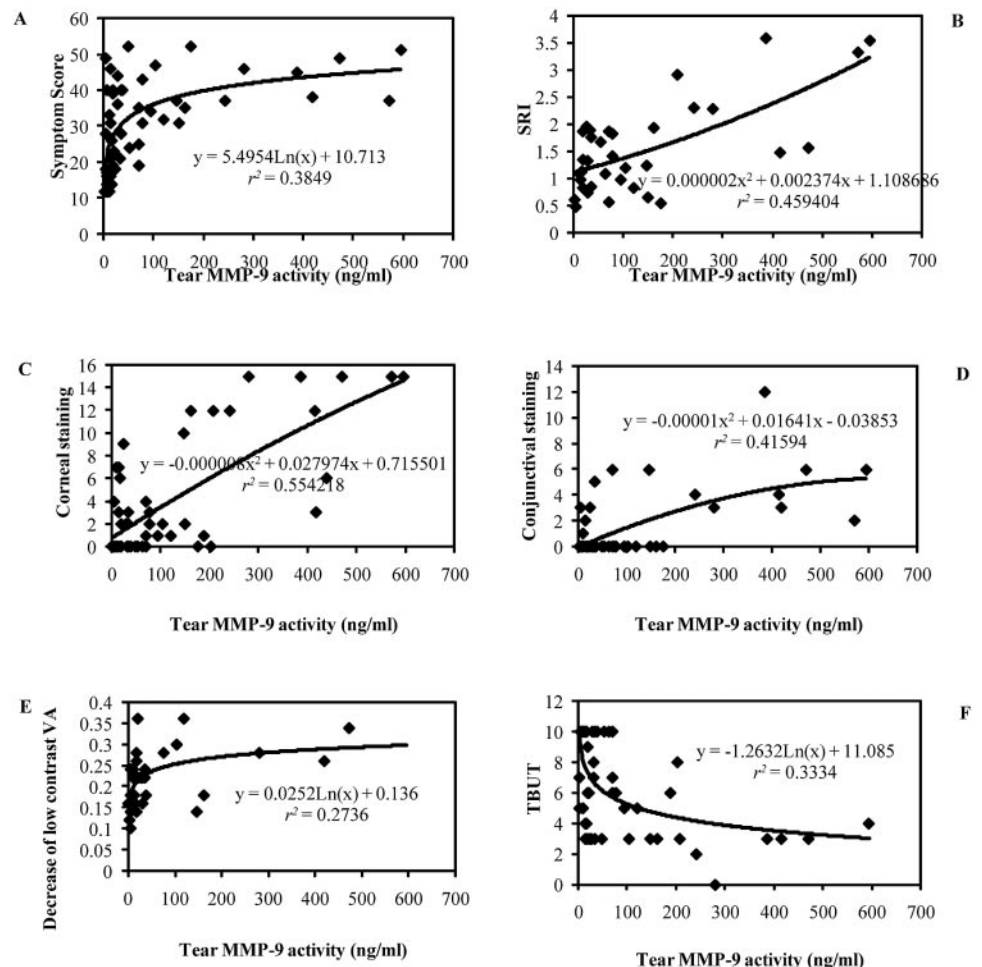


FIGURE 3. Correlation of tear MMP-9 activity in the study population ($n = 64$) with (A) symptom severity score, $P < 0.001$; (B) SRI score, $P < 0.001$; (C) corneal fluorescein staining score, $P < 0.001$; (D) conjunctival fluorescein staining score, $P < 0.001$; (E) decrease in low-contrast visual acuity, $P = 0.002$; and (F) fluorescein TBUT, $P < 0.001$. r^2 = coefficient of determination.

significantly with responses to the two questions about blurred symptoms on the OSDI questionnaire than did 100% high-contrast acuity.

Because of its ability to reveal clinicomorphologic correlations at the cellular level, the confocal microscope is becoming an increasingly valuable clinical tool. The normal superficial corneal epithelial cell was previously reported to have a dark cell nucleus and cytoplasm with a bright border.³² Previous confocal microscopic studies of dry eyes found a decreased density of corneal nerves and superficial corneal epithelia, but not of basal cells.^{33,34} In the present study, a correlation was found between tear MMP-9 activity and the percentage area of those abnormal superficial corneal epithelial cells. This change in the optical characteristics of the superficial epithelia may signal an early stage of epithelial desquamation induced by desiccating stress and increased tear protease activity.

Stimulated production of MMP-9, as well as cytokines that stimulate MMP-9 production (IL-1, IL-6, TNF- α , and TGF- β 1) by the ocular surface epithelia was confirmed at the transcriptional level by semiquantitative real-time PCR in our study. Similar results were also observed by Aragona et al. (IOVS 2008;49:ARVO E-Abstract 123). The increased tear film osmolarity that accompanies DTS is recognized as a proinflammatory stimulus.³⁵ Exposure of cultured human corneal epithelial cells to medium of increasing osmolarity has been found to stimulate production of MMP-9 and several inflammatory cytokines, such as IL-1 β and TNF- α (Li D, et al. IOVS 2002;43:ARVO E-Abstract 1981; Luo L, et al. IOVS 2003;44:ARVO E-Abstract 1026). Those inflammatory cytokines, in turn, have been shown to stimulate the production of a variety of MMPs, including MMP-9 by corneal epithelium.^{36,37}

Among its various activities, MMP-9 is known to activate precursor IL-1 β and latent TGF- β 1 into their active forms. Among several proteases, MMP-9 was found to be the most efficient activator of precursor IL-1 β .^{4,38} Therefore, the increase of MMP-9 activity on the ocular surface can amplify the chronic immune-based inflammation in DTS. Indeed, MMP-9 has been demonstrated in a previous study to speed cornea epithelial regeneration by modulating the inflammatory response in the healing cornea.³⁹ These findings indicate that DTS is capable of initiating an escalating cycle of cytokines and proteinases that can have deleterious consequences for the ocular surface.

Our finding of highly increased IL-6 gene expression in the conjunctival epithelium is consistent with several previously reported studies in both Sjögren- and non-Sjögren-associated aqueous tear deficiency.^{3,40,41} Significantly elevated IL-6 concentrations in tear fluid of patients with dry eye have been found in association with increased epithelial expression of IL-6.^{42,43} It should be noted that it was reported in another study that out of a spectrum of ocular surface diseases, including dry eye, IL-6 was found to be elevated only in eyes with conjunctivochalasis.⁴⁴ Of interest, this study found that the concentration of MMP-9 was elevated in all the ocular surface conditions compared with concentrations in control eyes. This finding supports the concept that elevation of tear MMP-9 is a common feature of ocular surface epithelial diseases, regardless of cause¹⁵ and is consistent with our finding that the mean MMP-9 activity in tears was increased in the variety of conditions associated with DTS noted in Table 3 and that tear MMP-9 activity showed strong correlation with the severity of corneal and conjunctival epithelial disease.

MMP-9 is secreted as a latent proenzyme that requires extracellular activation to be functional. In vitro, MMPs can be activated by chemical and physical agents, such as aminophenylmercuric acetate (AMPA), low pH, and heat. In vivo, MMPs are generally activated by other proteinases. MMP-9 has been reported to be activated by plasmin and more efficiently

by MMP-3.^{10,45,46} A previous study by our group found an increase in MMP-3 mRNA transcripts in cultured human corneal epithelial cells treated with IL-17 by real-time PCR using 1 μ g of RNA.⁴⁷ Using the same method, we were unable to detect MMP-3 transcripts in the conjunctival epithelium in our present study; however, a lower amount of total RNA was obtained by impression cytology which may have decreased sensitivity. Indeed, in a previous study, detection of MMP-3 transcripts by real-time PCR with 2 μ g of RNA obtained from retinoic-acid-treated cultured human conjunctival epithelial cells was reported.⁴⁸ Other possible explanations for not detecting MMP-3 are that it is produced by stromal cells or inflammatory cells that are in low density in the cytology samples, or MMP-3 production is lower in the conjunctival than in the corneal epithelium.⁴⁹⁻⁵² For the tear MMP-9 activity assay, we initially attempted to measure levels of the active MMP-9 enzyme in tear fluid without AMPA treatment; however, the levels were found to be too low to permit statistical comparison.

In summary, we found increased mean tear MMP-9 activity in all groups of patients with DTS, which was confirmed at the gene transcriptional level. In the DTS3 and -4 groups, the mean tear MMP-9 activity was found to be significantly higher than that in the other groups of patients with DTS. The MMP-9 activities were also found to correlate strongly with clinical parameters. This minimally invasive and sensitive MMP-9 assay is capable of evaluating the vital role of MMP-9 and may be clinically helpful for diagnosing, classifying, and monitoring DTS. It may prove to be an important clinical parameter in a future dry eye clinical trial.

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