ARTICLE

Reduced tear thrombospondin-1/matrix metalloproteinase-9 ratio can aid in detecting Sjögren's syndrome etiology in patients with dry eye

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Abstract

Differentiating patients with Sjögren's syndrome (SS)-associated dry eye from non-SS dry eye is critical for monitoring and appropriate management of possible sight- or life-threatening extraglandular complications associated with SS. We tested whether reduced tear levels of immunoregulatory thrombospondin (TSP)-1, which also inhibits matrix metalloproteinase (MMP)-9, would reflect SS pathogenesis aiding the identification of patients with SS-dry eye. Total of 61 participants, including healthy controls ($n = 20$), patients with non-SS dry eye ($n = 20$) and SS-dry eye ($n = 21$) were enrolled prospectively. Tear TSP-1 and MMP-9 levels were measured using a custom magnetic bead-based multi-plex assay in a masked manner. Analyte concentrations were assessed further according to ocular surface and tear film parameters. Relative to median tear TSP-1 (308 ng/ml) and MMP-9 (1.9 ng/ml) levels in the control group, significantly higher proportion of patients with SS-dry eye than non-SS had lower tear TSP-1 levels (55% vs. 29%, odds ratio [OR] = 3, 95% confidence interval [CI] = 1.64 to 5.35, $p < 0.05$) and higher tear MMP-9 levels (65% vs. 24%, OR = 5.8, 95% CI = 4.46 to 19.81, $p < 0.05$), respectively. The tear TSP-1/MMP-9 ratio was significantly reduced in patients with SS-dry eye compared to non-SS ($B = -2.36$, 95% CI = -3.94 to $-0.0.79$, $p < 0.05$), regardless of tear MMP-9 levels. Patients with a lower ratio were 2.3 times more likely to have SS (OR = 0.28, 95% CI = 0.1 to 0.75, $p < 0.05$). This ratio showed significant inverse correlations with clinical parameters (conjunctival and corneal staining scores). Our results denote that tear TSP-1/MMP-9 ratio can be useful in identifying patients with dry eye with underlying SS and used as a screening test.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
Differentiating patients with Sjögren's syndrome (SS)-associated dry eye from non-SS dry eye is critical for monitoring and appropriate management of possible sight- or life-threatening extraglandular complications associated with SS.

The high prevalence and multiple phenotypes and etiologies of dry eye in general make screening patients with underlying SS challenging.

WHAT QUESTION DID THIS STUDY ADDRESS?

Whether reduced tear levels of immunoregulatory thrombospondin (TSP)-1/ matrix metalloproteinase (MMP)-9 would reflect SS pathogenesis and aid in the identification of patients with SS-related dry eye.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

This study demonstrates for the first time the feasibility of quantifying tear TSP-1 levels. These results also indicate that determining tear TSP-1/MMP-9 ratio can be a better diagnostic tool in identifying patients with dry eye with underlying SS than tear MMP-9 or TSP-1 levels alone.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

The tear TSP-1/MMP-9 ratio can be used to screen patients with dry eye for the presence of underlying SS. Such an option can help guide clinical decisions regarding use of appropriate therapeutics to relieve clinical symptoms and prevent detrimental disease progression toward systemic complications. Additionally, a screening test can be a useful tool to stratify patients in clinical trials designed to develop new therapeutics for SS.

INTRODUCTION

Sjögren's syndrome (SS) is a highly prevalent rheumatic condition that destroys exocrine glands, leading to dry eye and dry mouth. SS is mediated by autoantibody production and can lead to many serious extraglandular ocular or systemic manifestations, including B-cell non-Hodgkin's lymphoma with high morbidity and mortality rates.[1–4] Currently, an estimated four million Americans are affected by SS.[5] The diagnosis of SS is complex due to diverse symptoms and signs and high false-negative results of antibody testing. The diagnosis is delayed by an average of 6.5 years from the onset of symptoms,[6] leading to delays in appropriate monitoring and management of the complications.[7] Of note, therapeutic interventions with biological agents are more likely to be beneficial when initiated within the first 5 years of disease onset.[6] Therefore, early diagnosis of SS is crucial.

The predominant clinical symptoms of dryness of the ocular surface and oral mucosa result from inflammatory infiltration of the lacrimal (main and accessory) and salivary glands, respectively.[2] A comparative study of salivary and lacrimal gland biopsy specimens obtained from patients with SS demonstrated that lacrimal glands have increased inflammatory infiltrates more than salivary glands.[8] In this study, about 19% of patients had increased inflammatory infiltrates only in their lacrimal glands, whereas salivary gland biopsies were normal. The study suggested that lacrimal glands are more likely to show inflammation earlier during the disease process

relative to salivary glands. Another more recent study of a large longitudinal cohort of patients with primary SS found that a dry eye diagnosis preceded systemic complications on average by a decade.[9] About one in 10 patients with clinically significant dry eye have underlying SS.[10,11] Considering that there are about 16 million Americans with clinician-diagnosed dry eye,[12] it is impractical and costly to test each patient to assess for the presence of underlying SS. Therefore, a screening test can be cost efficient and reduce delays in diagnosis to initiate timely therapeutic intervention.

Thrombospondin (TSP)-1 is a glycoprotein with immunoregulatory properties that is readily expressed by ocular surface epithelial cells.[13,14] In humans, a polymorphism in the *Thbs1* gene corresponding to a reduced expression of TSP-1 is associated with the development of a chronic dry eye condition.[15] In mice, TSP-1 deficiency was noted to lead to the development of SS and related ocular surface findings.[16,17] Anti-inflammatory activities of TSP-1 also include its ability to inhibit pro-inflammatory matrix metalloproteinase 9 (MMP-9), the latter of which has been implicated in the pathophysiology of dry eye.[18–20] Although a lateral flow immunoassay for rapid detection of tear MMP-9 is approved by US Food and Drug Administration (FDA) as a point-of-care test to aid in the diagnosis of dry eye, clinical trials have shown conflicting results regarding diagnostic utility of this modality.[19] The nonquantitative nature of the assay limits positivity to tears containing MMP-9 levels above the specified threshold (≥ 40 ng/ml). Importantly, this assay does not help distinguish dry eye subtypes.

In this study using a quantitative assay, we evaluated the TSP-1 and MMP-9 levels in tears from patients with SS-related versus non-SS dry eye versus controls to determine the utility of these analytes in identifying patients with dry eye with underlying SS.

MATERIALS AND METHODS

Subjects

This prospective case–control clinical study was approved by the Johns Hopkins University Institutional Review Board, Baltimore, MD, and adhered to the tenets of the Declaration of Helsinki. The details of the study participants, the methodology and clinical results have been published previously[21] in which decreased levels of goblet cell-specific gel forming MUC5AC were reported in patients with SS-related dry eye. Briefly, participants in the study included patients over the age of 50 years with clinically significant dry eye. Age-matched healthy individuals with no known history of ocular surface disease or dry eye or any autoimmune or inflammatory systemic diseases were included as controls. Study participants were recruited from the Ocular Surface Diseases and Dry Eye Clinic at The Wilmer Eye Institute, The Johns Hopkins University, Baltimore, MD. Participating patients were not on any prescription eye drops prior to enrollment. Use of any over-the-counter tears was held for 24 h prior to testing. Exclusion criteria for the study included a current or known history of contact lens wear or other ocular surface diseases where dry eye was a secondary diagnosis, such as graft-versus-host disease, mucous membrane pemphigoid, and atopic keratoconjunctivitis. Patients who were on other prescription eyedrops (such as for glaucoma) or patients who had any ocular surgery within the prior 3 months were excluded.

Three groups of study participants were formed: (1) patients with dry eye with definitively diagnosed SS (SS dry eye; $n = 20$, in whom a diagnosis was made according to 2012 American College of Rheumatology criteria with either a positive minor salivary gland biopsy or positive serology[22]); (2) patients with clinically significant dry eye but no definitively diagnosed underlying SS (non-SS dry eye; $n = 21$ with negative serology *and* a negative minor salivary gland biopsy); (3) subjects with no dry eye (controls; $n = 20$, no symptoms or clinical findings of dry eye or other ocular surface diseases, and no known history of systemic autoimmune disease with a negative review of systems).

All patients were evaluated in an identical manner. We designed a detailed review-of-systems questionnaire specifically for this study. Participants were first asked to complete this questionnaire, which inquired about the presence of dry eye, previous diagnosis of SS or other autoimmune diseases, and medication history, along with the Ocular Surface Disease Index (OSDI) symptom questionnaire. A total OSDI score and three subscores (1: ocular symptoms subscore [questions 1 to 5], 2: vision-related function subscore [questions 6 to 9], and 3: environmental triggers subscore [questions 10 to 12]), each ranging from 0 to 100, were calculated as previously described.[23] Then, clinical testing was performed in the following order: non-invasive tear break-up time (TBUT), tear osmolarity, tear sampling, Schirmer's test without anesthesia, and ocular surface staining using lissamine green for conjunctiva and fluorescein for the cornea. Noninvasive TBUT was measured using the Tear Stability Analysis System incorporated into the Tomey Top-Ref- Keratometer RT-7000 (Tomey, Phoenix, AZ) corneal topography machine.[24] The TearLab Osmolarity System (TearLab Corporation) was used to measure tear osmolarity. Tear film collection was performed 5 min after conducting a tear osmolarity test using single-use 1-μl microcapillary tubes. Tear samples were diluted 1:10 in 0.5 microL Cytokine Assay Buffer (Merck Millipore, Millipore Iberica) in a microtube and were labeled and stored at −80°C. De-identified, frozen tear samples were sent on dry ice to Boston University, Boston, MA, for analyses. Grading of ocular surface staining was performed according to a SICCA scoring system.[25] Adequate time was allotted between tests to allow the tear film to recover.

Tear TSP-1 and MMP-9 analysis

Tear samples diluted in phosphate-buffered saline (PBS) were analyzed in a masked manner using custom magnetic bead-based multi-plex assay (MilliporeSigma) and Drop Array platform (Curiox Biosystems). Standard curves for each analyte were generated using six known concentrations and mean fluorescence intensity corresponding to each concentration were plotted. Using a five-parameter logistic curve fitting procedure, lower and upper limits of detection were determined for each analyte. Data were analyzed using xPONENT for MAGPIX software (Luminex). Three replicates for each standard concentration were analyzed. Very low intra-assay (within-run, plate-specific) imprecision was indicated by low percent coefficient of variation (%CV) values for each standard curve. A good proportional relation between fluorescence intensity and standard concentration was indicated by R^2 value of 1. Low chi-square values (<0.04) indicated that the data are a good match to a proportional fit. Detection limit for each analyte was determined (Figure 1a). This
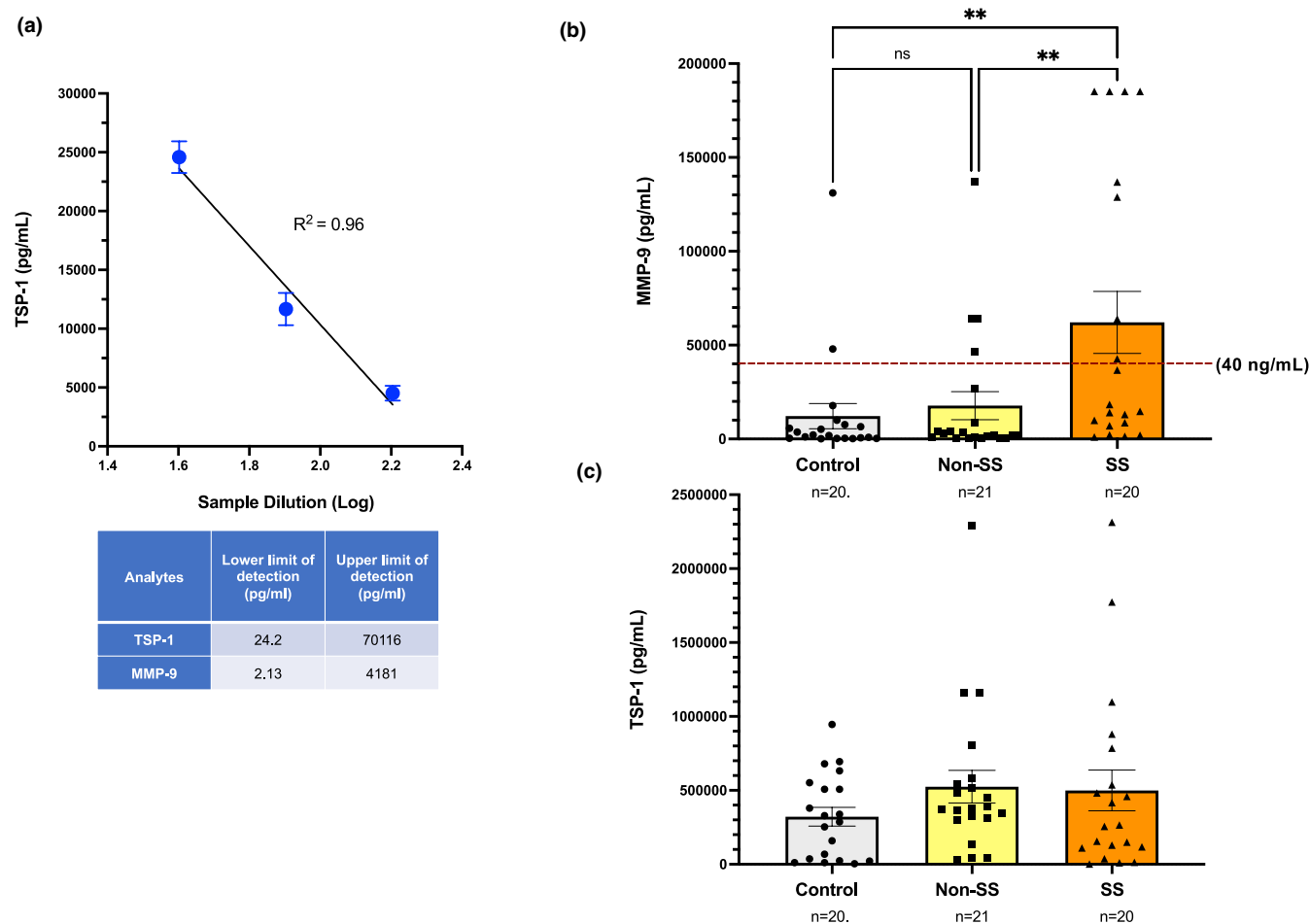
FIGURE 1 Tear TSP-1 and MMP-9 levels: assay validation and comparisons among study groups. Tear concentrations of TSP-1 detected over three dilutions of a sample from a healthy volunteer indicated a strong linear relationship. Lower and upper assay detection limit for each analyte is indicated (a). Comparison of mean tear MMP-9 (b) and TSP-1 (c) concentrations among healthy participants in control group and patients with non-SS or SS dry eye. The number in parentheses below each column indicates the number of participants in that group, and each circle (control), square (non-SS) and triangle (SS) represents an individual participant. Data are expressed as mean ± SEM. Statistical analysis was performed using a Kruskal-Wallis test with Dunn's correction for multiple comparisons **$p < 0.005$, ns: not significant. The dashed line in panel (b) indicates cut-off limit of rapid immunoassay (InflammaDry) test. SS, Sjögren's syndrome.

work was accomplished through the services provided by the Boston University Analytical Instrumentation Core.

Statistical analysis

Data received for tear analyte levels were assigned to three groups (SS, non-SS, and controls) after unmasking the associated diagnosis. Distribution of clinical parameters and tear TSP-1 and MMP-9 levels in three groups were tested for normality using the Shapiro–Wilk test. Based on the absence of normal distribution and the small sample size for continuous variables, global p value was determined using the Kruskal-Wallis test. The Dwass-Steel-Critchlow-Fligner multiple comparisons post hoc procedure was used to examine the pairwise difference. Categorical variables (e.g., gender) among

study groups were compared using a chi-square test. A Kruskal-Wallis test with Dunn's test to correct for multiple comparisons was used to compare tear TSP-1 and MMP-9 levels. Comparison of TSP-1:MMP-9 ratio between groups was made using a two-tailed Mann–Whitney U test. A Fisher's Exact test was used to compare frequencies of TSP-1, MMP-9, and TSP-1:MMP9 ratio among subgroups. To evaluate associations, regression analysis was performed on log-transformed data of TSP-1, MMP-9, or TSP-1:MMP-9 ratio. For multiple or logistic regression, after controlling for age and sex as required, continuous independent variables were standardized as a z-score so that the beta coefficient or odds ratio (OR) is per standard deviation change in the variable. Values of p less than 0.05 were considered statistically significant. To evaluate the accuracy of tear TSP-1/MMP-9 ratio to discriminate the SS and non-SS dry eye

groups, receiver of operating characteristic (ROC) curve analysis was performed. All statistical analyses were performed using SAS Institute 2013 Base SAS 9.4 (SAS Institute) and GraphPad Prism 9.0 for Mac (GraphPad Software). Data management and analytical support were provided by Biostatistics and Epidemiology Data Analytics Center, Boston University School of Public Health.

RESULTS

Demographics, tear film, and ocular surface parameters of the study population

A total of 61 participants were included in this study. Demographic features and clinical findings evaluated in each of the study groups are summarized in Table 1. The average age was 62 ± 8 years with no significant differences among groups. There were 47 women (77%) and 14 men (23%), and the proportion of women across the three groups did not differ significantly. Ocular symptoms as determined by the OSDI as well as the ocular staining scores for both the cornea and conjunctiva were significantly higher among both dry eye groups compared to those in the control group. Tear volume, as measured by a Schirmer's test, was significantly lower in both dry eye groups compared to the control group. The mean value for TBUT was significantly reduced only in patients with SS dry eye. Last, the conjunctival staining score was significantly higher in participants with SS dry eye compared to non-SS dry eye or the control group.

Tear TSP-1 and MMP-9 levels are inversely correlated in patients with SS dry eye

To validate the accuracy of analyte detection with our assay, dilutional linearity was first determined using three different dilutions of the tear sample from a single healthy volunteer using three replicates for each analyte. Considering that TSP-1 is expressed by epithelial cells of normal healthy ocular surface tissue,[14] we expected to detect some TSP-1 and minimum concentrations of MMP-9, if any. As expected, we detected 88.75 pg/ml (0.088 ng/ml) of MMP-9 in 1:40 dilution of the tear sample while at higher dilutions, MMP-9 levels were below the lower detection limit of the assay (2.13 pg/ml). This low MMP-9 concentration is expected in healthy volunteers in the absence of inflammation. At all three tested dilutions of tears, we successfully detected TSP-1 within the detection range of the assay. As indicated in Figure 1a, a strong linear relationship was noted in TSP-1 concentrations over

TABLE 1 Summary of demographic features and clinical parameters of the study participants

Parameter	Control (n = 20)	Non-SS dry eye (n = 21)	SS dry eye (n = 20)	Global p value	Control versus non-SS dry eye	Control versus SS dry eye	Non-SS versus SS-dry eye
Age (mean±SD)	60.4±5.7	63.2±7.9	63.6±10.3	0.582	0.5648	0.7444	0.9790
Gender (F/M)	F: 14 (70%) M: 6 (30%)	F: 18 (85%) M: 3 (14%)	F: 15 (75%) M: 5 (25%)	0.472			
OSDI score (0–100)	4.6±4.0	48.7±22.4	40.0±21.3	<0.0001	<0.0001	<0.0001	0.4222
TBUT (noninvasive), s	3.8±4.0	4.0±3.6	1.8±2.8	0.048	0.8049	0.1982	0.0430*
OSM (mOsm/L)	302.2±10.7	304.9±16.0	305.7±19.0	0.855	0.9842	0.9564	0.8197
Schirmer's test (mm)	19.3±8.0	13.1±7.8	9.1±8.0	0.0003	0.0165	0.0007	0.1632
Corneal staining score (0–6)	0.2±0.4	3.3±1.4	4.0±1.5	<0.0001	<0.0001	<0.0001	0.1540
Conjunctival staining score (0–6)	0.1±0.2	3.5±2.0	5.1±1.4	<0.0001	<0.0001	<0.0001	0.0228*

Abbreviations: OSDI, ocular surface disease index symptom questionnaire; OSM, osmolarity; SD, standard deviation; SS, Sjögren's syndrome; TBUT, tear break up time.

*Statistically significant.

three dilutions ($R^2 = 0.96$). This result suggests that there is no significant negative interference caused by sample matrix in our assay.

In our study, the average tear MMP-9 level in the control group was 12.2 ng/ml (Figure 1b), and the median value was 1.9 ng/ml. Significantly higher levels of tear MMP-9 were detected in the group with SS dry eye compared to the control and non-SS dry eye group. The proportion of individuals with tear MMP-9 levels above the control group median level was significantly higher in the SS dry eye group compared to non-SS dry eye (Table 2). We also evaluated tear concentration of TSP-1. Although we did not detect significant differences in mean values of tear TSP-1 levels among the three groups (Figure 1c), as shown in Table 2, the proportion of individuals with tear TSP-1 levels below the control group median (308 ng/ml) was significantly higher in the SS dry eye group compared to the non-SS dry eye group, supporting the inverse correlation between tear TSP-1 and MMP-9 levels in patients diagnosed with SS.

Tear TSP-1/MMP-9 ratio is reduced in patients with SS dry eye

We next determined the ratio of tear TSP-1/MMP-9 levels and compared this ratio among two study groups with dry eye. As shown in Figure 2a, median tear TSP-1/MMP-9 ratio was significantly reduced in patients with dry eye with a diagnosis of SS compared to those without underlying SS. The median ratio of the SS dry eye group but not the non-SS dry eye group was significantly reduced also when compared to the control group (10.4 vs. 69, Mann–Whitney U test, $p = 0.018$, 99.8 vs. 69 $p = 0.684$). The same change was observed when the control group median ratio (69) was used as a reference to determine the proportion of individuals with lower tear TSP-1/MMP-9 ratio. As shown in Table 3, a significantly higher proportion of patients with SS dry eye had a tear TSP-1/MMP-9 ratio below the control group median. Interestingly, in both dry eye groups, we detected several individuals with tear MMP-9 levels within a previously reported normal range of 3 to 40 ng/ml.[26] The median tear TSP-1/MMP-9 ratio

among this subset of individuals was also significantly reduced when dry eye was associated with a SS diagnosis (Figure 2b). Consistent with this finding, we detected a significantly increased proportion of individuals with a reduced tear TSP-1/MMP-9 ratio in the SS dry eye group compared to the non-SS dry eye group relative to the control group median (Table 3).

Ocular surface damage and low tear volume are associated with low tear TSP-1/MMP-9 ratio

Analysis of correlations between tear MMP-9 levels with clinical parameters in this study detected a significant positive association with corneal and conjunctival staining scores (Table 4). Conjunctival epithelial expression of MMP-9 in dry eye has been reported by other investigators.[27] In addition, a significant negative association was detected between tear MMP-9 levels and tear volume as measured with unanesthetized Schirmer's test. Other parameters, like TBUT, osmolarity, or OSDI scores, did not show significant correlation with tear MMP-9. Similarly, the tear TSP-1/MMP-9 ratio showed significant associations with ocular staining scores and tear volume as shown in Figure 3.

Low tear TSP-1/MMP-9 ratio is associated with SS diagnosis

A multiple linear regression model was built to evaluate the associations between tear MMP-9 or TSP-1/MMP-9 ratio as dependent variables each and clinical signs as independent variables while controlling for age and sex. Among all clinical measures of dry eye, a one-unit increase in conjunctival ($B = 2.02$, 95% CI $= 1.2$ to 3.4, $p = 0.009$) or corneal staining ($B = 1.97$, 95% CI $= 1.16$ to 3.35, $p = 0.014$) score was associated with two-fold increase in tear MMP-9 levels. Similarly, a two-fold reduction in TSP-1/MMP-9 ratio was associated with each unit increase in conjunctival ($B = 0.5$, 95% CI $= 0.26$ to 0.94, $p = 0.0323$) and corneal staining ($B = 0.49$, 95%

TABLE 2 Frequencies of abnormal tear analyte levels relative to control group median values in a prospective study comparing tear film inflammatory markers in patients with non-SS versus SS dry eye

		Non-SS dry eye	SS dry eye	OR	95% CI	Fisher's exact
MMP-9	Above median	48% (10/21)	90% (18/20)	9.8	4.463 to 19.81	$p < 0.0001$
TSP-1	Below median	24% (5/21)	55% (11/20)	3.9	2.140 to 7.079	$p < 0.0001$

Abbreviations: CI, confidence interval; OR, odds ratio; SS, Sjögren's syndrome.

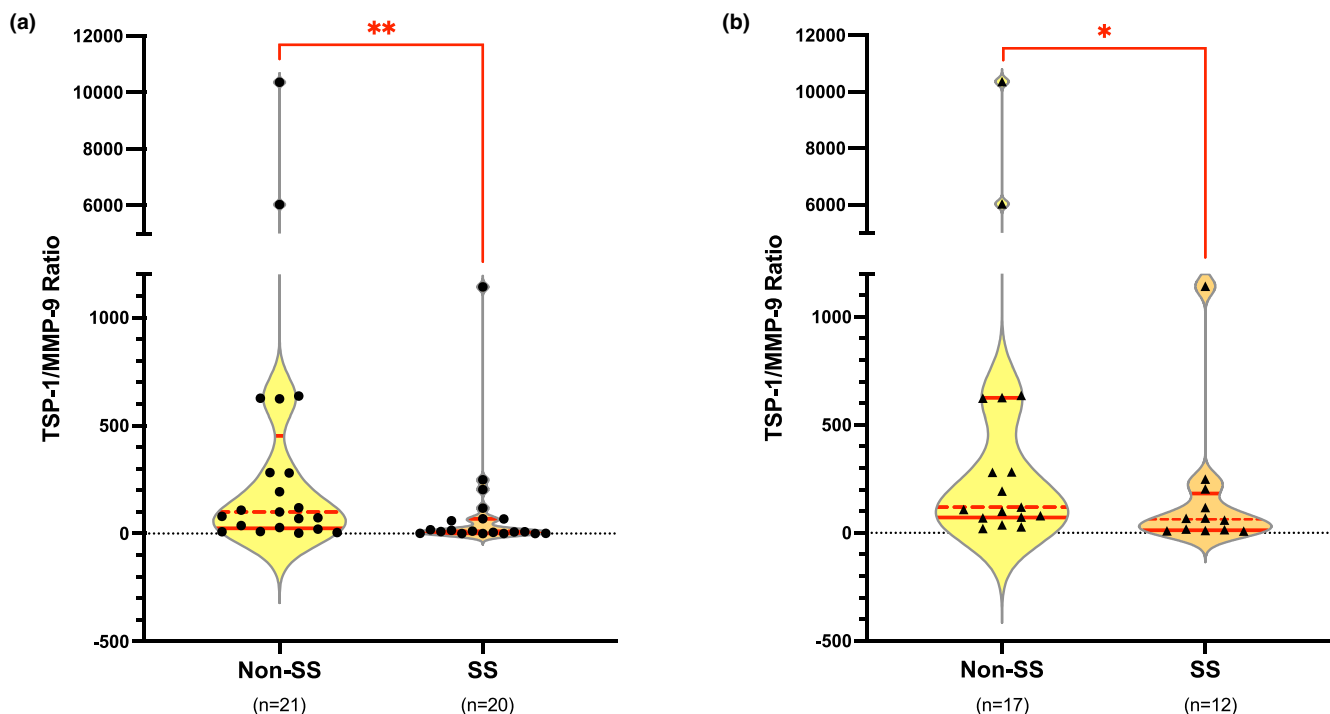(Median values in control group MMP-9 = 1.9 and TSP-1 = 308 ng/ml).

FIGURE 2 Tear TSP-1/MMP-9 ratio is reduced in patients with SS dry eye as compared to non-SS dry eye. Tear TSP-1/MMP-9 ratio was compared in all samples (a) and in samples with tear MMP-9 less than 40 ng/ml (b) between two dry eye groups. The number in parentheses below each group indicates number of participants included in that group. Violin plots of frequency distribution of tear TSP-1/MMP-9 values with each symbol (circle in a and triangle in b) represent individual data point in each group. Each violin plot is presented with a median (middle dashed line) and quartiles (top and bottom solid lines). Minimum and maximum values are shown as the bottom and top of each violin plot. Statistical analysis was performed using a Mann–Whitney *U*-test (two-tailed) **p = 0.0043 and *p = 0.0267. SS, Sjögren's syndrome.

TABLE 3 Frequency of TSP-1:MMP-9 ratio below the median determined in control group in a prospective study comparing tear film inflammatory markers in patients with non-SS versus SS-dry eye

Tear MMP-9 levels	Non-SS dry eye	SS-dry eye	OR	95% CI	Fisher's exact
All samples	33% (7/21)	80% (16/20)	8.0	2.014 to 27.09	*p* = 0.0044
<40 ng/ml	15% (3/17)	67% (8/12)	11.5	5.841 to 22.691	*p* < 0.0001

Abbreviations: CI, confidence interval; OR, odds ratio; SS, Sjögren's syndrome.

(Median TSP-1:MMP-9 ratio in control group 69).

TABLE 4 Spearman's correlation coefficient of tear MMP-9 levels with clinical parameters in a prospective study comparing tear film inflammatory markers in patients with non-SS versus SS-dry eye

	TBUT	OSM	OSDI	Schirmer's test	Fluorescein staining score	Lissamine green staining score
n	59	61	61	61	61	61
MMP-9	−0.1082 (0.415)	−0.0804 (0.538)	0.1689 (0.193)	−0.4233 (0.001)*	0.3532 (0.005)*	0.3430 (0.007)*

p values are included in parenthesis.

Abbreviations: OSDI, ocular surface disease index symptom questionnaire; OSM, osmolarity; SS, Sjögren's syndrome; TBUT, tear break up time.

*Statistically significant.

CI = 0.25 to 0.93, *p* = 0.031). Furthermore, using regression differences in mean values for conjunctival and corneal staining scores, tear MMP-9 levels and tear TSP-1/MMP-9 ratio were examined among patients with dry eye with and without SS diagnosis after adjusting for age and gender. Whereas significantly higher mean

conjunctival staining scores ($B = 1.60$, 95% CI $= 0.49$ to 2.72, $p = 0.0061$) and mean tear MMP-9 levels ($B = 1.8$, 95% CI $= 0.62$ to 2.99, $p = 0.0039$) were detected in patients with a diagnosis, significantly lower mean values for tear TSP-1/MMP-9 ratio were detected in these patients ($B = -2.36$, 95% CI $= -3.94$ to $-0.0.79$, $p = 0.0044$). Together, these results denote an inverse correlation between the TSP-1/MMP-9 ratio and the conjunctival staining score.

Furthermore, a logistic regression model adjusted for age and gender was analyzed using the SS diagnosis as a dependent variable with conjunctival and corneal staining scores, tear MMP-9 and TSP-1/MMP-9 ratio as independent variables. Patients with a higher conjunctival staining score (OR $= 2.77$, 95% CI $= 1.27$ to 6.04, $p = 0.0104$) and tear MMP-9 levels (OR $= 2.63$, 95% CI $= 1.06$ to 6.53, $p = 0.0367$) were more likely to have underlying SS. Moreover, patients with a lower tear TSP-1/MMP-9 ratio were 2.3 times more likely to have SS (OR $= 0.28$, 95% CI $= 0.1$ to 0.75, $p = 0.0118$). Together, these results clearly support an association between reduced tear TSP-1/MMP-9 ratio and SS diagnosis.

Diagnostic value of tear TSP-1/MMP-9 ratio in differentiating SS versus non-SS dry eye

Given that a reduced tear TSP-1/MMP-9 ratio was significantly associated with the diagnosis of SS, we evaluated the diagnostic value of this ratio in differentiating SS versus non-SS dry eye using ROC curve analysis. As indicated in Figure 4, the area under ROC curve (AUROC) is significantly different from 0.5 and allows discrimination between SS-dry eye from control or non-SS dry eye. Such discrimination was also applicable to subset of individuals with tear MMP-9 levels below 40 ng/ml (AUC $= 0.75$, SE $= 0.098$, 95% CI $= 0.554$–0.936, $p = 0.027$). However, distributions of ratio values in non-SS dry eye and the
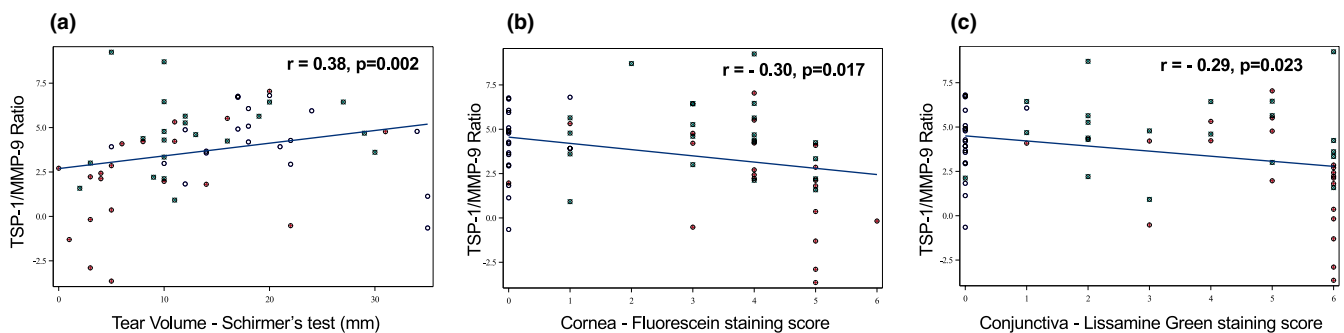

FIGURE 3 Correlation of tear TSP-1/MMP-9 ratio with clinical parameters tear volume and ocular surface staining scores. Scatter plots of tear TSP-1/MMP-9 ratio and (a) Tear volume, (b) Corneal staining score and (c) Conjunctival staining score. Spearman's correlation coefficient and p values corresponding to each plot are indicated.
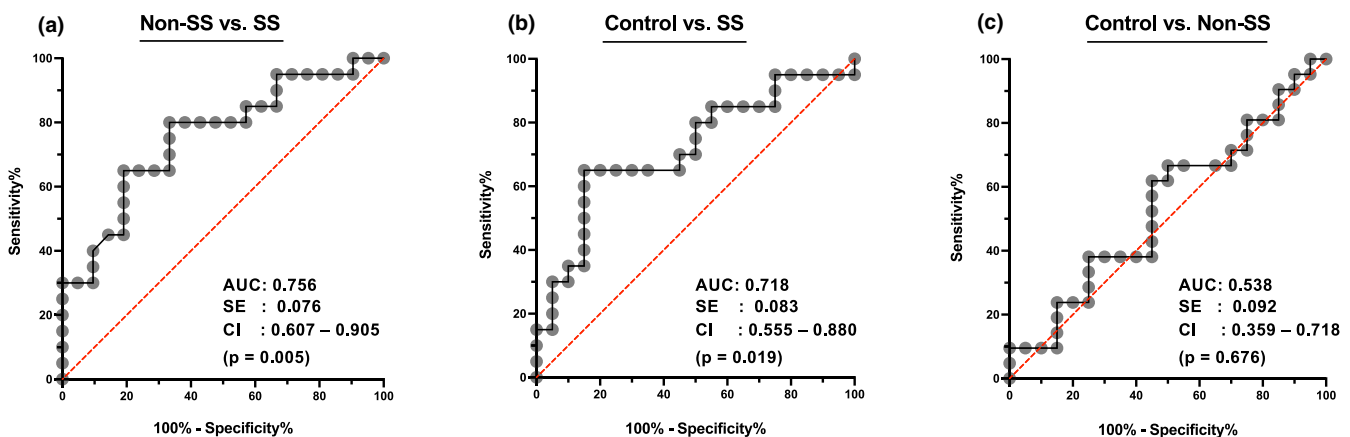

FIGURE 4 Diagnostic value of tear TSP-1/MMP-9 ratio. ROC curve analysis of ratios among control ($n = 20$), SS ($n = 20$), and non-SS ($n = 21$) groups shows the distribution of values between (a) non-SS versus SS, (b) control versus SS, and (c) control versus non-SS groups. True-positive rate (sensitivity%) is plotted with function of the false-positive rate (100-specificity%) for different cutoff points. Area under the receiver of operating characteristic (ROC) curve (AUROC), Standard error (SE), 95% confidence interval (CI), and p value compared to the AUC of 0.5 (red diagonal line). SS, Sjögren's syndrome.

control group were not different from 0.5. Therefore, there is evidence that the tear TSP-1/MMP-9 ratio does have an ability to distinguish between SS and non-SS dry eye groups. We also compared the sensitivity and specificity of tear TSP-1/MMP-9 ratio with that of tear MMP-9 level alone using their respective median values (Tables 2 and 3) as threshold values. Tear TSP-1/MMP-9 ratio provides 67% specificity and 80% sensitivity (true-positive rate) with a likelihood ratio (LR) of 2.4, whereas tear MMP-9 level provides 52% specificity and 90% sensitivity with LR of 1.9. These results confirm that tear TSP-1/MMP-9 ratio provides improved specificity and has the potential to serve as a biomarker to screen patients with dry eye with a suspected diagnosis of SS from non-SS dry eye with high sensitivity and specificity.

DISCUSSION

This prospective, masked study found that tear TSP-1/MMP-9 ratio is reduced in patients with SS-related dry eye as compared to non-SS dry eye and there is an inverse correlation between tear TSP-1 and MMP-9 levels as well as the TSP-1/MMP9 ratio and conjunctival staining score. Tear TSP-1/MMP-9 ratio can be useful as a screening test to identify patients with dry eye with underlying SS. Considering the frequency of the disease and the devastating ocular and systemic manifestations of SS with disease chronicity, identifying SS as the underlying cause of dry eye is important for appropriate clinical monitoring and timely therapeutic interventions. Among several inflammatory mediators detected in the tear film of patients with dry eye, MMP-9 is well known and previously approved by the FDA as a point of care testing to aid in diagnosis of dry eye (InflammaDry, Quidel). However, this test is not designed to identify SS and cannot differentiate among various underlying etiologies of dry eye. Our results indicate that tear TSP-1 (inhibitor of MMP-9 activation) levels are inversely correlated with tear MMP-9 levels and patients with dry eye with underlying SS have a significantly reduced tear TSP-1/MMP-9 ratio. Our findings suggest a potential use of tear TSP-1/MMP-9 ratio in screening patients with dry eye to identify those suspected of SS as an underlying cause.

To our knowledge, this is a first study to report the presence of TSP-1 in human tears. Our assay successfully detected both TSP-1 and MMP-9 at picogram levels. It is noteworthy that the detection limit for tear MMP-9 levels in our assay (0.002 ng/ml) is 2000-fold lower than the cutoff limit (20 ng/ml) of a point-of-care diagnostic test used in clinic (InflammaDry, Quidel). Although mean values for tear TSP-1 levels did not differ between study groups, molar concentration of TSP-1 relative to MMP-9 differed significantly between the two dry eye groups. It has been reported previously that the inhibitory effect of TSP-1 on MMP-9 activation occurs in a dose-dependent manner.[18,20] Thus, reduced TSP-1 level relative to MMP-9 level supports inadequate inhibition of MMP-9 activity in tears of patients with SS dry eye. These results are in agreement with reported increased MMP-9 expression in the conjunctival epithelium of SS patients[27] and decreased *conjunctival* TSP-1 expression associated with a genetic variant rs1478604 of TSP-1-encoding gene in individuals that develop chronic dry eye.[15] It is currently not known if extrinsic factors like prolonged use of certain therapeutics that contribute to the development of dry eye may result in reduced TSP-1 expression in ocular epithelial cells. Our study provides a basis for potential future longitudinal studies to shed light on extrinsic factors that may contribute to the development of SS by altering ocular surface TSP-1 expression.

Elevated expression of MMP-9 in ocular surface epithelial cells and tears under inflammatory conditions is well-documented.[19,27–31] In addition, increased expression of MMP-9 driven by inflammatory mediators is implicated in corneal matrix damage observed in ocular surface disease.[30] Consistently, ocular surface damage detected via corneal and conjunctival staining scores correlated with both increased MMP-9 message as well as tear MMP-9 levels (InflammaDry positive = >40 ng/ml).[27,29] Such correlation is also likely in TSP-1 deficient mice that are known to develop SS and associated ocular surface damage,[16,17] as increased active MMP-9 levels are reported in tissues derived from these mice.[20,32] These findings, together with the inhibitory activity of TSP-1, are consistent with the significant correlation detected in our study between low TSP-1/MMP-9 ratio and increased ocular staining scores.

The rapid immunoassay method to detect tear MMP-9 levels (InflammaDry) is a qualitative test designed to detect positive signal in samples containing MMP-9 levels greater than or equal to 40 ng/ml and negative for levels less than 40 ng/ml. Based on tear MMP-9 levels detected in our study, about 71% (29/41) of patients with clinically significant dry eye would test negative by rapid immunoassay. However, a reduced tear TSP-1/MMP-9 ratio below the control median value was detected in a significantly higher proportion of patients with SS dry eye as compared to non-SS dry eye regardless of their tear MMP-9 levels (Table 3). Thus, reduced TSP-1/MMP-9 ratio in tears not only helps to accurately reflect increased MMP-9 activity implicated in causing the damage to ocular surface epithelia, but also allows distinction between aqueous-deficient dry eye subtypes by identifying underlying SS pathology. Furthermore, ROC curve analysis provides evidence for the high accuracy in distinguishing SS dry eye from non-SS dry eye and indicates the advantage of improved

specificity of determining tear TSP-1 levels in addition to MMP-9 levels. In fact, our findings are consistent with a previously reported study that detected significantly increased MMP-9 immunostaining in conjunctival epithelial cells derived from patients with SS dry eye compared to those with non-SS dry eye.[33] Although other investigators have reported an imbalance in tear MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 as a potential cause of ocular surface damage observed in other pathologies,[34] this study did not evaluate patients with SS. Although ocular surface epithelial cell damage is common to dry eye caused by different etiologies, it has been suggested that different molecular pathways lead to ocular surface damage in different forms of dry eye.[27] Inhibition of MMP-9 activity by TSP-1 differs from that achieved by TIMP-1, in that TSP-1 blocks processing/activation of pro-MMP-9, whereas TIMP-1 binds the activated MMP-9.[20,35] It remains to be determined if these mechanistic differences also reflect differences in pathologies underlying dry eye associated ocular surface damage.

Reduced density of mucin-secreting goblet cells is a well-known manifestation of SS-related ocular surface damage.[36,37] We have reported significantly reduced tear MUC5AC (goblet cell-derived mucin) in dry eye-associated SS as compared to non-SS dry eye and that reduced tear MUC5AC levels are associated with increased conjunctival staining score.[21] The current study was performed with tears collected from the same participants and therefore allowed us to evaluate whether reduced tear TSP-1/MMP-9 ratio is also associated with lower tear MUC5AC levels. Indeed, among patients with a lower tear TSP-1/MMP-9 ratio than the control group mean, the proportion of patients with lower tear MUC5AC levels (below the previously used threshold value of 60 ng/ml) was significantly higher when dry eye was associated with SS versus non-SS causes (59% vs. 38%, respectively, OR = 2.3, 95% CI = 1.32 to 4.12, $p = 0.0045$). Taken together, our findings suggest a congruence between reduced goblet cell density and MUC5AC noted in SS dry eye with reduced TSP-1/MMP-9 ratio and their association with an increased conjunctival staining score.

Many previous studies aimed to identify clinical signs or patient-reported symptoms that may help distinguish dry eye caused by SS concluded that no single ocular diagnostic test can be helpful.[38–41] Although validation in a larger group of patients and controls is necessary, we propose tear TSP-1/MMP-9 ratio as a much-needed valuable tool to screen patients with dry eye to initiate work-up for an ultimate diagnosis of SS.

AUTHOR CONTRIBUTIONS

S.M. and E.K.A. wrote the manuscript. S.M. and E.K.A. designed the research. Boston University Analytical Instrumentation Core performed the research. S.M. analyzed the data.

CONFLICT OF INTEREST

Authors S.M. and E.K.A. are listed as inventors on a related patent application (Application No. 701586-191100PL01) submitted by Boston University School of Medicine.

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