

Reduced Levels of Tear Lacritin Are Associated With Corneal Neuropathy in Patients With the Ocular Component of Sjögren's Syndrome

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PURPOSE. To determine whether levels of endogenous tear protein, lacritin, are linked to altered corneal innervation and dry eye severity in patients with Sjögren's syndrome (SS).

METHODS. Clinical data were obtained from 10 SS and 10 age-matched controls. Enzyme-linked immunosorbent assay was used to assess total tear lacritin extracted from Schirmer strips. Western blot was used to detect active lacritin monomer (~25 kDa), active lacritin fragment (~12-15 kDa), and inactive tissue transglutaminase-generated lacritin (≥40 kDa). In vivo confocal microscopy was used to assess nerve fiber density (NFD) and length (NFL). Relationships between nerve morphology and tear lacritin were examined by Spearman correlation. Diagnostic performance of tear lacritin was analyzed using receiver operating characteristic.

RESULTS. Active tear lacritin was significantly reduced in SS patients (3.72 ± 5.62 [SS] versus 18.17 ± 4.57 ng/100 ng total tear protein [controls]; $P < 0.001$), while inactive lacritin was increased ($84.99\% \pm 11.15\%$ [SS] versus $51.04\% \pm 12.03\%$ [controls]; $P < 0.001$). Nerve fiber density (21.70 ± 18.93 vs. 31.80 ± 9.35 ; $P = 0.03$) and NFL (4.18 ± 3.44 vs. 6.54 ± 2.47 ; $P < 0.05$) were significantly decreased in SS patients compared to controls. Reduced NFL ($r = 0.74$, $P < 0.01$) and NFD ($r = 0.70$, $P < 0.01$) were highly correlated with reduced tear lacritin. Similarly, total tear lacritin was highly correlated with Schirmers ($r = 0.77$, $P < 0.01$), ocular staining ($r = -0.80$, $P < 0.01$), and corneal sensitivity ($r = 0.81$, $P < 0.01$). Tear lacritin showed equivalent or better diagnostic performance compared to traditional clinical measures for SS (100.00% sensitivity, 85.71% specificity, cutoff = 14.50 ng/100 ng tear protein).

CONCLUSIONS. Reduced tear lacritin levels in SS patients are highly correlated with clinical signs of dry eye, as well as decreased NFD and NFL. Lacritin and its components provide excellent diagnostic sensitivity and specificity in SS.

Keywords: lacritin, corneal innervation, Sjögren's syndrome, dry eyes

Sjögren's syndrome (SS) is a systemic disorder characterized by dryness of the eye and mouth that is attributed to lymphocytic infiltration of the lacrimal and salivary glands, respectively. Despite powerful immunosuppressive and immunomodulatory therapies, autoimmune-mediated, aqueous-deficient dry eye disease is highly recalcitrant to treatment and there is no cure. In light of the growing number of patients suffering from autoimmune disorders with symptoms of ocular dryness and the limited number of therapeutic options available, the focus of our work is to identify the contributing mechanisms that underlie aqueous-deficient dry eye pathogenesis.

An important yet often overlooked component of dry eye disease is altered innervation of the ocular surface.^{1,2} Loss of neuronal inputs upsets the complex reflex network connecting the ocular mucosal tissues (e.g., cornea, limbus, conjunctiva) and the tear-secreting machinery (e.g., lacrimal glands) that maintain ocular surface homeostasis. Furthermore, there is growing evidence that innervation itself is a negative modulator

of inflammation^{3,4} and a positive regulator of progenitor cell-mediated regeneration.⁵

Lacritin is an endogenous tear glycoprotein released apically from human lacrimal acinar cells that was discovered in a cDNA screen of expressed lacrimal proteins and identified as a novel secretion-enhancing factor.⁶ Lacritin is detected in tears as an active monomer of ~25 kDa, an active proteolytically cleaved fragment of ~12 to 15 kDa, and inactive, tissue transglutaminase-generated dimers, trimers, and larger polymers (>40 kDa).⁷ In recent years, the therapeutic potential of lacritin has been gaining attention for its prosecretory, mitogenic properties^{8,9}; and most recently, the cleavage-potential fragment of lacritin has been shown to be bactericidal.¹⁰ Lacritin promotes basal tearing when topically added to eyes of normal rabbits¹¹ and rescues cultured human corneal epithelial cells from inflammatory cytokine stress, including stress induced by tears from dry eye patients.¹² Consistent with in vitro studies, lacritin has been reported to be decreased in patients with various forms of dry eye disease.^{13,14} In our own work, we showed reduced levels of both lacritin

monomer and the 12- to 15-kDa fragment in tears of SS patients; however, the total amount of lacritin in tears was not explored, nor was the relative expression of active (<40 kDa) versus inactive (>40 kDa) species.¹⁵

Based on these data, we hypothesized that reduced levels of tear lacritin are functionally linked to altered integrity and innervation of the ocular surface in SS patients. Using tear proteins eluted from Schirmer strips, we are the first to quantify the amount of total lacritin and its three major isoforms in tears of SS patients and age-matched controls. In the same cohort, we used *in vivo* confocal microscopy (IVCM) to assess changes in corneal innervation and examine the relationships between corneal nerve morphology and tear lacritin levels. Our studies revealed profound reductions in corneal nerve fiber density and length that were highly correlated with reduced levels of active lacritin and increased levels of inactive lacritin in SS tears. These results provide the first evidence of an association between tear lacritin and altered corneal innervation, supporting the potential for a functional link that will be further explored in preclinical and clinical studies of lacritin as a treatment for aqueous-deficient dry eye.

MATERIALS AND METHODS

Human Subject Recruitment and Sample Collection

All aspects of the human subject studies presented in this manuscript followed the tenets of the Declaration of Helsinki and were approved by the University of California-San Francisco (UCSF) Committee for Human Research prior to subject recruitment. Informed consent was obtained from all subjects and all study activities were Health Insurance Portability and Accountability Act compliant. Clinical data were obtained from SS patients ($n = 10$) and age-matched controls ($n = 10$). All SS patients met diagnostic criteria established by the Sjögren's International Collaborative Clinical Alliance (SICCA).¹⁶ Briefly, each participant with SS tested positive for at least two of the three primary outcome variables: an ocular surface staining score (OSS) greater than or equal to 3; a labial salivary gland focus score greater than or equal to 1 focus/4 mm²; and presence of autoantibody anti-SSB/La and/or -SSA/Ro or both antinuclear antibody (ANA) titer ($\geq 1:320$) and rheumatoid factor (RF). These SICCA classified cases and controls were used as "gold standards" for diagnostic performance assessment. Outcome variables included unanesthetized Schirmer test (a measure of tear secretion), the OSS (a measure of ocular surface integrity),¹⁷ tear breakup time (TBUT, a measure of tear stability), Cochet Bonet aesthesiometry (a method of corneal sensitivity testing [CST]), and the Ocular Surface Disease Index (OSDI, an established questionnaire used to assess severity of dry eye-associated symptoms). Exam procedures adhered to the protocol established by SICCA¹⁷ with the addition of asking each patient to complete the OSDI questionnaire prior to examination and performing CST using Cochet Bonet at the beginning of each exam. Schirmer strips collected from SS ($n = 9$) and control ($n = 7$) patients were stored in a -80°C freezer until analysis. At the time of analysis, strips were immersed in 30 μL PBS for 20 minutes at room temperature and eluted by centrifugation, and the tear proteins were assessed by ELISA and Western blot. Tear protein concentration was assessed by the BCA assay (Thermo Scientific BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin as a protein standard.

Materials

Lissamine Green and fluorescein sodium were obtained from Leiter's Pharmacy and Compounding Center (San Jose, CA, USA).

Ocular surface integrity was assessed using the OSS developed by SICCA. Alcon Schirmer Standard Tear Test Strips (Fort Worth, TX, USA) were used to assess unanesthetized tear secretion. Enzyme-linked immunosorbent assay and Western blots for lacritin were carried out using antibodies produced from the peptide EDASSDSTGADPAQEAGTS (Pep Lac N-Term), corresponding to the N-terminus of mature human lacritin (amino acids 1-19 without signal peptide) synthesized >85% purity and conjugated to keyhole limpet hemocyanin (KLH) by Bio-Synthesis, Inc. (Lewisville, TX, USA). New Zealand white rabbits were immunized in three boosts with Pep Lac N-Term-KLH. Final antiserum (anti-Pep Lac N-Term) was collected on day 70.

ELISA Methods

For assay of tear samples, 100 ng total tear protein was coated in each well. To generate a standard curve of recombinant lacritin, each plate contained triplicate wells to which 2, 4, 6, 8, 10, 12, 14, or 16 ng protein was added. Wells were washed, blocked with PBS-Tween (PBS with 0.3% Tween-20 [PBS-T]), and then incubated for 1 hour at 37°C with 100 μL anti-Pep Lac N-Term antiserum diluted 1:3000 in PBS-T. After washing three times with PBS-T, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (MP Biomedicals, Solon, OH, USA) diluted 1:1000 in PBS-T was added for 1 hour (37°C). Plates were washed three times with PBS-T, and then bound antibody was measured after incubation for 10 minutes with 100 μL OPD (o-phenylenediamine dihydrochloride) substrate (Acros Organics, Geel, Belgium) by absorbance at 415 nm (model 680; Bio-Rad, Hercules, CA, USA).

Western Blotting

Tear samples were loaded on Any kD Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad), electrophoresed at 200 V, and transferred to nitrocellulose (Protran BA 83; Whatman, Dassel, Germany). Blots were blocked with PBS-T, incubated with anti-Lac Pep N-Term (1:1000 dilution in PBS-T) for 1 hour at room temperature, rinsed with PBS-T, and incubated for 1 hour at room temperature with HRP-conjugated goat anti-rabbit IgG (MP Biomedicals) diluted 1:5000 in PBS-T. Blots were rinsed with PBS-T and developed via chemiluminescence with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

Quantification of Active and Inactive Lacritin Species Using Densitometry

The signal intensity of lacritin bands was determined by multiplying the area of the band with the average pixel intensity to provide volume-sum intensity for each band. The signal intensity of the 12- to 15-kDa, 25-kDa, and 40-kDa bands from each individual's tear sample was quantified using ImageJ (<http://imagej.nih.gov/ij>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The signal intensity value of each band was expressed as area pixel density. Data were expressed as the relative percentage of lacritin species by dividing the signal intensity of each lacritin species (i.e., lacritin cleaved fragment [15 kDa], lacritin monomer [26 kDa], or inactive lacritin polymers >40 kDa) by the total signal intensity of all lacritin species.

In Vivo Confocal Microscopy

Images of corneal nerves were collected using the Nidek Confoscan 4 (Nidek Technologies, Fremont, CA, USA) *in vivo* scanning confocal microscope equipped with a $\times 40$ lens. Images were obtained from the epithelial to endothelial surface of the central cornea ($460 \times 345 \mu\text{m}$). To maintain masking, each set of images was renamed before selecting the series of

frames that corresponded to the subepithelial compartment and contained images of the subbasal nerve plexus. Three trained observers assessed four parameters of nerve morphology: nerve fiber density (NFD), nerve fiber length (NFL), nerve branch density (NBD), and tortuosity using CC Metrics, a semiautomated nerve analysis program developed by Rayaz Malik, University of Manchester, Manchester, United Kingdom.

Statistical Analysis

All analyses are based on readouts obtained from single eyes randomly chosen from each patient. Demographic variables, tear lacritin levels, corneal nerve morphology, and clinical measures of ocular surface disease (i.e., OSDI, Schirmer, OSS, TBUT, and CST) were compared between SS and age-matched controls. Group differences in sex and ethnicity were tested by Fisher's exact test; Schirmer score, OSDI, OSS, CST, TBUT, percent expression of tear lacritin isoforms, and features of nerve fiber morphology were examined by Wilcoxon exact test. Differences in age and total tear lacritin assessed by ELISA were compared by *t*-test. Correlations among variables were assessed by Spearman correlation and reported as correlation coefficients along with their corresponding *P* values to provide information on the strength and reliability of the correlation, respectively.¹⁸ Of these measures, integer and continuous variables were summarized as mean and standard deviation (SD), categorical and binary variables as the accounted number of subjects. Western blot data were normalized by the three-component sum of lacritin species for each sample¹⁹; then normalized values were presented as percentages. Performance of clinical readouts and lacritin-based tear measurements as diagnostic parameters to identify patients with the ocular component of SS was assessed by receiver operating characteristic (ROC) curves using the RStudio platform (Boston, MA, USA), version 0.98.1102. Area under the curve (AUC) was estimated by R package pROC to determine the 95% confidence intervals (95%CI).²⁰ The ROC cutoff for each of these variables was optimized to maximize the sensitivity and specificity. The optimized performances were compared to the performance of clinical tests and associated cutoffs currently used to diagnose the signs and symptoms of SS-associated dry eye, including Schirmer score ≤ 5 , OSS ≥ 3 , OSDI ≥ 20 , and TBUT < 10 seconds. The mean values and 95% confidence intervals of sensitivity and specificity for each clinical test were calculated from 2000 bootstrap runs using the R package Boot.²¹

RESULTS

Objective and Subjective Clinical Measures of Dry Eye Are Altered in SS Patients

Clinical data were obtained from 10 SS patients and 10 age-matched controls. There were nine females and one male in each group. The average ages were 58.2 (SD = 8.44) and 56.5 (SD = 8.71) in the control and SS groups, respectively. Most of these patients were Caucasians, seven in the control group and nine in the SS group. Statistical comparison of demographics confirmed that these two groups did not differ significantly with respect to sex, age, or ethnicity. Other than TBUT and symptoms of dryness (OSDI), each of the clinical tests routinely used to assess the signs of ocular surface disease in patients with SS was significantly different compared to that in control patients (Table 1).

Overall Lacritin and Its Active Isoforms Are Reduced in the Tears of SS Patients

As a naturally occurring tear glycoprotein with prominent prosecretory, cytoprotective, mitogenic, and bacteriocidal

TABLE 1. Subjective and Objective Measures of Sjögren's Syndrome (SS) Dry Eye

Clinic Characteristics	Control	SS	P Value
	Mean (SD)	Mean (SD)	
Schirmer tear test (STT)	21.57 (8.64)	5.89 (4.20)	<0.001
Tear breakup time (TBUT)	5.86 (2.67)	3.44 (3.54)	0.14
Ocular staining score (OSS)	1.43 (0.98)	6.11 (3.02)	0.001
Corneal sensitivity testing (CST)	5.58 (0.38)	3.22 (1.30)	<0.001
Ocular Surface Disease Index (OSDI)	17.12 (13.95)	39.23 (28.58)	0.07

properties, we hypothesized that lacritin was reduced in the tears of SS patients with aqueous-deficient dry eye. Using tear proteins collected by Schirmer strips and eluted using PBS, we quantified overall lacritin levels using ELISA, as well as the relative expression of a fully active cleaved fragment of lacritin (~15 kDa), active lacritin monomer (~23–25 kDa), and larger molecular weight inactive lacritin species complexed with tissue transglutaminase (~40–75 kDa) using Western blot analysis (Fig. 1). As shown in Figure 1, the total amount of lacritin visualized in Western blots using anti-lacritin antibodies was significantly greater in control samples compared to SS patients when the same concentration of total tear protein was run in each lane. The observation of reduced lacritin in Western blots of SS tears was supported by quantitation of total tear lacritin using ELISA (reported as ng lacritin/100 ng total tear protein, bottom row of Fig. 1). Interestingly, a number of tear samples from SS patients showed higher molecular weight (inactive) complexes of lacritin by Western blot while the ELISA analysis did not detect these complexes (last two lanes of Fig. 1), suggesting that they may not be accessible to anti-lacritin antibodies under conditions used for the ELISA.

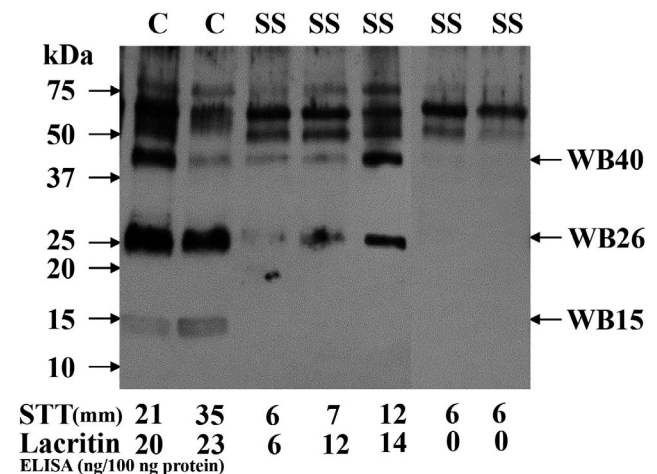


FIGURE 1. Western blot and ELISA analysis of human tear samples for lacritin. Human tear samples from patients with Sjögren's Syndrome (SS) and age-matched controls (C) were eluted from Schirmer tear test strips and total protein was determined by the BCA assay. Schirmer tear test (STT) values measured in millimeters (mm) were recorded prior to elution. Tear protein samples were normalized to 200 μ g/mL, and 4 μ g of each sample was loaded on SDS PAGE, transferred to nitrocellulose, challenged with anti N-terminal lacritin antibodies, and developed by chemiluminescence. Tear lacritin concentrations determined by ELISA, as described in Materials and Methods, are presented as ng lacritin per 100 ng total protein. WB15 is the proteolytically cleaved fragment of lacritin; WB26 is lacritin monomer; and WB40 represents the tissue transglutaminase-generated dimers, trimers, and larger polymers of lacritin above 40 kDa. Representative data from five eyes are shown.

Western blot results indicated that the level of the cleaved fragment, migrating at approximately 15 kDa (WB15), was 12.93% ($P < 0.01$) lower in SS versus control tears while lacritin monomer, migrating at approximately 26 kDa on Western blots (WB26), was 21.02% lower than in control eyes ($P < 0.01$). Perhaps most compelling, inactive lacritin cross-linked by tissue transglutaminase in tears⁷ and migrating as multiple bands at and above 40 kDa on Western blots (WB40) was 33.95% higher in SS patients compared to controls ($P < 0.01$) (Table 2). Thus, in contrast to reduced levels of active cleaved fragment, monomer, and total tear lacritin in SS patients, complexes of inactive lacritin were significantly increased.

Deficiency in Tear Lacritin Predicts the Severity of Nerve Damage and Ocular Surface Disease

Studies exploring the effects of dry eye on corneal innervation are gaining momentum, in part due to the technological advancements of IVCN. We used images of the central cornea captured using IVCN to assess NFD, NFL, NBD, and tortuosity. Among these features, NFD and NFL were significantly reduced in the corneas of SS patients, with nerves consistently noted to be less dense and shorter than those of control patients (Table 3). Interestingly, both the density and length of nerves were significantly correlated with ocular surface staining (Spearman correlation coefficients = -0.62 [$P = 0.01$] and -0.66 [$P < 0.01$], respectively), supporting the hypothesis that nerves provide an essential function to maintain corneal epithelial integrity.

Along with the association between corneal nerve loss and ocular surface disease, corneal innervation was similarly correlated with reduced levels of tear lacritin (Fig. 2). Among the readouts of tear lacritin, overall levels assessed by ELISA were the most highly correlated with NFD and NFL (Spearman correlation coefficients = 0.70 [$P < 0.01$] and 0.74 [$P < 0.01$], respectively). In contrast, the inactive component, WB40, was negatively correlated with fiber density and length ($r = -0.61$ and -0.62 , $P = 0.01$ and < 0.01 , respectively).

We also noted strong correlations between tear lacritin and many of the traditional, objective, diagnostic measures of dry eye (Fig. 3). Specifically, reduced lacritin measured by ELISA was positively correlated with Schirmer score (Spearman $r = 0.77$, $P < 0.01$), negatively correlated with OSS (Spearman $r = -0.80$, $P < 0.01$), and positively correlated with CST (Spearman $r = 0.81$, $P < 0.01$). In contrast, reduced TBUT (Spearman $r = 0.49$, $P = 0.05$) and subjective symptoms of dryness OSDI (Spearman $r = -0.52$, $P = 0.06$) were poorly correlated with a low overall level of lacritin. Similarly, the increased presence of inactive lacritin complexes (WB40) was highly correlated with low Schirmer score (Spearman $r = -0.87$, $P < 0.01$), increased OSS (Spearman $r = 0.78$, $P < 0.01$), and reduced CST (Spearman $r = -0.92$, $P < 0.01$), whereas

TABLE 2. Comparison of Total Lacritin and Percent Expression of Lacritin Species

Lacritin	Control Mean (SD)	SS Mean (SD)	P Value
ELISA*	18.17 (4.57)	3.72 (5.62)	<0.01
WB15†	14.65 (7.54)	1.72 (5.15)	<0.01
WB26†	34.31 (10.84)	13.29 (10.95)	<0.01
WB40†	51.04 (12.03)	84.99 (11.15)	<0.01

* Total tear lacritin measured by ELISA and expressed as ng/100 ng total protein.

† Percent expression of 15-kDa, 26-kDa, and >40-kDa lacritin species assessed by Western blot (WB) and quantified by densitometry.

TABLE 3. Comparison of Corneal Nerve Fiber Morphology

Nerve Features	Control Cohort	SS Cohort	P Value
	Mean (SD)	Mean (SD)	
Nerve fiber density (NFD)	31.80 (9.35)	21.70 (18.93)	<0.05
Nerve fiber length (NFL)	6.54 (2.47)	4.18 (3.44)	<0.05
Nerve branch density (NBD)	10.20 (7.22)	6.07 (13.01)	0.07
Tortuosity coefficient (TC)	21.05 (9.35)	22.04 (15.77)	0.99

correlations with TBUT (Spearman $r = -0.59$, $P = 0.02$) and OSDI (Spearman $r = 0.42$, $P = 0.14$) were minimal or insignificant. Together, these results demonstrated a profound decrease in overall levels of lacritin in tears of SS patients that occurred in conjunction with an equally significant decrease in the ratio of active to inactive lacritin. These changes in tear lacritin are highly correlated to objective measures of clinically significantly dry eye but minimally correlated with symptoms of dryness and TBUT. To our knowledge, these results are the first to demonstrate statistically significant associations between ocular surface disease severity, altered corneal innervation, and decreased levels of tear lacritin in a cohort of SS patients versus age-matched controls.

Tear Lacritin Levels Are Equivalent to or Better Than Standard Clinical Measures for Diagnosing SS-Associated Dry Eye

Compared to current clinical readouts used to assess the signs and symptoms of SS dry eye, lacritin and its isoforms performed as well as or better than traditional diagnostic tests to differentiate SS dry eye from controls. For example, using optimized cutoff values for total lacritin, lacritin monomer, and inactive lacritin species, the patients were correctly identified with a sensitivity of 100.00% (set by bootstrap) and specificities of 85.71% (95% CI, as reported in Table 4). The OSS, currently the primary objective clinical test used to diagnose the ocular component of SS, had a sensitivity of 88.89% (95%CI: 66.67–100.00%) and a corresponding specificity of 85.71% (95% CI: 57.14–100.00%) when using the cutoff of ≥ 3 ,

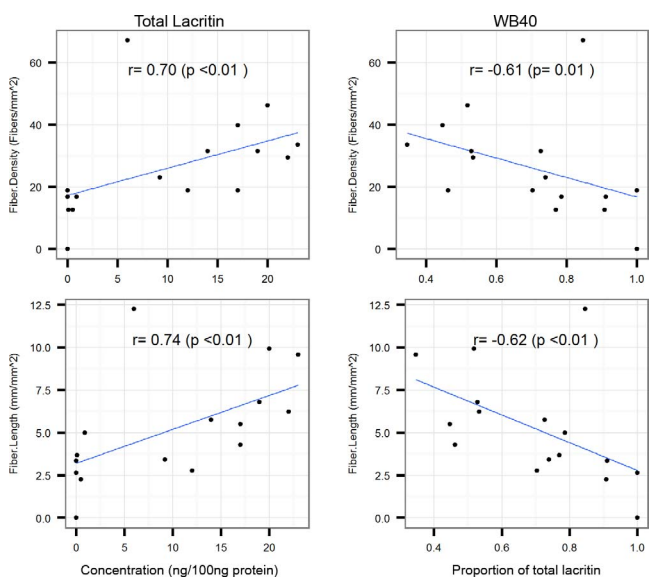


FIGURE 2. Correlation between tear lacritin levels and corneal nerve features. Spearman correlation coefficients and corresponding P values are provided. Data points represent a single eye of each patient.

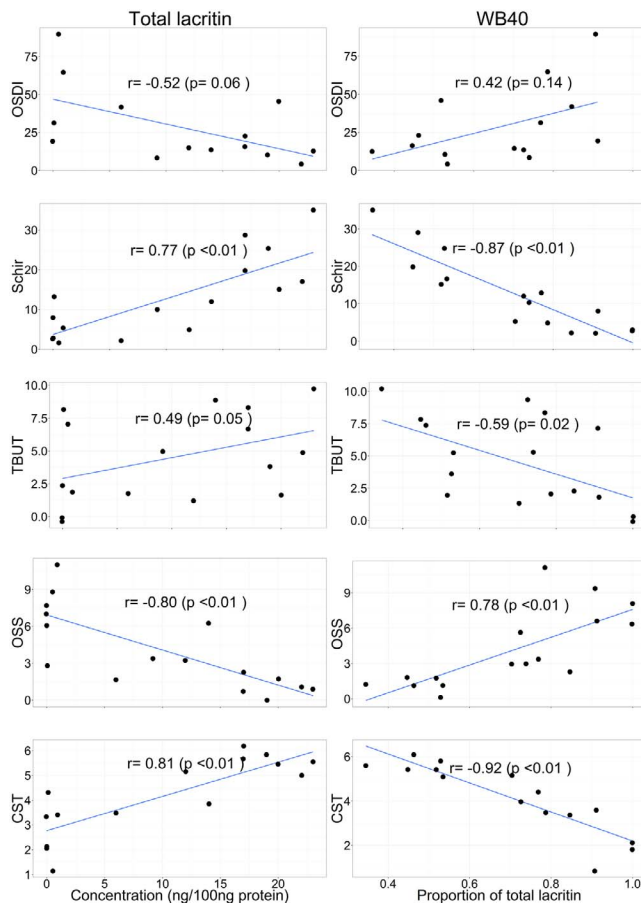


FIGURE 3. Correlation between total tear lacritin levels and clinical parameters used to assess dry eye. Spearman correlation coefficients and corresponding *P* values are provided. Data points represent a single eye of each patient.

as specified by the current American College of Rheumatology diagnostic criteria. As such, the sum of sensitivity and specificity for OSS was 11.11% lower than that of total tear lacritin and its inactive isoform (WB40), which had sums equal to 185.71%. It was also noted that the AUCs of lacritin ELISA (AUC = 0.98) and inactive lacritin WB40 (AUC = 0.97) were slightly higher than any of the clinical parameters used to differentiate SS patients from the healthy controls.

Finally, it was found that clinically defined cutoff values for traditional diagnostic tests (standard cutoffs) were different from the values optimized by the sum of sensitivity and specificity through ROC curve analysis (optimized cutoff). For example, 5 mm of wetting in 5 minutes on the Schirmer tear test (a classification criterion proposed by SICCA and others) provided a sensitivity of 50% and specificity of 100%. Alternatively, an optimized cutoff of 14 mm provided a sensitivity of 78% and specificity of 86%, suggesting that a higher Schirmer score may substantially improve the sensitivity of Schirmer testing with minimal compromise to its specificity.

DISCUSSION

We found significantly reduced levels of total tear lacritin, lacritin monomer, and the active cleaved fragment of lacritin in the aqueous-deficient eyes of SS patients compared with age-matched controls. Reduced levels of total tear lacritin were highly correlated with altered morphology of corneal nerves, low Schirmer scores, increased corneal staining, and reduced corneal sensitivity in SS patients. These data demonstrate an important association between the level of tear lacritin and altered homeostasis of the chronically inflamed cornea where reduced tear secretion and altered innervation contribute to ocular surface damage.

Interestingly, loss of active lacritin in SS samples was complemented by an equally profound increase in lacritin's inactive form that exists in higher molecular weight forms cross-linked by tissue transglutaminase. Tissue transglutaminase is a negative regulator of monomeric lacritin bioactivity,⁷

TABLE 4. Diagnostic Performance of Tear Lacritin and Clinical Test Using Standard and Optimized Cutoffs

Tested Variables	Diagnostic Performance of Tear Lacritin				
	AUC (95%CI)	Cutoff (95%CI)	Sensitivity, % (95%CI)	Specificity, % (95%CI)	Sensitivity + Specificity
Lacritin					
ELISA	0.98 (0.94–1.00)	14.50 (5.05–16.50)	100.00 (-)*	85.71 (57.14–100.00)	185.71
WB15	0.94 (0.80–1.00)	2.64% (2.62%–6.90%)	88.89 (66.67–100.00)	100.00 (-)	188.89
WB26	0.94 (0.84–1.00)	29.82% (10.76%–34.07%)	100.00 (-)	85.71 (57.14–100.00)	185.71
WB40	0.97 (0.89–1.00)	62.72% (61.05%–79.26%)	100.00 (-)	85.71 (60.00–100.00)	185.71
Diagnostic performance of traditional clinical tests					
OSDI					
Standard	0.80 (0.55–1.00)	20	57.14 (20.00–100.00)	85.71 (57.14–100.00)	142.85
Optimized		18.05 (12.03–53.10)	71.43 (37.50–100.00)	71.43 (42.86–100.00)	142.86
Schirmer					
Standard	0.96 (0.89–1.00)	5	50.00 (26.32–71.41)	100.00 (-)	150.00
Optimized		14.00 (7.50–16.50)	77.78 (44.44–100.00)	85.71 (57.14–100.00)	179.47
OSS					
Standard	0.95 (0.87–1.00)	3	88.89 (66.67–100.00)	85.71 (57.14–100.00)	174.60
Optimized		2.50 (1.50–4.50)	88.89 (66.67–100.00)	85.71 (57.14–100.00)	174.60
TBUT					
Standard	0.72 (0.45–0.99)	10	100.00 (-)	0.00 (-)	100.00
Optimized		3.00 (1.00–9.00)	66.67 (33.33–100.00)	85.71 (57.14–100.00)	152.38
CST					
Optimized	0.89 (0.67–1.00)	4.00 (3.25–5.55)	77.78 (55.00–100.00)	100.00 (-)	177.78

(-) Sensitivity or specificity kept constant for resampling of 2000 runs by bootstrap.

and the dramatic increase in lacritin's inactive cross-linked forms suggests that tear tissue transglutaminase may play an important role in SS-associated dry eye disease. It is also interesting to note that tear lacritin ELISA analysis does not detect the inactive forms of lacritin observed in Western blot analysis. One possible explanation for this observation is that the ELISA is done under nondenatured conditions while the Western blot analysis denatures proteins prior to SDS gel electrophoresis, thereby exposing antigen-binding sites that may be inaccessible in the cross-linked native state. This observation suggests that the ELISA analysis detects only active forms of tear lacritin, thus extending the use of Schirmer strips and ELISA to quantify lacritin tear levels as a diagnostic biomarker for aqueous tear deficiency.

Data from our lab and others indicate that 95% of dry eye patients are selectively deficient in lacritin,¹³⁻¹⁵ suggesting that its replacement may have therapeutic benefits. Using the autoimmune regulator knockout (*AIRE* KO) mouse model of SS-associated, aqueous-deficient dry eye, we have published data demonstrating significant improvements in tear secretion and ocular surface integrity of the cornea following 3× daily topical application of recombinant lacritin for 3 weeks.¹⁵ Although the exact mechanism by which topical lacritin promotes tearing is not understood, our recent investigation in the *AIRE* KO mouse suggests that corneal sensory nerves are directly or indirectly involved (McNamara NA, et al. *IOVS* 2015;56:ARVO E-Abstract 4808). Lacrimal tear secretion is tightly regulated through corneal innervations,²² and loss of epithelial integrity in dry eye provides tear lacritin direct access to syndecan 1-expressing neuronal cells within the corneal epithelium and subbasal nerve plexus.¹¹ Signaling via lacritin's receptor syndecan-1 has been shown to regulate neuron growth in *Caenorhabditis elegans*,²³ further supporting a functional link between lacritin tear levels, corneal innervations, and neuronal stimulation of lacrimation in the setting of dry eye.

Measurements of corneal nerves using IVCN have been successfully employed for the diagnosis and stratification of corneal neuropathy due to diabetes²⁴ and other conditions of the eye such as postcorneal transplantation, refractive surgeries,²⁵ and contact lens-associated disorders.²⁶⁻²⁸ However, studies examining corneal sensitivity and corneal nerves by IVCN in SS patients have presented conflicting results.^{27,29} Studies have reported SS patients as having reduced,^{1,30} similar,^{31,32} or increased³³ subbasal nerve density when compared to controls with variable levels of corneal sensation.^{1,31} This variability may be attributed to use of different instruments and/or selection of patients at various stages of disease severity where nerves might be in different phases of damage or compensatory regeneration. While this interpretation remains to be validated, it is conceivable that IVCN could provide a clinical measure of corneal denervation that indicates the physiological state of nerves and predicts the symptoms and severity of disease. Our results demonstrated significant decreases in corneal NFD and NFL in SS patients compared to age-matched controls. Moreover, fiber density and fiber length were both highly correlated with the traditional clinical parameters used to diagnose and assess the severity of dry eye in human patients (data not shown).

The current study provides evidence to support the use of tear lacritin measured by ELISA as a diagnostic marker for SS-associated dry eye. Compared to standard clinical tests, the diagnostic performance of tear lacritin was similar or superior to Schirmer testing and ocular surface staining as assessed using the optimized AUC. Tear lacritin was also highly correlated to altered morphology of corneal nerves, with levels of inactive lacritin in complex with tissue transgluta-

minase proving to be the form most significantly altered in SS patients compared to controls. Further investigation of the mechanisms and consequences associated with reduced tear lacritin and altered innervation may provide significant clinical implications for maintaining or restoring ocular surface health in autoimmune-mediated dry eye.

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