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Topical administration of lacritin peptide for the treatment of canine keratoconjunctivitis sicca

Eliza A. Gaylord

James Madison University

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Topical Administration of Lacritin Peptide for the Treatment of Canine Keratoconjunctivitis Sicca

An Honors Program Project Presented to the Faculty of the Undergraduate College of Integrated Science and Technology James Madison University

by Eliza Ann Gaylord

April 11th 2017

Accepted by the faculty of the Department of ISAT, James Madison University, in partial fulfillment of the requirements for the Honors Program.

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PUBLIC PRESENTATION
This work is accepted for presentation at the ISAT Senior Capstone Symposium on April 21st, 2017.
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ABSTRACT

Keratoconjunctivitis sicca (KCS), also known as dry-eye disease, causes a deficiency of tears in both humans and canines. Due to the lack of effective therapeutics for the treatment of dry-eye disease, there is a market potential for a novel secretion enhancing factor. Lacritin, a naturally occurring tear glycoprotein, increases basal tearing in rabbits when topically applied to the ocular surface and shows potential as a dry-eye therapeutic. This study aims to characterize Lacritin as a biomarker of dry-eye disease in canines with KCS. A total of 46 canine tear samples, 24 normal and 22 diagnosed with KCS, were obtained through a collaboration with the Virginia-Maryland College of Veterinarian Medicine and transported to James Madison University (JMU) for analysis. A bicinchoninic acid assay (BCA) was used to determine total tear protein in the samples. An indirect enzyme-linked immunosorbent assay (ELISA) was then used to determine the total tear Lacritin concentration in each sample using previously cloned and purified canine Lacritin as a standard. Total tear protein of the samples was normalized and the proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis with canine Lacritin antibodies was used to visualize Lacritin proteins and densitometry was used to determine the amount of the active Lacritin monomer (~18 kDa) band present. This study demonstrated that dry-eye samples had a significantly higher total protein concentration than normal samples, but a significantly lower
relative intensity of the monomeric Lacritin band than normal samples. There was no significant
difference in total Lacritin concentration between dry-eye and normal canines, as determined by
the ELISA. Thus, the lower relative intensity of monomeric Lacritin present in canines with dry-
eye suggests that monomeric Lacritin has potential as a biomarker of KCS, and should
researched further in the future.

INTRODUCTION

Background

Keratoconjunctivitis Sicca (KCS) is a prevalent ocular disease also known as dry eye
syndrome. It occurs in both human and canine populations, and is characterized by an unstable
tear film with decreased aqueous tear production. Symptoms include irritation to the eye, such
as burning, dryness, itching, scratching, and stinging (Merck Manual, 2003). Even though KCS
is the most common eye disease, affecting 4% of the general canine population (Williams et al.,
2008), it is under-diagnosed and poorly understood. KCS in canines is most commonly caused
by immune-mediated inflammation in the lacrimal glands, which results in decreased aqueous
tear production. Similar to that in canines, dry eye syndrome in humans can arise from
numerous etiologies and affects over 25 million Americans (Seifert et al., 2012). Sjögren’s
Syndrome, a form of dry eye, is similar to canine KCS, as it has a strong autoimmune component
and results in the destruction of the lacrimal and salivary gland tissues (Coursey et al., 2014). As
dry eye disease is often immune-mediated, both humans and canines typically require therapy
throughout their lives.

As KCS must be addressed with a tear replacement therapy until the patient’s
endogenous tear production has improved to a level adequate to alleviate symptoms, tear
supplementation requires a frequent, lifelong treatment. Therefore, to treat patients
appropriately, it is necessary to determine the underlying cause of the KCS. Currently, dogs
diagnosed with immune-mediated KCS receive the topical immunosuppressive medication
Cyclosporine A (Optimmune® 0.2% Ophthalmic Ointment) to increase aqueous tear production.
Though many canines respond to therapy, they are dependent on this treatment with tear
supplements for the remainder of their lives, and may experience persistent ocular discomfort,
corneal opacification leading to vision loss, recurrent corneal ulceration and other complications.
In human patients, the long-term therapies for dry eye include tear replacement and topical
cyclosporine A (Restasis® 0.05% Ophthalmic Suspension) (Kymionis et al., 2008). Though this treatment results in increased tear production, patients have experienced minor adverse effects, such as a burning sensation at the time of topical application (Sall et al., 2000). Therefore, the substantial subset of both canine and human patients that fail to respond to cyclosporine A treatment necessitates further research to identify a novel tear stimulating treatment.

Lacritin, a naturally occurring human tear glycoprotein approximately 12.3kDa in length (Ma et al., 2008), was discovered and characterized as a novel secretion enhancing factor from the human lacrimal gland (Sanghi et al., 2001). This prosecretory mitogen consists of 119 amino acids and is found in the tear film of various species, including human, non-human primate, horse, dog and rodent (Laurie et al., 2012). Characterized as a lacrimostimulant, topically applied Lacritin to rat and monkey lacrimal acinar cells in culture results in stimulated tear secretion (Sanghi et al., 2001). Additionally, Lacritin is the only prosecretory protein downregulated in patients suffering from dry eye (Srinivasan et al., 2012), including those with Sjögren’s Syndrome and dryness related to contact lens wear (Aluru et al., 2012). Synthesized in both human (Samudre et al., 2011) and canine (McKown et al., 2015) forms, recombinant Lacritin was topically applied to the eyes of normal rabbits three times daily for two weeks and demonstrated increased basal tear secretion throughout the two weeks and one week after treatment was ended (Samudre et al., 2011). Thus, Lacritin’s potential to stimulate lacrimal tissue to secrete tears, combined with the finding of decreased tear Lacritin concentrations under dry eye conditions, promotes the investigation of Lacritin as a novel therapy for dry eye diseases.

Researchers from James Madison University (JMU), Walter Reed Army Medical Center (WRAMC), Eastern Virginia Medical School (EVMS), UVA, and other institutions established a consortium to study Lacritin as a potential therapeutic for the treatment of dry eye. Through the 4-VA Collaboration at JMU, a partnership was initiated with the Virginia-Maryland Regional College of Veterinary Medicine (VMCVM), where canine tear samples were collected. A canine Lacritin ortholog gene was cloned, sequenced, and a recombinant canine Lacritin protein was expressed in E. coli and purified at JMU. Canine Lacritin polyclonal antibodies were created and a canine Enzyme-Linked Immunosorbent Assay (ELISA) was developed to determine the concentration of Lacritin in tear samples. Thus, this study aimed to investigate if Lacritin is a biomarker of canine dry-eye disease and therefore is a candidate for a future canine KCS replacement therapy.
Summary of Previous Work

N-terminal Antiserum Specificity Accomplished by Alan C. Tate, Honors Thesis, 2014

Both normal and KCS canine tear samples were collected at the Virginia-Maryland Regional College of Veterinary Medicine. Two α-Lacritin human polyclonal rabbit antibodies were used to detect Lacritin in canine tears: an N-terminal antibody corresponding to the first 19 amino acids of the human Lacritin N-terminus and the C-terminal antibody corresponding to a recombinant truncation mutant without the first 65 amino acids of the human Lacritin N-terminus. Tear wicks were eluted and assayed to find total tear protein concentrations. An indirect ELISA and Western blot (Figure 1) were performed to analyze Lacritin concentrations. The ELISA standard curve was generated using recombinant human Lacritin (pLAC). The C-terminal antibody did not detect Lacritin, while the N-terminal antibody did.

![ELISA and Western Blot Results](image)

**Figure 1.** ELISA and western blot results. (A) Lacritin concentrations in canine tear samples quantified with ELISA. (B) Selected tear samples were separated by SDS PAGE and visualized with western blotting. Hu is human tears, OD is right eye and OS is left eye (Tate, Honors Thesis 2012).

To improve the antibody sensitivity to Lacritin, the canine Lacritin gene was sequenced (Figure 2), cloned into the bacterial expression vector pTYB2, and purified through chitin affinity column and DEAE chromatography (Figure 2). The amino acid sequence was determined and used to produce the N-terminal and C-terminal antiserums (Figure 2).
Figure 2. Purified recombinant canine Lacritin DNA gel, purified recombinant canine Lacritin SDS gel, and purified recombinant canine Lacritin amino acid sequence (Tate, Honors Thesis 2012). (A) Lane 1: 100 bp DNA ladder. Lane 2: amplified PCR product. (B) Lane 1: molecular weight marker. Lane 2: cleared cell lysate prior to chitin affinity chromatography. Lane 3: fraction extracted after chitin affinity chromatography. Lane 4: DEAE Sepharose column, canine Lacritin 140 mM NaCl PBS elution. (C) Amino acid sequence of recombinant canine Lacritin. N-terminal antibody (lightly shaded region) and C-terminal truncated antibody (darker shaded region).

ELISA Development Accomplished by Alison M. Enghauser, Honors Thesis, 2015

Canine Lacritin N-terminal antibodies were synthesized with above 90% purity and conjugated to keyhole limpet hemocyanin/bovine serum albumin (KLH/BSA) by Bio-Synthesis, Inc. (Lewisville, TX). Two New Zealand white rabbits (6924 and 6925) were immunized with canine Lacritin and bled over a 10-week period to collect serum (Appendix C). IgG antibodies from the α-cLACRT NT antiserum were purified with Protein A coupled with agarose beads (Bio-Rad Laboratories, Hercules, CA). An indirect ELISA was developed and titrated with the antibodies.

Both normal and KCS canine tear samples were collected at the Virginia-Maryland Regional College of Veterinary Medicine. Tear wicks were eluted and assayed to determine total tear protein. An indirect ELISA and Western blot (Figure 3,4) were performed to analyze Lacritin concentrations in tear samples using the 10 week α-cLACRT NT PA purified antiserum (6924 FB PANT) and a secondary antibody (HRP-conjugated goat α-rabbit IgG).
Figure 3. **Indirect ELISA of Lacritin in canine tears.** Summary of canine tear Lacritin in normal (blue) and dry eye (red) tear samples as determined by indirect ELISA (Enghauser, Honors Thesis 2014).

Figure 4. **Western blot analysis of normal and dry-eye canine tears.** Dry eye tear samples 66, 59, and 44 (OD/OS) and normal tear sample A1 (OD/OS). The band corresponding to monomeric canine Lacritin is seen at ~18 kDa. Higher molecular weights are cross-linked variations of Lacritin. (Enghauser et al., 2014)
Clinical Study Accomplished by Katherine E. Kelly, Honors Thesis 2016

Tear samples were collected from canines being treated at Virginia-Maryland College of Veterinarian Medicine and transported to JMU for analysis. At JMU, the tears were eluted from the wicks and assayed to determine total protein concentrations. An indirect ELISA was developed and optimized to quantitate total tear Lacritin. SDS-PAGE was used to visualize the tear total protein profile. Western blot analysis was used to visually determine monomeric and higher order complexes of canine Lacritin in tear samples. A total of 64 tear samples were analyzed with 32 samples from healthy dogs and 32 samples from dogs clinically diagnosed with dry eye. Canines with KCS had a significant decrease in tear film Lacritin as determined by ELISA analysis. Western blot analysis detected prominent bands in healthy tears at approximately 18 kDa corresponding to monomeric canine Lacritin that were absent or reduced in tears from dry eye dogs. This study provides clinical data that supports the application of Lacritin as a topical therapeutic for the treatment of dry eye disease (Figure 5).
Figure 5. Western blot analysis of dry-eye and normal samples. Western Blot analysis of the samples with their STT values and Lacritin concentrations as determined by the indirect ELISA listed below. An STT value below 15 indicates dry eye, while above indicates normal tearing levels. The band corresponding to monomeric canine Lacritin is seen at ~18 kDa. Higher molecular weights are cross-linked variations of Lacritin. N = Dry eye tear samples and N = Normal tear samples. Lacritin values are ng per 50 ng total tear protein as determined by the ELISA.
This study aimed to analyze the amount of Lacritin present in both normal and dry-eye canine tear samples. Prior to sample arrival to JMU, canine Lacritin polyclonal antibodies were titrated to maximize detection for assays, and the canine indirect ELISA was optimized to reliably determine the concentration of Lacritin in tear samples.

**MATERIALS AND METHODS**

**Collection and Elution of Tears**

Both normal and KCS canine tear samples were collected at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech by Julie Disney, DVM. Canines were either previously diagnosed with dry-eye or had a Shirmer Tear Test (STT) performed to determine the rate (mm/min) of tear production and generate an STT value. An STT value of 15mm/min or higher was classified as normal, while an STT value of 14mm/min or lower was classified as dry-eye. Wicks were applied to the canine’s ocular surface for approximately 1 minute and stored in microcentrifuge tubes at -20°C. A total of 46 samples were collected, 24 normal and 22 diagnosed with KCS. Of the 24 normal canines, only one was on treatment; of the 22 KCS canines, eleven were on treatment. Samples were divided into 6 sets (I-VI) and labeled with the canine identification number and oculus dexter (OD) for right eye or oculus sinister (OS) for left eye. Tears were eluted from the wicks with 30μL of filter-sterilized 1X phosphate buffered saline (FSPBS) and incubated for 20 minutes at 37°C. Eluted tears were spun at 13,000 rpm for 5 minutes, total volume was recorded, and samples were then stored at -20°C for future analysis.

**Bicinchoninic Acid Assay**

The Thermo Scientific Pierce BCA Protein Assay Kit was used to determine the total tear protein concentration in the samples compared to a protein standard. BCA samples were prepared in microcentrifuge tubes by combining 4 μL of the eluted sample with 20 μL FSPBS. Duplicate standards and samples were plated in a 96 well microtiter plate, incubated at 37°C for 30 minutes, and then measured at 570 nm in a spectrometer. Standard protein concentrations versus absorbance values were graphed to generate a linear trend line and the coefficient of determination. The total tear protein concentration of each sample was calculated.
Antibody Specificity and Titration

To determine the optimal dilutions of both primary and secondary antibodies for the ELISA standard curve, 10-week Final Bleed N-Terminal Protein A purified primary antibodies (6924 FB PANT) from rabbit 6924 were titrated against recombinant canine Lacritin. In a 96 well microtiter plate, previously cloned and purified lyophilized recombinant canine Lacritin (cLAC) was diluted with coating buffer from 0.1 to 1000 ng/mL. In the same microtiter plate, 6924 PANT FB antibodies were diluted with a 1X PBS and 0.3% Tween20 solution (PBST) from 1:200 to 1:12800. The secondary antibody, HRP-conjugated goat α-rabbit IgG, was diluted in PBST to 1:400 and applied to the wells. The absorbance values were plotted and analyzed; the dilution that produced the most linear standard curve was chosen as the optimal dilution used for the ELISA.

Indirect Enzyme-Linked Immunosorbent Assay

The indirect ELISA was used to determine the total Lacritin concentration in the samples compared to a Lacritin standard. To generate a standard curve for comparison, cLAC was resuspended with 100μL of deionized water (dH2O) and diluted to 32ng/mL with coating buffer (4.53mL 1.0M NaHCO3, 1.82mL 1.0M Na2CO3, 93.65mL dH2O). The standard 32ng/mL stock was diluted and plated (100μL) in triplicate in a 96 well microtiter plate at 32, 24, 16, 12, 8, 6, 4, 3, 2, 1.5, 1, 0.5, and 0 ng concentrations. Plates were duplicated and incubated at 4°C for 24 hours.

Wells were washed three times with 300μL of PBST, and excess liquid was expelled. Plates were coated with 300μL of 1% w/v blocking buffer (0.2g BSA, 20mL PBS) and incubated for 35min at 37°C. Wells were washed three times with 300μL PBST, and excess liquid was expelled. Primary antibody (6924 FB PANT) was diluted at 1:3200 in PBST and then 100μL was pipetted into each well. Plates were incubated for 35min at 37°C. Wells were washed three times with 300μL PBST, and excess liquid was expelled. Secondary antibody (HRP-conjugated goat α-rabbit IgG) was diluted at 1:400 in PBST and then 100μL was pipetted into each well. Plates were incubated for 35min at 37°C. Wells were washed three times with 300μL PBST, and excess liquid was expelled. Secondary antibody (HRP-conjugated goat α-rabbit IgG) was diluted at 1:400 in PBST and then 100μL was pipetted into each well. Plates were incubated for 35min at 37°C. Wells were washed three times with 300μL PBST, and excess liquid was expelled. Substrate (6.0mL 0.1M C6H5O7, 6.5mL 0.2M Na2HPO4, 12.5mL dH2O, 10μL H2O2, 10mg o-phenylenediamine dihydrochloride) was prepared using a vortex and 100μL was pipetted into each well. Plates were incubated at room temperature in the dark for
10 min and then measured at 415 nm in a spectrometer. Standard Lacritin concentrations versus absorbance values were graphed to generate a linear trend line and the coefficient of determination. The total Lacritin protein concentration of each sample was calculated.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis & Western Blots

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting with cLAC antibodies were used to analyze the abundance of monomeric Lacritin in tear samples. Samples were normalized with PBS and 4 μL of 6x loading dye was added to each sample. Samples were boiled for 5 minutes, and 10 μL of each sample and 7 μL of protein ladder (Precision Plus Protein Kaleidoscope Standards, BioRad) were loaded into the gel (Any kD™ Mini-PROTEAN® TGX™ Precast Gel). Tris-glycine buffer (BioRad) was added, and the gel was run at 200 volts; the gel was stopped when the loading dye front reached the bottom.

The samples were transferred onto nitrocellulose membrane with a trans-blot apparatus (Appendix C). The blot was run at 100 volts for one hour. Membranes were removed and stored at 4°C overnight in aluminum foil. The membranes were washed for ten minutes with PBST four times, and primary antibody at a 1:1000 dilution in PBST was added for one hour at room temperature. During this incubation, the membranes were agitated constantly by a shaker. The membranes were washed for ten minutes with PBST four times, and secondary antibody at a 1:5000 dilution in PBST was added for one hour at room temperature. During this incubation, the membranes were agitated constantly by a shaker. The membranes were washed twice for fifteen minutes with PBST, and then wicked and stored.

Membranes were taken to a dark room and film was developed via the chemiluminescent reaction between the horseradish peroxidase and ECL substrate (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific Inc.). Membranes were submersed in substrate for one minute and wrapped in Saran wrap. The membranes were placed face down on a sheet of x-ray film for an appropriate exposure time (10-25 seconds). Films were bathed in successive baths of developing solutions (developer, deionized water, fixer, and deionized water) for 30-second intervals. Solution baths were constantly agitated.
**Densitometry**

Densitometry was used to determine the amount of the active Lacritin monomer (~18 kDa) band present. Previously exposed x-ray films were imaged using the gel documentation system (Image Lab software, Bio-Rad), and the relative signal intensity of the Lacritin monomer band was quantified.

**Statistical Analysis**

An unpaired t-test was performed using the mean, standard deviation and the number of samples to compare dry-eye and normal data and determine a level of significance. A p-value less than or equal to 0.05 between samples was considered a significant difference as it was unlikely to be a chance event.
RESULTS

Bicinchoninic Acid Assay

The total tear protein concentration of the untreated dry eye samples (Table 1) had a range of 824.9 to 2510.2 µg/mL, an average of 1664.5 µg/mL and a median of 1688.8 µg/mL. Two untreated dry-eye samples had total tear protein concentrations further than three standard deviations from the average, and thus were excluded from the data (SD = 597.6 µg/mL). The total tear protein concentration of the treated dry eye samples (Table 2) had a range of 928.2 to 2106.9 µg/mL, while the average was 1439.9 µg/mL and the median was 1335.8 µg/mL. The total tear protein concentration of the untreated normal samples (Table 3) had a range of 615.8 to 1758.6 µg/mL, while the average was 1235.1 µg/mL and the median was 1207.1 µg/mL. The total tear protein concentration of the treated normal sample (Table 4) was 1542.2 µg/mL. All assays had a standard curve with a coefficient of determination (R²) value above 0.99 (Figure 6), and the raw data is shown in Appendix A.

Figure 6. BCA analysis of canine tears. BCA assay standard curve for Set I of the canine tear samples.
Table 1. **Dry-eye total tear protein concentrations for untreated dry-eye canines.** Sample identifier (OD = right, OS= left), STT value, and total tear protein concentrations in untreated dry-eye canines.

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<th>Sample</th>
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<th>Total Protein (ug/mL)</th>
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Table 2. **Dry-eye total tear protein concentrations for treated dry-eye canines.** Sample identifier (OD = right, OS= left), STT value, and total tear protein concentrations in treated dry-eye canines.

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Table 3. Normal total tear protein concentrations for untreated normal canines. Sample identifier (OD = right, OS= left), STT value, and total tear protein concentrations in untreated normal canines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>STT Value</th>
<th>Total Protein (ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 131440</td>
<td>19</td>
<td>1758.6</td>
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<td>853.1</td>
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</tr>
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<td>OD 141920</td>
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</tr>
<tr>
<td>OD 140688</td>
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<td>1068.3</td>
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</table>

Table 4. Normal total tear protein concentrations for treated normal canines. Sample identifier (OD = right, OS= left), STT value, and total tear protein concentrations in normal canines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>STT Value</th>
<th>Total Protein (ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 140818</td>
<td>24</td>
<td>1542.2</td>
</tr>
</tbody>
</table>
**Antibody Specificity and Titration**

Lacritin signal was detected at concentrations greater than 0.1 ng/mL (Figure 7) by 6924 PANT FB. The titration determined that 6924 PANT FB diluted in PBST to 1:3200 and secondary antibody diluted in PBST to 1:400 produced the most linear standard curve, and was used for the ELISAs.

![Figure 7. Titration of 6924 FB PANT antibodies.](image)

**Indirect Enzyme-Linked Immunosorbent Assay**

Lacritin concentrations were quantified using a 32ng Lacritin per 50ng total tear protein standard curve. Each plate was done in duplicate, and each sample was assayed in triplicate. Any samples with a concentration higher than 32ng Lacritin per 50ng total tear protein were not included to prevent extrapolation error. The Lacritin concentration of the untreated dry-eye samples (Table 5) had a range of 7.7 to 29.0ng Lacritin per 50ng total tear protein, while the average was 18.0ng Lacritin per 50ng total tear protein and the median was 22.1ng Lacritin per 50ng total tear protein. The Lacritin concentration of the treated dry-eye samples (Table 6) had a range of 17.3 to 23.8ng Lacritin per 50ng total tear protein, while the average was 19.2ng Lacritin per 50ng total tear protein and the median was 18.2ng Lacritin per 50ng total tear protein. Two samples had Lacritin concentrations that exceeded the assay’s maximum boundary.
of 32ng per 50ng total tear protein, and thus, were excluded from the data to prevent extrapolation errors (Table 6). The Lacritin concentration of the untreated normal samples (Table 7) had a range of 15.1 to 28.8ng Lacritin per 50ng total tear protein, while the average was 22.0ng Lacritin per 50ng total tear protein and the median was 22.3ng Lacritin per 50ng total tear protein. The Lacritin concentration of the treated normal sample (Table 8) was 23.5ng Lacritin per 50ng total tear protein. All assays had a standard curve with an R² value above 0.98 (Figure 8), and the raw data is shown in Appendix B.

![Figure 8. Indirect ELISA Standard Curve.](image)

The total tear Lacritin concentrations plotted as a standard curve with a coefficient of determination above 0.99.
Table 5. Dry-eye total Lacritin concentrations for untreated dry-eye canines. Sample identifier (OD = right, OS= left), STT value, and total Lacritin concentrations in untreated dry-eye canines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>STT Value</th>
<th>Lacritin (ng/50 ng total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 140939</td>
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</tr>
<tr>
<td>OD 139969</td>
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<td>10.1</td>
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<td>OS 139969</td>
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<td>OD 140221</td>
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<td>OS 140221</td>
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<td>OD 112296</td>
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<td>7.7</td>
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<td>OS 112296</td>
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</tr>
<tr>
<td>OS 140688</td>
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<td>11.3</td>
</tr>
</tbody>
</table>

Table 6. Dry-eye total Lacritin concentrations for treated dry-eye canines. Sample identifier (OD = right, OS= left), STT value, and total Lacritin concentrations in treated dry-eye canines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>STT Value</th>
<th>Lacritin (ng/50 ng total protein)</th>
</tr>
</thead>
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<td>OS 140932</td>
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<td>17.3</td>
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<tr>
<td>OD 133815</td>
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<td>--</td>
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<tr>
<td>OS 136979</td>
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<td>OS 140818</td>
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<td>20.2</td>
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<td>OD 140932</td>
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</table>
Table 7. Normal total Lacritin concentrations for untreated normal canines. Sample identifier (OD = right, OS= left), STT value, and total Lacritin concentrations in untreated normal canines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>STT Value</th>
<th>Lacritin (ng/50 ng total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 131440</td>
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<td>OD 141938</td>
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<tr>
<td>OD 140688</td>
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</tr>
</tbody>
</table>

Table 8. Normal total Lacritin concentrations for treated normal canines. Sample identifier (OD = right, OS= left), STT value, and total Lacritin concentrations in treated normal canines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>STT Value</th>
<th>Lacritin (ng/50 ng total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 140818</td>
<td>24</td>
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</tr>
</tbody>
</table>
Western Blots and Densitometry

The monomeric Lacritin band (~18kDa) was visualized via western blotting (Figure 9). The boldness of the band indicated how much Lacritin was detected; thus, thicker bands represented samples with more Lacritin present. Additionally, banding was present above the 20kDa marker. This indicated crosslinking of higher weight molecules occurred, which caused the formation of protein complexes. One sample in Set II was excluded, as the same sample was repeated later in Set VI. The Lacritin concentration of two samples from Set IV were excluded, as determining their values would have required extrapolation from the standard curve.
Figure 9. Visualization of monomeric Lacritin in canine samples. Western blot analysis of the samples. Tear status, STT value, and Lacritin concentrations (ng/50ng) determined with the ELISA are depicted within each lane. The band corresponding to monomeric canine Lacritin is seen at ~18 kDa. Higher molecular weights are cross-linked variations of Lacritin. DE = Dry eye tear samples, N = Normal tear samples, E = Excluded tear sample due to extrapolation. Lacritin values are ng per 50 ng total tear protein as determined by the ELISA.
Densitometry of the western blot determined that the relative intensity of the untreated dry-eye samples (Table 9) had a range of 2.50x10^6 to 2.40x10^8, while the average was 6.43x10^7 and the median was 2.68x10^7. The relative intensity of the treated dry-eye samples (Table 10) had a range of 4.04x10^7 to 2.42x10^8, while the average was 1.01x10^8 and the median was 5.65x10^7. The relative intensity of the untreated normal samples (Table 11) had a range of 3.43x10^7 to 2.40x10^8, while the average was 1.25x10^8 and the median was 1.18x10^7. The relative intensity of the treated normal sample (Table 12) was 2.30x10^8.

**Table 9. Relative intensity of Lacritin monomer band in untreated dry-eye canines.** Sample identifier (OD = right, OS= left), STT value, and densitometry to determine the relative intensity of the canine Lacritin monomer band in untreated dry-eye samples.

<table>
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<tr>
<th>Sample</th>
<th>STT Value</th>
<th>Relative Intensity (*10^6)</th>
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**Table 10. Relative intensity of Lacritin monomer band in treated dry-eye canines.** Sample identifier (OD = right, OS= left), STT value, and densitometry to determine the relative intensity of the canine Lacritin monomer band in treated dry-eye samples.

<table>
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<th>Sample</th>
<th>STT Value</th>
<th>Relative Intensity (*10^6)</th>
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</tr>
<tr>
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<td>49.6</td>
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</table>
Table 11. Relative intensity of Lacritin monomer band in untreated normal canines. Sample identifier (OD = right, OS= left), STT value, and densitometry to determine the relative intensity of the canine Lacritin monomer band in untreated normal samples.

<table>
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<th>Sample</th>
<th>STT Value</th>
<th>Relative Intensity (*10^6)</th>
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<td>OD 140688</td>
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</table>

Table 12. Relative intensity of Lacritin monomer band in treated normal canines. Sample identifier (OD = right, OS= left), STT value, and densitometry to determine the relative intensity of the canine Lacritin monomer band in treated, normal samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>STT Value</th>
<th>Relative Intensity (*10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 140818</td>
<td>24</td>
<td>230.1</td>
</tr>
</tbody>
</table>

**STT Comparison**

Schirmer Tear Test (STT) values were plotted against the total tear protein concentration, total Lacritin concentration, and relative intensity of the untreated samples (Figure 10, 12, 14) and of the treated samples (Figure 11, 13, 15). The STT is used to measure tearing rates in
humans and canines as a means to diagnose dry-eye syndrome. In canines, 15mm/min or above indicates normal tear production, whereas anything below that rate indicates dry-eye.

**Figure 10. STT values versus total tear protein for untreated canines.** A scatterplot of STT values against total tear protein in untreated dry-eye and normal canines.

**Figure 11. STT values versus total tear protein for treated canines.** A scatterplot of STT values against total tear protein in treated dry-eye and normal canines.
Figure 12. **STT values versus Lacritin concentration for untreated canines.** A scatterplot of STT values against Lacritin concentration in untreated dry-eye and normal canines.

Figure 13. **STT values versus Lacritin concentration for treated canines.** A scatterplot of STT values against Lacritin concentration in treated dry-eye and normal canines.
**Figure 14. STT values versus relative intensity for untreated canines.** A scatterplot of STT values against relative intensity of the Lacritin monomer band (~18kDa) visualized with a western blot for untreated dry-eye and normal canines.

**Figure 15. STT values versus relative intensity for treated canines.** A scatterplot of STT values against relative intensity of the Lacritin monomer band (~18kDa) visualized with a western blot for treated dry-eye and normal canines.
DISCUSSION

In previous research, the canine Lacritin gene was sequenced, cloned, and purified. The amino acid sequence was used to produce canine N-terminal antiserum, which were titrated for use in an indirect ELISA. Western blot analysis revealed monomeric Lacritin banding was present in normal canine samples, but faint or absent in dry-eye canine samples.

In this canine clinical study, 46 tear samples – 24 samples from normal canines and 22 samples from canines diagnosed with KCS – were collected by Julie Disney, DVM, through collaboration with the Virginia-Maryland College of Veterinarian Medicine. Samples were transported to James Madison University (JMU) for analysis, where total tear protein and Lacritin concentration was determined. Monomeric Lacritin was visualized and the relative intensity was quantified with western blotting and densitometry.

Prior to or at the time of collection, canines were designated a tear status of normal or dry-eye. Traditionally, patients are diagnosed with dry-eye using the Shirmer Tear Test (STT) and assigned an STT value, which measures tear production in mm/min. Canines with an STT value less than 15mm/min are diagnosed with dry-eye, while canines with an STT value greater or equal to 15mm/min are classified as normal. As determined by this study, many canines classified as dry-eye had STT values of 15mm/min or higher, signifying normal tear production despite the diagnosis. However, this increase in STT value may have been observed due to several variables. Several of the patients were diagnosed previous to collection and had been on treatment for several months or years. Therefore, treatment may have inflated STT values in canines with dry-eye tear statuses. To examine the relationship between treatment and STT value in future studies, data should be collected solely from untreated canines.

As determined with BCA analysis, the total tear protein concentration in untreated dry-eye canines was significantly greater than the total tear protein concentration in untreated normal canines (p-value: 0.0152), with a mean of ~1665µg/mL for dry-eye and ~1235µg/mL for normal samples. This increase may have been the result of several variables. Canines diagnosed with dry-eye often have irritation and inflammation in their eyes due to dryness or other dry-eye related symptoms. Thus, there are potentially higher amounts of immune response proteins located in and around their tear ducts. Additionally, as there is less tearing at the ocular surface, the concentration in the tear samples may have been higher due to less liquid present at collection.
The Lacritin concentration in untreated dry-eye canines was not significantly different to the Lacritin concentration in untreated normal canines (p-value: 0.0626). The mean Lacritin concentration in untreated dry-eye canines was ~18ng/50ng total protein, while the mean Lacritin concentration in untreated normal canines was ~22ng/50ng total protein. As the p-value was 0.0126 away from indicating a significant difference between Lacritin concentrations in untreated dry-eye and normal canines, additional clinical data should be collected and analyzed.

Densitometry analysis revealed that the relative intensity of the monomeric Lacritin band in untreated dry-eye canines was significantly lower than the relative intensity of the monomeric Lacritin band in untreated normal canines (p-value: 0.0190), with a mean of $6.43 \times 10^7$ for dry-eye and $1.25 \times 10^8$ for normal. Therefore, this indicated that normal canines had more abundant monomeric Lacritin bands than dry-eye canines. While this data indicates a significant difference, while the ELISA data does not, additional clinical data should be collected and analyzed. Additionally, Lacritin complexes were detected higher than 18kDA, indicating cross-linked Lacritin. Tissue transglutaminase is known to cross-link Lacritin and causes the formation of higher molecular weight complexes, visualized with western blotting; this form of Lacritin is shown to be inactive (Velez, 2013).

Overall, a canine Lacritin detection and quantification system was developed to analyze the total protein, the total Lacritin concentration, and the relative intensity of monomeric Lacritin in clinical canine samples. Dry-eye samples had a significantly higher total protein concentration than normal samples, but a significantly lower relative intensity of the monomeric Lacritin band than normal samples. There was no significant difference in total Lacritin concentration between dry-eye and normal canines, as determined by the ELISA. Thus, the lower relative intensity of monomeric Lacritin present in canines with dry-eye suggests that monomeric Lacritin has potential as a biomarker of KCS, and should researched further to determine if Lacritin holds promise as a potential therapeutic for dry-eye syndrome.
REFERENCES


Kelly, K.E. (2016). *Clinical study of canine tear Lacritin as a treatment for dry eye.* Unpublished B.S., James Madison University, USA.


Tate, A. (2012). *Canine clinical study for tear lacritin as a treatment for dry eye.* Unpublished B.S., James Madison University, USA.


## APPENDIX A

### BCA Canine Samples 11-29-16 SET 1

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<th>Sample</th>
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<th>OD 131440</th>
<th>OS 92754</th>
<th>OS 138958</th>
<th>OS 131440</th>
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![Graph showing the relationship between protein concentration and absorbance with the equation y = 0.4896x and R² = 0.9979.](image-url)
## BCA Canine Samples 11-29-16 SET II

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<th>Conc (mg/mL)</th>
<th>Corrected</th>
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<td>0.008</td>
<td>0.091</td>
<td>0.17137</td>
</tr>
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<td>OS 123271</td>
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### Graph

\[ y = 0.531x \]

\[ R^2 = 0.9951 \]

---

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<th>ST DEV</th>
<th>corrected</th>
<th>mg/mL</th>
<th>ug/mL</th>
<th>6x</th>
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### BCA Canine Samples 11-30-16 SET III

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<th>Corrected</th>
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<td>0.166</td>
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### Absorbance vs. Protein Concentration

\[ y = 0.5285x \]

\[ R^2 = 0.9916 \]

---

**Sample**

**ABS 1**

**ABS 2**

**AVG**

**ST. DEV.**

**Corrected**

**mg/mL**

**ug/mL**

**6x**
**BCA Canine Samples 11-30-16 SET IV**

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<th>corrected</th>
<th>mg/mL</th>
<th>ug/mL</th>
<th>6x</th>
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**y = 0.5205x**

**R² = 0.9943**
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BCA Canine Samples 12-1-16 SET VI

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\[
y = 0.5139x \\
R^2 = 0.9959
\]
APPENDIX B

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<th>Treatment?</th>
<th>Y, N, NA</th>
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Set I  11-30-2016 Standard Curve 32 ng/mL, 6924 FB PANT (1:3200), Secondary (1:400), OPD

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\[ y = 0.0309x + 0.072 \]
\[ R^2 = 0.98058 \]

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### Graph

**Equation:**

\[ y = 0.0344x + 0.0449 \]

**R²:** 0.98706

### Table: Sample Results

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<th>Tear Status</th>
<th>Treatment?</th>
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### Set III 12-1-2016 Standard Curve 32 ng/mL, 6924 FB PANT (1:3200), Secondary (1:400), OPD

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**Graph: OD 415**

\[ y = 0.0365x + 0.0732 \]

\[ R^2 = 0.97498 \]

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\[
y = 0.0292x + 0.0529 \\
R^2 = 0.98598
\]

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y = 0.031x + 0.0257
R^2 = 0.98515

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**Graph:**

- Equation: $y = 0.0329x + 0.0749$
- $R^2 = 0.99487$

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**Graph:**

- **Equation:** \( y = 0.0352x + 0.0897 \)
- **R\(^2\):** 0.98356

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<tr>
<td>OS 102140</td>
<td>0.713</td>
<td>0.707</td>
<td>0.797</td>
<td>0.739</td>
<td>0.050</td>
<td>18.446</td>
<td>DE</td>
<td>Y</td>
<td>Y</td>
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<tr>
<td>OD 112296</td>
<td>0.312</td>
<td>0.340</td>
<td>0.304</td>
<td>0.319</td>
<td>0.019</td>
<td>6.505</td>
<td>DE</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>OS 112296</td>
<td>0.414</td>
<td>0.347</td>
<td>0.335</td>
<td>0.365</td>
<td>0.043</td>
<td>7.830</td>
<td>DE</td>
<td>N</td>
<td>N</td>
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</table>
Set VI 12-2-2016 Standard Curve 32 ng/mL, 6294 FB PANT (1:3200), Secondary (1:400), OPD

<table>
<thead>
<tr>
<th>ng CI Lac</th>
<th>abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
<th>average</th>
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<tbody>
<tr>
<td>32</td>
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<td>1.317</td>
<td>1.177</td>
<td>1.288</td>
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<tr>
<td>24</td>
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<td>1.040</td>
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<td>0.779</td>
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<td>12</td>
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<td>0.553</td>
<td>0.592</td>
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<td>8</td>
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<td>0.438</td>
<td>0.382</td>
<td>0.414</td>
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<tr>
<td>6</td>
<td>0.356</td>
<td>0.304</td>
<td>0.302</td>
<td>0.321</td>
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<td>4</td>
<td>0.225</td>
<td>0.233</td>
<td>0.211</td>
<td>0.223</td>
</tr>
<tr>
<td>3</td>
<td>0.164</td>
<td>0.148</td>
<td>0.147</td>
<td>0.153</td>
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<td>0.136</td>
<td>0.113</td>
<td>0.130</td>
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<td>1.5</td>
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<td>0.089</td>
<td>0.093</td>
<td>0.094</td>
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<td>0.096</td>
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<td>0.093</td>
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<tr>
<td>0.5</td>
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<td>0</td>
<td>0.066</td>
<td>0.061</td>
<td>0.067</td>
<td>0.065</td>
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</table>

\[
y = 0.0394x + 0.067 \\
R^2 = 0.99144
\]

<table>
<thead>
<tr>
<th>Sample</th>
<th>ABS 1</th>
<th>ABS 2</th>
<th>ABS 3</th>
<th>AVG</th>
<th>ST DEV</th>
<th>ng per 50 ng</th>
<th>Tear Status</th>
<th>Treatment?</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 128357</td>
<td>0.812</td>
<td>0.768</td>
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<td>0.031</td>
<td>18.503</td>
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<td>NA</td>
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<tr>
<td>OS 128357</td>
<td>0.785</td>
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<td>0.802</td>
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<tr>
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<td>0.948</td>
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<td>0.226</td>
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<td>NA</td>
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<tr>
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<td>0.507</td>
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<td>OD 132730</td>
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<tr>
<td>OS 132730</td>
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<td>0.830</td>
<td>0.828</td>
<td>0.824</td>
<td>0.060</td>
<td>19.213</td>
<td>DE</td>
<td>Y</td>
</tr>
<tr>
<td>OD 140932</td>
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<td>0.718</td>
<td>0.709</td>
<td>0.731</td>
<td>0.040</td>
<td>16.844</td>
<td>DE</td>
<td>Y</td>
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</table>
## Set VI

**12-2-2016 Standard Curve 32 ng/mL, 6924 FB PANT (1:3200), Secondary (1:400), OPD**

<table>
<thead>
<tr>
<th>ng clAc</th>
<th>abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
<th>average</th>
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</thead>
<tbody>
<tr>
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<td></td>
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<td>1.043</td>
<td>0.984</td>
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<td>12</td>
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<td>0.580</td>
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</tr>
<tr>
<td>8</td>
<td>0.439</td>
<td>0.449</td>
<td>0.455</td>
<td>0.448</td>
</tr>
<tr>
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<td>0.186</td>
<td>0.294</td>
<td>0.309</td>
<td>0.263</td>
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<tr>
<td>4</td>
<td>0.228</td>
<td>0.240</td>
<td>0.220</td>
<td>0.229</td>
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<tr>
<td>3</td>
<td>0.171</td>
<td>0.145</td>
<td>0.144</td>
<td>0.153</td>
</tr>
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<td>2</td>
<td>0.131</td>
<td>0.114</td>
<td>0.144</td>
<td>0.130</td>
</tr>
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<td>1.5</td>
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<td>0.091</td>
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<td>0.090</td>
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<tr>
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<td>0.084</td>
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<td>0.055</td>
<td>0.050</td>
<td>0.058</td>
<td>0.054</td>
</tr>
</tbody>
</table>

![Graph showing the standard curve with the equation y = 0.0375x + 0.0726 and R² = 0.97266.](image)

### Sample Data

<table>
<thead>
<tr>
<th>Sample</th>
<th>ABS 1</th>
<th>ABS 2</th>
<th>ABS 3</th>
<th>AVG</th>
<th>ST DEV</th>
<th>ng per 50 ng</th>
<th>Tear Status</th>
<th>Treatment?</th>
<th>Y, N, NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 128357</td>
<td>0.836</td>
<td>0.885</td>
<td>0.867</td>
<td>0.863</td>
<td>0.025</td>
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<tr>
<td>OS 128357</td>
<td>0.836</td>
<td>0.949</td>
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<td>0.866</td>
<td>0.072</td>
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<tr>
<td>OD 140688</td>
<td>0.898</td>
<td>0.914</td>
<td>0.922</td>
<td>0.911</td>
<td>0.012</td>
<td>22.366</td>
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<td>NA</td>
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<tr>
<td>OS 140688</td>
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<td>0.504</td>
<td>0.515</td>
<td>0.518</td>
<td>0.016</td>
<td>11.886</td>
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<tr>
<td>OD 122730</td>
<td>0.794</td>
<td>0.800</td>
<td>0.792</td>
<td>0.795</td>
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<td>OD 140932</td>
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<td>0.743</td>
<td>0.046</td>
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<td>Y</td>
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</tr>
</tbody>
</table>
Antibody Production Schedule

October 30, 2013

Robert L. McKown
James Madison University

Dear Dr. McKown,
Your full service Custom Antibody Project immunization protocol has been initiated on 10/29/2013. The following table outlines the bleed schedule:

<table>
<thead>
<tr>
<th>Project No.</th>
<th>Immunogen Name</th>
<th>Rabbit Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1412-1</td>
<td></td>
<td>BSYN 6924 and 6925</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bleed No.</th>
<th>Date</th>
<th>Yield (serum/rabbit)</th>
<th>Project duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0(pre-immune)</td>
<td>10/29/13</td>
<td>2-3ml</td>
<td>0 days</td>
</tr>
<tr>
<td>1(week 6)</td>
<td>12/10/13</td>
<td>15ml</td>
<td>42 days</td>
</tr>
<tr>
<td>2(week 8)</td>
<td>12/24/13</td>
<td>15ml</td>
<td>56 days</td>
</tr>
<tr>
<td>3(week 10)</td>
<td>01/07/14</td>
<td>15ml</td>
<td>70 days</td>
</tr>
</tbody>
</table>

The project can be continued beyond Bleed No. 3. We can continue the boost/bleed maintenance schedule of the project for a charge of $200/month/rabbit. The project may be continued until the entire available antigen is used. At any time, you may also request exsanguination of any rabbit for an additional 30-100ml of crude serum. The charge for this service is $1/ml (max. charge $100/rabbit) of crude serum shipped. There is a shipping charge for any additional shipments past the 3rd bleed.

Bleed Nos. 0, 1, and 2 will be shipped together a few days after the 8th week bleed. The last bleed will be shipped to you following the 10th week unless affinity purification was added to the project. You must complete the necessary analysis on this shipment of serum and confirm by fax or e-mail your desire to continue with the project within three days of termination (day 75). We will terminate your project if we do not hear from you prior to day 75.

If you desire to continue the project or wish to exsangunante an animal, please give the appropriate payment instructions with your request (i.e. PO number).

Should you have any questions regarding our services, please call 1-800-227-0627 ext. 103, e-mail to antibody@biosyn.com or fax your concerns to the Antibody Department.

Sincerely,

Antibody Department
BIOSYNTHÈSE, INC.
Dry-Eye Syndrome

- Occurs in humans and canines
- Chronic disease
- Affects over 25 million Americans and 4% of canine population
What is Lacritin?

- Tear film protein
- Secretion enhancing factor
- 119 amino acids, \(~12.3\) kDa

Summary of Previous Work

- Canine Lacritin gene sequenced, cloned, and purified
- Development and titration of N-terminal antiserum
- Development of indirect ELISA
- Western blot analysis

EGDSSDPAPGAAADPGGLTPAADPAAPPQKAQQEPEGSTPHGEDQSPLKS
LVSRGLTLHGLEGAEKRLDQGRQFRNLYDRGAEFGRNLKQLVPQFN
Project Outline and Purpose

*investigate Lacritin as a potential biomarker for dry-eye syndrome*

Collection  Elution  BCA Assay  Indirect ELISA  Western Blot  Densitometry

Tear Collection and Elution
**BCA Assay**

- Graph showing the relationship between protein concentration and absorbance (OD), with the equation $y = 0.5139x$ and $R^2 = 0.99586$.

**Indirect ELISA**

- Graph showing the relationship between canine lactatin concentration and absorbance (OD), with the equation $y = 0.0394x + 0.067$ and $R^2 = 0.9144$.
Western Blotting and Densitometry

Results and Key Takeaways

- Untreated dry-eye canines have significantly more total protein in their tears
- Untreated dry-eye canines have significantly less monomeric Lacritin in their tears
- Lacritin has potential as a biomarker of dry-eye syndrome
In the Future...

• Human Clinical Trials
• Topical Application in Humans
• Bring to Market

THANK YOU!

• Robert Mckown, Ph.D.
• Ron Raab, Ph.D.
• Stephanie Stockwell, Ph.D.
• Julie Disney, DVM
• Allie Enghauser
• Katie Kelly
• McKenzie Patrick
• Brooke Justis
• Jessica Cornell
• Casey Coburn
• Family and Friends
ANY QUESTIONS?