

Tear film lacritin concentrations in dogs with keratoconjunctivitis sicca

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## ABSTRACT

**Background:** Keratoconjunctivitis sicca (KCS) is a chronic ocular disease of both dogs and humans that can result in ocular discomfort, corneal opacification, and vision loss. Lacritin, a protein found in the tears of many species, has been shown to play a role in lacrimation and corneal health. Because of its role as a potential lacrimostimulant, assessment of endogenous lacritin levels could reveal a correlation between lacritin and tear production in the dog.

**Objectives:** To determine if tear lacritin concentrations are decreased in canine eyes affected by KCS.

**Animals:** 58 client-owned dogs (tear samples from 55 eyes with normal tear production and 55 eyes diagnosed with KCS).

**Methods:** All eyes underwent an ophthalmic exam, including Schirmer Tear Testing (STT), anterior segment assessment, and tear sample collection. Tear samples were evaluated for their total protein concentrations via BCA assay and lacritin concentrations via ELISA.

**Results:** Total protein of canine tears is increased in KCS-affected eyes as compared to normal eyes. Tear lacritin as a component of total tear protein is significantly decreased in tears from KCS-affected eyes. When measured as a concentration (mass per volume of aqueous tears), lacritin is not significantly different between KCS-affected eyes and normal eyes, nor were they strongly correlated to STT values.

**Conclusions and Clinical Importance:** Total tear protein levels were significantly increased in canine KCS. When quantified as a proportion of total tear sample protein, tear lacritin levels are decreased in KCS-affected eyes. Relative to tear volume, tear lacritin levels are not significantly different between KCS-affected eyes and normal eyes. Assessment of lacritin supplementation in canine KCS is warranted to evaluate potential effects on lacrimation and ocular surface health.

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## GENERAL AUDIENCE ABSTRACT

Keratoconjunctivitis sicca describes a syndrome of inadequate pre-ocular tear film, due to decreased quantity or quality of tears on the ocular surface. A multitude of clinical signs and sequelae result, including ocular discomfort, ocular discharge, and corneal opacification that may lead to vision loss. Current therapies aim at improving endogenous tear production; standard therapy, however, may not significantly improve disease in up to 30% of canine patients and 40% of human patients. Development of additional therapies could improve the vision and comfort of patients with KCS.

Lacritin, a protein found in the tears of many species, has been shown to play a role in lacrimation and corneal health. Because of its role as a potential stimulant of tear production, assessment of endogenous lacritin levels could reveal a correlation between lacritin and tear production in the dog. This project aims to compare tear lacritin concentrations in dogs with normal tear production versus dogs with KCS.

Tear samples were collected from normal dogs ( $n=55$ ) and those affected with KCS ( $n=55$ ). Samples were analyzed for their total tear protein levels via BCA assay and for their lacritin levels via ELISA analysis. Total tear protein levels and tear lacritin levels were compared to Schirmer Tear Test results, a routine method of diagnosing KCS in dogs. Tear lacritin was significantly decreased in KCS, relative to the quantity of total tear proteins. As a portion of the total volume of tears, lacritin was not significantly different in KCS-affected eyes as compared to normal eyes. Further investigation is warranted to determine the effects lacritin supplementation may have on canine KCS, in regard to tear production and clinical signs of KCS.

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## CHAPTER 1: LITERATURE REVIEW

### A. Keratoconjunctivitis sicca: Pathogenesis

The precorneal tear film serves many purposes: maintains optical clarity and contributes to refraction and vision, removes foreign matter from the cornea and conjunctiva, lubricates the cornea and conjunctiva, provides nutrients to the avascular cornea, and contains antimicrobial proteins to maintain local surface flora.<sup>1</sup> It is comprised of three layers, including an outer lipid layer, middle aqueous layer, and inner mucin layer. As the primary contributor to the functions of the tear film, the middle aqueous layer is the primary area of concern in canine dry eye diseases. The aqueous portion of the tear film provides glucose, electrolytes, oxygen, and water to the cornea; lubricates the corneoconjunctival surfaces; removes waste products such as carbon dioxide and lactate; and flushes away debris and bacteria.<sup>2</sup> This layer is formed by aqueous secretions from the lacrimal gland, as well as the gland of the third eyelid in domestic species. Contributions to the aqueous layer by each of these glands varies between species and from animal to animal within a species; it is well-established that both glands are important in the production of aqueous tears in the dog.<sup>3</sup> As such, disease or removal of either gland has the potential to result in a decrease of aqueous tear production.

Delivery of tears to the ocular surface generally occurs in two forms: basal tear secretion and reflex tearing. Basal tear secretion is a persistent, low-level production of tears, which provides the adequate tears for normal function of the precorneal tear film.<sup>1</sup> Basal tear secretion is measured via Schirmer tear testing, preceded by application of topical anesthetic to prevent induction of reflex tearing; this test is typically referred to as the Schirmer tear test II (STTII). Adequate basal tearing as measured by STTII in the dog is reported to range from 6.2+/-3.1mm/min<sup>4</sup> to 11.6+/-6.1mm/min.<sup>5</sup> Reflex tearing results in an increase in tear production in response to a noxious stimulus, such as light, foreign material, or other sources of irritation.<sup>1</sup> Schirmer tear testing is also utilized in evaluating reflex tearing, but in the absence of topical anesthetic so the contact of the strip incites a noxious stimulus on the corneal and conjunctival surfaces (Schirmer tear test I, or STTI). Adequate STTI measurements in the dog are reported to range from 20.2+/-3mm/min<sup>4</sup> to 21+/-4.2mm/min.<sup>5</sup> Interpretation of any Schirmer tear test value is accompanied by clinical evaluation of the patient, as discussed below.

Lacrimation requires not only functional glands to supply aqueous tears, but also an intact neurologic pathway to stimulate release of aqueous

tears when appropriate. The afferent arm that stimulates reflex lacrimation (tearing in response to a stimulus) involves the sensory innervation that cranial nerve V (trigeminal nerve) supplies to the conjunctiva and cornea. The need for an intact afferent arm of this pathway is important to remember in our clinical assessment of tear production, as the Schirmer Tear Test I is a measure of reflex tear production (i.e. aqueous tear production in response to a mechanical stimulus). The efferent arm, stimulating the lacrimal glands to secrete aqueous tears, is provided by parasympathetic neurons that originate from the parasympathetic nucleus of the facial nerve. The fibers travel through the facial canal of the petrous temporal bone with cranial nerve VII (facial nerve), then join with branches of the trigeminal nerve to ultimately reach the lacrimal gland and stimulate tear secretion.<sup>6</sup>

Keratoconjunctivitis sicca (KCS) is a dry eye syndrome that occurs with relative frequency in both canine and human patients. It is characterized by an unstable tear film or decrease in aqueous tear production and some combination of the accompanying clinical signs, including conjunctival hyperemia, blepharospasm, mucoid to mucopurulent ocular discharge, and keratitis (corneal neovascularization, pigmentation, and fibrosis). Effective

treatment of KCS requires identification of the underlying cause, of which there are many.

In the dog, immune-mediated disease is the most common etiology of KCS. Inflammation of and subsequent damage to the lacrimal glands results in decreased aqueous tear production; histologic studies demonstrate infiltration of lymphocytes and plasma cells in affected lacrimal glands.<sup>7,8</sup>

Interference with the neurologic pathway for lacrimation can also result in dry eye disease, termed neurogenic KCS. This results from a loss of direct innervation to the lacrimal tissue (cranial nerve VII), such as from traumatic injury or otitis media/interna.<sup>9,10</sup>

Other causes of KCS exist in the dog, though they are considered less common. This includes drug-induced KCS, which results in transient or permanent decreases in tear production due to administration of a medication. Sulfonamide antimicrobials<sup>11</sup>, the NSAID etodolac<sup>12</sup>, topical atropine<sup>13</sup>, and pre-anesthetic/anesthetic agents<sup>14-16</sup> have each been documented to negatively affect tear production through a variety of mechanisms. Congenital alacrima, a disease characterized by either lacrimal

gland agenesis or lacrimal gland hypoplasia, has also been reported as an etiology of KCS in young dogs, particularly in the Yorkshire Terrier.<sup>17</sup> A form of congenital KCS, in conjunction with ichthyosiform dermatosis, has also been identified in the Cavalier King Charles Spaniel, though causative pathology in the lacrimal gland has not yet been identified.<sup>18</sup>

Dry eye syndromes in humans can also result from numerous etiologies, and are often multifactorial in cause.<sup>19</sup> Similar to the most common cause of KCS in dogs, Sjögren's syndrome is an immune-mediated disease that occurs in humans, resulting in destruction of both lacrimal and salivary tissue.<sup>20</sup> Parallels between etiologies of canine and human KCS are significant, as novel therapies developed for use in one species may have successful application in the other.

## **B. Keratoconjunctivitis sicca: Epidemiology**

KCS is a common ophthalmic disease in dogs, with the literature citing a prevalence of 1-4% in the general canine population.<sup>21,22</sup> Certain breeds have been noted to be at increased risk, including the Cavalier King Charles Spaniel, English Bulldog, Lhasa Apso, Shih Tzu, West Highland White Terrier, and Pug, among others.<sup>2,9,11,17,22</sup>

In human patients, dry eye syndromes are similarly prevalent, particularly in older and female patients. In a population of US veterans examined at two Veterans Affairs eye clinics, 12% of male and 22% of female patients were diagnosed with dry eye syndrome.<sup>23</sup> The etiologies of human dry eye syndromes are also varied; however, an immune-mediated disease in humans, Sjögren's Syndrome, is similar to canine KCS. This syndrome is characterized by lymphocyte infiltration of the lacrimal and salivary glands, which leads to destruction of these secretory tissues.<sup>24</sup>

### **C. Keratoconjunctivitis sicca: Diagnosis and Clinical Signs**

Diagnosis of KCS in veterinary medicine is typically made via the Schirmer Tear Test (STT), which measures aqueous tear production by placement of a test strip into the conjunctival fornix for one minute. In dogs, a STT I value of  $\geq 15$ mm/min indicates adequate reflex tear production. A patient may have early or subclinical KCS if their STT I ranges from 11-14mm/min, in which case other clinical factors must be considered in establishing a diagnosis and recommending therapy. In such cases, presence of clinical signs or ophthalmic exam findings associated with KCS (e.g. corneal neovascularization or pigmentation) would prompt therapy, whereas

repeat STT I at a later date might be recommended in an otherwise asymptomatic case. A STT I measurement of 0-10mm/min results in a definitive diagnosis of KCS and therapy is recommended.<sup>2</sup>

The STT II, which measures basal tear production, is not frequently used in the diagnosis of KCS in veterinary medicine. Rather than being utilized in a preliminary evaluation, it may be used as a secondary diagnostic for patients that do not have a straightforward diagnosis of KCS. This test is performed similarly to an STT I; however, prior to placement of the strip, reflex tearing is suppressed via use of topical anesthetic. Topical anesthetic is applied to the ocular surface, and allowed adequate time (typically one minute) to take effect prior to drying of the conjunctival sac with a cotton-tipped applicator. The test strip is then placed into the conjunctival fornix for one minute, as for the STT I. Reference ranges for the STT II in dogs are lower than those for the STT I, with reports ranging from  $6.2+/-3.1\text{mm/min}^4$  to  $11.6+/-6.1\text{mm/min}^5$ .

Various complications may arise from altered tear homeostasis in veterinary patients. Assessment of bacterial flora in normal dogs and those with KCS indicated that KCS-affected canines are more likely to have

bacterial overgrowth of the conjunctival surface. They are more likely to have greater numbers of bacterial species, as well as more likely to harbor potentially pathogenic bacteria such as *Pseudomonas spp.*<sup>25</sup>

Patient comfort is difficult to assess in veterinary KCS patients, but is an important factor in assessing adequacy of management of KCS in human medicine. Human KCS patients often report ocular discomfort, described as a gritty feeling with or without a sensation that foreign material is entrapped underneath the eyelids.<sup>26</sup> As with dogs, human patients may also demonstrate signs of corneal damage and are at greater risk of surface infections.<sup>27,28</sup>

#### **D. Keratoconjunctivitis sicca: Current Treatments**

Regardless of etiology, KCS must be addressed with tear replacement (lacrimomimetic) therapy until the patient's endogenous tear production has improved to a level adequate to alleviation of symptoms. Unfortunately, tear supplementation requires extremely frequent therapy, as often as hourly, to ameliorate dry eye discomfort. Because this is often not realistic, it is essential to determine the underlying cause of the patient's KCS and treat appropriately, with the goal of restoring lacrimal function.

Due to the complicated composition of tears, the ideal theoretical solution to KCS would be to improve the patient's ability to produce their own normal tears, rather than attempt to replace the biological substance with artificial tears. Therefore, lacrimostimulants aimed at improving a patient's endogenous tear production are a mainstay of therapy.

In dogs, lacrimostimulants can take several forms, depending on the suspected root pathology of the patient's KCS. In the case of immune-mediated KCS in dogs, the gold standard of therapy is topical immunosuppressive medications – more specifically, the calcineurin inhibitor cyclosporine A (Optimmune® 0.2% Ophthalmic Ointment). Originally derived from a fungus (*Tolyphocladium inflatum*) for use in preventing rejection of organ transplants, cyclosporine A inhibits T-cell activation. In CD4+ T-helper lymphocytes, the cyclosporine molecule first binds to intracellular cyclophilin. The cyclosporine-cyclophilin complex then binds to calcineurin, thus preventing induction of interleukin-2 by calcineurin. This leads to impaired T-helper and T-cytotoxic proliferation.<sup>2,29</sup> Topical application of cyclosporine A has been demonstrated to improve tear production in 75-100% of eyes and decrease clinical signs such as

corneal pigmentation in 67-80% of cases.<sup>30-32</sup> Increases in tear production begin within 3 to 56 days of initiation of cyclosporine A therapy, with a mean increase of 8.9mm/min in KCS-affected dogs.<sup>30</sup> Canine patients which have STT values ranging from 0 to 2mm/min have a decreased response to therapy, with only 59% of these patients having a STT increase of 5mm/min or greater.<sup>30</sup> Despite significant success with cyclosporine A therapy, lifelong treatment is typically necessary. In one study, decreasing the frequency or discontinuation of cyclosporine therapy in 20 dogs resulted in a need to return to twice daily administration in 11 dogs, once daily administration in two dogs, every other day administration in one dog, and discontinuation of therapy in only 6 dogs.<sup>31</sup> An additional calcineurin inhibitor, tacrolimus, has been demonstrated to provide a similar, and in some cases superior, response to that of cyclosporine A<sup>33,34</sup> and is also commonly used to manage KCS in dogs. Treatment with either topical cyclosporine or tacrolimus has been demonstrated to improve clinical signs of KCS, such as ocular discharge and keratitis, even in patients who do not respond with an increase in STT values.<sup>30,31</sup>

More recently, a cyclosporine-impregnated device intended for subconjunctival/episcleral implantation has been described. Episcleral

implantation of this silicone-based device in dogs diagnosed with KCS was demonstrated in a pilot study to increase STT for up to 300-330 days, as well as improve clinical signs (such as corneal neovascularization, corneal opacity, and ocular discharge) for up to 480-540 days.<sup>35</sup> This implant, while not a permanent cure, does provide alternative options for animals that are refractory or resistant to traditional topical therapy.

As neurogenic KCS has a different pathophysiology than the more common immune-mediated disease, different therapy is also necessary in order to effectively stimulate tear production. Because it is a lack of nerve stimulus which results in loss of tear production in this condition, the goal of therapy is to mimic the normal stimulus with medications. Pilocarpine is a direct-acting parasympathomimetic drug and, as such, can supply the parasympathetic stimulus needed for lacrimation. Administration may be performed topically with a dilute pilocarpine ophthalmic solution, or orally. Unfortunately, when administered systemically, this medication may be associated with signs of systemic toxicity (salivation, vomiting, diarrhea, bradycardia) in some dogs<sup>10,36</sup>, even before satisfactory lacrimation responses are obtained in some cases. Topical administration frequently results in blepharospasm and miosis secondary to an acidic pH of 3.5-5.5<sup>37</sup>

and induction of uveitis<sup>36,38-40</sup>, and may not provide satisfactory improvements in STT values or clinical signs of KCS.<sup>38</sup>

Long-term therapies for dry eye in humans are similar to those of canines, including tear replacement and topical cyclosporine.<sup>41</sup> A phase III clinical trial demonstrated that cyclosporine A (Restasis® 0.05% Ophthalmic Suspension) results in increased aqueous tear production, decreases the patient's need for supplemental artificial tears, and reduces clinical signs.<sup>28</sup> Schirmer tear test values increase in approximately 59% of patients, with 15% increasing to  $\geq 10\text{mm}/5\text{ min}$ .<sup>24</sup> Unfortunately, approximately 25% of patients experience minor adverse effects (particularly a burning sensation at the time of topical application), leading 2.2% of patients to discontinue use of Restasis®.<sup>28</sup>

Symptomatic care also plays an important role in the management of dry eye diseases in both canine and human patients, particularly in cases that do not respond or have a sub-optimal response to lacrimostimulant therapy. Lacrimomimetics, products which serve to temporarily replace the deficient tear film, are available over-the-counter in abundance; in 2011, the artificial tear market was estimated to be worth \$55.4 billion.<sup>42</sup> Unfortunately, these

products remain on the surface of the eye only transiently and do not adequately resolve clinical signs; the necessary frequency of administration to manage a dog with uncontrolled KCS is simply not feasible for most pet owners. Other medications may be used on a case-by-case basis, such as mucolytic medications to reduce the severity ocular discharge, or antimicrobial therapy to manage bacterial overgrowth secondary to KCS.

#### **E. Keratoconjunctivitis sicca: Prognosis**

In dogs, 20-25% of suspected immune-mediated cases do not respond to cyclosporine or tacrolimus, or respond sub-optimally with only marginal improvements in tear production.<sup>31,33</sup> These patients are resigned to lifelong adjunctive treatment with tear supplements and may experience persistent ocular discomfort, corneal opacification leading to vision loss, recurrent corneal ulceration, and other complications. Unsatisfactory responses to medical therapy may necessitate surgical management in order for the patient to have adequate quality of life. Partial permanent tarsorrhaphy, which decreases the size of the palpebral fissure, provides patients additional protection of the ocular surface and may help preserve any tears that are produced.<sup>2</sup> Unfortunately, this procedure does not provide additional tear production or other lubrication to the ocular surface.

Parotid duct transposition (PDT) surgery has been described in the dog as a means of providing additional lubrication to the ocular surface via salivation. Saliva and tears share very similar composition, with the most clinically relevant difference being a higher mineral content in saliva. Short-term complications of the procedure may result from operative trauma, such as inadvertent twisting of or damage to the duct can prevent the flow of saliva to the ocular surface, or poor wound healing, such as surgical wound dehiscence or infection. Long-term complications can also result in failure to deliver saliva to the ocular surface, such as retraction of the parotid papilla from the conjunctival sac, obstruction of the duct with sialoliths or fibrosis, or sialoadenitis. Even without complete surgical failure such as those listed above, other long-term complications often occur: overproduction of saliva resulting in periocular salivary staining and moist dermatitis; mineral deposition in the cornea, conjunctiva, and eyelids leading to ocular discomfort; stromal abscessation; and salivary intolerance.<sup>2,43</sup> One retrospective study found high owner satisfaction with this procedure, with 90% of owners indicating that they would pursue PDT surgery again. This same study, however, also identified a relatively high complication rate, with 50% of eyes undergoing surgery experiencing complications and 39% of those with complications necessitating further surgical procedures, including

enucleation.<sup>43</sup> Furthermore, surgical procedures may not be an option for all patients or owners, often due to poor anesthetic candidacy or financial limitations.

Though numerous strategies exist for the management of keratoconjunctivitis sicca in the dog, veterinary ophthalmologists are still commonly presented with patients that respond poorly to the therapies available. Development of additional therapies may aid in further reduction of these poorly responding cases, thus improving our ability to manage this uncomfortable and potentially blinding disease.

## **F. Lacritin: A New Avenue to Explore**

Lacritin is a protein found in the tear film of various species, including human, non-human primate, horse, dog, and rodent.<sup>44-47</sup> The full scope of lacritin's functions is still being elucidated, but recent in vitro studies suggest that it is a lacrimostimulant with several other ancillary functions. When applied topically to rat and monkey lacrimal acinar cells in culture, lacritin stimulates tear secretion.<sup>48,49</sup> It also demonstrates bactericidal effects.<sup>50</sup> Lacritin exerts numerous effects on corneal epithelial cells, including exerting cytoprotective effects on stressed corneal

epithelium,<sup>51</sup> cytoprotective effects on corneal epithelial cells exposed to the common ophthalmic preservative benzalkonium chloride,<sup>52</sup> protection for corneal epithelial cells against lipopolysaccharide-induced cell death,<sup>53</sup> and promoting corneal epithelial wound healing when bound to an elastin-like polypeptide nanoparticle.<sup>54</sup> These are all potentially important properties for managing clinical consequences of dry eye.

In healthy adult humans, there does not appear to be a significant difference in tear lacritin levels between males and females or among those aged 18 to 52 years of age.<sup>55</sup> Interestingly, tear lacritin monomer concentrations are decreased in several dry eye conditions. In humans, lacritin is decreased in patients with dry eye syndromes, including those with Sjögren's syndrome, dry eye related to contact lens wear, and blepharitis.<sup>44,56-61</sup> In humans with Sjögren's syndrome, reduced tear lacritin concentrations have also been correlated to decreased Schirmer tear test values, clinical signs of dry eye such as ocular staining, decreased corneal sensitivity, and decreased corneal nerve fiber density and nerve fiber length.<sup>62</sup> Preliminary analysis of canine tears have demonstrated that tear lacritin concentrations are decreased in dogs with KCS as compared to dogs with normal tear production.<sup>63</sup>

Because of lacritin's potential to stimulate lacrimal tissue to secrete tears, combined with the finding of decreased tear lacritin concentrations under dry eye conditions, lacritin is being investigated as a therapy for dry eye diseases.<sup>64</sup> Recombinant lacritin proteins have been synthesized in both human<sup>65</sup> and canine<sup>63</sup> forms. Human recombinant lacritin, when applied to the eyes of normal rabbits three times daily for two weeks, demonstrated that this therapy was well-tolerated and resulted in increased basal tear secretion that was sustained not only throughout the two week trial period, but also for one week following the discontinuation of therapy.<sup>45</sup> Recombinant lacritin solution was also evaluated in a dry-eye model of mice, which were treated three times daily for three weeks. Over the course of the treatment period, basal tear production increased and signs of keratitis improved significantly; the lacrimal glands of treated eyes also demonstrated decreased inflammation on histopathology.<sup>44</sup> As a potential clinical therapeutic agent, however, recombinant lacritin presents logistical problems, due to the need for reconstitution from a lyophilized powder into a solution shortly before to use. For either human or veterinary patients, this is impractical for long-term use.

A synthetic peptide, Lacripep<sup>TM</sup>, representing the active region of the lacritin protein, has been developed for further evaluation as a potential therapeutic. The functions of Lacripep<sup>TM</sup> parallel those of lacritin, including cytoprotection of stressed corneal epithelial cells, as well as restoration and reduction of signs of keratitis in a dry-eye model of mouse.<sup>66</sup> Preliminary pharmacokinetic studies have shown that Lacripep<sup>TM</sup> is well-tolerated and does not accumulate in the blood or plasma when applied as a topical ophthalmic solution (personal communication, GW Laurie). While maintaining these physiologically beneficial properties of lacritin, Lacripep<sup>TM</sup> is stable in solution over long periods. If therapy with a lacritin analog proves beneficial in dry eye syndromes, a reproducible synthetic product such as Lacripep<sup>TM</sup> may be advantageous for larger-scale production and distribution.

## **G. Detecting Lacritin: The Enzyme-Linked Immunosorbent Assay**

In order to determine if lacritin deficiency has any role in keratoconjunctivitis sicca in the dog, it must first be established if lacritin concentrations are decreased in the tears of dogs affected by KCS as compared to dogs with normal tear production. Measurements of tear lacritin

concentrations can be determined via an enzyme-linked immunosorbent assay (ELISA) specific to detecting canine lacritin.

In general, ELISAs are utilized to detect specific antigens or antibodies in a given sample. Enzyme-labeled antibodies and antigens are used to detect the targeted biological molecule. First, the sample is placed into a well, where antigens are coated to the well surface. A specific primary antibody is then utilized to bind to the targeted biological molecule, followed by a secondary enzyme-linked antibody to bind to the primary antibody. Lastly, substrate for the enzyme is added to the well, resulting in a color change that can be quantified.<sup>67</sup>

The ELISA provides a means of detecting and quantifying a target molecule, but limitations exist as well. A specific antibody that can bind to the target molecule must be developed for each target molecule. Additionally, the enzymatic reaction which occurs in the final step of the ELISA can continue to occur beyond the desired timeframe, requiring that each plate must be read expediently and accurately in order for data from different plates to remain comparable.<sup>67</sup>

## **H. Conclusion and Research Justification**

Keratoconjunctivitis sicca in dogs is a chronic disease that requires long-term medical therapy and management of various complications that have the potential to cause pain and vision loss. For patients that don't respond to currently available therapies, this can result in chronic discomfort, vision loss, and even loss of the affected eye. Additional therapeutic options could alleviate those non-responsive patients, as well as reduce some of the frustrations of pet owners and clinicians alike.

This proposed study will investigate lacritin in the tear film of dogs. We hypothesize that lacritin will be decreased in the tear film of KCS-affected canine eyes as compared to the tear film of normal canine eyes. Additionally, we hypothesize that total tear protein will be increased in the tear film of KCS-affected canine eyes as compared to the tear film of normal canine eyes.

## CHAPTER 2: TEAR FILM LACRITIN CONCENTRATIONS IN DOGS WITH KERATOCONJUNCTIVITIS SICCA

### A. Introduction

Canine keratoconjunctivitis sicca is a common ocular disease of dogs that can result in significant ocular discomfort and potentially vision-threatening complications. Diagnosis of dry eye diseases in human medicine is relatively complex and typically incorporates a combination of objective and subjective measures; patient-reported symptoms play a significant role in diagnosis and treatment.<sup>68</sup> As patient-reported symptoms are unavailable in veterinary medicine, diagnosis of KCS relies upon the presence of clinical signs of the disease and a decreased Schirmer Tear Test (<15mm/min). Current available medical therapies for the most common type of KCS (immune-mediated destruction of lacrimal tissue) are successful in improving tear production and reducing clinical signs in a majority of cases<sup>30-32</sup>, but therapies are limited in those patients who do not respond.

Investigation into additional therapies for KCS is warranted, for the benefit of both veterinary and human patients. Tear lacritin content has been shown to be decreased in various dry eye conditions in humans<sup>44,56-61</sup>, but a large-scale evaluation of lacritin has not been undertaken in canine KCS.

Promising *in vitro* effects of lacritin<sup>48-54</sup> and demonstration of decreased tear lacritin in canine KCS could implicate lacritin as a prime potential therapy for dogs with KCS that is non-responsive to traditional therapies. We evaluated total tear protein concentration and lacritin concentration in the tears of both normal dogs and those with a diagnosis of KCS. We hypothesized that total tear protein would be increased in the tears of KCS-affected eyes, while lacritin would be decreased in the tears of KCS-affected eyes.

## B. Materials and Methods

### *Animals and study design*

Canine patients presenting to the Virginia-Maryland College of Veterinary Medicine Veterinary Teaching Hospital between May 2015 and November 2016 that had a Schirmer tear test I (STTI) performed were candidates to be included in the study, provided owner consent could be obtained and adequate time for tear collection was available in the allotted appointment time. Patients were excluded if they had significant active ocular disease other than keratoconjunctivitis sicca. Dogs with ocular conditions that could potentially lead to excessive tearing, such as corneal ulceration or other painful processes were excluded. Additionally, patients

with STTI values of <2mm/min were excluded due to the absence of adequate tears for collection. Historical data collected included signalment information, history of ocular disease, and current ophthalmic medications. All patients received an ophthalmic examination performed by 1 of 2 board-certified ophthalmologists (IPH or JPP) or 1 resident (JLD). In all cases, this examination included at minimum STTI measurements and examination of the ocular surface and adnexa with a Finoff transilluminator and via slitlamp biomicroscopy; in most cases, this also included rebound tonometry and indirect ophthalmoscopy.

Eyes were categorized as having KCS if their STTI value was <15mm/min or if they had been previously diagnosed with KCS (via STT <15mm/min) and had since been started on treatment. Eyes were categorized as normal if their STTI value was ≥15mm/min and had no clinical signs consistent with KCS. Informed owner consent was obtained prior to inclusion in the study.

#### *Tear sample collection and storage*

After ophthalmic examination and STTI measurements were completed and owner consent obtained, a tear sample was collected from

each eye (or from a single eye if the fellow eye was not a candidate due to significant ocular disease as outlined above or previous enucleation). This was performed in the absence of topical anesthetic and prior to the application of any topical medications or saline rinse. Patients were gently manually restrained while an absorbent wick was placed in the lacrimal lake for approximately 30 seconds, while minimizing contact with the ocular surface. After collection, samples were stored in a -80°C freezer until batch analysis was performed.

#### *Tear sample elution*

Tear samples were removed from the -80°C freezer and allowed to reach room temperature. To aid in removal of all protein from the absorbent wick, 30 $\mu$ L PBS (sterilized via 0.2 $\mu$ m filter) was applied to each wick and allowed to incubate for 20 minutes. The eluted sample was removed from the wick and collected in an Eppendorf tube via centrifugation at 13K rpm for 5 minutes. After elution, each eluted sample volume was measured and recorded.

*Total Tear Protein Measurement: Bicinchoninic acid assay*

Each sample was analyzed for total protein concentration via bicinchoninic acid (BCA) protein assay kit, used as recommended by the manufacturer (Thermo Fisher Scientific, Rockford, IL). On a 96 well microtiter plate, 10uL of each tear sample was plated in duplicate. Each plate also contained a standard curve of known protein concentrations of bovine serum albumin, also plated in duplicate. All plates were incubated for 30 minutes at 37°C, then read at 570nm in a spectrometer (model 680; Bio-Rad, Hercules, CA). The standard protein concentrations were graphed against the absorbance values and a line of best fit was applied to the data. Based on the equation of the line of best fit, each sample's total tear protein concentration was calculated from their absorbance values.

*Tear Lacritin Measurement: Indirect enzyme-linked immunosorbent assay*

Tear samples were analyzed for lacritin measurements in two separate batches. All samples were analyzed on two plates, in triplicate on each plate (for a total of 6 wells per sample). Included on each plate was a standard curve of recombinant canine lacritin, to allow subsequent calculation of lacritin concentrations in the tear samples. To generate this standard curve, known recombinant canine lacritin concentrations were used to coat each

plate in triplicate. In batch 1, the standard curve was plated in triplicate wells of 0, 2, 4, 6, 8, 10, 12, 14, and 16ng lacritin protein. In the batch 2, the standard curve was extended to include triplicate wells of 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, and 32ng lacritin protein. To assess lacritin content of eluted tear samples in both batches, 50ng total tear protein was coated overnight into triplicate wells for each sample, on two separate plates (for a total of 6 wells per sample).

Wells were washed three times with PBS-Tween (PBS with 0.3% Tween-20 [PBS-T]), blocked with 1% bovine serum albumin in PBS, and then incubated for 1 hour in batch 1 and 35 minutes in batch 2 at 37°C. After washing three times with PBS-T, 100 $\mu$ L primary antibody (6924 FB PANT) diluted in PBS-T (1:6400 for batch 1, 1:3200 for batch 2) was added and plates were incubated for 1 hour in batch 1 and 35 minutes in batch 2 at 37°C. Plates were again washed three times with PBS-T, and then incubated for 1 hour in batch 1 and 35 minutes in batch 2 at 37°C with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG diluted in PBS-T (1:1200 for batch 1, 1:400 for batch 2). After three final washes with PBS-T, bound antibody was measured after incubation for 10 minutes with 100 $\mu$ L OPD

substrate (Acros Organics, Geel, Belgium) by absorbance at 415nm (model 680; Bio-Rad, Hercules, CA).

*Tear Lacritin Detection: Western blot*

Tear samples were loaded on Any kD Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad), electrophoresed at 200V, and transferred to nitrocellulose (Protran BA 83; Whatman, Dassel, Germany). The blots were blocked with PBS-T, and then incubated with the primary antibody (6924 FB PANT) in a 1:1,000 dilution in PBS-T for one hour at room temperature. The blots were then washed a second time with PBS-T and incubated with HRP-conjugated goat anti-rabbit IgG diluted 1:5,000 in PBS-T. The blots were washed with PBS-T and developed via chemiluminescence with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc, Rockford, IL, USA).

*Statistical Analysis*

A two-eye design was utilized for data analysis in the study. This allows accountability for two data points (two eyes) coming from a single subject (one dog). Failure to do so would disregard the circumstances unique to the individual subject which could potentially affect both eyes; this can

lead to an overstatement of conclusions, with a falsely high degree of precision (falsely high confidence intervals, falsely low p-values, and higher likelihood of false positives).<sup>69-72</sup> As such, appropriate statistical methods were performed in this study to take this into consideration.

In this study, lacritin concentrations were quantified and statistically evaluated in two ways: (1) on a weight-by-weight basis, and (2) on a weight-by-volume basis. Previous studies evaluating lacritin concentrations have utilized a weight-by-weight method, in which lacritin was reported in a nanogram amount per 50 or 100 nanograms of total sample protein. Because of potential confounding effects of total sample protein being different in KCS-affected versus normal dogs, we also evaluated lacritin concentrations via a weight-by-volume method. In this method, we report lacritin concentrations in a microgram amount per milliliter of tear sample.

Normally distributed data were summarized as means ± standard deviation, while data with a lognormal distribution were summarized as medians accompanied by their ranges. Effects of KCS status and batch number on total sample protein concentrations and tear lacritin concentrations were assessed using mixed model ANOVA. Effects of

lacrimostimulant therapy and sex on tear lacritin concentrations were assessed using mixed model ANOVA. Effect of age on tear lacritin concentrations was assessed using mixed model ANCOVA.

## C. Results

A total of 110 tear samples were collected from 58 dogs. This included 4 intact females, 28 spayed females, 3 intact males, and 23 neutered males. The mean  $\pm$  SD age was  $7.3 \pm 3.7$  years, with no significant difference ( $p=0.0882$ ) in age between the normal (mean  $6.76 \pm 3.58$  years) and KCS-affected populations (mean  $8.3 \pm 3.7$  years). Breeds included mixed (n=14), Miniature Dachshund (n=5), Shih Tzu (n=4), Siberian Husky (n=4), Cavalier King Charles Spaniel (n=3), Yorkshire Terrier (n=3), Chihuahua (n=2), English Bulldog (n=2), French Bulldog (n=2), German Shepherd (n=2), Miniature Schnauzer (n=2), Pug (n=2), Toy Poodle (n=2) and 1 each of the following: Australian Shepherd, Beagle, Bichon Frise, English Springer Spaniel, Golden Retriever, Labrador Retriever, Lhasa Apso, Maltese, Miniature Pinscher, Miniature Poodle, and Standard Poodle. Samples were analyzed in two large batches: batch 1 (n=64) in spring 2016 and batch 2 (n=46) in fall 2016.

Of the 110 eyes from which samples were collected, 55 canine eyes were diagnosed with KCS and 55 canine eyes were not. Of the eyes diagnosed with KCS, 29 of the eyes were currently on lacrimostimulant therapy for KCS (topical cyclosporine or tacrolimus) while 26 were not on any lacrimostimulant therapy. A total of 29 samples were excluded from analysis of lacritin measurements for various reasons, as follows: a total of 17 samples had lacritin levels that exceeded the standard curve on the ELISA; due to potential inaccuracies in extrapolated data beyond the standard curve, these samples were excluded from lacritin analysis. Additionally, 1 sample was excluded from lacritin analysis as its absorbance measured more than 2 standard deviations below the absorbance of the “0” point on the standard curve (the control containing no lacritin). This resulted in a total of 92 samples included in tear lacritin analysis on a weight-by-weight basis. Eluted sample volume was not recorded in 8 samples due to inadvertent error; after centrifugation, 3 additional samples yielded only the volume of eluent (phosphate-buffered saline) that had been applied for sample elution. Without a sample volume, tear lacritin concentrations ( $\mu\text{g/mL}$ ) could not be measured in these samples. As such, a total of 81 samples were included in tear lacritin analysis on a weight-by-volume basis.

### *Schirmer Tear Test I values*

All non-KCS eyes (n=55) had a Schirmer Tear Test I value  $\geq 15$  mm/min. Of the KCS eyes, 29 were on lacrimostimulant therapy and 26 were not. Of those on lacrimostimulant therapy, 14 responded to treatment (STTI  $\geq 15$  mm/min) and 15 did not (STTI  $< 15$  mm/min). A significant difference ( $p=<0.0001$ ) was detected between STTI values for normal eyes (mean  $21.73 \pm 3.93$  mm/min) and for KCS eyes (mean  $11.47 \pm 6.52$  mm/min). A significant effect of lacrimostimulant therapy was detected ( $p=0.0005$ ), with STT values being significantly higher in KCS eyes on therapy (mean  $13.67 \pm 7.77$  mm/min) as compared to those not on therapy (mean  $8.78 \pm 2.93$  mm/min).

### *Total tear sample protein measurements*

A significant difference ( $p<0.0001$ ) was detected in the total sample protein concentration between tear samples from normal eyes (median 5,548.94  $\mu\text{g}/\text{mL}$ , range 2,200.12 to 31,992.0  $\mu\text{g}/\text{mL}$ ) and tear samples from KCS-affected eyes (median 14,354.47  $\mu\text{g}/\text{mL}$ , range 3,074.24 to 77,607.77  $\mu\text{g}/\text{mL}$ ). No significant effect of batch analysis was detected ( $p=0.1155$ ), with batch 1 median total sample protein of 7,067.79  $\mu\text{g}/\text{mL}$  (range 2,615.35 to 65,344.0  $\mu\text{g}/\text{mL}$ ) and batch 2 median total sample protein of 6,071.65

$\mu\text{g/mL}$  (range 2,200.12 to 77,607.77  $\mu\text{g/mL}$ ). When comparing tear total protein to STT as a continuous variable, no significant correlation between the two was detected.

### *Lacritin measurements*

On a weight-by-weight basis, tear lacritin concentrations differed significantly between disease groups ( $p<0.0001$ ). In normal eyes, mean tear lacritin concentration was  $17.5707 \pm 5.7022 \text{ ng lacritin}/50\text{ng total protein}$ . In KCS-affected eyes, mean tear lacritin concentration was  $11.7279 \pm 8.0962 \text{ ng lacritin}/50\text{ng total protein}$ . No significant difference was detected in KCS-affected eyes receiving lacrimostimulant therapy (mean  $11.5564 \pm 7.0893 \text{ ng lacritin}/50\text{ng total protein}$ ) as compared to KCS-affected eyes receiving no lacrimostimulant therapy (mean  $10.7512 \pm 8.7386 \text{ ng lacritin}/50\text{ng total protein}$ ), with a p-value of 0.6757). Tears from KCS eyes that responded to lacrimostimulant therapy ( $\text{STT} \geq 15\text{mm/min}$ ) did not have significantly different lacritin levels as compared to tears from KCS eyes that did not respond to lacrimostimulant therapy ( $\text{STT} < 15\text{mm/min}$ ) ( $p=0.2941$ ). A significant effect of batch analysis was detected ( $p<0.0001$ ). Mean lacritin concentration in batch 1 was  $9.2503 \pm 5.1016 \text{ ng lacritin}/50\text{ng total protein}$ , while batch 2 was  $20.4064 \pm 5.1723 \text{ ng lacritin}/50\text{ng total protein}$ .

protein. There was no significant effect of age ( $p=0.3760$ ) or sex ( $p=0.3556$ ) on tear lacritin concentrations when measured on a weight-by-weight basis.

On a weight-by-volume basis, tear lacritin concentrations did not differ significantly between disease groups ( $p=0.3904$ ). In normal eyes, median tear lacritin concentration was 2,022.64  $\mu\text{g/mL}$  (range 295.9 to 10,034.2  $\mu\text{g/mL}$ ). In KCS-affected eyes, median tear lacritin concentration was 2,295.54  $\mu\text{g/mL}$  (range 106.1 to 12,621.2  $\mu\text{g/mL}$ ). No significant difference was detected in KCS-affected eyes receiving lacrimostimulant therapy as compared to KCS-affected eyes receiving no lacrimostimulant therapy ( $p=0.9601$ ). KCS eyes that responded to lacrimostimulant therapy did not have significantly different lacritin concentrations than KCS eyes that did not respond to lacrimostimulant therapy ( $p=0.5934$ ). A significant effect of batch analysis was detected ( $p=0.0056$ ). Median lacritin concentration in batch 1 was 1,472.58  $\mu\text{g/mL}$  (range 106.06 to 12,533.59  $\mu\text{g/mL}$ ), while batch 2 was 2603.54  $\mu\text{g/mL}$  (range 832.06 to 12,621.22  $\mu\text{g/mL}$ ). A significant effect of age was detected ( $p=0.0009$ ), with an increase of 258.8  $\mu\text{g/mL}$  of lacritin for every 1-year increase in age. No significant effect of sex was detected ( $p=0.6042$ ). When comparing tear

lacritin to STT as a continuous variable, no significant correlation was detected.

#### *Dogs with unilateral keratoconjunctivitis sicca*

Six dogs were included in the study in which tears were collected from one eye that was affected by KCS as well as the fellow eye that had adequate tear production. The KCS-affected eyes had a significantly lower STT value (mean 10.9mm/min) than their counterparts (mean 20.6mm/min) with normal tear production ( $p=0.006$ ). Tears from these 6 KCS-affected eyes had a total protein concentration median of 17,842.99  $\mu\text{g}/\text{mL}$  (range 6,968.85 to 65,344  $\mu\text{g}/\text{mL}$ ), while their fellow normal eyes had a total protein concentration median of 9,735.03  $\mu\text{g}/\text{mL}$  (range 5,074.43 to 14,910  $\mu\text{g}/\text{mL}$ ); the difference between these values approached significance with a p-value of 0.1199. When measured on a weight-by-weight basis, lacritin concentration between the normal eyes (mean 20.0307ng/50ng tear protein  $\pm$  5.6613) and KCS eyes (mean 12.2499ng/50ng tear protein  $\pm$  9.4662) were not significantly different ( $p=0.0694$ ). Lacritin concentrations were not significantly different between these eyes on a weight-by-volume basis ( $p=0.8812$ ), as the tears from the 6 KCS-affected eyes had a lacritin concentration median of 3,249.64  $\mu\text{g}/\text{mL}$  (range 1,378.57 to 6,784.61

$\mu\text{g/mL}$ ), while their fellow normal eyes had a lacritin concentration median of  $3,324.97 \mu\text{g/mL}$  (range  $2,182.44$  to  $6,961.21 \mu\text{g/mL}$ ).

## D. Discussion

When measured as a proportion of total tear protein (weight-by-weight) in this study, a significant decrease in lacritin was noted in KCS-affected animals. This contrasts with measurements of lacritin as a proportion of tear volume (weight-by-volume), in which no significant difference was detected between KCS-affected eyes and normal eyes. Previous studies of human tears<sup>44,57-59,61,62</sup>, which measured lacritin on a weight-by-weight basis, identified decreased lacritin or lacritin precursor in the tears of dry eye-affected individuals. These studies did not evaluate lacritin on a weight-by-volume basis, so comparisons between weight-by-weight and weight-by-volume measurements could not be made. Only one of these studies<sup>57</sup> reported total tear protein, which was significantly decreased in dry eye-individuals as compared to normal individuals. In the present study, total tear protein was significantly increased in KCS-affected canine eyes. We feel this may be attributed to various alterations to normal ocular surface health in KCS, such as a decrease in the aqueous portion of the tear film and/or increase in other proteins within the tears due to a more

inflammatory environment on the ocular surface; regardless of the underlying mechanism, the difference between total tear protein in canine KCS and human KCS warrants further consideration.

Alterations in numerous tear proteins have been correlated with human dry eye disease<sup>73,74</sup>, in addition to other diseases such as infectious keratitis<sup>75</sup>, blepharitis<sup>61</sup>, keratoconus<sup>76</sup>, and diabetes mellitus.<sup>77</sup> No other specific tear proteins were analyzed in the present study, so no further conclusions could be made in this regard. Total protein of tear samples is not traditionally used as an indicator of ocular surface health, and to the author's knowledge there is not an established reference range for total protein of canine tear samples. Few studies report total tear protein concentrations. In humans, total tear protein concentration has been variably reported, ranging from 7.51mg/mL<sup>78</sup> to  $13.04 \pm 3.46\mu\text{g}/\text{mL}$ <sup>79</sup> in normal adults. In normal dogs, only a single report of tear protein concentration could be identified: 4.45-4.52mg/mL if collected via microcapillary tube, and 54.15-54.5mg/mL if collected via Schirmer tear strip.<sup>80</sup> Interestingly, the aforementioned canine study found a significant difference in tear protein concentration depending on collection method, but further protein analysis was not performed to determine the source for this increased protein level. Our study's findings

(median 5,548.94 µg/mL, range 2,200.12 to 31,992.0 µg/mL) more closely resembled that of the tears collected via microcapillary tube. This could be due to similarities in collection method, as the microcapillary tube (as used by Farias *et al*) and polyester wick (as used in our study) had minimal contact with the ocular surface as compared to a Schirmer tear strip, which has intentional contact with the ocular surface. Contact with the ocular surface could result in a sample that contains not just tear fluid, but also cells and debris from the ocular surface. Given that the type of KCS focused on in this study is due to a deficiency of aqueous tears, it is not surprising that KCS tear samples had higher protein concentrations. If the quantity of solute (protein) remains constant, a decrease in solvent (aqueous tears) would result in an increase in total concentration. Additionally, human patients with KCS have been demonstrated to have increases in numerous inflammatory cytokines in their tears.<sup>81-92</sup> Though this has not been well studied in dogs, an increase in inflammatory proteins may be contributing to an increase in tear protein levels in canine KCS as well.

Tear film osmolarity has been evaluated in the human and, less commonly, in the veterinary literature. In human medicine, tear film osmolarity has been proposed as perhaps the best indicator of dry eye

disease and even as a means of monitoring response to therapy.<sup>93-97</sup> Tear film osmolarity has not been thoroughly evaluated in veterinary species; however, a point-of-care instrument for measuring tear film osmolarity demonstrated poor repeatability and technical difficulties in canine patients with KCS.<sup>98</sup> This same study also demonstrated a counterintuitive relationship between STT and tear osmolarity in dogs with KCS, as lower osmolarity values were measured with decreasing STT (the opposite of which has been reported in humans). While further work is needed to improve methodology, tear film osmolarity and total protein concentrations may provide additional information regarding alterations in the content of the tear film in dry eye conditions in both physician and veterinary medicine.

Variability in total tear protein led us to examine methods of quantifying tear lacritin, with measurements on a weight-by-weight basis presenting lacritin as a proportion of the total protein of a given tear sample. This is a common and accepted method in the literature for quantifying many tear proteins, including lacritin.<sup>47,55,62,63</sup> Seifert *et al* reported that lacritin levels, as nanograms lacritin per 100ng total sample protein, were not significantly different between healthy men and women, or by age group. McNamara *et al* also reported lacritin as nanograms lacritin per

100ng total sample protein in a study of Sjögren's syndrome patients, identifying that tear lacritin levels are correlated to clinical signs of dry eye, as well as decreased nerve fiber length and density in the cornea.

Only one other study could be identified in the literature that presents tear protein on both a weight-by-weight and weight-by-volume basis.<sup>99</sup> In that study, numerous tear proteins were found to be significantly decreased in tears from dry eye subjects; however, Versura *et al* found agreement in these decreases in both weight-by-weight and weight-by-volume measures. It should be noted that the dry eye subjects evaluated by Versura *et al* did not exhibit significant aqueous tear deficiency, as determined by Schirmer tear measurement, which contrasts with our KCS study population. The present study data indicated that tear lacritin concentrations were significantly lower in KCS-affected eyes when evaluated on a weight-by-weight basis, while a weight-by-volume assessment indicated no significant difference between normal eyes and KCS-affected eyes. This lack of agreement poses the question of which method should be pursued in future studies, or if different information may be gleaned from each type of measurement, thereby making both methods of potential benefit. A weight-by-weight approach would provide information on lacritin (or any target tear

protein) in relation to all of the proteins present on the ocular surface.

Biologic materials do not always function independently; rather, they can have interactions with other biologic materials that can alter their function, thereby increasing or decreasing their effects. Some tear proteins may act synergistically to promote antimicrobial activity<sup>100</sup>, while others could interact with mucins to maintain stability of the tear film.<sup>101</sup> As such, knowledge of lacritin as it relates to tear protein content could point to similar interactions between lacritin and other proteins. Assessing numerous proteins in the tear film concurrently could provide additional information regarding potential interactions between many biologic substances within a tear sample.

A weight-by-volume approach, on the other hand, maintains a constant denominator across all samples. This reduces the influence of variations in total tear protein content between normal and KCS eyes, allowing for straightforward comparison between samples. This also may offer insight into clinical applications of lacritin, as topical supplementation of lacritin would be formulated as a solution with a weight-by-volume concentration (such as a  $\mu\text{g/mL}$  or  $\mu\text{M}$  measurement). Indeed, current *in vivo* reports describe topically applied lacritin in weight-by-volume

measures.<sup>44,45,53</sup> Knowledge of lacritin in respect to tear volume could provide a starting point for the concentration of supplementation. Until our knowledge of lacritin *in vivo* progresses, we suggest reporting tear protein levels in both weight-by-weight and weight-by-volume methods side-by-side. This will allow for lacritin (or other target tear proteins) to be assessed both as a proportion of all tear proteins and as a portion of the aqueous tears.

While KCS is typically a bilateral disease in dogs, it is not exclusively so. An intriguing sub-population within this study is a group of 6 dogs that had normal tear production in one eye and were diagnosed with KCS in the other eye; the data from these 6 dogs was analyzed as a subset of the overall population. The pattern of increased total tear protein concentration in KCS-affected eyes persisted, with the difference in these eyes approaching significance, despite the small sample size ( $p=0.1199$ ). The trends identified for all study samples persisted for these patients. When measured on a weight-by-weight basis, lacritin was decreased in the KCS-affected eyes (approaching significance at  $p=0.0694$ ); on a weight-by-volume basis, however, lacritin concentrations were not significantly different ( $p=0.8812$ ). This suggests that the ocular surface conditions in KCS that contribute to

alterations in tear lacritin levels can occur in one eye without affecting the fellow eye of the same animal.

Shortcomings of this study include adjustments made in the ELISA protocol between batch 1 and batch 2. The changes made were an adjustment to the sample incubation period and an adjustment to the ELISA standard curve. Internal laboratory testing (performing ELISAs on known concentrations of canine lacritin) demonstrated that shortening of the incubation periods from 1 hour (the standard in batch 1) to 35 minutes (the standard in batch 2) did not significantly alter the ability of the ELISA to accurately detect lacritin concentrations, so the incubation period for the second batch was shortened to 35 minutes. It is possible that the altered incubation period could result in altered absorbance values on the ELISA with our clinical samples, though the basic principles of ELISAs would suggest that shortening the incubation period would decrease absorbance values rather than increase them. Decreased absorbance values would then lead to decreased lacritin levels in batch 2, which was not the case.

Fifteen of 64 (23%) of samples analyzed in batch 1 had absorbance values above the upper end of the standard curve, which correlated to 16 ng

lacritin/50 ng of total tear protein concentration; 10 of these 15 samples were from normal eyes, while 5 samples were from eyes affected by KCS.

Because of this, the standard curve was extended to 32 ng lacritin/50 ng of total tear protein concentration for batch 2. Tear lacritin concentrations were found to be significantly higher in batch 2 than batch 1 ( $p<0.0001$  for weight-by-weight analysis,  $p=0.0056$  for weight-by-volume analysis), which is, at least in part, explained by the aforementioned change in the standard curve although additional factors may also have resulted in this finding. Due to the potential for inaccuracy in attempting to extrapolate beyond the standard curve, these 15 samples were excluded from lacritin analysis.

Because a substantial number of samples had absorbance values higher than the standard curve in batch 1, the standard curve utilized in batch 2 was extended to 32ng, resulting in all but 2 samples (1 from a normal eye and 1 from a KCS-affected eye) in batch 2 falling within the standard curve of known lacritin levels. When each batch is analyzed independently, slightly different results are obtained. If lacritin is assessed on a weight-by-weight basis, data from both batch 1 and batch 2 demonstrate a significant decrease in lacritin from KCS affected eyes ( $p=0.0002$  and 0.0076 for batch 1 and 2, respectively). When evaluating lacritin on a weight-by-volume basis, data from batch 1 demonstrates no significant difference between KCS and

normal eyes ( $p=0.6725$ ); batch 2 data, however, indicate that lacritin concentration is significantly higher in KCS-affected eyes (0.0107). While the extension of the standard curve led to differences in methodology between batches, we felt this was necessary to avoid loss of data due to samples reading beyond the top end of the lacritin standard curve, as occurred in batch 1. Extending the curve in the second batch analysis allowed us to capture as many samples as possible on this standard curve of lacritin, which we suspect was the most influential factor for the difference between batches. In the future, we suggest use of a standard curve that extends to at least 32ng to ensure accurate data capture for as many samples as possible.

The ELISA in this study also provided us with tear lacritin values on a weight-by-weight basis, as this has been the standard practice in the literature. As previously discussed, a weight-by-volume interpretation of the data was also of interest. In order to obtain data that presented lacritin in a weight-by-volume manner as well, we mathematically converted the tear lacritin values from a weight-by-weight value to weight-by-volume using the original sample volume collected from the canine eyes. This relies upon accurate measurements of those original volumes and subtraction of the

30µL eluent added to the sample prior to centrifugation from the polyester wick; this was controlled to the best of our abilities by measuring these small sample volumes (ranging from 30 to 50µL, including the addition of a 30µL eluent prior to centrifugation) with appropriately sized pipettes that are routinely calibrated for accuracy. This conversion to weight-by-volume measurements also assumes that the volume and protein content of the samples remained unchanged over the storage period. Samples used in this study were stored at -80°C for anywhere from several days to 9 months. Storage was necessary in order to maximize our sample numbers and still perform batch analysis, though storage may alter the volume and protein content of samples. One study evaluated the effect of a four-year long storage period on serum sample volumes of 500µL and concluded that storage at -70°C does not have a significant effect on sample volumes.<sup>102</sup> Another study evaluating storage of tears in various conditions demonstrated that storage at -70°C was better than storage at -20°C for obtaining accurate protein measurements; however, even at -70°C, significant decreases in protein levels could be detected if stored for 6 months or longer.<sup>103</sup> Though sample analysis within 4 months was not always possible in this study, it would be recommended to do so whenever possible within the constraints of future studies.

## CHAPTER 3: CONCLUSIONS AND FURTHER RESEARCH

Total tear protein levels were demonstrated to be increased in KCS-affected eyes. While certain inflammatory proteins are known to be increased in the tears of humans with KCS, this has not been thoroughly investigated in dogs. Additional assessment of specific proteins in the tears of KCS-affected dogs could reveal similar increases to those of humans, which may account for the increase in total tear protein documented in this study. Tear film osmolarity is also more commonly utilized in diagnosis and management of dry eye diseases in humans; refining techniques for measuring tear film osmolarity dogs could allow for use of this technique in veterinary medicine as well.

This study provided a thorough analysis of tear lacritin content of canine tears in both normal and KCS-affected dogs. It also provided two methods of examining the data, so that lacritin content may be evaluated as a component of total tear protein and aqueous tear volume. As one of these methods concluded that lacritin was decreased in the tears of KCS-affected eyes and one concluded that lacritin was not significantly different in KCS-affected eyes, lacritin's role as a potential lacrimostimulant therapy for

canine KCS is unclear from this study alone. We feel both methods of evaluation are valuable as we learn more about lacritin's functions and potential for application in medicine in the years to come. Regardless of lacritin's function as a stimulant of tear secretion in dogs, investigation of lacritin supplementation as a therapeutic is still warranted. Because dogs with KCS are inherently at risk of bacterial overgrowth and corneal ulceration, other potential biologic properties of lacritin (bactericidal effects, cytoprotection for corneal epithelial cells, promotion of corneal epithelial wound healing)<sup>49-52</sup> could still be of value in dogs with KCS. Investigation into the effects of lacritin supplementation on these consequences of canine KCS is warranted, especially if success is found in studies of humans with dry eye disease.

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## APPENDIX: FIGURES

Figure 1. Median and range of total tear protein ( $\mu\text{g/mL}$ ) for normal eyes and KCS-affected eyes.

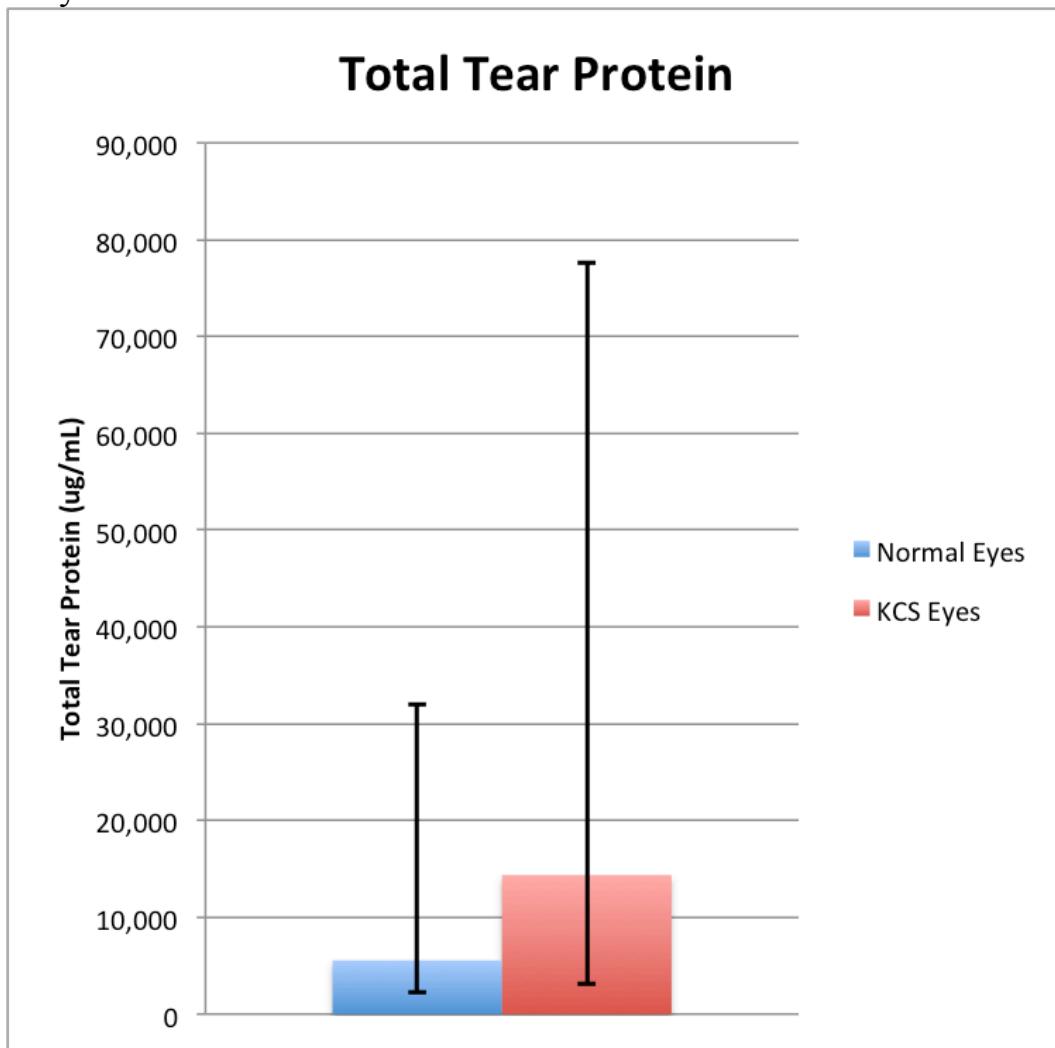


Figure 2. Total tear protein ( $\mu\text{g/mL}$ ) for normal eyes and KCS-affected eyes as compared to Schirmer Tear Test values.

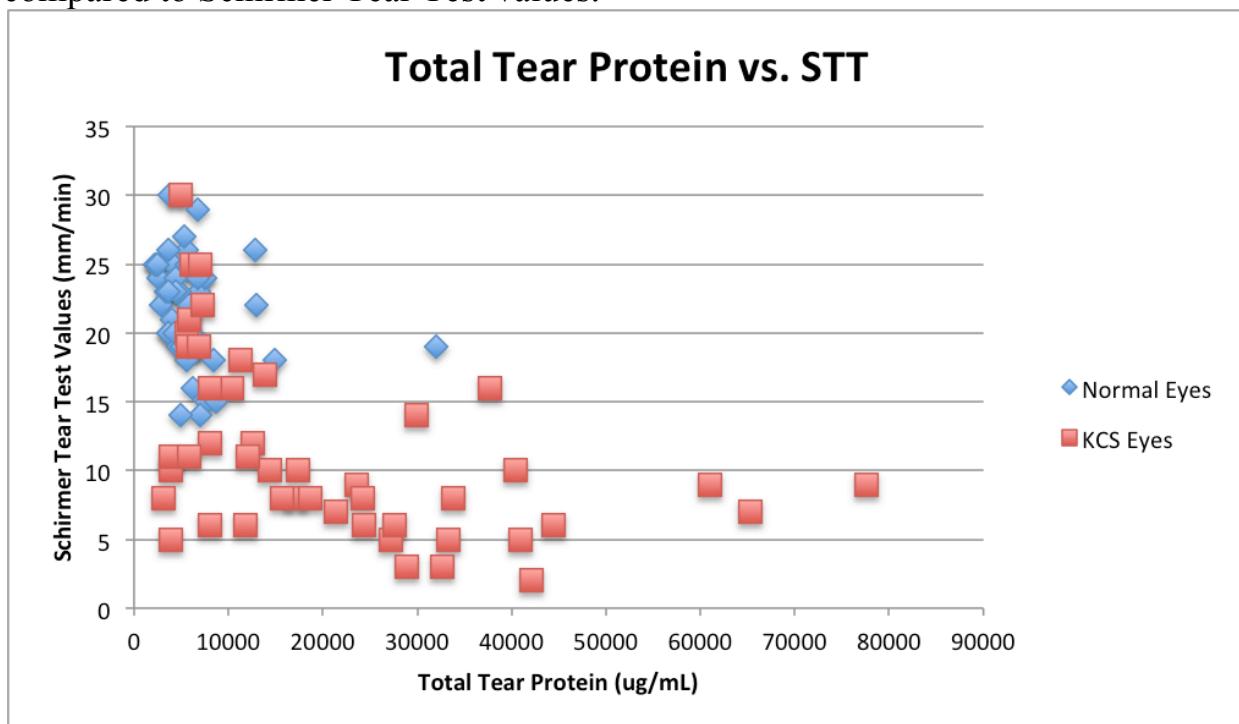


Figure 3. Mean  $\pm$  standard deviation tear lacritin content (ng lacritin/50ng total tear protein) for normal eyes and KCS-affected eyes.

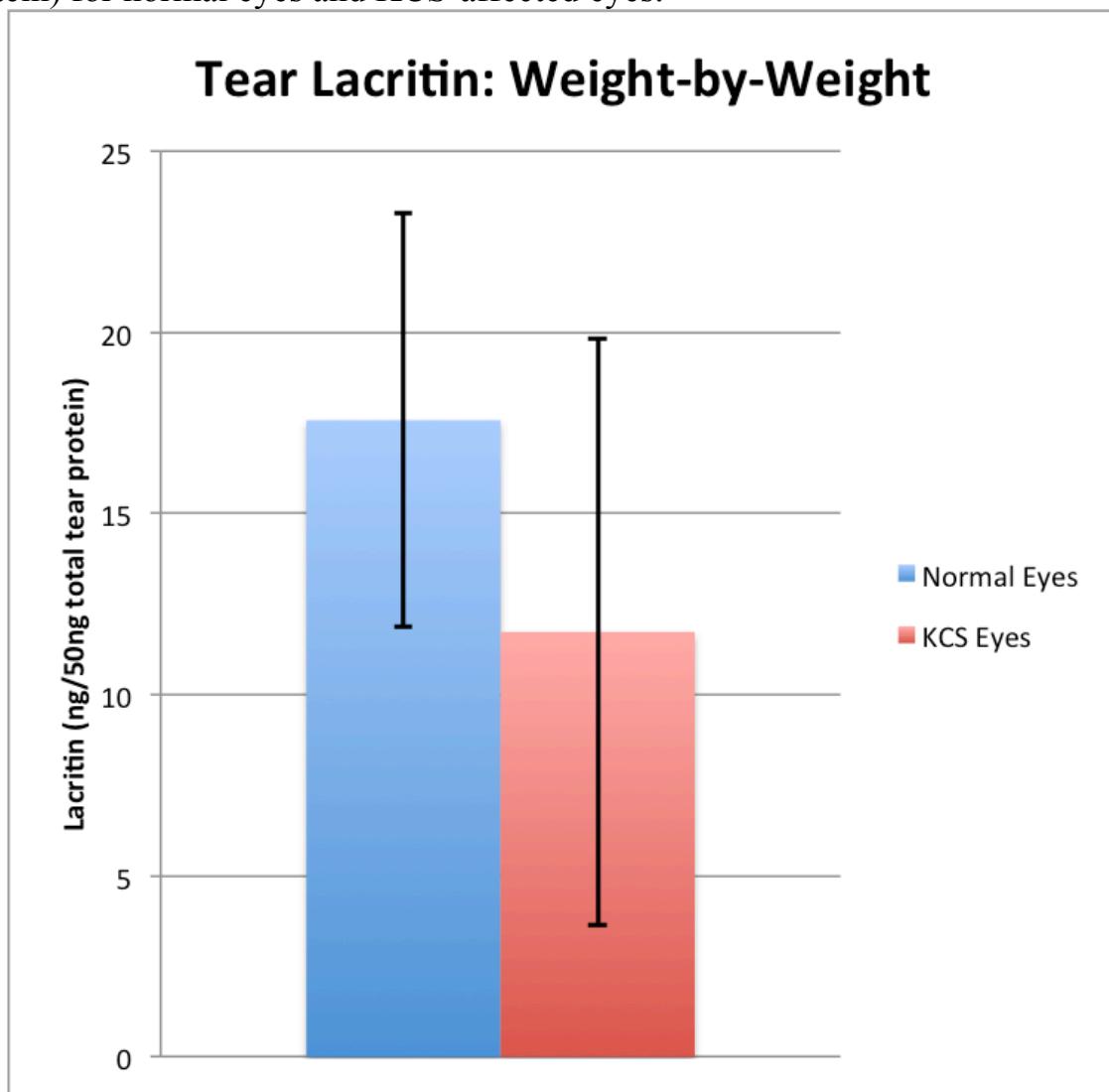


Figure 4. Tear Lacritin Content (ng lacritin per 50ng total tear protein) for normal eyes and KCS-affected eyes as compared to Schirmer Tear Test values.

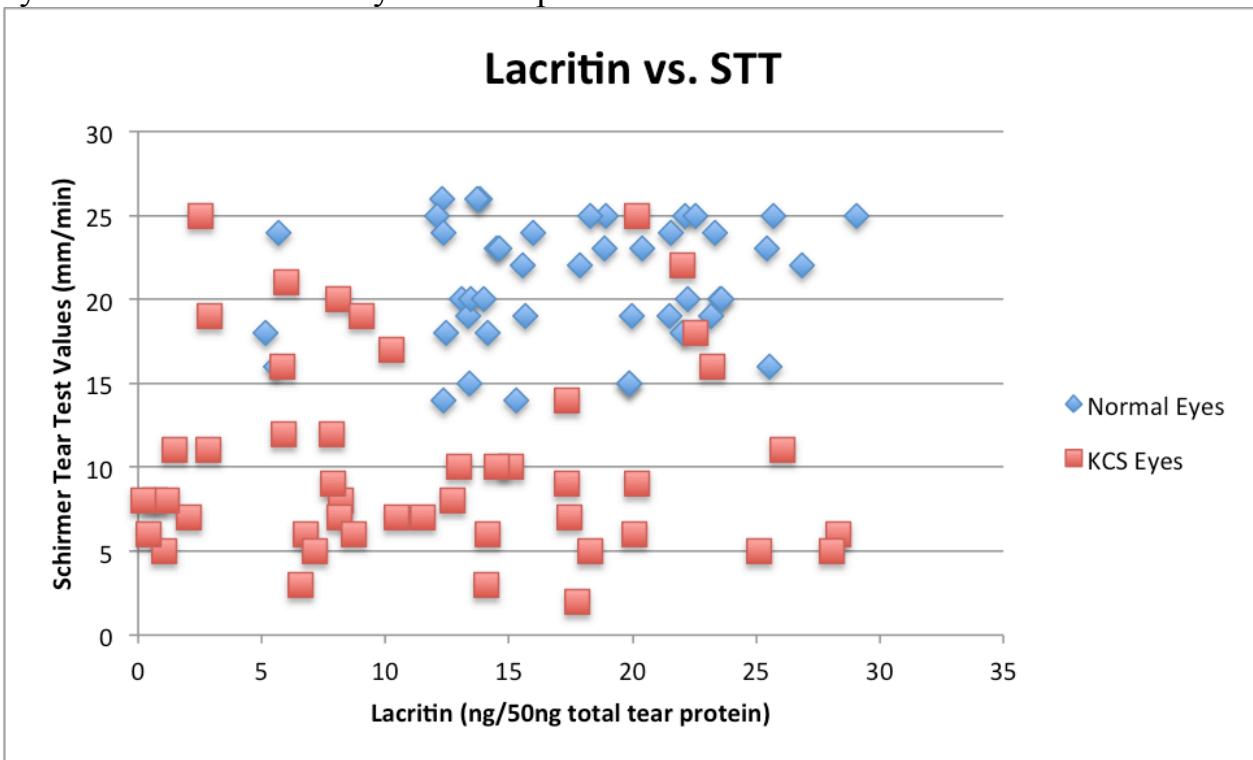


Figure 5. Median and range of tear lacritin concentration ( $\mu\text{g/mL}$ ) for normal eyes and KCS-affected eyes.

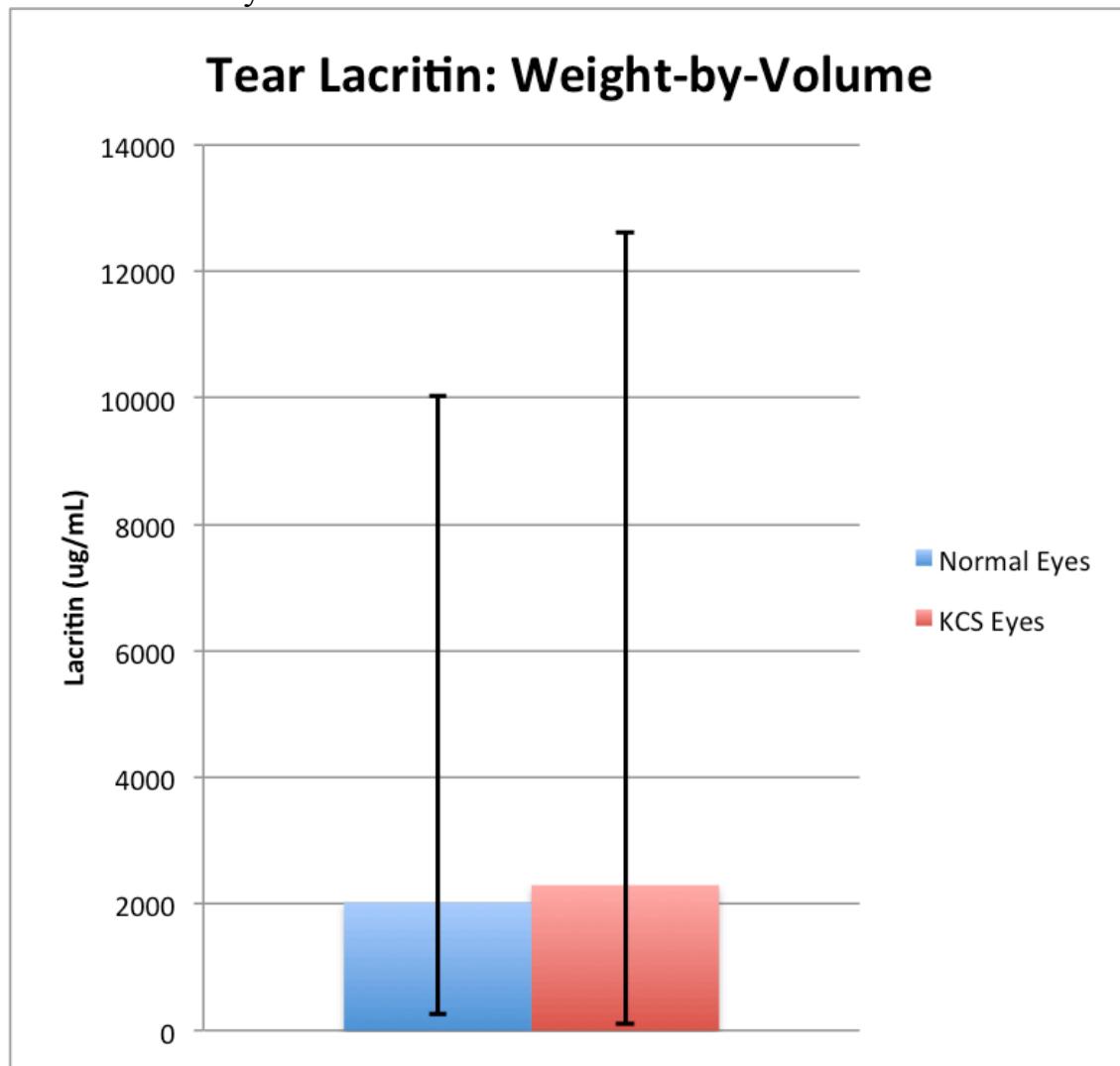


Figure 6. Tear lacritin concentration ( $\mu\text{g/mL}$ ) for normal eyes and KCS-affected eyes as compared to Schirmer Tear Test Values.

