

# Tear Lacritin Levels by Age, Sex, and Time of Day in Healthy Adults

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**PURPOSE.** Several small proteomic studies suggest that the prosecretory tear protein lacritin may be selectively downregulated in dry eye syndrome and in blepharitis, yet little information is available about normal baseline levels. This study assessed lacritin levels in tears from healthy individuals and addressed whether they differ according to sex, age, or time of day.

**METHODS.** Rabbit antibodies against lacritin N-terminal peptide EDASSDSTGADPAQEAGTS (Pep Lac N-Term) were generated and characterized against human recombinant lacritin and N-65 truncation mutant. Basal tears were collected from 66 healthy individuals ranging in age from 18 to 52 years, and at four times during one 24-hour period from 34 other individuals. Lacritin levels were then analyzed by ELISA and Western blotting.

**RESULTS.** Anti-Pep Lac N-Term bound lacritin, but not truncation mutant N-65 that lacks the N-terminal antigenic site. Tear lacritin levels followed a normal distribution with a mean of  $4.2 \pm 1.17$  ng/100 ng total tear protein. Levels differed little by age or sex, and decreased slightly between 4 and 8 hours in a 24-hour cycle. Tear-blocking effects were minimal, as suggested by spiking of tears with recombinant lacritin.

**CONCLUSIONS.** Anti-Pep Lac N-Term-detectable lacritin comprises  $\sim 4.2$  ng/100 ng total tear protein in healthy individuals, with no significant differences between males and females or among individuals between 18 and 52 years old. Levels decrease slightly in the late afternoon. These findings provide a baseline for future immunodiagnostic studies of lacritin in dry eye and other ocular diseases. (*Invest Ophthalmol Vis Sci* 2012;53:6610-6616) DOI:10.1167/iovs.11-8729

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Lacritin is a 12.3 kDa human tear glycoprotein that is prosecretory, mitogenic,<sup>1</sup> and antimicrobial (McKown RL, et al. *IOVS* 2010;51:ARVO E-Abstract 4181) and that promotes sustained basal tearing in rabbits.<sup>2</sup> Lacritin acutely augments constitutive but not stimulated lacrimal acinar cell secretion, even when prior treatment with interferon- $\gamma$  and TNF has neutralized the response to carbachol (Fujii, et al. *IOVS* 2011;52:ARVO E-Abstract 3714).

Lacritin's ability to stimulate tear production makes it an interesting protein to study for its potential involvement in dry eye syndrome and other eye-related diseases. Dry eye affects the lives of over 25 million Americans, yet it is poorly understood and lacks sensitive early-stage diagnostics. Current tests are more appropriate for later disease stages, making difficult the diagnosis of patients with mild to moderate symptoms.<sup>3</sup> Moreover, tests such as Schirmer strips, ocular surface staining, and tear film breakup time are still not uniformly applied<sup>4</sup> (although standardization has improved with publication of the International Dry Eye Workshop report<sup>5</sup>), and new devices to assess tear osmolarity show promise,<sup>6</sup> although not in isolation.<sup>7</sup> Development of an assay to help diagnose both early-onset and later dry eye, recognizing that there may be different etiologies, would be of great benefit.

Dry eye syndrome and other associated conditions are believed to correlate with changes in specific protein content of the ocular surface.<sup>8</sup> Some small proteomic studies suggest that lacritin is one of only 4% to 5% of the tear proteome that is downregulated in dry eye or dry eye-related conditions.<sup>9</sup> Lacritin levels measured by mass spectroscopy analysis of tear samples were 7-fold less from 11 individuals with contact lens-related dry eye than from 10 users of contact lenses with normal eye conditions.<sup>10</sup> Sensitivity, inability to provide relative tear concentrations, and lack of information on normal baseline levels or whether lacritin levels are subject to time-of-day variation are all limitations of these studies. Blepharitis is characterized by inflammation of the eyelid and dry eye conditions. A study using electrospray-quadrupole-time-of-flight mass spectrometry (ESI-Q-TOF) identified several changes in tear proteins.<sup>11</sup> Lacritin was one of nine proteins downregulated by  $\sim 50\%$ .<sup>11</sup>

Quantitation of major tear proteins has been studied by gel electrophoresis (lactoferrin, tear-specific prealbumin, and lysozyme)<sup>12-14</sup> and by size-exclusion chromatography combined with enzymatic assays and immunologic methods (lysozyme, IgA, IgG, albumin, and lactoferrin).<sup>15</sup> More recent studies have employed a variety of mass spectrometry-based methods.<sup>16-21</sup> Antibody-based methods have also been used to quantify tear proteins, including sandwich ELISA<sup>22</sup> or sandwich ELISA following size-exclusion high-performance liquid chromatography to assess levels of several major tear proteins.<sup>23,24</sup> Membrane arrays have comprehensively docu-

mented tear cytokines,<sup>25,26</sup> and a new microfluidic chip approach shows promise.<sup>27</sup>

To date, no assay has been developed to quantitate tear lacritin levels. In this study we established an indirect lacritin ELISA that is based on the new N-terminal-specific anti-lacritin antibody (anti-Pep Lac N-Term) and screened normal basal tear samples from 66 healthy individuals of different ages and both sexes. Tears were also collected four times during a 24-hour period from 34 others to assess time-of-day variation. This analysis of lacritin in normal healthy individuals sets the stage for future analysis of lacritin in dry eye.

## METHODS

### Lacritin Peptide, Anti-N-terminal Anti-lacritin Antisera, and Recombinant Lacritins

Peptide EDASSDSTGADPAQEAGTS (Pep Lac N-Term), corresponding to the N-terminus of mature human lacritin (amino acids 1–19 without signal peptide), was synthesized (>85% purity) and conjugated to keyhole limpet hemocyanin (KLH) by Bio-Synthesis, Inc. (Lewisville, TX). New Zealand white rabbits were immunized in three boosts with Pep Lac N-Term-KLH. Final antiserum (anti-Pep Lac N-Term) was collected on day 70. Preimmune serum was collected before immunization (Bio-Synthesis, Inc.).

Recombinant human lacritin was generated and purified from the lacritin-intein fusion plasmid pLAC.<sup>28</sup> Lacritin lacking 65 amino acids from the N-terminus (N-65) (McKown RL, et al. *IOVS* 2010;51:ARVO E-Abstract 4181) was generated out of pLAC using the forward primer 5'-GGGAATCCATATGAAATCCATAGTGGAGAAAAGT-3' and reverse primer 5'-GGGAATCCATATGTATATCTCCTTCTTAAAG3-3'. Recombinant proteins were expressed in *E. coli*, affinity purified on chitin beads (New England Biolabs, Inc., Ipswich, MA), eluted without intein tag,<sup>28</sup> and then further purified on DEAE-Sepharose (GE Healthcare, Little Chalfont, UK), as previously described.<sup>2</sup> Purified proteins were freeze-dried and stored at –80°C until use.

### Enzyme-Linked Immunosorbent Assay

To assess anti-Pep Lac N-Term specificity, plates were coated overnight with 100 µL lacritin or N-65 diluted 0, 50, or 100 ng/mL in coating buffer (0.017 M NaHCO<sub>3</sub>, 0.015 M Na<sub>2</sub>CO<sub>3</sub>, pH 9.6).<sup>29</sup> For assay of tear samples, 100 ng total tear protein was coated in each well. To generate a standard curve of recombinant lacritin, each plate contained triplicate wells to which was added 0, 0.5, 1, 2, 4, 6, or 8 ng protein. Wells were washed, blocked with PBS-Tween (PBS with 0.3% Tween-20 [PBS-T]), and then incubated for 1 hour at 37°C with 100 µL anti-Pep Lac N-Term antiserum or preimmune serum diluted 1:200 in PBS-T. After washing three times with PBS-T, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (MP Biomedicals, Solon, OH) diluted 1:1,000 in PBS-T was added for 1 hour (37°C). Plates were washed three times with PBS-T, and then bound antibody was measured after incubation for 10 minutes with 100 µL OPD substrate (Acros Organics, Geel, Belgium) by absorbance at 415 nm (model 680; Bio-Rad, Hercules, CA). The same ELISA protocol was used for determining human tear lysozyme concentrations with lysozyme from human milk (Sigma-Aldrich, St. Louis, MO) for the standard curve and rabbit anti-human lysozyme polyclonal antibodies (MP Biomedicals) diluted 1:200 in PBS-T for detection.

### Western Blot

Recombinant lacritin, N-65, or tear samples were loaded on 4% to 20% Mini-PROTEAN TGX precast gels (Bio-Rad), electrophoresed at 200 V, and transferred to nitrocellulose (Protran BA 83; Whatman, Dassel, Germany). Blots were blocked with PBS-T, incubated with anti-Lac Pep N-Term (1:500 dilution in PBS-T) for 2 hours at room temperature,

rinsed with PBS-T, and incubated for 2 hours at room temperature with HRP-conjugated goat anti-rabbit IgG (MP Biomedicals) diluted 1:10,000 in PBS-T. Blots were rinsed with PBS-T and developed via chemiluminescence with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Rockford, IL).

### Tear Collection

Tear collection followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at Walter Reed Army Medical Center, where tears were collected, and at James Madison University, where the tears were analyzed. Sixty-six subjects aged 18 to 52 years voluntarily enrolled after explanation of the nature and potential risks of the study. Subjects wearing contact lenses were instructed to remove their lenses and wait at least 5 minutes before proceeding with tear collection. After one drop of 0.5% proparacaine was instilled on the ocular surface (Lossen VI, et al. *IOVS* 2010;51:ARVO E-Abstract 6279), excess fluid around the eye was blotted dry, and patients were instructed to sit with eyes gently closed for 2 minutes. Tears were collected from the lower cul-de-sac of the left eye into a 2 × 10 mm polyester fiber rod (Transorb Wick; Filtrona, Richmond, VA), as previously described.<sup>30–32</sup> Collection was for a time sufficient to obtain fluid without irritation (3–5 minutes per consent form; actual time 1–2 minutes). Each wick with tears was placed into a modified micropipette tip in a 1.5 mL Eppendorf tube for –70°C storage. Tears were subsequently eluted from wicks with 30 µL PBS per wick for 20 minutes, followed by centrifugation for 10 minutes at 16.2g. Eluted tear samples were stored at –70°C. Protein concentration was assessed by the BCA assay (Thermo Scientific BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL) using bovine serum albumin as a protein standard.

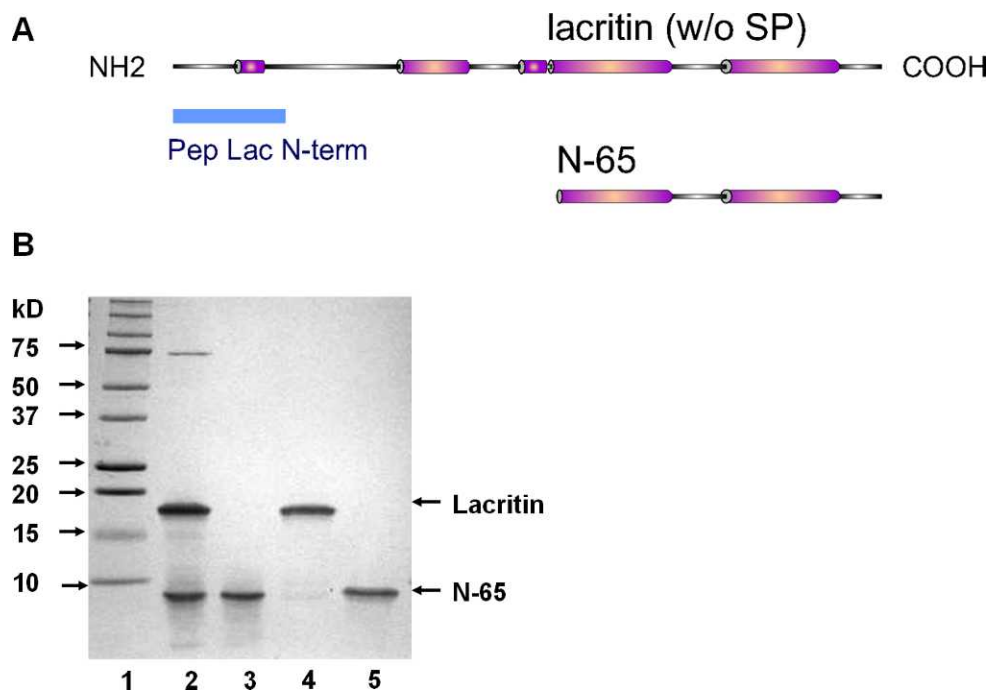
### Statistical Analysis

All tear samples were assayed in triplicate on each of two or three separate plates. Statistical analytical details have been published previously (see Supplemental Methods, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8729/-/DCSupplemental>).

## RESULTS

### Purification of Recombinant Lacritins and Characterization of Anti-Pep Lac N-Term

Tear lacritin is a 119 amino acid glycoprotein with several alpha helices, including a C-terminal amphipathic alpha helix.<sup>28</sup> Truncation mutant N-65 lacks 65 amino acids from the N-terminus (Fig. 1). Recombinant lacritin and N-65 were generated as intein fusion proteins that make possible purification on chitin beads and on column release of each from the intein tag. Release yields two predominant Coomassie blue-stainable bands of 18 and 6 kDa, plus a minor band of 75 kDa (Fig. 1B, lane 2). Further purification on DEAE-Sepharose produced a single protein band of 18 kDa (Fig. 1B, lane 4) that has been confirmed as lacritin by mass spectroscopy analysis (data not shown). Intact recombinant lacritin was the antigen for the first anti-lacritin polyclonal antibody that was suitable for immunolocalization and ELISA, but not for Western blotting.<sup>1</sup> Subsequent discovery of alternative splice variants affecting the C-terminus<sup>9,33</sup> presented a need for domain-specific antibodies capable of monitoring lacritin levels and lacritin integrity in normal and dry eye tears. Rabbits were immunized with synthetic peptide (Pep Lac N-Term-KLH) corresponding to the first 19 amino acids of tear lacritin, a region lacking from N-65 (Fig. 1A). Increasing amounts of lacritin or N-65 were adsorbed to ELISA plates or separated by SDS-PAGE and transferred to nitrocellulose. Anti-Pep Lac N-Term detected lacritin, but not N-



**FIGURE 1.** Purification of recombinant lacritin and deletion variant N-65. (A) Diagram of lacritin without signal peptide shown with boxed regions of predicted alpha-helices, and N-65 truncation mutant lacking 65 amino acids from the N-terminus. Also shown is the location of the Pep Lac N-term antigen. (B) SDS-PAGE with Coomassie stain: *lane 1*, molecular weight standards; *lane 2*, eluant from chitin affinity chromatography of lacritin; *lane 3*, DEAE-Sepharose 140 mM NaCl flow-through lacritin; *lane 4*, DEAE-Sepharose 140 mM NaCl step elution of lacritin; *lane 5*, DEAE-Sepharose-purified N-65.

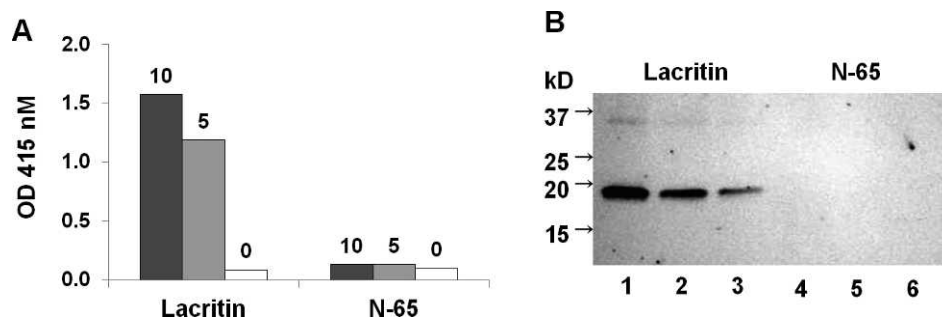
65, at coating levels of 5 and 10 ng (Fig. 2A), and in blots of 100, 200, or 400 ng lacritin but not of N-65 (Fig. 2B). No lacritin or N-65 titer was detected in the preimmune serum (data not shown). Thus anti-Pep Lac N-Term displays N-terminal domain specificity and appears to be highly sensitive.

### Lacritin Tear Analysis

ELISA plate-to-plate variability is controllable by the inclusion of internal standards. Accordingly, each tear-coated plate included wells of 0.5 to 8 ng added lacritin from which a standard curve could be generated (Fig. 3A).  $R^2$  values were required to exceed 0.97<sup>34</sup> and calculated lacritin tear values to have at least two standard curve data points on either side of the calculated value. Plates and samples that did not meet the required criteria were removed from the population. These criteria reduced the number of tear samples reported from 66 to 58. The capacity of tear lacritin to compete with other tear proteins for adsorption to microwell plastic<sup>25</sup> was a concern. We chose a coating level per well of only 100 ng total protein

of tears, a level in keeping with 70% to 80% coating efficiency<sup>35</sup> according to a binding capacity of ~154 ng/well. This coating level was also appropriate for the small volume of collectable tears per individual. Nonetheless, it was possible that lacritin's nonpolar side chains might not be available for hydrophobic adsorption to microwell plastic. To address this issue, increasing amounts of recombinant lacritin were spiked into recombinant lacritin or into human tears (Fig. 3B). Detection of lacritin by ELISA increased linearly when recombinant lacritin was added to either recombinant lacritin or tears with a fixed concentration of lacritin, suggesting that tear lacritin is likely adsorbed to microwell plastic with minimal interference from other tear constituents.

We next compared lacritin levels in normal human tears using in-plate standard curves to express values as nanograms of lacritin per 100 ng total tear protein or as percent lacritin. A minimum of six determinations for each individual (three wells per plate from two plates) were used for statistical analysis. Tear lacritin values for the population of normal and relatively young individuals in this study followed a normal distribution



**FIGURE 2.** Specificity of anti-Pep Lac N-Term antibodies for full-length lacritin, and lack of reactivity with N-65. (A) ELISA of anti-Pep Lac N-Term antibodies against 0, 5, and 10 ng of lacritin or N-65. (B) Western blot of decreasing amounts of purified lacritin and N-65 incubated with anti-Pep Lac N-Term antibodies and developed via chemiluminescence: *lanes 1 and 4*, 400 ng; *lanes 2 and 5*, 200 ng; *lanes 3 and 6*, 100 ng.

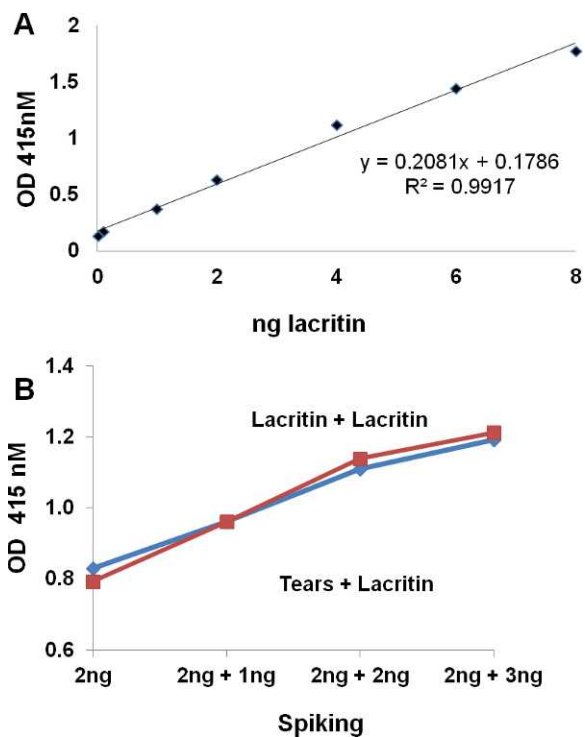


FIGURE 3. ELISA standard curve and spiking experiments. (A) Example of a lacritin standard curve replicated in each ELISA plate. (B) Two spiking experiments. In one of these, increasing amounts of lacritin were added to a human tear sample with an observed lacritin concentration of 2 ng. In the second experiment, increasing amounts of lacritin were added to 2 ng lacritin. In both experiments, mixtures were analyzed by ELISA.

with a mean of  $4.2 \pm 1.17$  ng/100 ng total tear protein (Fig. 4). Little apparent difference between the sexes was detected (Table). Note that the standard deviation values reported here are not estimates of variation in the corresponding population, as they include variations attributed to the number of replicates and plates used on each patient's sample. Moreover, the number of males and females tested (Table) was not the

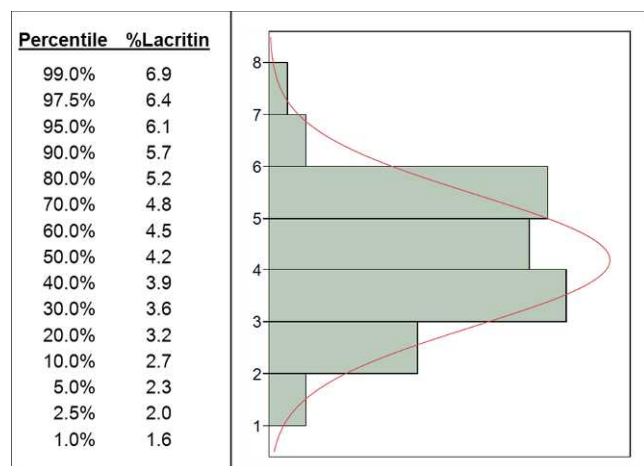


FIGURE 4. Normal distribution fit and normal distribution-based percentiles for percent lacritin (ng lacritin/100 ng total tear protein) in tears of a healthy population. Percentile values are given to indicate ranges of patient-wise lacritin values that might be considered unusual with respect to the population represented in this study.

TABLE. Lacritin in Normal Human Tears

	Male	Female	Combined
Number	43	15	58
Average age, y	32	33	33
Average lacritin*	4.11	4.47	4.20
Standard deviation	1.31	1.13	1.26
Maximum	7.52	6.85	7.52
Minimum	1.33	2.99	1.33

\* Average lacritin = ng lacritin/100 ng total tear protein.

same, and their ages were not evenly distributed from the youngest (18 years) to the oldest (52 years). In order to better visualize the distribution of percent lacritin as a function of age and sex, a scatter plot of all 58 individuals was generated (Fig. 5). This analysis confirms a lack of apparent difference by age or sex within this population.

Detection of Tear Lacritin by Western Blot

Lacritin pre-mRNA is subject to alternative splicing.<sup>33</sup> Once synthesized, 12 O- and 1 N-glycosylation sites are predicted.<sup>9</sup> In Coomassie blue staining of recombinant lacritin (Fig. 1B), the appearance of a ~6 kDa lacritin proteolytic fragment was noted. In Western blots of seven equally loaded tear samples, anti-Pep Lac N-Term detected a prominent 20 to 25 kDa band of variable width and intensity (Fig. 6). The breadth of the 20 to 25 kDa band is in keeping with O- and N-glycosylation<sup>1,9</sup> of 4 to 7 kDa, as calculated versus the 18 kDa mobility of unglycosylated bacterial recombinant lacritin.

Comparison to Tear Lysozyme

Lysozyme is a prominent tear component that serves as a useful comparative benchmark. Using the same ELISA approach and purchased human lysozyme and anti-human lysozyme antibodies, we assessed tear lysozyme levels in selected samples. Tear lysozyme ranged from 18 to 23 ng lysozyme/100 ng total tear protein with an average of ~20 ng lysozyme/100 ng total tear protein. Therefore the concentration of human tear lacritin can be estimated to be approximately one-fifth the concentration of human tear lysozyme by this analysis. Figure 7 shows an example of this analysis with two standard curves from which the concentration of lacritin was calculated at 3.6 ng lacritin/100 ng total tear protein and that of lysozyme at 22 ng lysozyme/100 ng total tear protein for the tear sample shown.

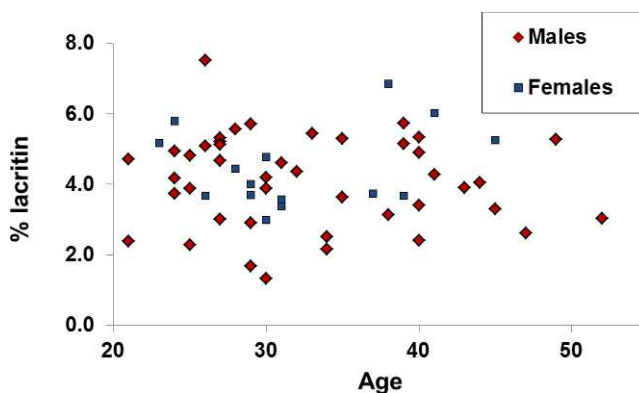
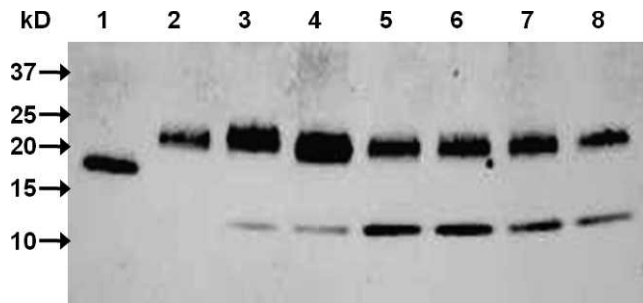


FIGURE 5. Distribution of percent lacritin (ng lacritin/100 ng total tear protein) for males and females by age. Percent lacritin was determined for 58 individuals (43 males and 15 females) and plotted as a function of age (18–52 years).



**FIGURE 6.** Western blot analysis of selected tear samples and recombinant lacritin. Here 200 ng of purified recombinant lacritin (lane 1) and 2  $\mu$ g total protein of tear samples from seven individuals (lanes 2-7) were analyzed by Western blot. Blots were incubated with anti-Pep Lac N-Term antibodies and developed by chemiluminescence. Recombinant lacritin made in *E. coli* (lane 1) is not glycosylated, while human tear lacritin is glycosylated (lanes 2-7).

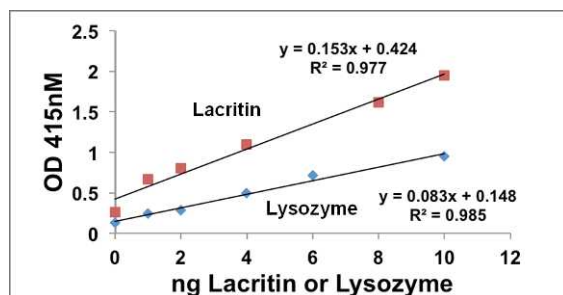
### Time-of-Day Study

Some tear proteins vary in amount over the daily cycle.<sup>14,36</sup> To determine if the same is true of lacritin, samples were collected from 34 additional individuals at time 0 hours (7:30-8:30 AM), 4 h (11:30 AM to 12:30 PM), 8 h (4:30-5:30 PM), and 24 hours (7:30-8:30 AM the following day) and assayed for lacritin (Fig. 8). Nested, mixed ANOVA points to significant differences across time ( $P = 0.0341$ ) that by Tukey's honestly significant difference (HSD) test may be represented by a slight decrease between 4 and 8 hours.

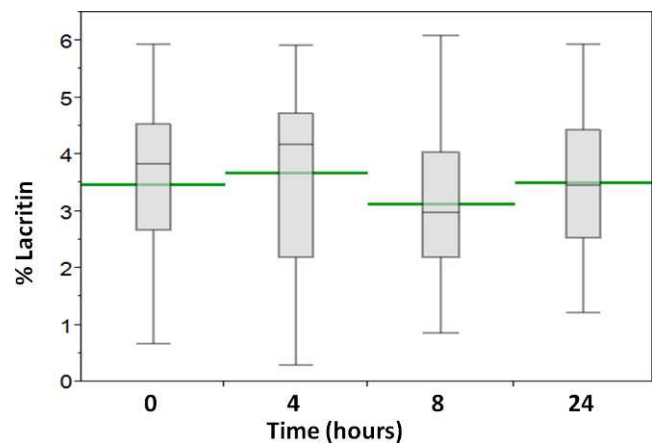
### DISCUSSION

We report that anti-Pep Lac N-Term antibody specifically binds prosecretory mitogen lacritin in basal human tears with minimal apparent blocking effect and that detected levels vary little by age, sex, or time of day. In most normal individuals, lacritin appears to represent 4.2 ng/100 ng total tear proteins by indirect ELISA, suggesting that lacritin may be at micromolar levels in tears. The range for percentage total protein for lysozyme from this analysis is consistent with previously published concentrations of tear lysozyme.<sup>37</sup>

Although levels varied little by age, samples were acquired from a relatively young group of normal individuals. Basal tear peroxidase decreases during menopause in women and around age 80 in men.<sup>38</sup> Similarly, PLA2G2A is significantly decreased in basal tears of 60- to 70-year-old women and 70-year-old



**FIGURE 7.** Example of standard curves for recombinant lacritin and human lysozyme used to determine protein concentrations in tear samples. Increasing concentrations of lacritin and human lysozyme were analyzed by ELISA with anti-Pep Lac N-Term and anti-human lysozyme, respectively. The concentration of lacritin was calculated at 3.6% of total protein and that of lysozyme at 22% of total protein for the same human tear sample.



**FIGURE 8.** Box plots for the percent lacritin averages over 24 hours. Each "box" represents the range covered by the middle 50% of individuals. The horizontal lines within each box represent the median value in each group, and the long horizontal lines that cross each box represent the mean value for each group.

men.<sup>39</sup> An earlier study of reflex tears from 20- to 82-year-olds, not distinguished by sex, reported a negative correlation of lysozyme and lactoferrin by age, whereas IgG and ceruloplasmin increased with age.<sup>22</sup> Thus tears from older women and men would be helpful for more thoroughly investigating the effect of age on lacritin levels.

The issue of tear protein in males versus females was recently queried in a proteomic study of reflex tears from 20 normal male and female individuals pooled by sex.<sup>16</sup> Lacritin, lipocalin, haptoglobin, mammoglobin B precursor, cystatin S precursor, and anti-alpha 1 trypsinogen were all reported to be greater in female tears. Additional studies with larger numbers of females, in particular tears from older women, would be helpful for more thorough investigation of the effect of age on tear lacritin levels.

The time-of-day variation of major tear proteins has been investigated.<sup>14,36</sup> We also looked at diurnal variation of lacritin over a 24-hour period. In that study, lacritin levels dropped slightly in late afternoon, but the change was minimal, so sampling time from patients would not be crucial for comparison with other tear samples or for lacritin determination. Some studies have demonstrated changes in total protein and lacrimal secretion when samples were taken from closed versus open eyes.<sup>26,40</sup> All of the samples in the current study were collected from open eyes. Additional studies could also investigate the amount of lacritin in closed eye samples.

Quantitative detection of a single protein in a complex fluid is optimally performed by sandwich ELISA or membrane array, in which an optimized coat of immobilized antibody captures a target protein for detection by a second antibody directed to a different epitope. An assay with known amounts of purified target protein then establishes a standard curve for extrapolation of target protein in complex fluids. Such an approach has been successfully employed to detect picogram/milliliter levels of angiogenin (ANG), epidermal growth factor (EGF), chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C-X-C motif) ligand 5 (CXCL5), chemokine (C-X-C motif) ligand 10 (CXCL10), interleukin 8 (IL-8), chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 4 (CCL4), insulin-like growth factor binding protein 2 (IGFBP2), TIMP metalloproteinase inhibitor 1 (TIMP-1), and TIMP metalloproteinase inhibitor 2 (TIMP-2) in normal reflex tears.<sup>26</sup> Although anti-Pep Lac N-Term was originally envisioned as the capturing antibody in a sandwich ELISA, generating a detector antibody has been

challenging through four separate attempts in rabbits and mice; however, the linearity of detection when increasing amounts of recombinant lacritin were spiked into whole tears implied that tear blocking was minimal and that the assay was sensitive. Moreover, the subsaturating quantity of tears added was appropriate for 70% to 80% adsorption.<sup>35</sup>

Subsequent studies will analyze lacritin levels in tears of dry eye, Sjögren's syndrome, and pre- and post-photorefractive keratectomy (PRK) surgery individuals as a potential new diagnostic for dry eye.

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