**Supplemental Methods**

**Statistical Analysis**

All replicate readings from a given plate were retained for the analysis if (1) the lacritin standard curve from the plate had an $R^2$ value greater than 0.97[1] and (2) all three replicate values for a subject on a given plate points resided within at least two points of the standard curve. Following McCulloch, Searle, and Neuhaus[2] the statistical analysis represents each subject's individual % lacritin reading from the ELISA as a mean value for the population from which the subject was drawn, plus a linear combination of random effects due to the unique characteristics of the subject, variations attributable to different ELISA plates, wells within the plate, and other uncontrollable factors. The model for the % lacritin reading of a randomly selected subject is:

$$\% \text{ lacritin} = \mu_{\text{population}} + \delta_{\text{subject}} + \delta_{\text{plate}} + \epsilon, \quad \text{where} \tag{1}$$

[1]

- $\mu_{\text{population}}$ is the average % lacritin value in the target population,
- $\delta_{\text{subject}}$ is the amount by which this particular subject deviates from the population average (a random quantity that varies randomly from subject to subject),
- $\delta_{\text{plate}}$ is the (random) deviation attributable to the effect of the particular plate on which the assay was run (including plate*subject interaction),
- $\epsilon$ is a random error deviation that is not accounted for all the other sources of variation, including the differences attributable to the particular cell on the plate in which the assay was run, short-term variations in instrumentation, etc.

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In accordance with this formulation, a variance component analysis was conducted using the statistical software package JMP v.9 (from the SAS Institute). The model and variance estimates were fit using standard least squares with restricted residual maximum likelihood estimation [2].

In calculating the standard error and confidence interval for a given patient's % lacritin value, the estimated standard error of a subject's average % lacritin is shown in equation (2) given a person's sample is run on P plates, with R replicates per plate.

$$patient\ std\ error = \sqrt{\frac{S^2_{plate}}{P} + \frac{S^2_{error}}{P*R}} \qquad (2)$$

The Distribution of % lacritin in a normal population is defined by two parameters: the mean for the entire population ($\mu_{\text{population}}$), and the population standard deviation ($\sigma_{\text{population}}$). The population mean can be estimated by the grand mean from the data. Therefore, the standard deviation is equal to:

$$\text{Population std dev} = \sigma_{\text{population}} = \sqrt{\sigma^2_{subjects} + \sigma^2_{\bar{L}}}, \qquad (3)$$

where the first term under the radical accounts for the random variation between subjects and the second term is the standard error of the patient averages that accounts for the imprecision of the patient's average measurement, based on the ELISA protocol (#plates, #replicates/plate, etc). This second term is estimated using equation (2). Hence, the estimate of the population standard deviation corresponding to the study data is shown in equation (4).

$$\text{Estimated population std dev} = S_{\text{population}} = \sqrt{S_{subjects}^2 + \frac{S_{plate}^2}{P} + \frac{S_{error}^2}{P*R}} \quad (4)$$

In accordance with this formulation, the variance component analysis results are summarized in Table 1.

Supplemental Table 1: Estimated variance components from statistical analysis

| Source of variation | Baseline Study (n = 58) Estimated standard deviation attributable (% lacritin) |
|---|---|
| $\delta_{\text{subject}}$ | $S_{\text{subject}} = 1.056$ |
| $\delta_{\text{plate}}$ | $S_{\text{plate}} = 0.715$ |
| $\epsilon$ | $S_{\text{error}} = 0.279$ |

This analysis revealed that subject-to-subject variation is the largest variability source in this study, followed by the plate-to-plate variation, and finally the pure random error variation ($\epsilon$). Since the $\delta_{\text{plate}}$ and $\epsilon$ terms represent variation that is attributable to the ELISA method, and not attributable to differences between patients, this variance breakdown suggests that the ELISA method can be a useful tool for distinguishing between patients with respect to their lacritin levels. Apart from differences between subjects, most of the variation comes from random differences between ELISA plates.

In calculating the standard error and confidence interval for a given patient's % lacritin value, it is assumed that that a given person's sample (taken at a particular point in time) is run on P plates, with R replicates per plate. Also assumed is that the sample preparation (dilutions, assay methods, reagent concentrations, etc) and data quality standards are the same as used in the protocol described in this study. Substituting the values from

Supplemental Table 1 into equation (2), the standard error for the lacritin level for individual patients using different protocols (#plates, #replicates/plate) can be calculated. This analysis shows that the greatest improvements in precision (greatest reductions in standard error) are made by running a given patient's sample on multiple plates, while only marginal improvements are made by running multiple wells within each plate. The greatest reduction in standard error is seen by increasing from one to two plates; further increases in the number of plates only marginally reduced the standard error. Based upon the protocol used in this study of two or three plates with three replicates per plate for each individual, a standard error at an approximate 95% confidence interval for the patient's true avg % lacritin can be calculated as:

$$\overline{L} \pm 1.96 * \textit{patient std error}, \tag{5}$$

where $\overline{L}$ is the average reading from the patient and the *patient std error* is from equation (2).

A normal distribution curve was superimposed on a histogram of the average % lacritin values for the 58 patients in the study (Figure 5). Using the Shapiro-Wilks goodness of fit test, the null hypothesis that this sample of patients comes from a normal distribution was not rejected ($p = 0.8543$). Hence, a normal distribution will be used to describe the mean lacritin readings in a population of healthy subjects from which our sample of 58 subjects was taken. This distribution describes the relative frequency of average lacritin values of the population of healthy patients, assuming those averages were determined using the same protocol as in the present study. The population mean can be estimated by the grand mean from the baseline study, which in this case is equal to 4.2%. The

population standard deviation for the protocol used in this study can be calculated from equation (4), where the values of $S_{subject}$, $S_{plate}$, and $S_{error}$ in (4) were taken from Table 1.

For the diurnal study, multiple samples were collected from 34 individuals ages 24-52 for the diurnal study at time 0, (7:30-8:30 am), 4 hr (11:30 am-12:30 pm), 8 hr (4:30 pm-5:30 pm), and 24 hr (7:30 am-8:30 am the following day) as described previously. These individuals were not included as part of the previous study. Total protein in the tear sample and determination of % lacritin in the tear sample by indirect ELISA were performed as described above. Samples were required to meet the criteria for analysis as described.

In accordance with McCulloch, Searle, and Neuhaus (2008), the individual readings of %lacritin were represented as follows

$$\%\ \text{lacritin} = \mu_{\text{population}} + \delta_{\text{subject}} + \delta_{\text{plate}} + \gamma_{time-of-day} + \epsilon, \quad (6)$$

[2]

Where the quantities $\mu_{\text{population}}$, $\delta_{\text{subject}}$, $\delta_{\text{plate}}$, and $\epsilon$ are defined as in equation (1), and where $\gamma_{time-of-day}$ represents the (non-random) effect of the time of day on the %lacritin level. An F-test was used to test the null hypothesis that there was no difference in average lacritin levels among the four time periods used in the study. This hypothesis was rejected (p = 0.0341). A follow-up pairwise comparison using Tukey's Honestly Significant Difference test was used to determine that there was a statistically significant drop in lacritin level between 4 and 8 hrs (see Figure 7).

References

1. Plikaytis BD, Turner SH, Gheesling LL, Carlone GM. Comparisons of standard curve-fitting methods to quantitate neisseria meningitidis group A polysaccharide antibody levels by enzyme-linked immunosorbent assay. *J.Clin.Microbiol.* 1991;29:1439-1446.

2. Muculloch CE, Searle SR, and Neuhaus JM. *Generalized, linear, and mixed models.* Noboken, NJ: John Wiley & Sons; 2008.