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Comparison of Low Abundance Biomarker Levels in Capillary-collected Non-stimulated Tears and Washout Tears of Aqueous-deficient and Normal Patients

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COMPARISON OF LOW ABUNDANCE BIOMARKER LEVELS IN CAPILLARY-COLLECTED NON-STIMULATED TEARS AND WASHOUT TEARS OF AQUEOUS-DEFICIENT AND NORMAL PATIENTS

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ABSTRACT

Purpose: For patients suffering from aqueous-deficient dry eye, non-stimulated (NS) tear collection can be challenging and time-consuming. Adding a small volume of sterile saline to the eye allows a "washout" (WO) tear collection that takes considerably less time. The primary goal of this project was to determine if WO tear collection is a viable alternative to NS tears for comparing the levels of common ocular surface biomarkers in patients with and without aqueous-deficient dry eye (AD). Stimulated (Stim) tears were also investigated as a second possible alternative to NS tears.

Methods: Two studies were conducted, tear cytokine levels being measured using a 27-Plex BioRad cytokine assay. In the first study, NS and WO tear samples were collected from 23 non-AD patients and 26 AD patients, group allocation being determined by Schirmer score (wetting length). WO tears were collected after instillation of 10µL sterile saline to the lower conjunctival fornix. T-tests, correlation analyses and Bland-Altman plots and analyses were performed to determine the agreement between cytokine levels in NS and WO samples. In the second study, matching NS, WO, and Stim samples were collected from 15 patients. ANOVA and post-hoc comparisons of means were performed for comparison of NS, WO, and Stim tear samples.

Results: In Study 1, 11 of 25 routinely detected cytokines showed a significant difference between NS tears of non-AD and AD patients. Five of the 11 also showed a

significant difference between WO tears of non-AD and AD patients. In Study 2, greater differences between NS and Stim tears than between NS and WO tears were clearly evident and Stim tears showed little potential to replace NS tears.

Discussion: The WO tear collection method appears to be a viable substitute for NS tear collection for many inflammatory biomarkers and in distinguishing major differences between non-AD and AD patients. Some subtle differences between non-AD and AD samples are lost in the WO method. Stim tear collection is not a reliable or practical alternative to NS tear collection and shows little potential to differentiate non-AD from AD dry eye patients.

Keywords: tear collection, non-stimulated, washout, cytokines, aqueous-deficient dry eye

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INTRODUCTION

Tear fluid analysis has the potential to provide a great deal of information about the health of the ocular surface. Many inflammatory biomarkers including cytokines and chemokines have been detected in tear fluid in dry eye and other diseases states. In the process of discovering this wealth of biomarkers, it has also been found that the tear fluid "matrix" (tear fluid and constituent ions, proteins, mucus and lipid components) is prone to producing artifacts in protein and other biomarker assays. The complex and variable nature of tears further complicates biomarker assays because these matrix effects have been shown to vary with sampling condition. Deliberate stimulation of tearing creates a profile progressively more dominated by lacrimal gland fluid. Invasive conditions such as a Schirmer strip result in a higher level of mucus and cellular debris. An important challenge for tear biomarker assessment is the collection of valid, useable, samples from aqueous-deficient (AD) dry eye patients. Microcapillary collection of non-stimulated (NS) tear samples often proves impractical in these cases.

The primary goal of this project is to determine if a "washout" (WO) tear collection method is a viable substitute for NS collection, in particular for patients for whom NS collection is impractical. To test this possibility, NS and WO tear biomarker profiles of aqueous deficient dry eye (AD) patients will be determined and compared to the profiles obtained from (non-AD) controls. Biomarker profiles will consist of the tear levels of 27 cytokines and associated inflammatory markers. A secondary goal will be to

investigate stimulated (Stim) tears as another alternative to NS tears. Stim tearing is known to modify the resident ocular surface biomarker profile, so this method would only be considered for recommendation should WO tears prove unsatisfactory[1-3]. The tear sampling methods are: (a) NS tear collection by polished micropipette (this represents tears that have flowed over the ocular surface and accumulated in the inferior marginal tear strip), (b) tears collected after instillation of a small volume (10 μL) of sterile saline, thereby constituting a washout (WO) of resident ocular surface biomarkers, and (c) stimulated (Stim) tear collection after indirect nasal stimulation of the sneeze reflex.

Background

Major Histocompatibility Complex (MHC) molecules are glycoproteins divided into two classes, each with different functions. Class I MHC molecules are found on almost all nucleated human cells, while Class II MHC molecules are only found on antigen-presenting cells (APCs). APCs can be macrophages, B lymphocytes, or dendritic cells. APCs take up antigens and then display the antigens on MHC class II molecules. APCs also function to induce the production of cytokines to activate the T helper (T_H) cells[4].

Humoral immune responses involve B cells interacting with antigens to produce antibody-secreting plasma cells. These antibodies bind to antigens to facilitate antigen removal and destruction[4]. Cell-mediated immune responses involve T_H cells and cytotoxic T lymphocytes (CTLs). T_H cells are activated by antigen-bound Class II MHC

molecules, which causes the release of cytokines that act on other cells to affect the immune response. CTLs are activated by Class I MHC molecules with antigen binding leading to destruction of the infected cells[4].

Innate immunity is nonspecific and does not require previous exposure to an antigen. It can include skin, lysosomal enzymes, and stomach acid. Adaptive immunity is highly specific, diverse, has memory, and self-nonself recognition. The specificity and diversity come from the production of highly specific antibodies that bind to specific antigens. A "memory" component develops from a subpopulation of long-lived memory B cells to enable a faster and larger immune response following a second exposure to the same antigen[5].

Cytokines are proteins that affect the intensity and duration of an immune response by affecting lymphocytes and many other cell types[4]. Chemokines are chemotactic cytokines. Chemokines can be divided into two basic groups, α-chemokines and β-chemokines. The α-chemokines are chemotactic for neutrophils while β-chemokines are chemotactic for monocytes, eosinophils, basophils, and lymphocytes[5].

Cytokine changes rarely occur in isolation. Changes in one cytokine can produce changes in multiple other cytokines. The complexity of inflammatory pathways and cytokine/chemokine interactions, upregulation and downregulation, mean that ideally multiple cytokines/chemokines should be assayed to yield a cytokine profile rather than assaying just one or two cytokines[6]. Tear cytometric bead-based assay has the potential to quantify cytokine/chemokine profiles of up to 100 biomarkers in single small tear samples. This is ideally suited to predictive modeling of disease states.

Many cytokines are important in tears and ocular surface disease. Those that are regularly cited as serving important roles include IL-2, IL-6, IL-8, IL-10, IL-17, IP-10, TNF-α, and IFN-γ[7]. IL-8 has both been reported to be significantly elevated in the tears of patients with mild-to-moderate dry eye syndrome, along with some other cytokines[8]. IL-8 is also elevated in tears of patients with atopic keratoconjunctivitis (AKC) and giant papillary conjunctivitis (GPC)[9]. IL-2 is elevated in patients with AKC, SAC (seasonal allergic conjunctivitis), and VKC (vernal keratoconjunctivitis), while IL-6 is elevated in patients with AKC, SAC, VKC, and Type I hypersensitivity. IL-10 has been found to be increased in patients with VKC and Type I hypersensitivity and IL-17 levels elevated in patients with Type IV hypersensitivity. TNF-α levels are increased in patients with AKC, VKC, and Type I hypersensitivity. IFN-γ is found to be elevated in tears of patients with AKC, SAC, VKC, and Type IV hypersensitivity[9].

Other cytokines, such as IL-1β, IL-1RA, G-CSF, and IL-12p70, have been shown to play a role in dry eye syndrome. Overproduction of IL-1β appears to occur in dry eye-associated inflammation [10]. Therefore, inhibition of this inflammation, via IL-1RA, may prove to be a viable treatment of dry eye syndrome[10]. Recently, G-CSF in AD-patient tears was shown to correlate with expression of pro-inflammatory conjunctival biomarkers[11]. IL-12p70 has been shown to be active in autoimmune diseases[10] and to correlate with dry eye severity[12].

Sack et al.[13] found that some proteins and cytokines, including IP-10, are higher in concentration in closed-eye tear fluid than in open-eye tear fluid. This supports the theory that IP-10 is produced by ocular epithelial cells[13]. Some doubt has been cast on

the validity of immunoassay-based tear IP-10 measurement due to possible assay artifacts[13].

As a chemokine that is involved in anti-angiogenic processes, IP-10 may contribute to corneal immune privilege, helping to prevent inflammatory responses that could threaten corneal transparency and result in loss of vision[14]. IP-10 inhibits the chemokine IL-8 and the growth factor basic Fibroblast Growth factor (bFGF), both of which exhibit the pro-angiogenic property of stimulating blood vessel growth[4]. Because IP-10 inhibits corneal vascularization, patients with corneal neovascularization would be expected to have lower IP-10 levels. It has been suggested that there may be two subsets of dry eye, one based on IP-10 upregulation and another on IL-8 upregulation, although this has yet to be proven. Yoon et al found that tear IP-10 levels and conjunctival IP-10 receptor (CXCR3) expression were elevated in Sjögren's dry eye relative to non-Sjögren's dry eye. This suggests an alternative mechanism to the much more commonly cited IL-8 increase in AD dry eye.[15]

Studies by De Paiva et al.[16] suggested that IFN-γ levels increase in dry eye to promote conjunctival squamous metaplasia. Based on this and the previously cited evidence, dry eye clearly involves an inflammatory component. IFN-γ originates from T lymphocytes and Natural Killer cells that are active in inflammation. The immunosuppressant, Cyclosporine, is thought to inhibit production of IFN-γ by T lymphocytes and therefore, help reduce the inflammation of dry eye[16].

Being in intimate contact with the ocular surface, tears can provide a great deal of information about ocular surface health. NS tears wash over the ocular surface at a slow rate and should therefore be subject to maximum interaction with the ocular surface

environment. NS tear are sometimes inappropriately defined as "basal" tears, because all tear collection methods induce some reflex tearing leading to tears that are not completely basal. Conversely, Stim tears, with a considerably faster flow-rate, represent a much greater input of lacrimal gland fluid with minimal ocular surface interaction. Differences between NS and Stim tear biomarker profiles can be useful in providing information about ocular surface-derived versus lacrimal gland-derived biomarkers. Several previous studies have demonstrated clear differences in protein profiles between NS and Stim tears[1], however some studies have used them in the study of dry eye. Because NS tear volume is limited, absorptive substrate tear collection methods have been widely tested as a possible alternative to microcapillary collection[17-20]. Collection by Schirmer strip results in more mucus, lipid, and cellular material in the collected sample and variable, incomplete, elution of proteins from the filter collection matrix[18, 21]. As a result Schirmer-derived "tear" samples are not directly comparable to any type of micropipette-collected tear sample.

NS tears typically flow at a rate of less than 1 μL/minute, making collection time a considerable challenge. For AD dry eye patients, NS tear flow rate can be much slower and, for severe AD cases, effectively impractical. This presents a significant logistical challenge when attempting to collect NS tear samples from AD patients for biomarker assay. One approach that has been proposed is to add a small volume of sterile isotonic solution to the conjunctival sac to "rinse out" the resident biomarkers and increase collected sample volume[22, 23]. To measure the validity of the WO method, its biomarker profile must be compared to that obtained from NS tears. An important issue with the WO method is to determine if the "just-quantifiable" NS biomarkers shift below

threshold in the WO profile. Another issue to observe is the consistency of the NS vs WO tear profiles within non-AD patients and within AD patients. For comparisons of the two patient groups, WO tears should be confirmed as viable alternative to NS tears in both. To ensure stimulated tearing does not occur with the WO method, secretory IgA levels, which decrease more or less linearly across a range of tear flow rates, can be measured[2]. Markoulli et al studied sIgA levels and found that WO tears do not appear to induce reflex tearing [24].

A second possible alternative to NS tear collection is to collect Stim tears. Indirect nasal stimulus of the sneeze reflex promotes reflex tearing without any direct effect on the ocular surface[1-3]. Stim tears present different challenges to those of WO tears. First, tear flow-rate may vary from patient to patient and even from session to session depending on the degree of stimulus induced. Second, AD patients would be expected to have lower Stim tear flow-rates than non-AD patients. This would be a potential source of sampling bias in which the reflex tear contribution would be greater in non-AD patients and less in AD patients. Third, Stim tears would be expected to contain a higher lacrimal gland contribution than NS tears or WO tears, thus representing a skewed profile[1-3]. NS and WO tears should be more representative of the resident ocular surface biomarker profile. Finally, the dilution effect of stimulated tearing on resident ocular surface proteins may reduce the levels of some ocular surface biomarkers to below their lower limit of quantification. However, if WO tears prove to be a non-viable alternative to NS tears, Stim tears would be the only remaining option.

Comparison of Tear Biomarker Profiles between Sampling Methods: NS vs WO Tears

Bland and Altman state that, in order for two methods to provide a reliable measure of the same parameter, they must have good correlation[25]. However, correlation can be artificially inflated if the data is highly variable[25]. A Bland-Altman (BA) plot of the difference between NS and WO levels of a cytokine versus the mean of NS and WO levels addresses the variability issue. It shows the level of agreement between collection methods and how it varies across the range of mean values. In general, if data points for the difference between methods fall within two standard deviations of the mean, the tests are considered to be measuring the same thing. However, an additional consideration is the ability of the BA plot to reveal bias, seen as systematic differences between test measurements. Fixed bias indicates that one method is consistently producing a higher value than the other, but the difference remains relatively constant across the full range of means. For example, if NS tear levels of a particular biomarker were 50 pg/mL higher than WO levels across the full range of means, this would be considered fixed bias and would show up on the BA plot as a horizontal line of best fit of the difference between values versus the means. On the other hand, proportional bias between the two methods would produce a regression line with definite, possibly significant, slope. This indicates that the agreement between the two methods varies as a function of the mean value for each patient. The most common presentation of proportional bias is a regression line with positive slope, indicating in the current context that, as the mean cytokine level increases, the difference between NS and WO levels also increases[25].

Specific Aims and Significance

Specific Aim 1 and the primary aim of this project is to determine if WO tear samples produce comparable tear cytokine profiles to NS tear samples in both non-AD and AD patients.

Specific Aim 2 is to determine if a second alternative to the NS tear collection method, stimulated tear collection, produces comparable cytokine profiles to NS tears in a direct comparison of NS, stimulated and WO tear sampling methods.

Working hypothesis: WO tear collection produces a cytokine profile that is representative of the profile seen in NS tears for both non-AD and AD patients.

Null hypothesis: WO tear collection produces a cytokine profile that is not representative of the profile seen in NS tears for non-AD and AD patients.

Rationale of Aims

Specific Aim 1: NS tear samples are difficult to collect from the majority of patients; in particular dry eye patients. The WO method is considerably faster and may enable collection of usable tear samples in much shorter times than currently required when collecting NS tears from AD dry eye patients. By comparing cytokine levels in WO tears and regular NS tears versus for the two patient groups, it will be possible to determine the extent to which the cytokine profile for WO tears is statistically comparable to that of NS tears. This will provide a measure of the validity of the WO method and the cytokines for which it can replace NS tear collection. If WO tear samples are comparable to NS tear samples, it will help facilitate sample collection in dry eye

biomarker evaluation studies, treatment studies, and other studies involving dry eye patients.

Specific Aim 2: A second possible alternative is stimulated tear collection, which will also be investigated as a possible substitute for NS tear collection. Stim tears are less likely to be valid substitute for NS tears because they are expected to skew the biomarker profile in ways not anticipated with WO collection. However, in the event that WO tear collection proves to be a nonviable alternative to NS sampling, stimulated tears may then be considered as the only viable remaining option. Some studies use Stim tears in the study of dry eye biomarkers.

METHODS

All aspects of this study adhered to the guidelines of the Declaration of Helsinki and were conducted with the approval of the University of Alabama at Birmingham Institutional Review Board. Tear samples were collected from non-AD patients and from AD patients. All patients underwent an ocular surface exam that included history, Ocular Surface Disease Index (OSDI) questionnaire, and multiple dry eye tests. Schirmer 1 test results were the single test results used to group patients into normal versus aqueous-deficient, because it directly addressed the low tear volume challenge of tear collection. A score of less than 10mm in 5 minutes was considered AD and score of 10mm or higher in 5 minutes was considered non-AD. Other dry eye tests were conducted have been shown to have poor standardization[26].

Tear Collection

The following tear collection techniques were performed. All samples were stored at -86°C until ready for use.

1. NS Tear Collection Method:

a. NS tears were collected using 10 μL glass microcapillary tubes (Drummond, Broomall, PA) placed just above the lower eyelid in the marginal strip.

b. NS tears were collected in 10 minute increments until a total of 6.5 μL of tears were collected using. Each 10-minute sample aliquot was stored 1 in 10 in Assay Buffer (PBS 0.05% Tween/1% BSA, pH 7.4, Teknova, Hollister, CA) containing EDTA-free antiprotease cocktail (Thermo-Fisher Pierce, Rockford IL) at a final concentration of 1x.

c. Whenever possible, patients were trained to self-collect tear samples, using a magnifying mirror to assist with micropipette placement. If patients were unable to self-collect, the study investigator collected the sample from the patient.

2. WO Tear Collection Method:

   a. 10 μL of sterile saline (Hudson RCI, Durham, NC) was instilled into lower conjunctival fornix using a calibrated digital pipette.

   b. The patient then closed his/her eyes for one minute and was instructed to keep their eyes still to prevent any reflex stimulation or loss of sample.

   c. A tear sample was then collected for 5 minutes or until 6.5 μL was collected (whichever came first) using 10μL microcapillary tubes. The sample was stored 1 in 10 in Assay Buffer.

   d. If the patient did not collect 6.5 μL within 5 minutes, a second 10 μL sterile saline aliquot was instilled and the process repeated.

e. The process of aliquot collection was continued until sufficient tear volume was collected. To allow for multi-aliquot losses, minimum total volume requirements were 7 μL minimum for 2 aliquots, 7.5 μL for 3, or 8 μL for 4 aliquots, etc. These increased amounts allowed for larger potential tear loss through evaporation.

3. Stim Tear Collection Method

   a. Stim tears were collected with 20 μL microcapillary tubes. A cotton Q-Tip was first inserted into the nose to stimulate the sneeze reflex and induce stimulated tear production. Tears were collected once stimulated flow was established.

   b. A total of 10 μL were collected, after 20 μL pre-collection, in < 3 min.

Tear Cytokine Assay

All tear samples were assayed using a BioRad 27-Plex Polystyrene Bead-based Assay (Bio-Rad, Hercules, CA). Each kit contains a set of assays for commonly cited inflammatory biomarkers. These are: Interleukin (IL)-1β, IL-1 receptor antagonist (RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic-fibroblast growth factor (FGF), Eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage (GM)-CSF, interferon (IFN)-γ, interferon gamma-induced protein (IP)-10, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). All assays steps were applied

according to the manufacturer's instructions. A Tecan Hydroflex® automatic Microplate Washer (Tecan Systems, San Jose, CA) with vacuum attachment was used to standardize all assay wash steps.

All assays were run on a Luminex 200 Cytometric Bead-based assay platform (Luminex Corporation, Austin, TX) using default instrument settings.

Study Design

Main Study: NS vs WO Tears of AD and non-AD Patients

NS and WO tears were always collected within the same session. To prevent any influence of WO fluid on NS tear samples, NS sample collection always preceded WO collection. Cytokine levels in NS and WO tears were compared to determine lower limits of quantification and the potential for WO tears to substitute for NS tears were investigated for each cytokine and for the overall cytokine profile. These procedures were also applied to compare cytokine levels between non-AD and AD patients.

NS vs WO vs Stim Tears

A subset of patients collected Stim tears for comparison with NS and WO tear samples. Although the groups were not separated for analysis, there were 8 non-AD and 7 AD patients in this subset. Stim tears were collected after NS and WO tears. A delay period was incorporated as necessary after WO collection to ensure that all WO fluid had drained from the eye. The primary purpose for collecting Stim tears was to test an alternative tear biomarker source in the event that WO sampling results were sub-

optimal. Because of the smaller patient number, the comparison of NS, WO, and Stim tears did not involve non-AD and AD group separation.

Statistical Analysis

NS vs WO Comparisons between Non-AD and AD Patients

To determine if WO tear samples mirror the biomarker profile of NS tear samples, Pearson correlations between NS and WO assays were evaluated. Bland-Altman plots were investigated for all consistently quantifiable cytokines to determine the level of agreement between collection methods. Paired *t*-tests were used to quantitatively test the difference between the NS and WO sampling methods.

After running the above analyses for the pooled NS and WO data, the analyses were re-run separately for AD and non-AD patients, the groups being defined based on Schirmer test scores. Satterthwaite *t*-tests and Hartley's folded *F*-tests were used to assess the between-group differences in mean and variance, respectively. Spearman rank-based correlations, Wilcoxon signed rank tests to assess distributional differences between NS and WO assays, and Kruskal-Wallis tests to assess distributional differences between the normal and AD groups were also conducted.

In an additional analysis to assess the ability of NS and WO tears to distinguish between AD and non-AD patients based on cytokine levels, the data was first normalized so that scale for assessment of differences was the same for each cytokine. This was achieved by standardizing the data for each cytokine to a mean of zero and standard

deviation to 1 for both NS and WO assays. By setting each AD and non-AD cytokine mean value to zero (e.g. zero overall mean for NS and WO IL-8 for the AD and non-AD groups), the difference between non-AD and AD levels would be shown as a distance either side of zero. Setting the overall standard deviation for each AD and non-AD cytokine data set to 1 (e.g. a total standard deviation of 1 for NS and WO IL-8 for the AD and non-AD groups) allows a direct comparison of the amount of total variance that is accounted for by AD versus non-AD differences for every cytokine. In other words, the total variance scale is fixed. This allows each cytokine profile to be compared and evaluated without overall levels of cytokines affecting the comparison. It produces a data point for NS non-AD, NS AD, WO non-AD, and WO AD for each cytokine. This standardization procedure is totally independent of any other previous statistical tests.

Pearson correlations were used to determine if age influenced AD or non-AD cytokine profiles,. Analysis of covariance (ANCOVA) models used NS and WO cytokine levels as dependent variable, AD status as group variable, and age as covariate.

Comparison of Cytokine Levels in NS, WO and Stim Tear Samples

Comparisons of the tear sampling methods were conducted using ANOVA and Tukey's test for global comparisons among means. In the case of biomarkers for which the Shapiro-Wilk test indicated non-normal data distribution, a Kruskal-Wallis One Way ANOVA on Ranks was substituted. If ANOVA indicated a lack of significant between-groups differences for any cytokine, paired t-test were instead used to compare NS vs WO, NS vs Stim, and WO vs. Stim results. For these two-group comparisons, any non-normally distributed cytokine data was analyzed by Wilcoxon's Signed ranks test.

RESULTS

NS vs WO Comparison Participant Information

Sixty-three pairs of NS and WO samples were collected with each eye independently assessed and grouped for normal versus AD. The data is summarized in Table 1.

Table 1 – NS vs WO Study: Non-AD and AD Patient Clinical Information

	Non-AD	AD
Number of Patients / Eyes	23 / 26	26 / 37
Age (years)	42.4 ± 18.8*	49.9 ± 13.9
Age Range (years)	21 – 75	21 – 72
Schirmer Test (mm/5 min)	22.6 ± 9.5	4.6 ± 2.4
OSDI Score	17 ± 21	30 ± 19
Tear Osmolarity (mOsm/L)	295 ± 13	301 ± 14
NIBUT (sec)	13.1 ± 7.0	10.1 ± 5.3
Fluorescein Score (Oxford)	0.8 ± 1.1	2.1 ± 2.0
Lissamine Green Score (Oxford)	1.0 ± 1.3	2.5 ± 2.0

*mean ± standard deviation, AD=Aqueous deficient, OSDI = Ocular Surface Disease Index, NIBUT = non-invasive tear break-up time.

NS vs WO Tear Cytokine Assay Results

Twenty-five cytokines were detected in at least 80% of the tear samples. The remaining two cytokines, FGF-basic and MIP-1β, were not quantifiable in the majority of tear samples and were therefore excluded from subsequent analyses. Table 2 shows NS and WO comparisons for all patients. Scatterplots of NS versus WO levels of all 25 cytokines showed significant correlations, 19 of these cytokines having correlation coefficients >0.75 ($R^2 \geq 0.56$). Because of the large number of cytokines, only selected plots are included in the Results section.

Table 2 - NS vs WO Tear Samples: All Patients; Paired Comparisons.

Cytokine	NS vs. WO Slope (Slope R^2, *p*)	NS Level (SD)	WO Level (SD)	NS – WO (SD)	B-A Slope (*p*-value)
IL-8	0.33 (0.29, <0.001)	316.6 (307.3)	195.7 (185.9)	120.9[B] (257.5)	0.62 (<0.0001)
IL-1β	0.57 (0.72, <0.001)	6.03* (7.92)*	4.28 (5.29)	1.74[B] (4.38)	0.43 (<0.0001)
VEGF	0.53 (0.47, <0.001)	435.9 (294.1)	354.3 (223.9)	81.57[B] (213.4)	0.32 (0.004)
IL-1RA	0.66 (0.56, <0.001)	10,195 (15,925)	9,197 (14,038)	997.3 (10662)	0.14 (0.14)
G-CSF	0.64 (0.66, <0.001)	34.38 (38.14)	29.64 (30.14)	4.74[W] (22.21)	0.26 (0.002)
IL-9	0.25 (0.16, <0.001)	49.02 (40.54)	34.14 (24.58)	14.88[B] (37.74)	0.68 (<0.0001)
Eotaxin	0.69 (0.48, <0.001)	111.1 (66.32)	90.91 (64.86)	20.17[B] (50.77)	0.03 (0.81)
IL-15	0.64 (0.62, <0.001)	7.63 (5.43)	5.89 (4.39)	1.74[B] (3.34)	0.23 (0.01)
GM-CSF	0.69 (0.45, <0.001)	192.4 (148.0)	162.7 (151.7)	29.72[W] (120.3)	–0.03 (0.79)
IL-12p70	0.78 (0.68, <0.001)	47.83 (20.13)	42.12 (19.03)	5.72[B] (11.48)	0.061 (0.44)
MCP-1	0.73 (0.60, <0.001)	108.3 (179.5)	88.01 (168.2)	20.32[W] (116.7)	0.07 (0.43)
TNF-α	0.78 (0.64, <0.001)	590.0 (367.9)	470.8 (358.2)	119.2[B] (230.5)	0.03 (0.73)

PDGF-bb	0.74 (0.60, <0.001)	16.80 (16.17)	14.57 (15.39)	2.24[W] (10.52)	0.06 (0.54)
MIP-1α	0.86 (0.79, <0.001)	92.75 (73.82)	77.56 (70.93)	15.19[B] (33.87)	0.04 (0.49)
IL-6	0.58 (0.56, <0.001)	51.55 (40.07)	43.09 (31.14)	8.46[B] (26.50)	0.29 (0.004)
IL-7	0.70 (0.68, <0.001)	95.28 (57.11)	79.04 (48.56)	16.23[B] (32.06)	0.18 (0.03)
IL-13	0.81 (0.70, <0.001)	17.21 (7.44)	16.02 (7.21)	1.18[B] (4.19)	0.08 (0.66)
IL-10	0.81 (0.69, <0.001)	110.5 (42.70)	97.13 (41.81)	13.41[B] (24.73)	0.02 (0.77)
IL-17	0.92 (0.92, <0.001)	11.98 (15.01)	10.07 (14.30)	1.91[B] (4.18)	0.05 (0.18)
RANTES	0.91 (0.79, <0.001)	115.8 (70.53)	105.3 (71.96)	10.49[B] (33.70)	–0.02 (0.73)
IL-4	0.76 (0.57, <0.001)	32.84 (19.17)	29.29 (19.27)	3.55[B] (13.34)	–0.005 (0.96)
IFN-γ	0.82 (0.62, <0.001)	496.7 (367.7)	446.5 (381.5)	50.25[W] (242.6)	–0.04 (0.64)
IL-2	0.78 (0.57, <0.001)	67.79 (45.29)	61.21 (46.32)	6.58[W] (31.89)	–0.03 (0.79)
IL-5	0.78 (0.58, <0.001)	9.27 (8.11)	8.36 (8.23)	0.91[W] (5.58)	–0.02 (0.86)
IP-10	0.36 (0.31, <0.001)	17,111 (14,281)	14,360 (9,061)	2,750[W] (11,791)	0.56 (<0.0001)

*Cytokine levels in pg/mL (± standard deviation), NS=non-stimulated, WO=washout, B-A Slope = slope of Bland-Altman plot regression line, SD=Standard Deviation.
[B] *p*-values from Wilcoxon Signed Rank test and paired *t*-test < 0.05.
[W] *p*-value from the Wilcoxon Signed Rank test only < 0.05.

FGF-basic and MIP-1β are not included because of low detection rate among patients. NS tears showed higher means for tear cytokine levels than WO tear samples (Table 2). NS tear samples also elicited a broader range of cytokine concentrations, causing paired t-tests and signed rank tests to show significant distributional differences between NS and WO samples. The potential of WO tears as a viable replacement for NS tear is determined in part by the scatter of data in the Bland-Altman plot and by the slope bias. If a cytokine shows no Bland-Altman plot bias, the WO cytokine level was

underestimating the NS level by a constant value, indicating that a simple scalar transformation would be required to estimate the NS level from the WO value. This would require, for example, the addition of 50 pg/mL to the WO level for an underestimate of 50 pg/mL. However, in cases of significant Bland-Altman slope bias, WO tears may not be a viable "proxy" measurement of NS levels of that cytokine (Table 2). Nine cytokines showed significant Bland-Altman bias: IL-8, IL-1β, VEGF, G-CSF, IL-9, IL-15, IL-6, IL-7, and IP-10. These analyses did not separate tears from AD and non-AD patients meaning that between-group bias differences were not assessed. To illustrate the difference between simple scatterplots and Bland-Altman plots to determine agreement between NS and WO measurement of tear cytokines, two examples are shown. The IL-2 and IL-6 scatterplots of NS vs. WO tears (Figures 1 and 2) both show significant correlations and almost identical correlation coefficients. However, the corresponding Bland-Altman plots (Figures 3 and 4) indicate stronger agreement between sampling methods for IL-2, the IL-6 plot showing more data points outside the ± 2 standard deviation range and significant Bland-Altman plot bias (also see Table 2).

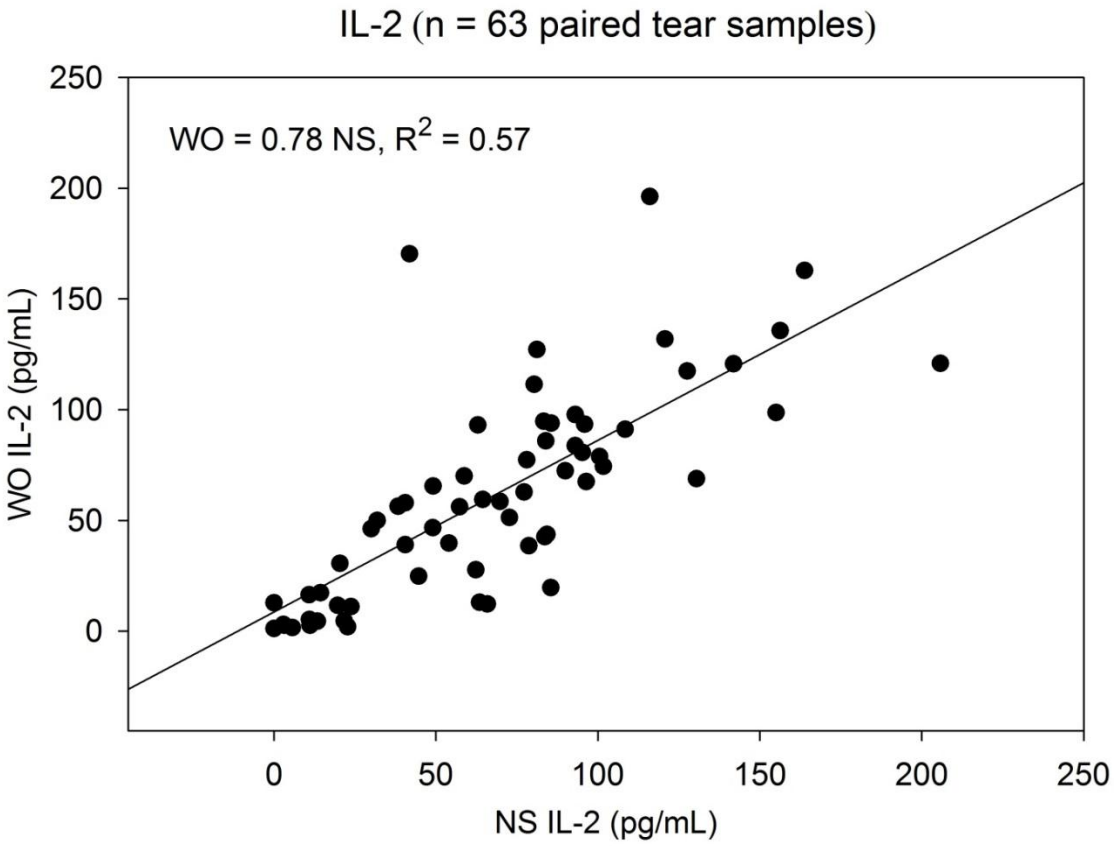

Figure 1: Scatterplot showing the correlation between NS and WO tear levels of IL-2 for the entire patient group. Slope of the linear regression line shows that WO tear IL-2 levels averaged 78% of NS levels and correlated strongly ($R^2 = 0.57$ ($p < 0.001$). NS = non-stimulated, WO = washout.

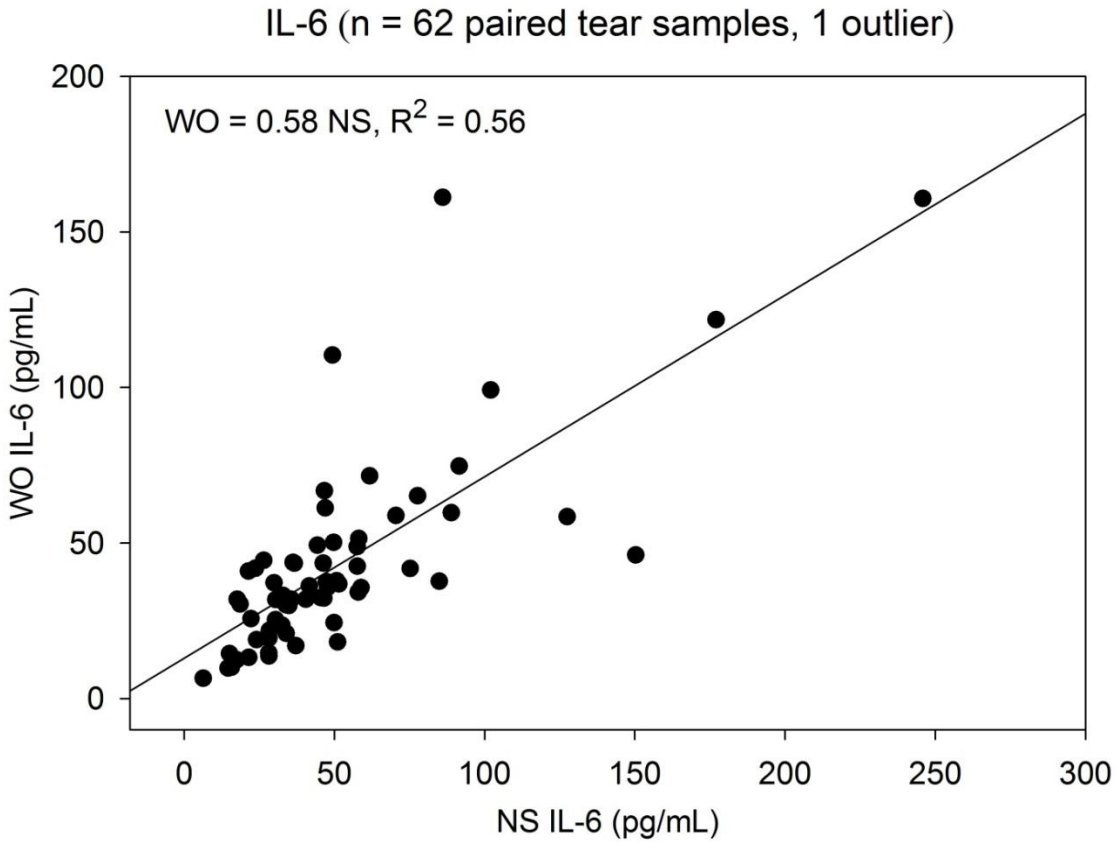

Figure 2: Scatterplot showing correlation between NS and WO tear levels of IL-6 for the entire patient group. Slope of the linear regression line shows that WO tear IL-2 levels averaged 58% of NS levels with a strong correlation ($R^2 = 0.56$, $p < 0.001$). NS = non-stimulated, WO = washout.

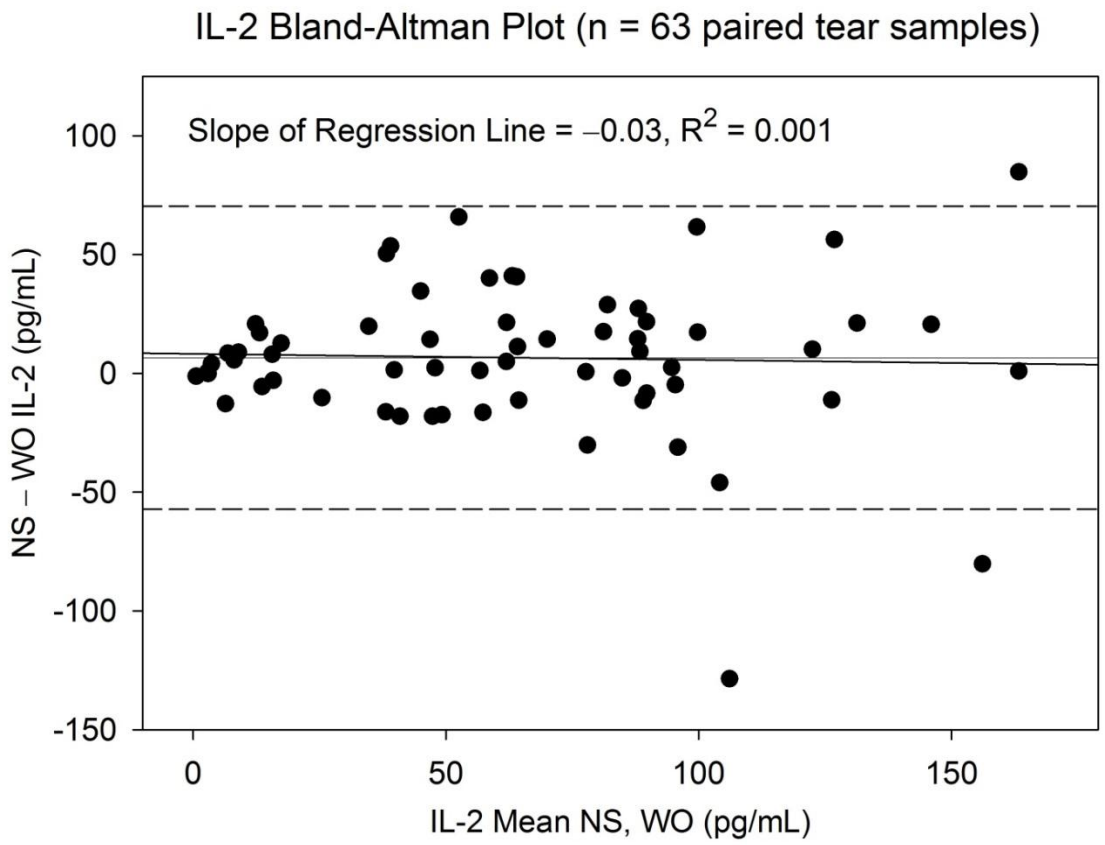

Figure 3: Bland-Altman plot showing difference between NS and WO tear IL-2 level vs. mean level for paired tear samples (entire patient group). Slope of the regression plot is negligible, indicating that the difference between NS and WO levels does not vary as a function of the mean. Dashed lines represent 95% confidence limits. Three data points fall outside the Bland-Altman range for agreement between tests. NS = non-stimulated, WO = washout.

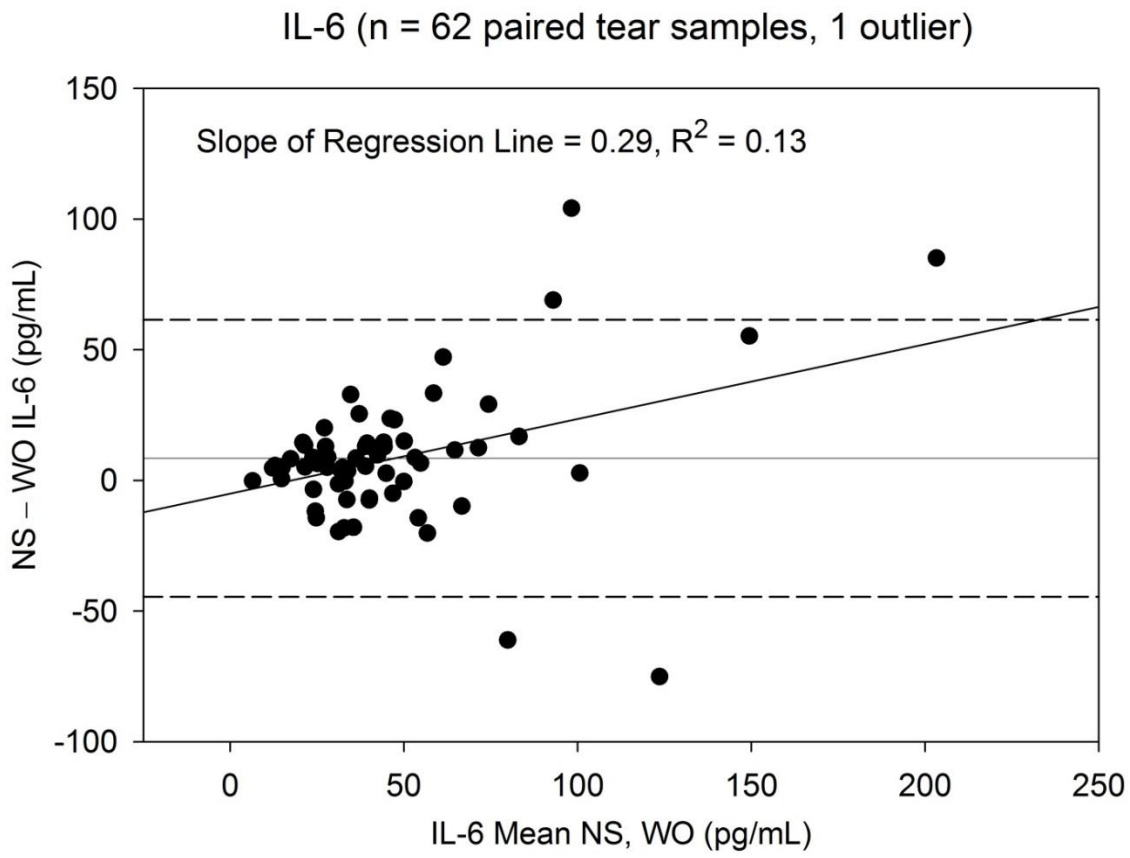

Figure 4: Bland-Altman plot showing difference between NS and WO tear IL-6 levels vs. mean level for paired tear samples (entire patient group). Slope of the regression plot is positive indicating that the difference between NS and WO levels increases as a function of the mean. Dashed lines represent 95% confidence limits. Five data points fall outside the Bland-Altman range for agreement between tests. NS = non-stimulated, WO = washout.

Schirmer Score (AD vs Non-AD) Effect on NS vs WO Tear Cytokine Levels

Schirmer scores were chosen to separate non-AD and AD patients for two reasons. First, AD patients should have lower overall tear volume, reflected in the Schirmer score, and should benefit most from the WO method. Second, the added saline would be expected to constitute a larger portion of the collected sample in AD patients than non-AD patients. For the nine cytokines showing significant Bland-Altman plot bias, the reason was higher variability of cytokine levels in AD samples. This variability

was evident in NS and WO samples for seven of the nine cytokines. These cytokines showed less variability in non-AD patients for both NS and WO assays.

NS vs WO Tear Comparisons for AD and Non-AD Groups

Table 3 shows 10 cytokines (IL-8, IL-1β, VEGF, IL-1RA, G-CSF, IL-9, Eotaxin, IL-15, GM-CSF, and IL-12p70) with significant differences between AD and non-AD NS samples by either the Kruskal-Wallis or Satterthwaite *t*-test. WO samples showed significant differences for five cytokines (IL-8, IL-1β, VEGF, IL-1RA, and G-CSF).

Table 3. Mean Cytokine Levels (SD) and Standardized Effect Sizes for Between Group Comparisons of Non-AD vs AD Patient NS and WO Tear Samples.

	Non-Stimulated			Wash-Out		
Cytokine	Normal (n = 26)	AD (n = 37)	ES,N ES,AD ES,Total	Normal (n = 26)	AD (n = 37)	ES,N ES,AD ES,Total
IL-8 **	193.80 (142.63)	403.30 (360.84)	–0.24 0.57 0.81^{B}	125.29 (67.22)	245.39 (224.45)	–0.50 0.04 0.54^{B}
IL-1β **	2.98 (1.95)	8.17 (9.69)	–0.32 0.45 0.77^{B}	2.75 (2.06)	5.35 (6.51)	–0.35 0.03 0.38^{B}
VEGF **	341.56 (178.81)	503.99 (341.42)	–0.21 0.41 0.62^{T}	284.76 (109.13)	404.53 (269.55)	–0.42 0.04 0.46^{T}
IL-1RA **	5,968.8 (7,043.0)	13,334.0 (19,680.0)	–0.25 0.24 0.49^{T}	4,942.9 (6,856.0)	12,358.0 (17,002.9)	–0.32 0.18 0.50^{B}
G-CSF **	25.30 (23.19)	40.77 (45.06)	–0.20 0.26 0.46^{K}	20.07 (15.78)	36.36 (35.76)	–0.35 0.13 0.48^{B}
IL-9 *	33.92 (19.11)	60.56 (48.42)	–0.22 0.56 0.78^{B}	28.74 (17.87)	38.26 (28.25)	–0.38 –0.10 0.28
Eotaxin *	91.33 (53.13)	124.96 (71.66)	–0.15 0.36 0.51^{T}	81.03 (58.90)	97.85 (68.68)	–0.30 –0.05 0.25
IL-15 *	6.12 (3.94)	8.61 (6.05)	–0.13 0.37 0.50^{K}	4.81 (3.40)	6.59 (4.85)	–0.39 –0.03 0.36
GM-CSF *	148.82 (110.35)	223.10 (164.12)	–0.19 0.30 0.49^{T}	164.50 (158.07)	161.49 (149.24)	–0.09 –0.11 –0.02
IL-12p70 *	42.17 (15.80)	51.81 (22.02)	–0.14 0.35 0.49^{T}	38.75 (14.44)	44.49 (21.56)	–0.32 –0.03 0.29
MCP-1 *	93.01	119.09	–0.03 0.12	84.70	90.34	–0.08 –0.05

	(184.79)	(177.42)	0.15[K]	(209.57)	(135.02)	0.03
TNF-α	489.17 (287.97)	660.85 (403.78)	–0.11 0.36 0.47	415.31 (287.46)	509.76 (399.78)	–0.31 –0.05 0.26
PDGF-bb	12.67 (10.29)	19.71 (18.86)	–0.19 0.26 0.45	12.94 (13.43)	15.72 (16.72)	–0.17 0.01 0.18
MIP-1α	76.43 (61.07)	104.22 (80.43)	–0.12 0.26 0.38	66.54 (61.79)	85.31 (76.58)	–0.26 0.00 0.26
IL-6	43.62 (22.08)	57.12 (48.46)	–0.10 0.27 0.38	36.53 (20.00)	47.71 (36.60)	–0.30 0.01 0.31
IL-7	84.74 (44.31)	102.68 (64.16)	–0.05 0.29 0.34	72.26 (31.17)	83.81 (57.71)	–0.28 –0.06 0.22
IL-13	16.25 (6.61)	17.88 (7.99)	–0.05 0.17 0.22	15.94 (6.33)	16.08 (7.85)	–0.09 –0.07 0.02
IL-10	105.20 (33.97)	114.29 (48.00)	0.03 0.25 0.22	96.47 (31.53)	97.58 (48.17)	–0.17 –0.15 0.02
IL-17	10.21 (14.19)	13.23 (15.64)	–0.06 0.15 0.21	8.99 (14.81)	10.83 (14.09)	–0.14 –0.01 0.13
RANTES	108.20 (65.40)	121.16 (74.33)	–0.03 0.15 0.18	105.01 (64.79)	105.55 (77.47)	–0.08 –0.07 0.01
IL-4	30.90 (15.98)	34.20 (21.24)	–0.01 0.16 0.17	31.57 (18.12)	27.68 (20.11)	0.03 –0.18 –0.20
IFN-γ	459.13 (302.11)	523.13 (409.56)	–0.03 0.14 0.17	469.12 (336.00)	430.55 (414.31)	–0.01 –0.11 –0.10
IL-2	63.23 (36.69)	70.99 (50.73)	–0.03 0.14 0.17	65.79 (43.25)	57.99 (48.68)	0.03 –0.14 –0.17
IL-5	8.56 (7.37)	9.76 (8.66)	–0.03 0.12 0.15	8.74 (8.03)	8.09 (8.47)	–0.01 –0.09 –0.08
IP-10	16,178.8 (18,535.2)	17,740.3 (10,749.0)	0.04 0.17 0.13	12,080.6 (7,032.0)	15,900.5 (10,005.1)	–0.31 0.01 0.32

N=Normal, AD=Aqueous deficient, ES = effect size, cytokine levels in pg/mL (± standard deviation). [T]*p*-value from Satterthwaite *t*-test for mean differences adjusting for unequal variances < 0.05. [K]*p*-value from Kruskal-Wallis nonparametric test for distributional differences < 0.05. [B]*p*-values from both Kruskal-Wallis and Satterthwaite *t*-tests < 0.05. ** Both NS and WO tears show a significant difference between Normal and AD group. *Only NS tears show a significant difference between Normal and AD group. FGF-basic and MIP-1β are not included because of low detection rate among patients.

Figure 5 shows the standardized cytokine levels in NS and WO tears for the non-AD and AD groups. The Z-score, or vertical distance, between non-AD and AD samples for each cytokine and tear sample type is the statistical "effect size." The larger the effect

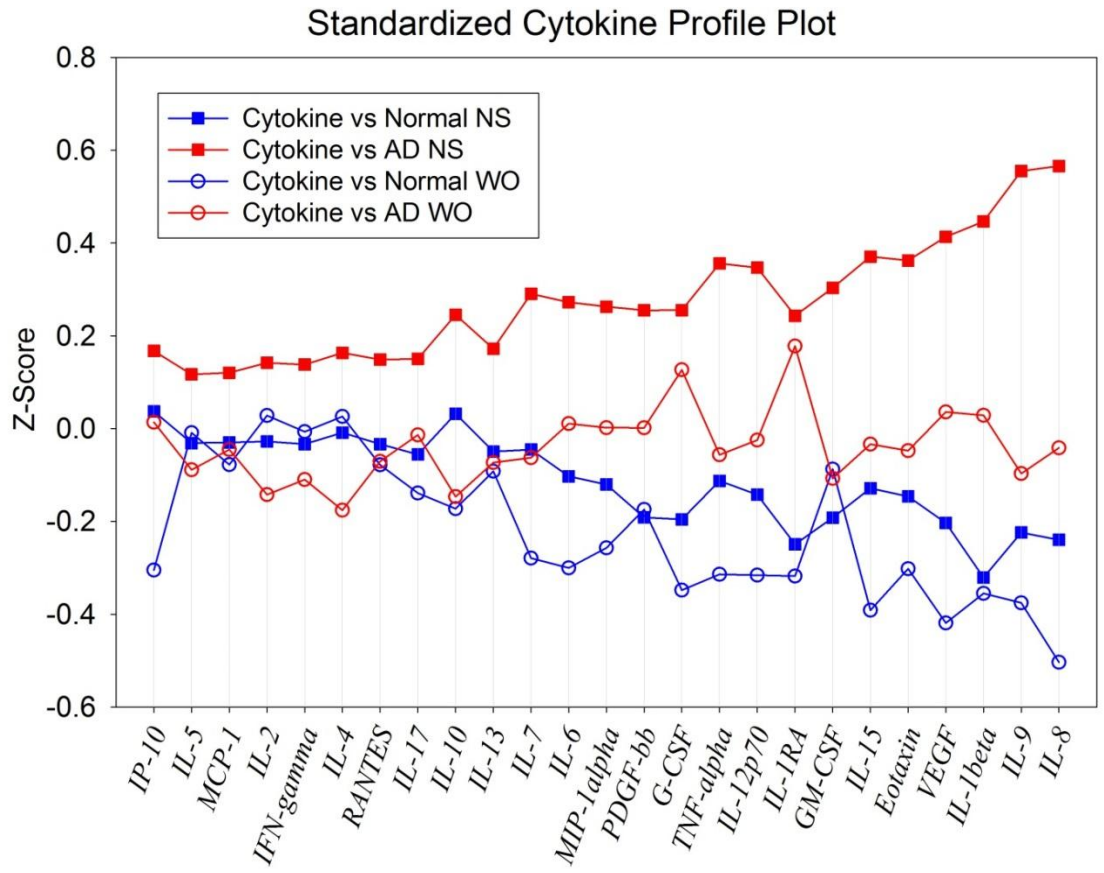

Figure 5: Tear data for each cytokine and collection method are standardized to have a mean of 0 and standard deviation of 1. This allows an equivalent determination across all cytokines of the ability to differentiate AD patients from non-AD using NS tear samples (filled symbols) and WO samples (open symbols). Cytokines are ordered on the X-axis by Z score difference between AD NS and Normal NS tear samples. AD=Aqueous deficient, NS=non-stimulated, WO= washout.

size, the more significant the difference between non-AD and AD assays. For the majority of cytokines, the Z-score for NS differences between AD and non-AD groups is greater than the corresponding WO Z-score. IL-8 and IL-1β elicited the greatest effect sizes for NS tears and also showed significant effect sizes for WO tears. The IL-8 correlation plots of NS versus WO tear levels for AD and non-AD groups (Figure 6) clearly demonstrate the substantial increase in scatter and range of values for the AD

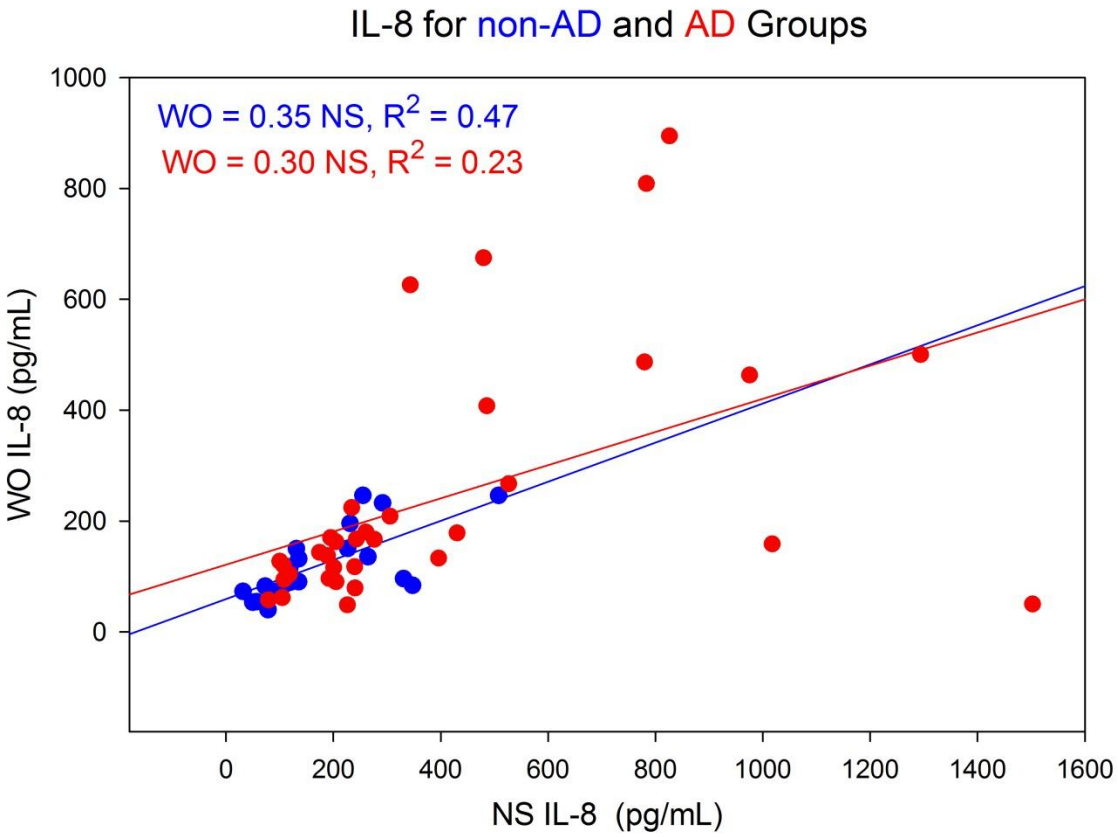

Figure 6: Scatterplot showing correlation between NS and WO tear levels of IL-8 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Slope of the linear regression lines shows that WO tear IL-8 levels averaged about one third of NS levels for both groups, correlations both being significant ($p < 0.001$ non-AD; $p < 0.005$ AD). NS = non-stimulated, WO = washout, AD=aqueous deficient.

group. However, with few exceptions, the WO IL-8 levels in AD patients are substantially higher than in non-AD patients. The corresponding IL-8 Bland-Altman plots (Figure 7) reveal the spread of differences between NS and WO levels in AD patients and show several points near or above the two standard deviation limit for difference between NS and WO IL-8 levels. This is a visual indicator of the lower effect size in differentiating AD from non-AD patients for WO tears. Significant positive proportional bias is present for both AD and non-AD groups.

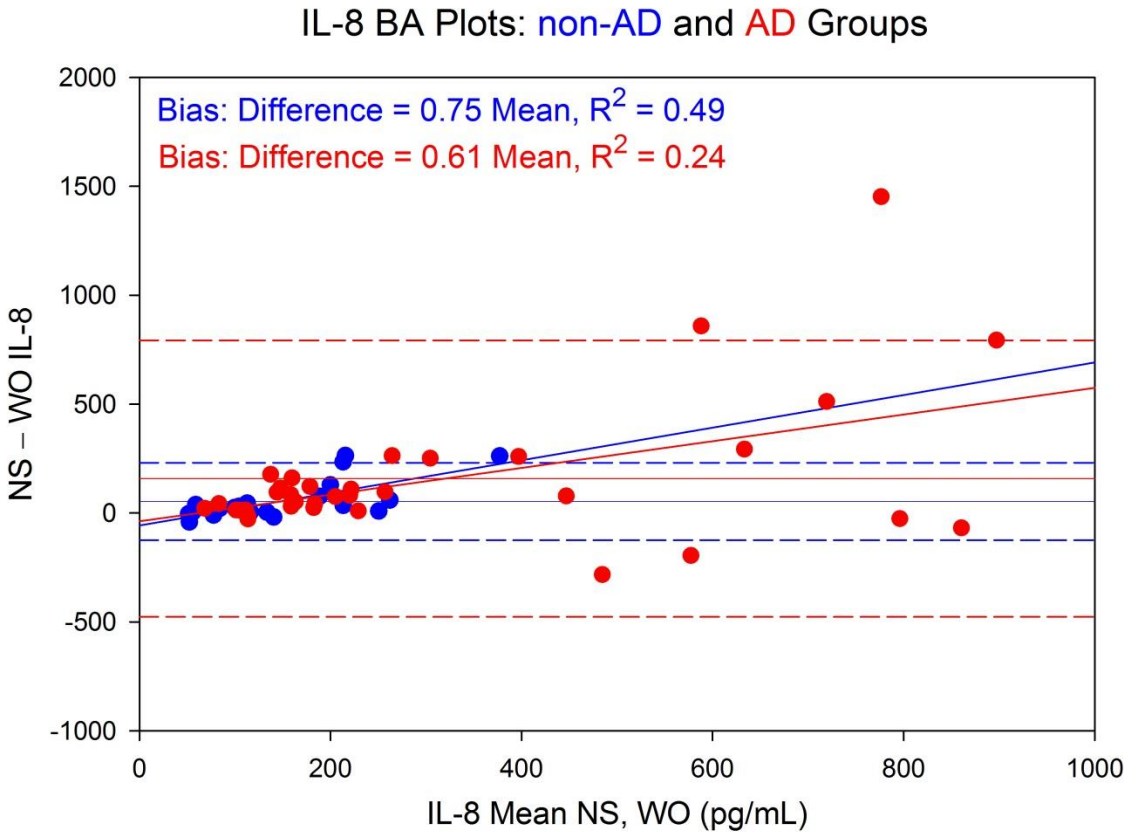

Figure 7: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-8 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear IL-8 levels is plotted against mean level for paired tear samples. Slope of the BA plot is positive for both groups and significant ($p<0.001$ non-AD; $p<0.003$ AD), showing an increasing difference with increasing mean. Dashed lines represent 95% confidence limits.

Based on the effect size for IL-1β in distinguishing AD from non-AD patients (0.77 for NS tears and 0.38 for WO tears), the statistical power to differentiate AD from non-AD patients is considerably higher in NS tears (Table 3 and Figure 5). Unlike the IL-8 scatterplots, IL-1β shows greater scatter within the non-AD than AD group according to the scatterplot correlations coefficients in Figure 8. The smaller range of tear IL-1β levels in the non-AD group makes this difficult to appreciate from the scatterplots. IL-1β Bland-Altman plots (Figure 9) demonstrate, as was the case for IL-8, that for most AD patients, NS IL-1β levels exceed WO levels; some by a wide margin. Significant

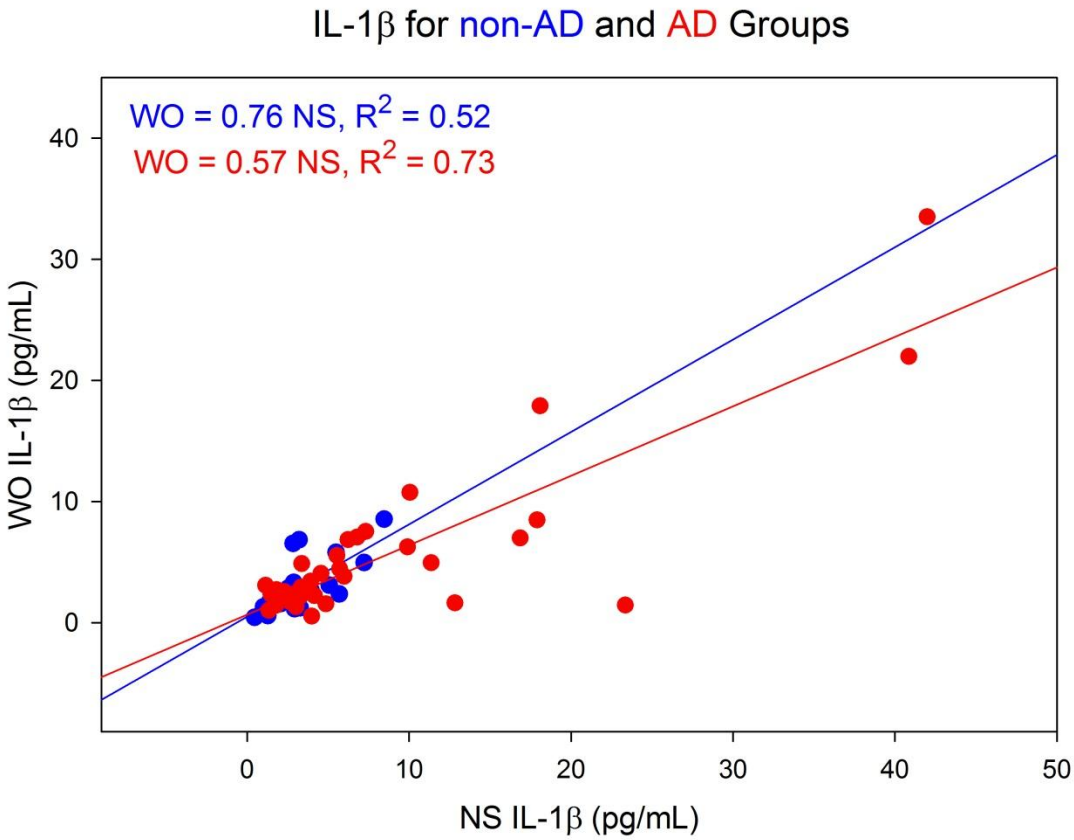

Figure 8: Scatterplot showing correlation between NS and WO tear levels of IL-1β for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Slope of the linear regression lines shows that WO IL-1β levels were closer to those of NS levels in the non-AD group. Correlations were both significant ($p < 0.001$ non-AD; $p < 0.001$ AD). NS = non-stimulated, WO = washout, AD = aqueous deficient.

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proportional plot bias is also present in the IL-1β Bland-Altman plots.

VEGF shows a smaller difference in effect size between NS and WO tears for distinguishing AD from non-AD patients between. Scatterplots show a strong correlation between NS and WO tear VEGF levels in the AD group that is more balanced between the lower and higher tear levels than was the case for IL-8 or IL-1β (Figure 10). The Bland-Altman plots for VEGF also show a more balanced difference between NS and average, the NS tear VEGF level in the AD group exceeds the WO level by approximately 100 pg/mL.

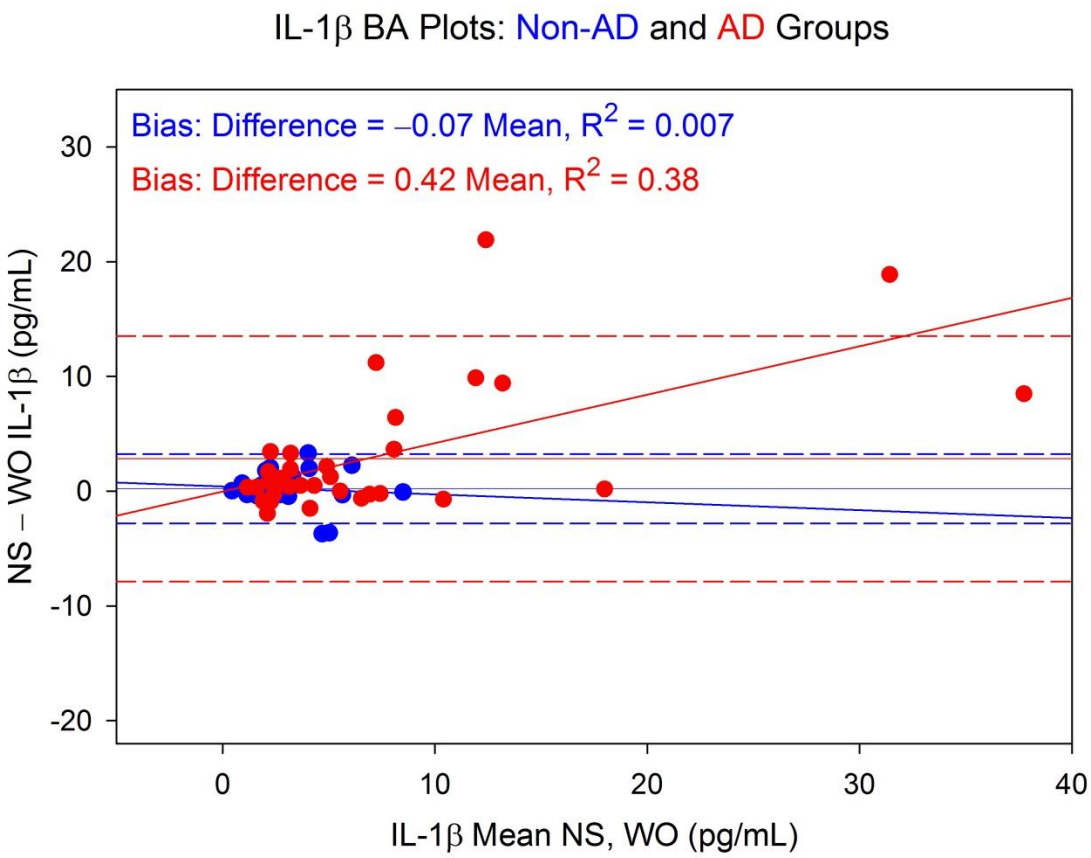

Figure 9: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-1β for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear IL-1β levels is plotted against mean level for paired tear samples. Slope of the BA plot is negative and not significant for the non-AD group, but positive and significant ($p<0.001$) for the AD group, showing an increasing difference with increasing mean. Dashed lines represent 95% confidence limits.

For cytokines, IL-8, IL-1β and VEGF, the difference between NS and WO effect size is due primarily to the AD group. However, IL-1RA and G-CSF show similar effect sizes for NS and WO tears (Figure 5, Table 3). The IL-1RA scatterplot shows greater scatter of NS versus WO IL-1RA levels in the non-AD group versus AD group (Figure 12). Bland-Altman plots for IL-1RA reveal no proportional plot bias and almost zero fixed bias for the AD group (Figure 13). Isolated data points also fall both above and below the two standard deviation mean difference range.

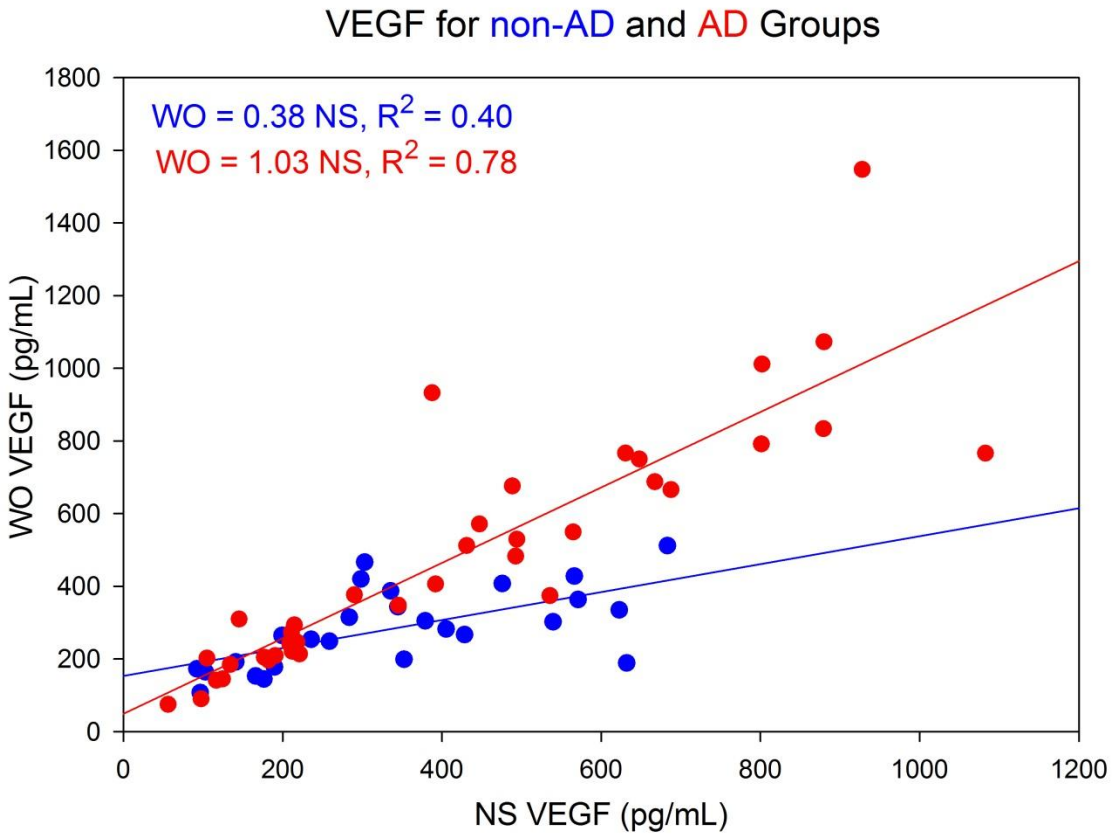

Figure 10: Scatterplot showing correlation between NS and WO tear levels of VEGF for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Slope of the linear regression line shows that WO tear VEGF levels averaged 63% of NS levels for both groups, correlation coefficients both being significant ($p < 0.001$). NS = non-stimulated, WO = washout, AD=Aqueous deficient.

G-CSF presents an interesting example of strongly and similarly correlated NS and WO tear levels for both the AD and non-AD groups (Figure 14) and a greater degree of Bland-Altman plot proportional bias in the non-AD group (Figure 15). The significant effect sizes for both NS and WO tears in differentiating AD from non-AD groups indicates that the mere presence of Bland-Altman plot proportional bias in either patient group does not necessarily preclude the ability to distinguish AD from non- AD patients with both tear sample types. This indicates that G-CSF may be a relatively strong biomarker for distinguishing patient groups.

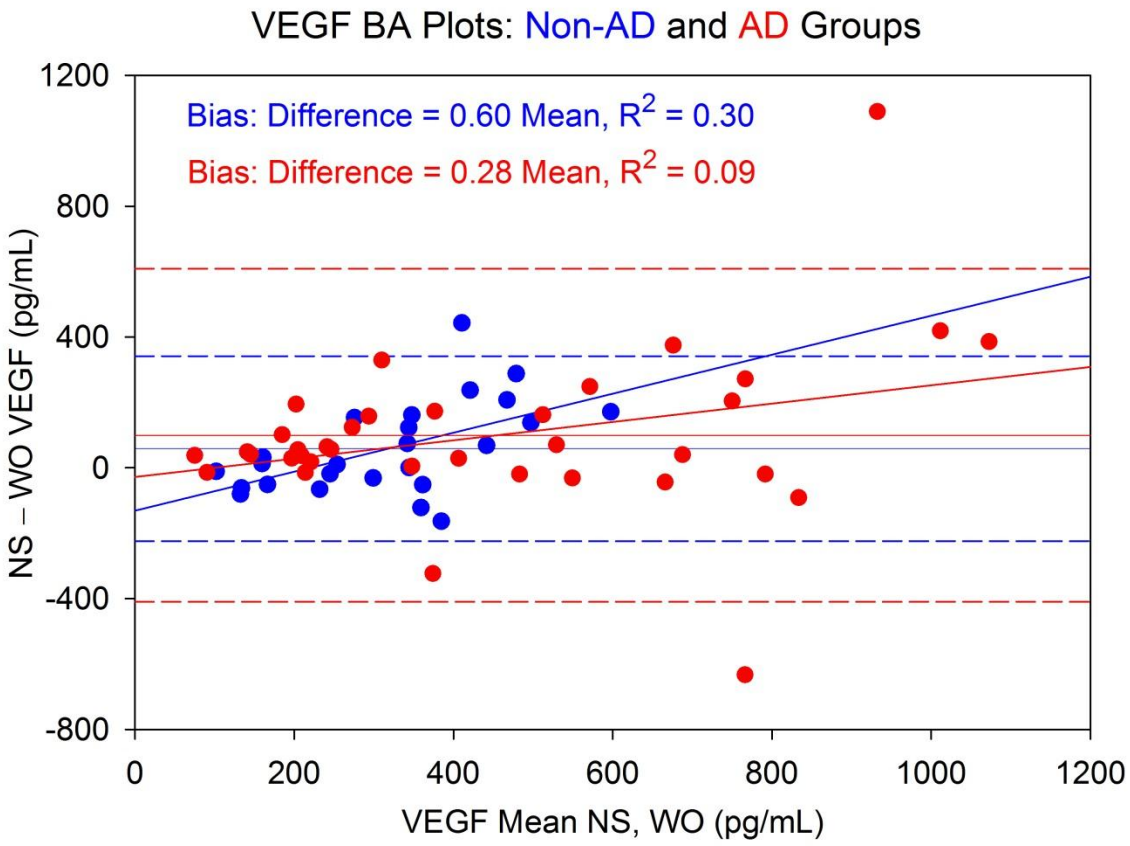

Figure 11: Bland–Altman (BA) plot showing NS versus WO tear levels of VEGF for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear VEGF levels is plotted against mean level for paired tear samples. Slope of the BA plot is positive for both groups, showing an increasing difference with increasing mean. Dashed lines represent 95% confidence limits.

Five cytokines, IL-9, Eotaxin, IL-15, GM-CSF, and IL-12p70, showed significant differences between non-AD and AD samples in NS tears only. In all cases, the main contributor to the effect size was higher NS levels in the AD relative to non-AD group. Bland-Altman plots for the five cytokines are shown in Figures 16-20 respectively. For IL-9 (Figure 16) and IL-15 (Figure 18), the lack of effect size for WO tears could be attributable in part to weak, but significant, Bland-Altman plot proportional bias in the AD group. In addition, an unusual trend of statistically significant negative Bland-Altman

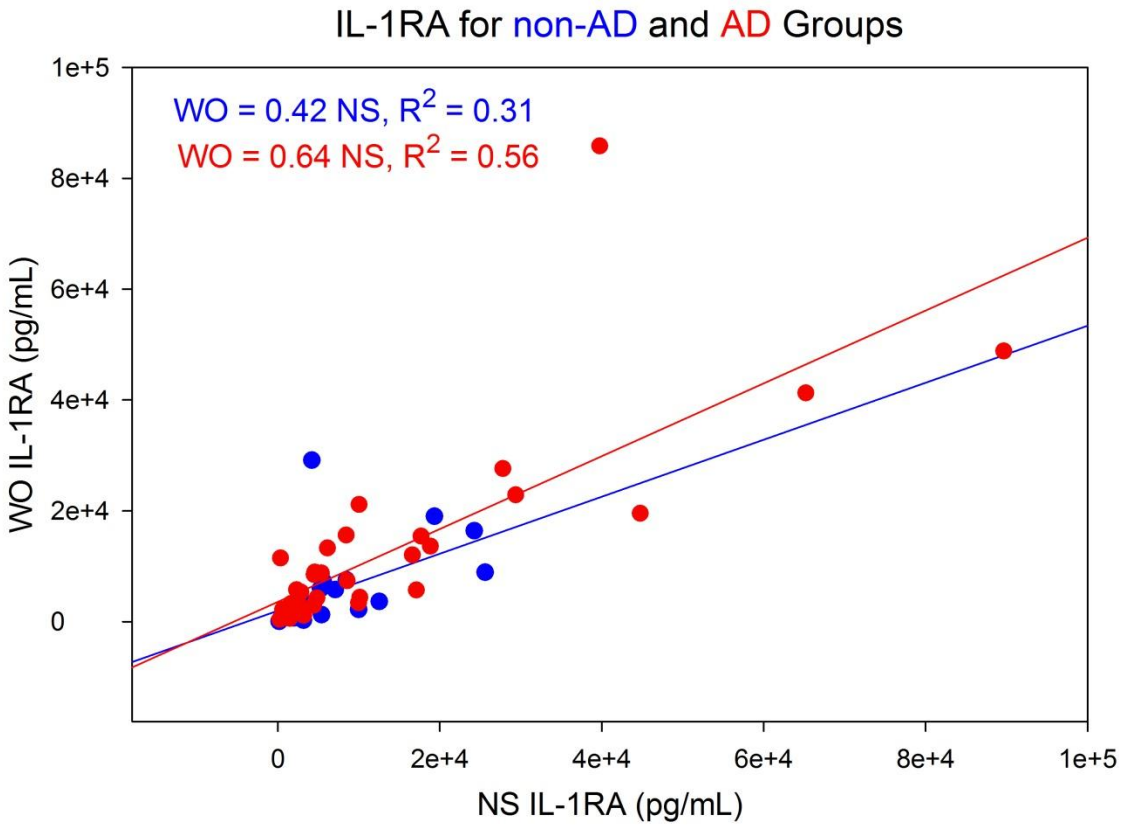

Figure 12: Scatterplot showing correlation between NS and WO tear levels of IL-1RA for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Slope of the linear regression line shows that WO tear IL-1RA levels were closer to NS levels for the AD group. Both correlation coefficients were significant ($p < 0.001$). NS = non-stimulated, WO = washout, AD=Aqueous deficient.

plot proportional bias in the non-AD group for GM-CSF is evident in Figure 19. This would contribute to a lower mean NS tear GM-CSF value in the non-AD group, making it more likely that the AD group level would be significantly higher.

The cytokines showing the least potential to differentiate non-AD from AD patients in either NS or WO tears were IL-4, IFN-γ, IL-2, IL-5, and IP-10 (Table 3). However, these cytokines lack Bland-Altman bias and do show a strong correlation between NS and WO assays for both AD and non-AD groups. Examples are shown for

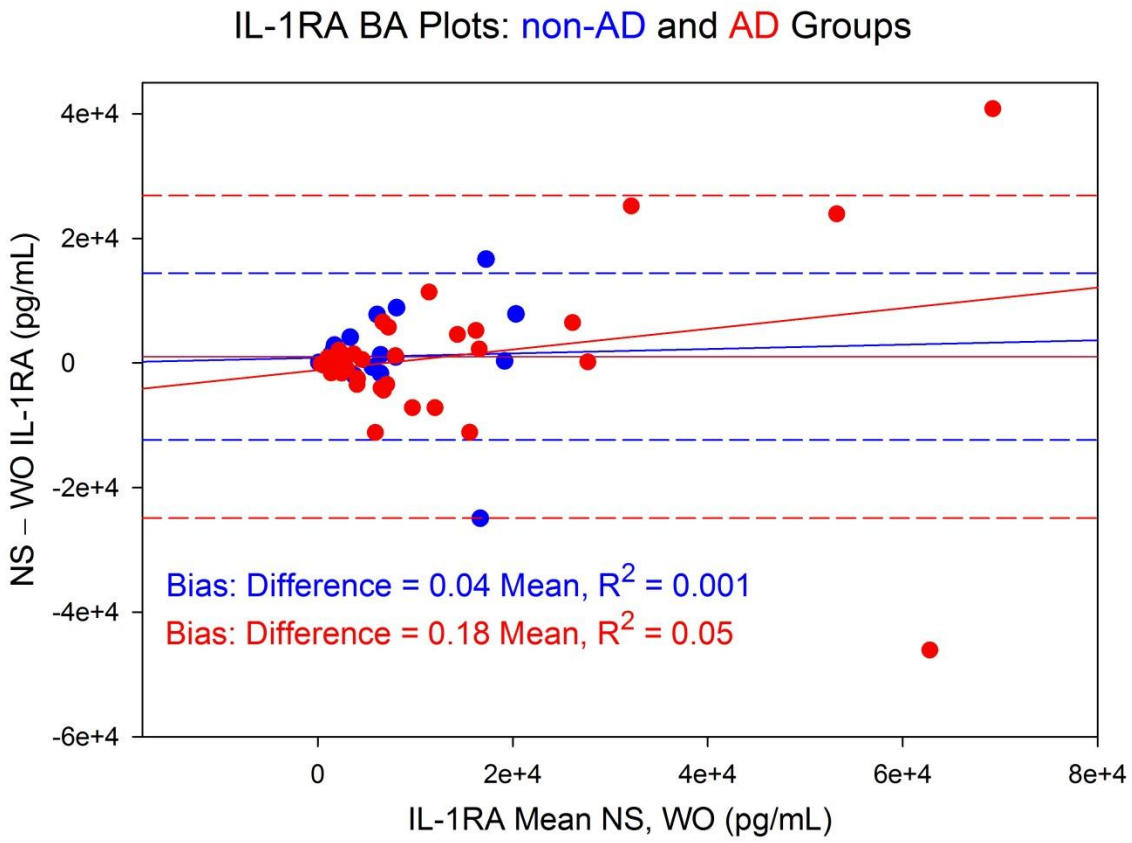

Figure 13. Bland–Altman (BA) plot showing NS versus WO tear levels of Il-1RA for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear IL-1RA levels is plotted against mean level for paired tear samples. Slope of the BA plot is positive for both groups, but not significant. Dashed lines represent 95% confidence limits.

IL-4 (Figures 21 and 22) and IL-5 (Figures 23 and 24) respectively. Therefore, these cytokines can be effectively measured in both NS and WO tears, but neither tear type can discriminate between non-AD and AD patients (Figure 7 and Table 3). In other words, regardless of tear collection method, IL-4, IFN-γ, IL-2, IL-5, and IP-10 do not appear to be useful biomarkers for AD dry eye according to the current study.

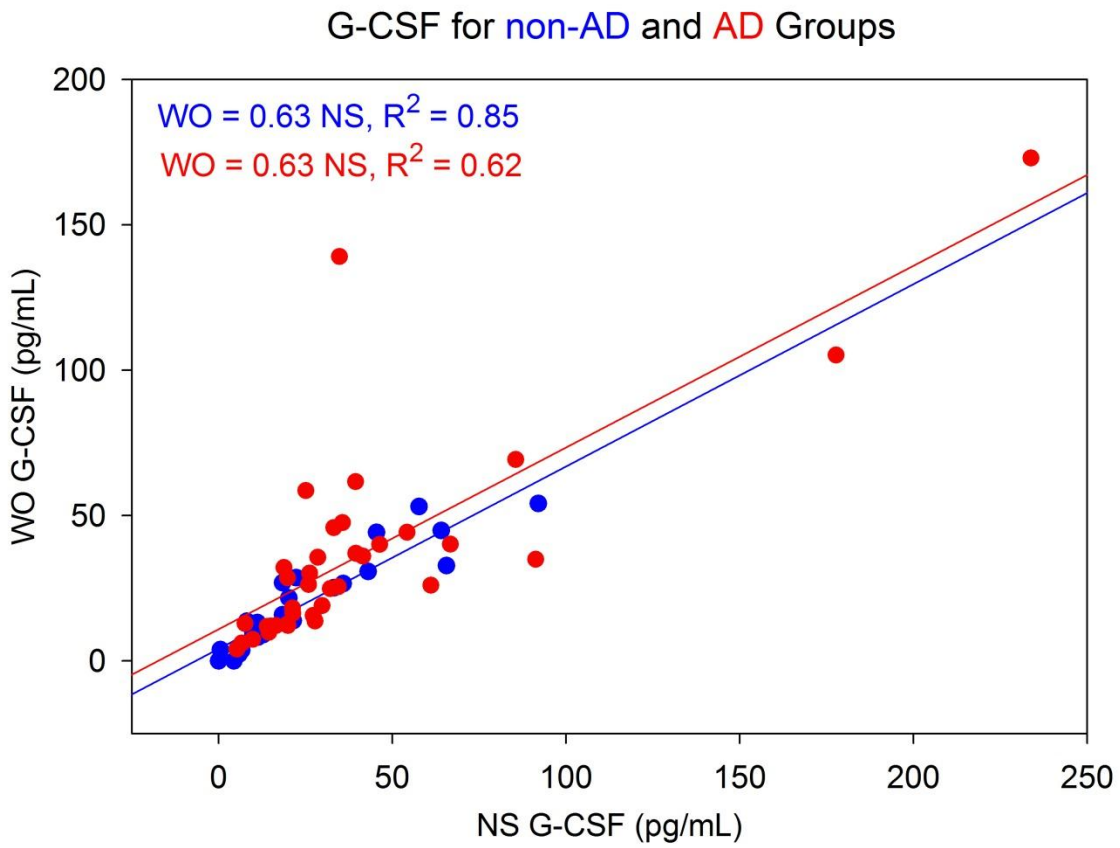

Figure 14: Scatterplot showing correlation between NS and WO tear levels of G-CSF for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Slope of the linear regression line shows that WO tear G-CSF levels averaged 63% of NS levels for both groups, correlation coefficients both being significant ($p < 0.001$). NS = non-stimulated, WO = washout, AD=Aqueous deficient.

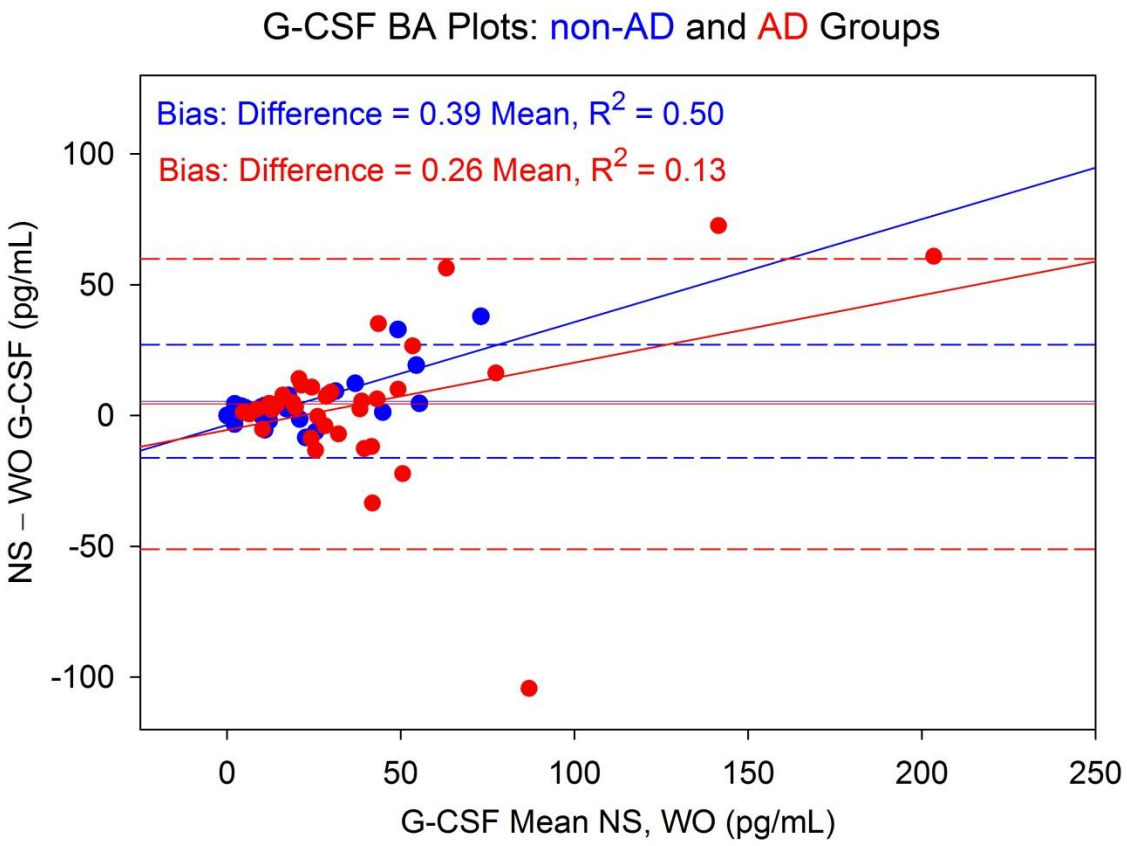

Figure 15: Bland–Altman (BA) plot showing NS versus WO tear levels of G-CSF for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear G-CSF levels is plotted against mean level for paired tear samples. Slope of the BA plot is positive for both groups, but significant only for the non-AD group ($p<0.001$). Dashed lines represent 95% confidence limits.

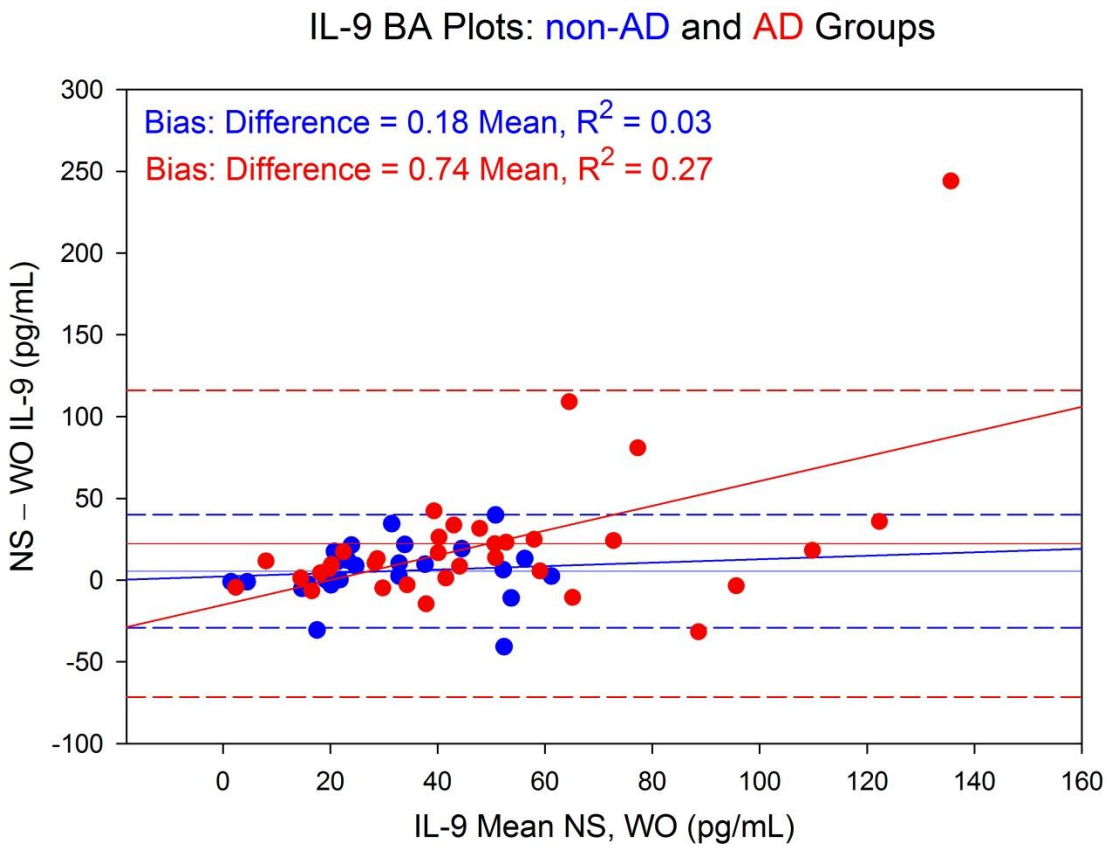

Figure 16: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-9 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear IL-9 levels is plotted against mean level for paired tear samples. Slope of the BA plot is negligible for the non-AD group, but positive and significant for the AD group ($p<0.001$). Dashed lines represent 95% confidence limits.

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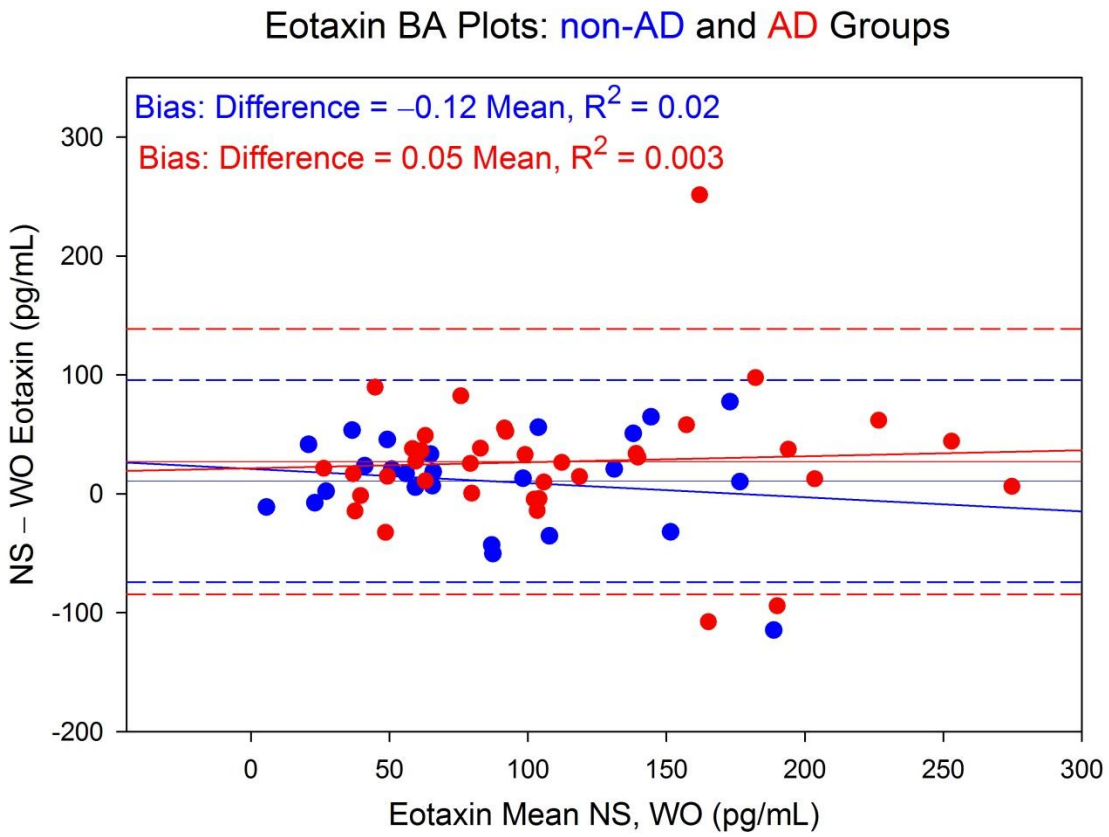

Figure 17: Bland–Altman (BA) plot showing NS versus WO tear levels of Eotaxin for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear Eotaxin level is plotted against mean level for paired tear samples. Slopes of the BA plots are small and not statistically significant for both groups. Dashed lines represent 95% confidence limits.

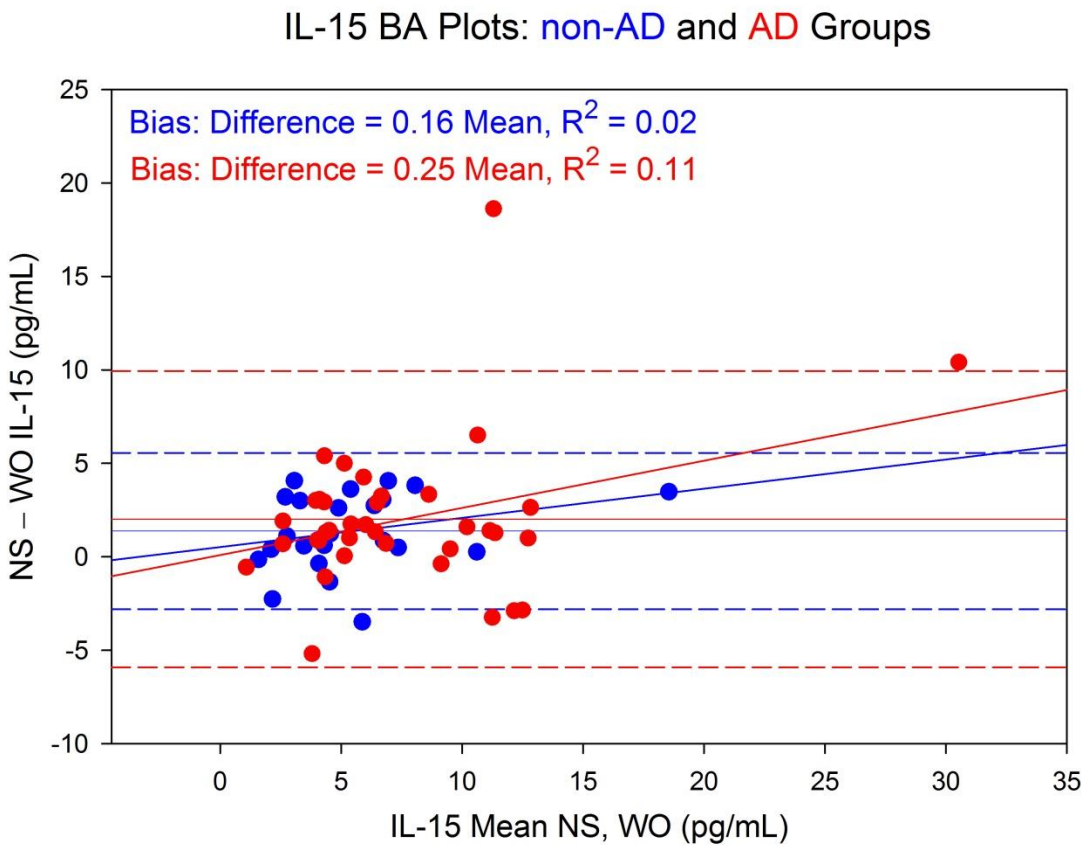

Figure 18: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-15 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear IL-15 levels is plotted against mean level for paired tear samples. Slope of the BA plot is negligible for the non-AD group, but positive and significant for the AD group. Dashed lines represent 95% confidence limits.

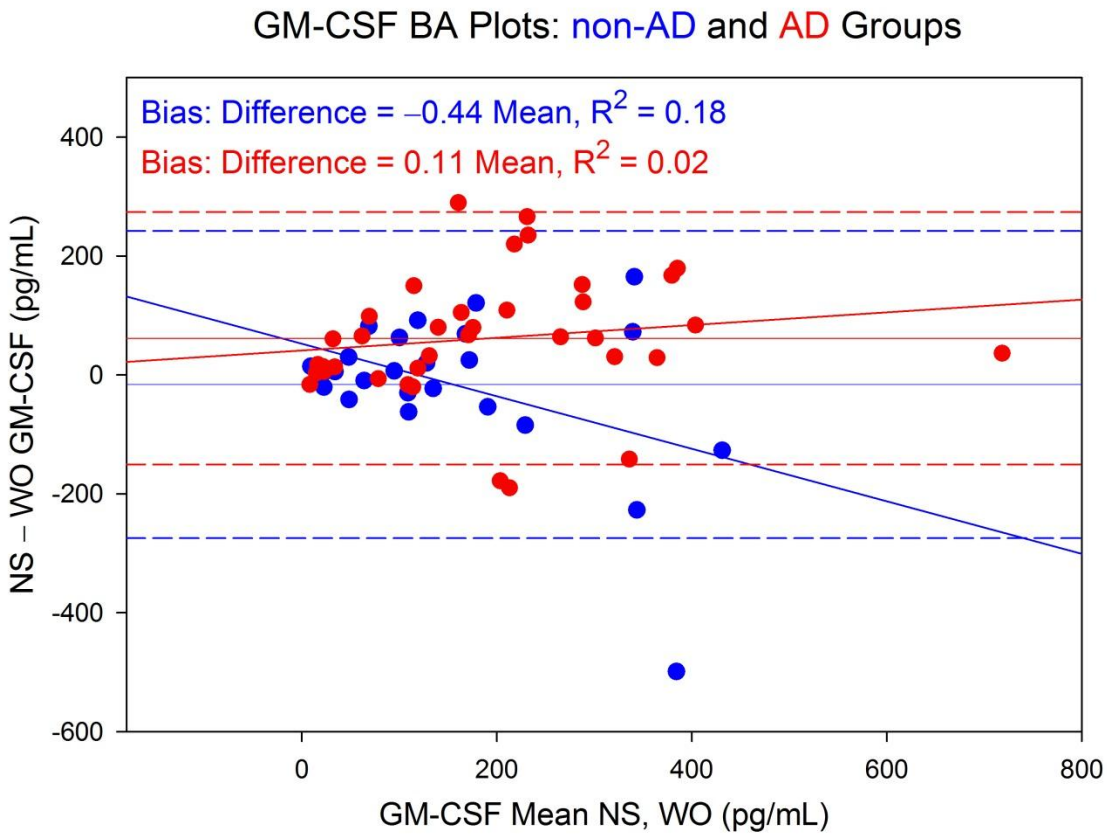

Figure 19: Bland–Altman (BA) plot showing NS versus WO tear levels of GM-CSF for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear GM-CSF levels is plotted against mean level for paired tear samples. Slope of the BA plot is negative and significant for the non-AD group, and negligible for the AD group. Dashed lines represent 95% confidence limits.

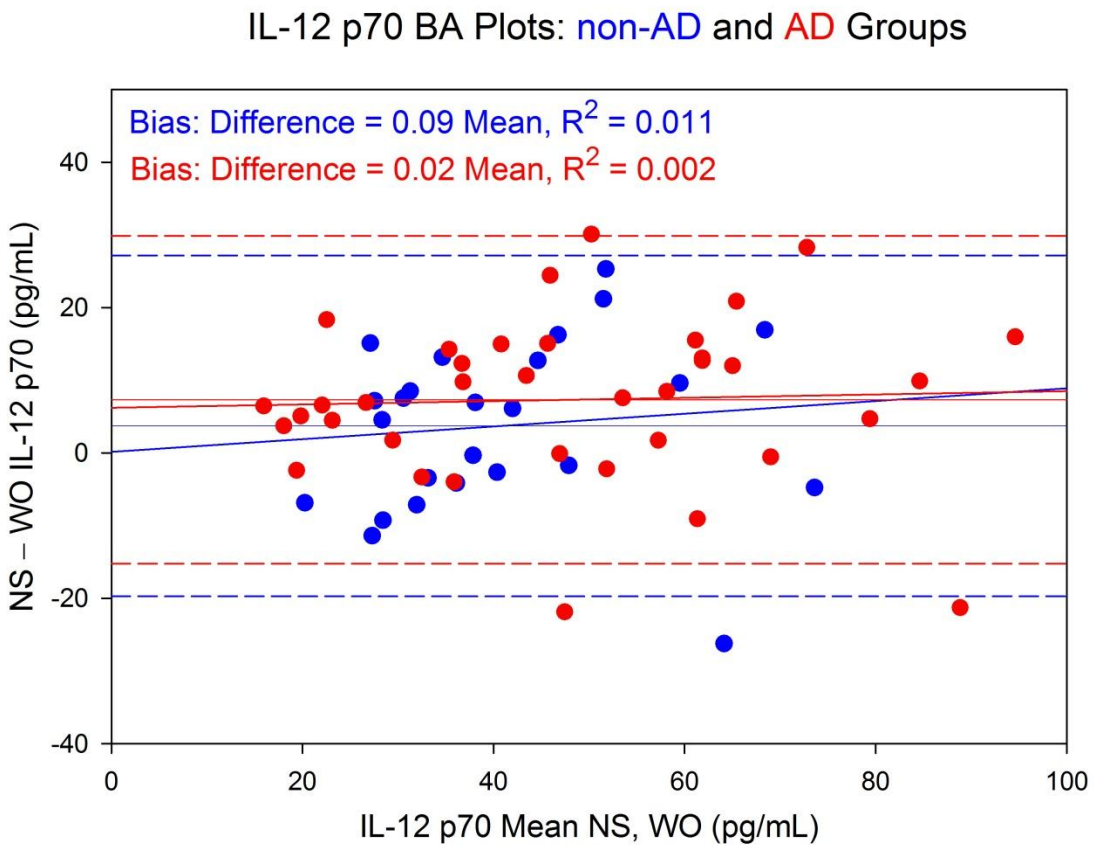

Figure 20: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-12p70 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear IL-12p70 levels is plotted against mean level for paired tear samples. Slope of the BA plots are negligible and not statistically significant for both groups. Dashed lines represent 95% confidence limits.

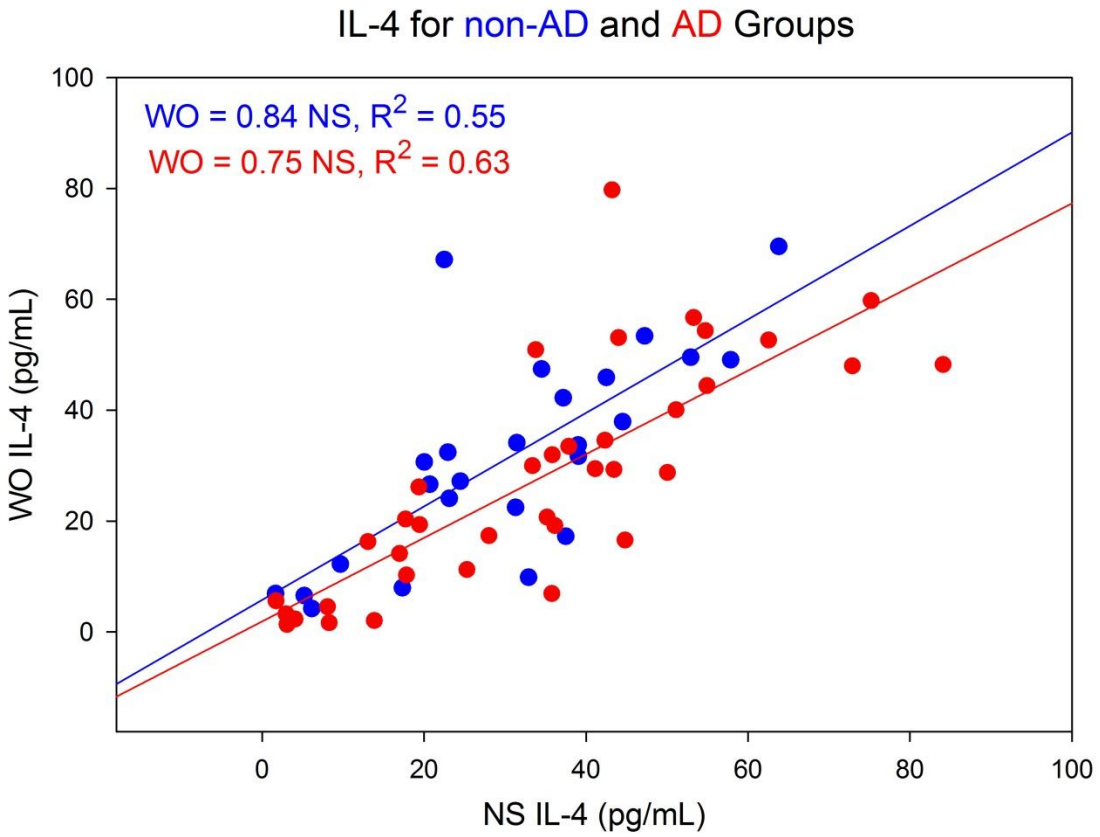

Figure 21: Scatterplot showing correlation between NS and WO tear levels of IL-4 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Slope of the linear regression line shows that WO tear IL-4 levels averaged 84% and 75% of NS respectively for non-AD and AD groups. Both correlation coefficients were statistically significant ($p < 0.001$). NS = non-stimulated, WO = washout, AD=Aqueous deficient.

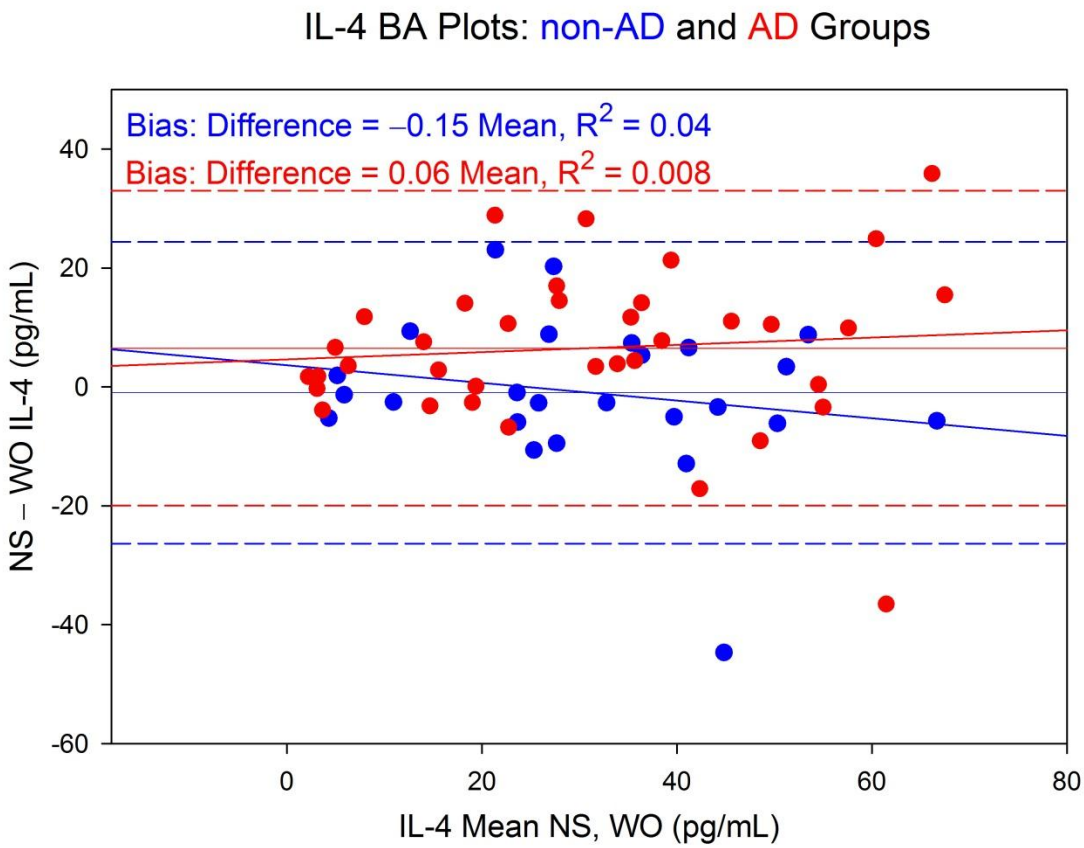

Figure 22: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-4 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear IL-4 levels is plotted against mean level for paired tear samples. Slope of the BA plot is small and not statistically significant for both groups. Dashed lines represent 95% confidence limits.

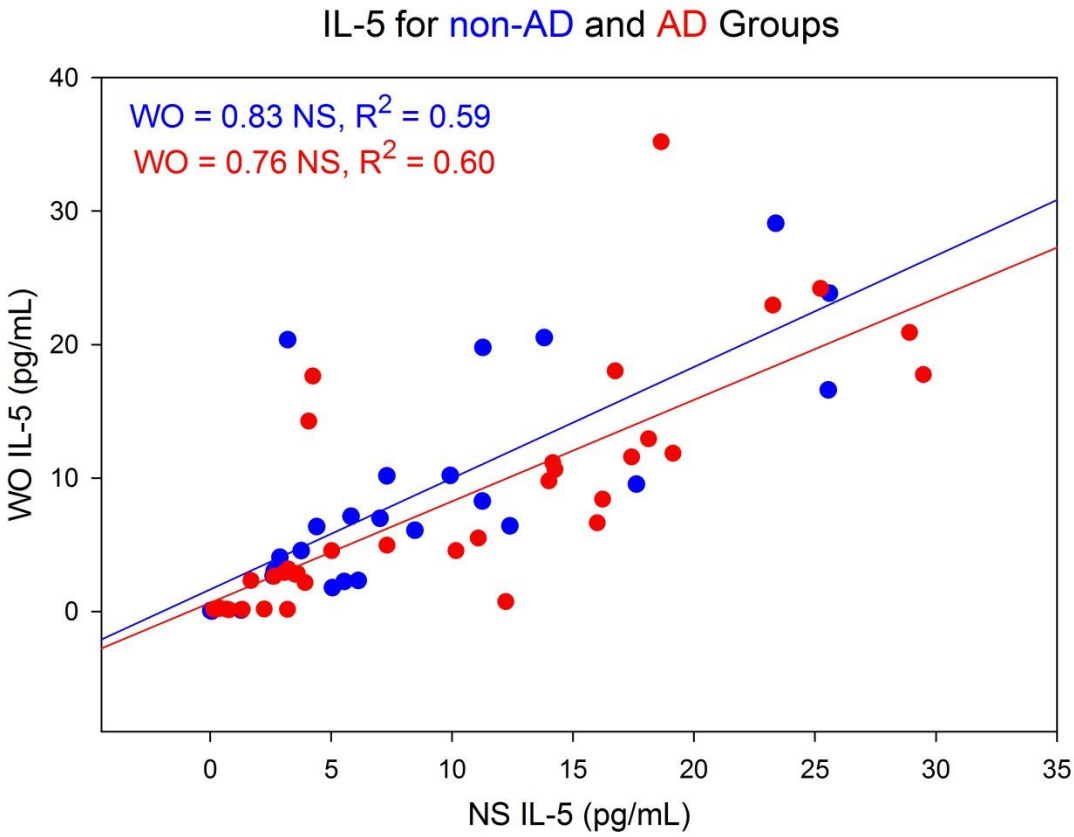

Figure 23: Scatterplot showing correlation between NS and WO tear levels of IL-5 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Slope of the linear regression line shows that WO tear IL-5 levels averaged 83% and 76% of NS respectively for non-AD and AD groups. Both correlation coefficients were statistically significant ($p < 0.001$). NS = non-stimulated, WO = washout, AD=aqueous deficient.

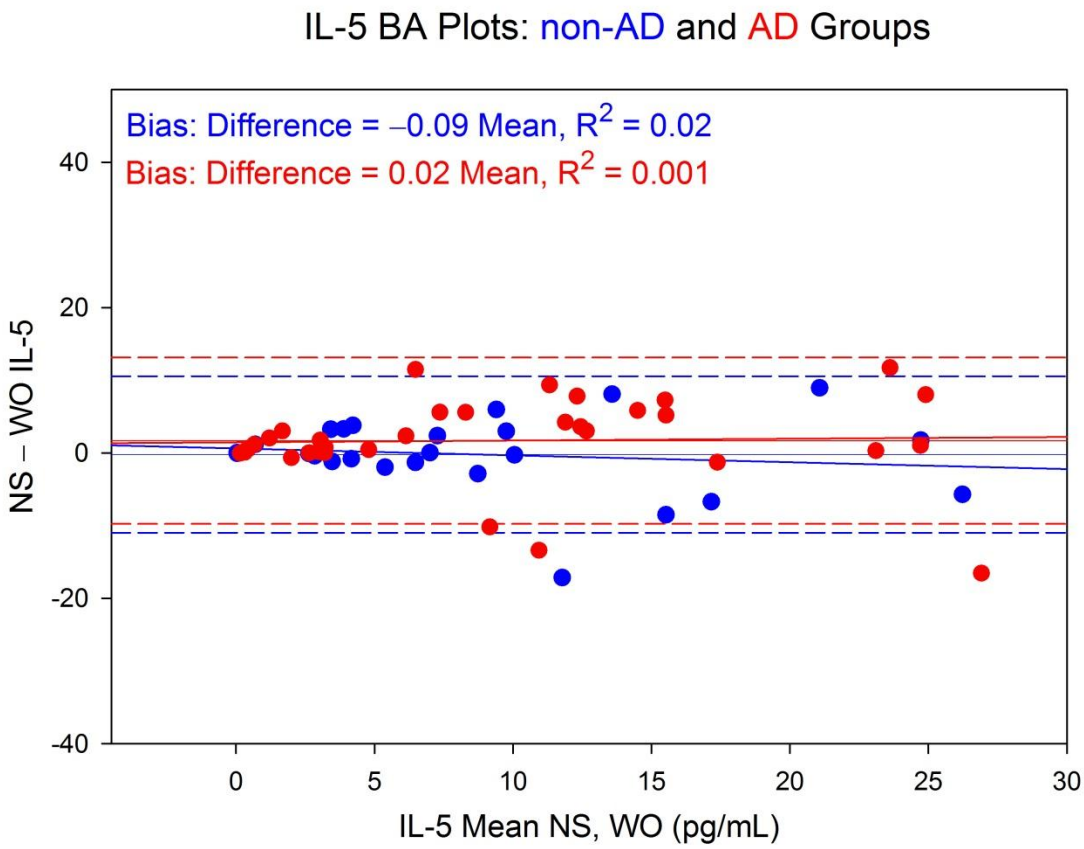

Figure 24: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-5 for normal (blue circles) and aqueous-deficient (AD, red circles) patient groups. Difference between NS and WO tear IL-5 levels is plotted against mean level for paired tear samples. Slopes of both BA plots are negligible and not statistically significant for both groups. Dashed lines represent 95% confidence limits.

Relationship between Schirmer Score and Tear Cytokine Levels

IL-8 is the most likely cytokine to be produced by ocular surface sources because its levels decrease significantly with increasing tear flow rate[6]. Therefore, this cytokine should be most likely to reveal any influence of Schirmer score and tear collection rate on tear cytokine levels. Figure 25 shows NS IL-8 versus Schirmer wetting length and Figure 26, the corresponding plot of WO IL-8 versus Schirmer wetting length. The lack of

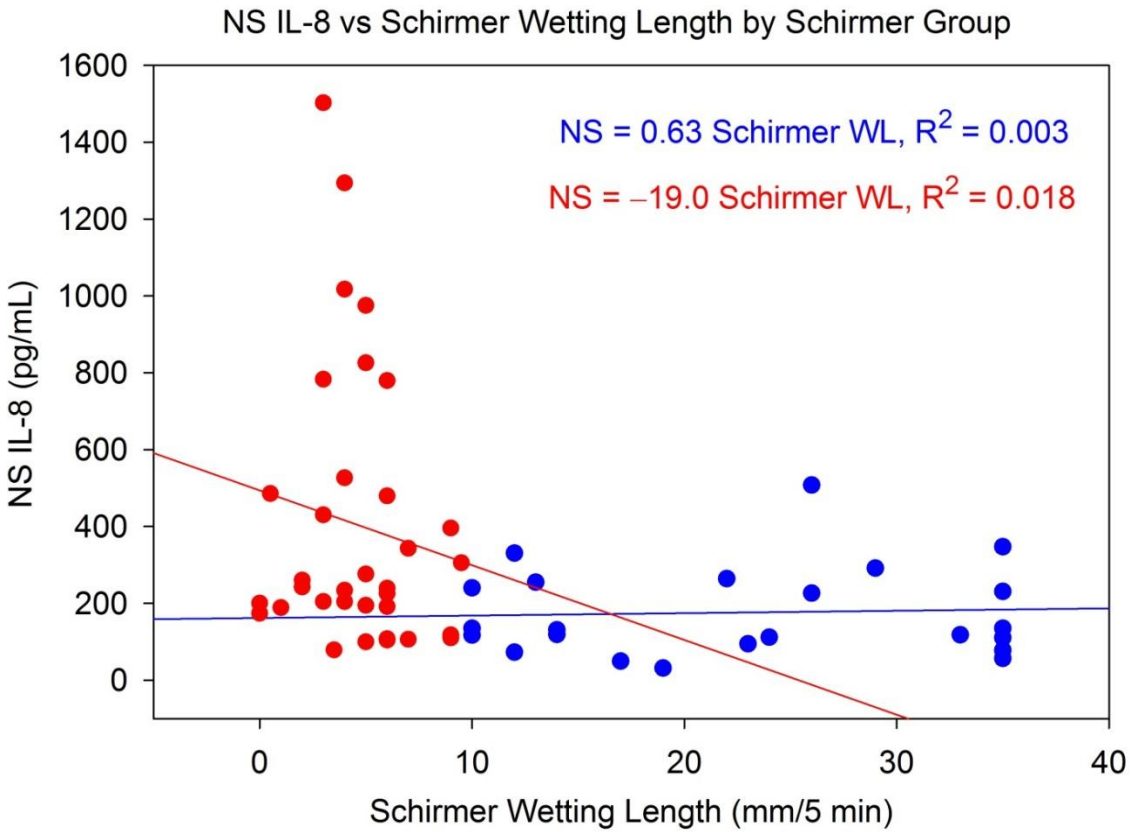

Figure 25: Relationship between NS tear IL-8 level and Schirmer test score (5 minute wetting length) for normal group (blue circles) and AD group (red circles). Neither correlation is statistically significant. WL = wetting length, NS = non-stimulated.

significant trends between cytokine level and Schirmer score within AD and non-AD groups indicates tear collection rate per se was not an important determinant of tear IL-8 levels.

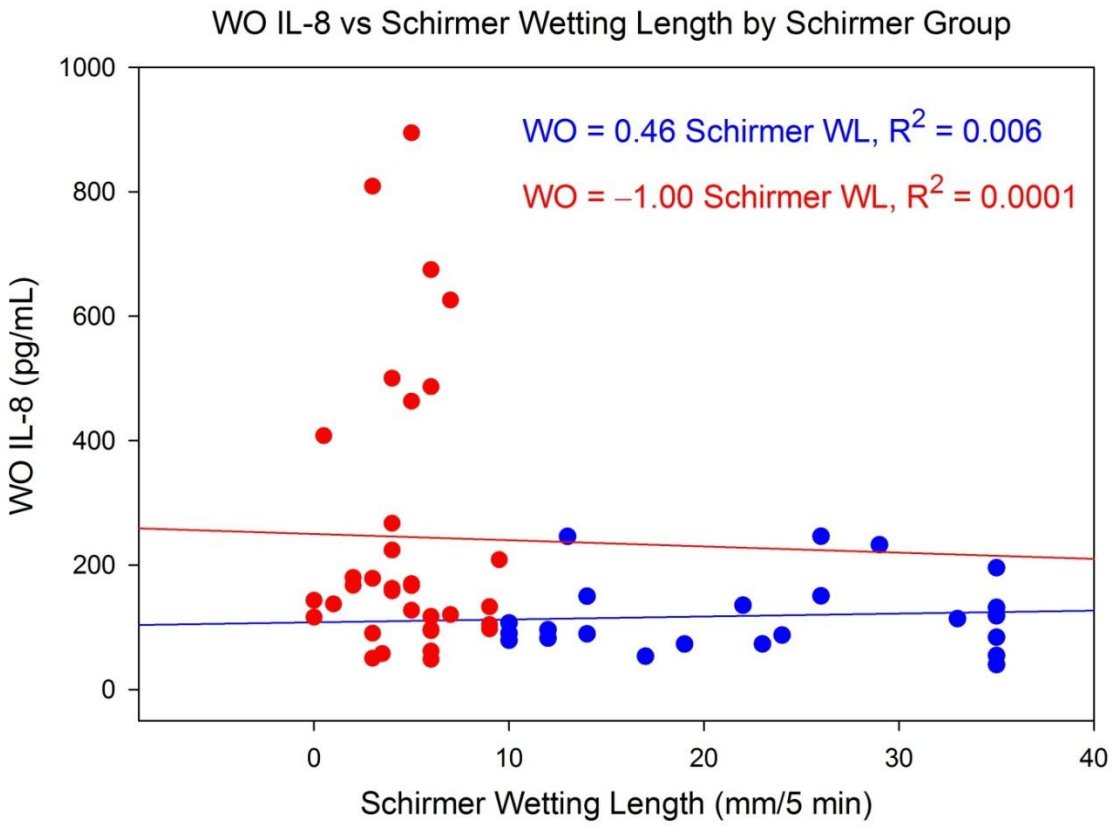

Figure 26: Relationship between WO tear IL-8 level and Schirmer test score (5 minute wetting length) for normal group (blue circles) and AD group (red circles). Neither correlation is statistically significant. WL = wetting length, WO = washout.

Age Effects

While the mean age of patients in the AD group was older than that of the non-AD group, the mean difference was not statistically significant ($p < 0.08$). However, to fully investigate potential age effects, age contributions to the AD vs non-AD differences in tear cytokine levels were tested. Analysis of covariance (ANCOVA) models with each tear cytokine assay as the dependent variable, AD status as group variable, and age as the covariate were performed. Pearson correlations were used to estimate the overall relationship of age with each cytokine assay. Patient age was significantly correlated to

both the NS ($R = 0.32$, $p < 0.01$) and WO ($r = 0.28$, $p < 0.02$) IL-1β assays. However, for the IL-1β NS assays, there were still significant differences between the AD and non-AD groups ($p < 0.03$) after adjusting for age, which remained a statistically significant predictor ($p < 0.04$). The overall model explained 16.7% of the variation in the IL-1β NS measurements with 6.7% being due to the AD vs. non-AD mean differences and age explaining 6.1% of the variation. A similar distribution of age and AD group status was found for the IL-1β WO assays.

For IL-1RA, age was significantly related to the WO assays ($R = 0.32$, $p < 0.01$), but age was not a significant factor in any of the ANCOVA models. In the case of IL-8, age was not significantly correlated with either the NS or WO assays. In the IL-8 ANCOVA models, the AD versus non-AD groups were still significantly different for both NS and WO assays after adjusting for age as a covariate.

In the case of G-CSF, patient age was significantly correlated to both the NS ($R = 0.28$, $p < 0.02$) and WO ($R = 0.25$, $p < 0.05$) assays. In the ANCOVA models, however, age was of marginal significance. For VEGF, age was not significantly correlated with either the NS or WO assays and was not a significant predictor in either ANCOVA model.

Interestingly, for IP-10, age was strongly related to NS assay results ($R = 0.33$, $p < 0.01$). However, this relationship appeared to be strongly influenced by outlier effects, the main contribution coming from a single patient with very high NS tear IP-10 levels. This also occurred in the ANCOVA models with both age and AD status being statistically significant. It is therefore unclear how age affects NS IP-10 measurements

because of the outlier effects. Age was not significantly related to IP-10 WO assays nor was age significant in the ANCOVA models.

To summarize, based on ANCOVA, age was not an important determinant of differences between the non-AD and AD groups. Significant differences between non-AD and AD groups remained after factoring out age effects for most dry eye-related cytokines. Therefore, despite the fact that the mean age of the AD group was older, age was not the primary cause of tear cytokine level differences between the AD and non-AD groups.

NS vs WO vs Stim Tear Comparison

Fifteen participants completed this study of all three tear sampling methods. While the study deliberately included both non-AD and AD patients, it was not intended as a second comparison of the two patient groups. The number of participants (n = 15) precluded a meaningful analysis for separate non-AD and AD groups. Results are shown in Table 4.

Table 4: Mean Cytokine Levels (SD) in NS, WO and Stim Tears of 15 Participants

Cytokine	NS level	WO level	Stim level	NS v WO	NS v Stim	WO v Stim
IL-8	574 (797)	340 (488)	125 (151)	nsd	P<0.05 (A,K,Tu)	nsd
IL-1β	4.01 (3.18)	3.40 (2.96)	2.58 (2.19)	P<0.01 (Wil)	nsd	nsd
VEGF	526 (311)	360 (143)	316 (132)	P<0.02 (t-test)	P<0.04 (t-test)	nsd
IL-1RA	8,358 (8,560)	4,170 (3,337)	790 (665)	nsd	P<0.05 (A,K,Tu)	P<0.05 (A,K,Tu)
G-CSF	38.2 (21.7)	29.7 (20.0)	10.9 (11.2)	nsd	P<0.05 (A,K,Tu)	P<0.05 (A,K,Tu)
IL-9	36.1 (23.6)	28.9 (15.5)	29.0 (31.7)	nsd	nsd	nsd

Eotaxin	96.1	78.4	74.8**	nsd	nsd	nsd
	(54.4)	(39.4)	(59.3)			
IL-15	4.67	3.29	2.51	P<0.01	P<0.01	t-test
	(2.77)	(2.73)	(2.25)	(t-test)	(t-test)	
GM-CSF	133	82.1	82.0	P<0.01	nsd	nsd
	(88.1)	(52.8)	(70.9)	(t-test)		
IL-12p70	41.3	40.4	43.8	nsd	nsd	nsd
	(13.3)	(10.5)	(18.1)			
MCP-1	142	94.9	15.9	nsd	P<0.05	P<0.05
	(164)	(134)	(21.5)		(A,K,Tu)	(A,K,Tu)
TNF-α	500	327	377	P<0.01	nsd	nsd
	(315)	(158)	(318)	(t-test)		
PDGF-bb	14.4	11.2	12.5	nsd	nsd	nsd
	(11.2)	(5.8)	(11.1)			
MIP-1α	82.8	60.9	50.5	P<0.04	nsd	nsd
	(70.2)	(31.8)	(43.1)	(t-test)		
IL-6	60.5	40.5	20.0	nsd	P<0.05	nsd
	(40.3)	(27.2)	(12.9)		(A,K,Tu)	
IL-7	108.2	88.5	87.9	nsd	nsd	nsd
	(52.6)	(36.0)	(36.9)			
IL-13	14.3	12.5	13.5	P<0.02	nsd	nsd
	(4.3)	(2.6)	(7.0)	(t-test)		
IL-10	115	102	101	nsd	nsd	nsd
	(32.6)	(24.9)	(35.4)			
IL-17	8.36	6.72	5.18*	nsd	P<0.05	nsd
	(9.42)	(12.0)	(10.6)		(A,K,Tu)	
RANTES	94.2	80.5	97.6	nsd	nsd	nsd
	(34.1)	(29.8)	(39.1)			
IL-4	25.6	20.4	12.2	nsd	P<0.05	
	(18.0)	(12.8)	(7.2)		(A,K,Tu)	
IFN-γ	404	266	179	nsd	P<0.05	nsd
	(259)	(174)	(129)		(A,K,Tu)	
IL-2	58.2	47.2	27.7	nsd	P<0.007	P<0.030
	(43.9)	(32.6)	(17.9)		(t-test)	(t-test)
IL-5	6.57	5.64	6.25	nsd	nsd	nsd
	(5.30)	(4.29)	(5.88)			
IP-10	21,316	20,601	16,458	nsd	nsd	nsd
	(21,823)	(19,362)	(24,259)			

SD = standard deviation, NS = non-stimulated, WO = washout, Stim = stimulated, Wil = Wilcoxon's signed ranks test, A, K-Tu = Kruskal-Wallis analysis of variance on ranks and Tukey's test, * IL-17 was undetectable in more than half of the stimulated tear samples, ** Eotaxin was undetectable in three stimulated tear samples.

For 19 of the 25 cytokines in Table 4, NS levels exceeded WO levels and WO levels in turn exceeded those of Stim tears. In five cases, the WO levels were more than

double Stim tear levels of the same cytokine. These include potential key dry eye biomarkers, IL-1RA, IL-8, and G-CSF. Eight of the cytokines listed in Table 4 showed significant differences between NS and Stim levels by ANOVA and Tukey's test for global comparisons among means. An additional three cytokines differed between NS and Stim tear samples by t-test only. There were seven cytokines that showed significant differences between NS and WO levels. However all seven comparisons were based on t-tests alone. ANOVA of NS versus WO data indicated that there were no significant differences among groups when all sources of variance were included. This was the case for all cytokines. An additional limitation with Stim tear samples was the failure to consistently detection some cytokines; most notably IL-17. Because IL-17 is a potential marker of autoimmune disease, the <50% detection rate of IL-17 in Stim tears would limit their use in detecting an autoimmune connection in affected patients.

Figure 27 compares the levels of key AD-predicting cytokines in WO and Stim tears to NS tears. The selected cytokines were found in the main NS vs WO study to differ significantly between AD and non-AD patients in NS tears (Table 3). The first five cytokines in Figure 27 were previously found to differentiate AD from non-AD patients in both NS and WO tears (Table 3) and are ordered in the figure by "predictive power" (IL-8 being "most predictive"). Three of these five cytokines demonstrated significantly lower levels in Stim tears relative to NS by ANOVA and Tukey's test (Table 4). The remaining 6 cytokines in Figure 27 only differentiated AD from non-AD patients in NS tears (Table 3). Again the cytokines are ordered by "predictive power".

The remaining cytokines for which NS vs WO vs Stim tear level comparisons were conducted did not show differences in NS tear levels between non-AD and AD

patients in the earlier NS vs WO tear study (Table 3). The levels of these cytokines in WO and Stim tears normalized to NS tears are shown in Figure 28. For five of the cytokines, Stim levels were significantly lower than NS levels by ANOVA and Tukey's test.

To further explore the potential of Stim tears to provide the same information as NS tears, correlation analyses and Bland-Altman analyses and plots were conducted on the "predictive" cytokines previously identified in the main NS vs WO study. These are the cytokines included in Figure 27. Scatterplots of NS versus WO and NS vs Stim levels of IL-8 are shown in Figure 29. A very poor correlation is evident between NS and Stim tear IL-8 levels, the slope of the regression line of best fit being close to zero.

IL-8 Bland-Altman plots (Figure 29) support the correlation scatterplots, showing a strong positive proportional bias for NS versus Stim IL-8 that is almost four times greater than for NS vs WO tears. Again, positive proportional bias indicates that both Stim and WO tears are underestimating NS levels progressively more as mean IL-8 level increases, but that the underestimation is disproportionately greater for Stim tears. A separate pilot study prior to the NS vs WO vs Stim tear comparison highlighted the potential influence of Stim tear collection rate or total collection volume on measured cytokine levels. Although the study was limited to a subset of the 27-Plex cytokines, it clearly demonstrated that tear IL-8 levels are strongly dependent on total Stim tear collection volume (Figure 31). The figure shows that as Stim tear flow-rate increases IL-8 levels decrease to less than half the initial value. Given the typical pattern of Stim tear collection, the primary determinant of collected volume is the Stim tear flow-rate established after nasal stimulus, which varies widely between individuals.

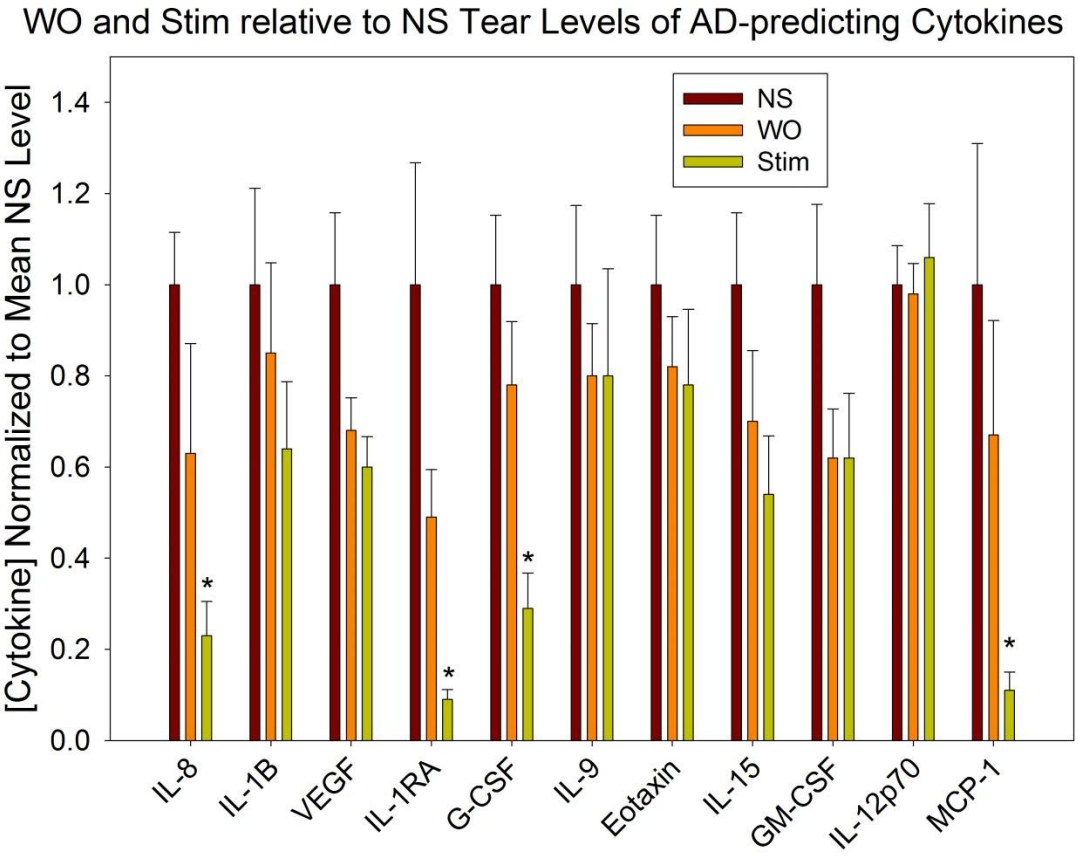

Figure 27: Levels of cytokines in WO and Stim tears of 15 patients normalized to the mean NS tear level for cytokines whose NS levels were previously shown (Table 3) to differentiate between AD and non-AD patients. Error bars show standard error of the mean. NS = non-stimulated, WO = washout, Stim = stimulated, AD = aqueous-deficient. * Cytokine level differs significantly from NS tear level by ANOVA and Tukey's test.

In the case of IL-1β, the NS vs Stim relationship shows wide scatter of data (Figure 32) and a low correlation coefficient for the line of best fit. Stim values substantially underestimate NS tear levels of IL-1β, averaging 22% of the NS level. Conversely, WO tear levels averaged 78% of NS IL-1β, with a statistically significant correlation coefficient for the regression line of best fit. According to the IL-1β Bland-Altman plot (Figure 33), Stim values actually exceeded NS in three cases for mid-range

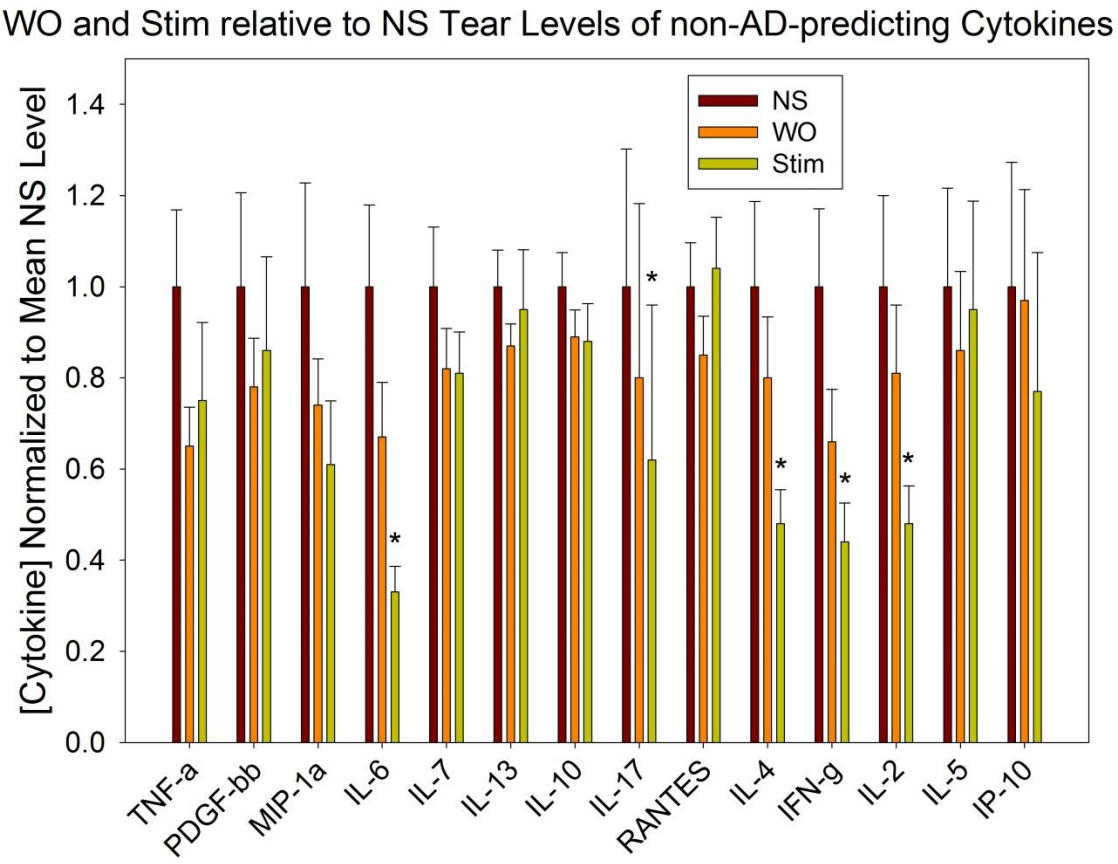

Figure 28: Levels of cytokines in WO and Stim tears of 15 patients normalized to the mean NS tear level for cytokines whose NS levels were previously shown (Table 3) not to differentiate between AD and non-AD patients. Error bars show standard error of the mean. NS = non-stimulated, WO = washout, Stim = stimulated, AD = aqueous-deficient. * Cytokine level differs significantly from NS tear level by ANOVA and Tukey's test.

means. However, for the higher means, NS values clearly exceeded those of Stim. Despite this variable trend, the NS vs Stim Bland-Altman plot showed positive, but not statistically significant, proportional bias.

VEGF showed an unusual scatterplot trend for NS vs Stim levels (Figure 34). While not statistically significant, Stim VEGF levels decreased as NS levels increased. Given the amount of scatter in the data, the plot suggests that VEGF levels in Stim tears

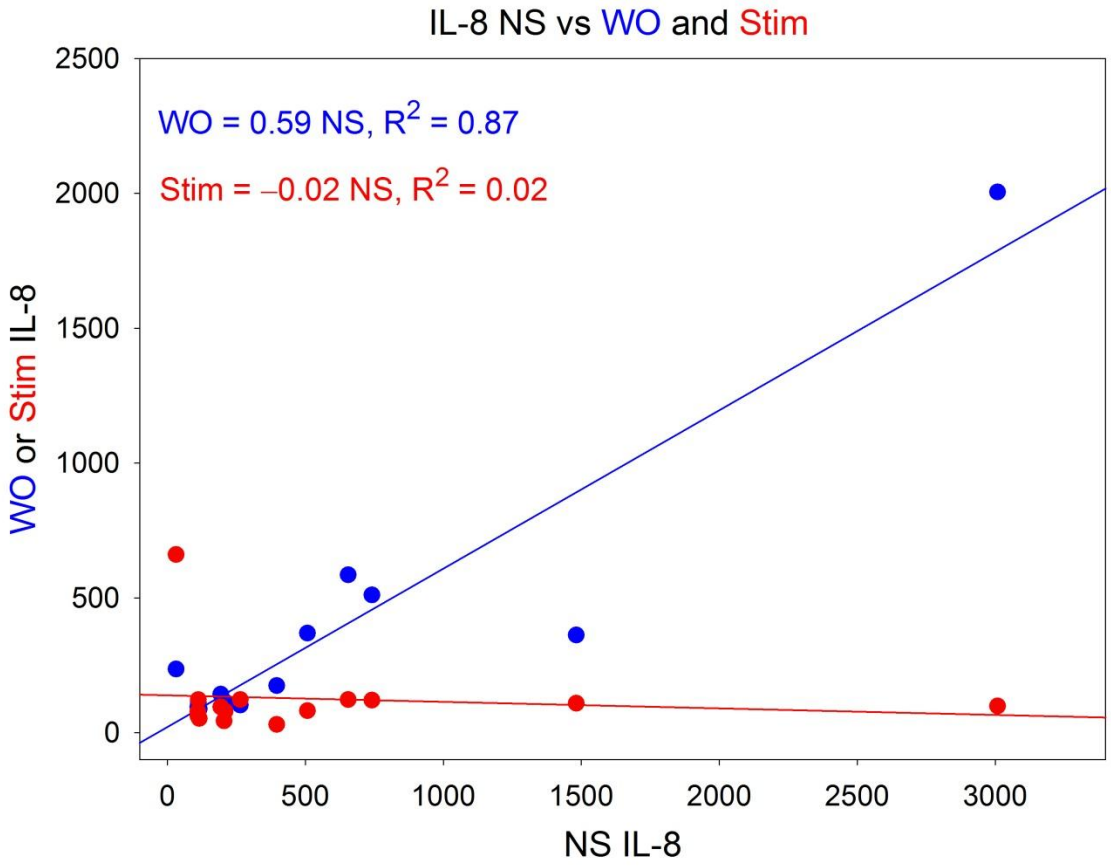

Figure 29: Scatterplot showing correlation between NS and WO tear levels of IL-8 (blue circles) and between NS and Stim levels (red circles). Slope of the linear regression line shows that WO tear IL-8 levels averaged 59% of NS. However, Stim IL-8 levels showed a slight negative slope with a very low correlation coefficient. Only the NS vs. WO correlation coefficient was statistically significant ($p < 0.001$). NS = non-stimulated, WO = washout, Stim=stimulated.

simply do not bear any meaningful relationship to NS levels. Conversely, WO tear VEGF shows a modest positive relationship with NS ($p<0.016$), although averaging only 28% of the NS value. Bland-Altman plots for VEGF (Figure 35) reveal strong positive proportional bias for both NS vs WO and NS vs Stim tears ($p<0.001$ in both cases). While the bias is greater for NS vs Stim, both tear sample types are clearly underestimating NS levels to a progressively greater extent with increasing NS tear VEGF concentration. IL-1RA is another example showing a negligible relationship

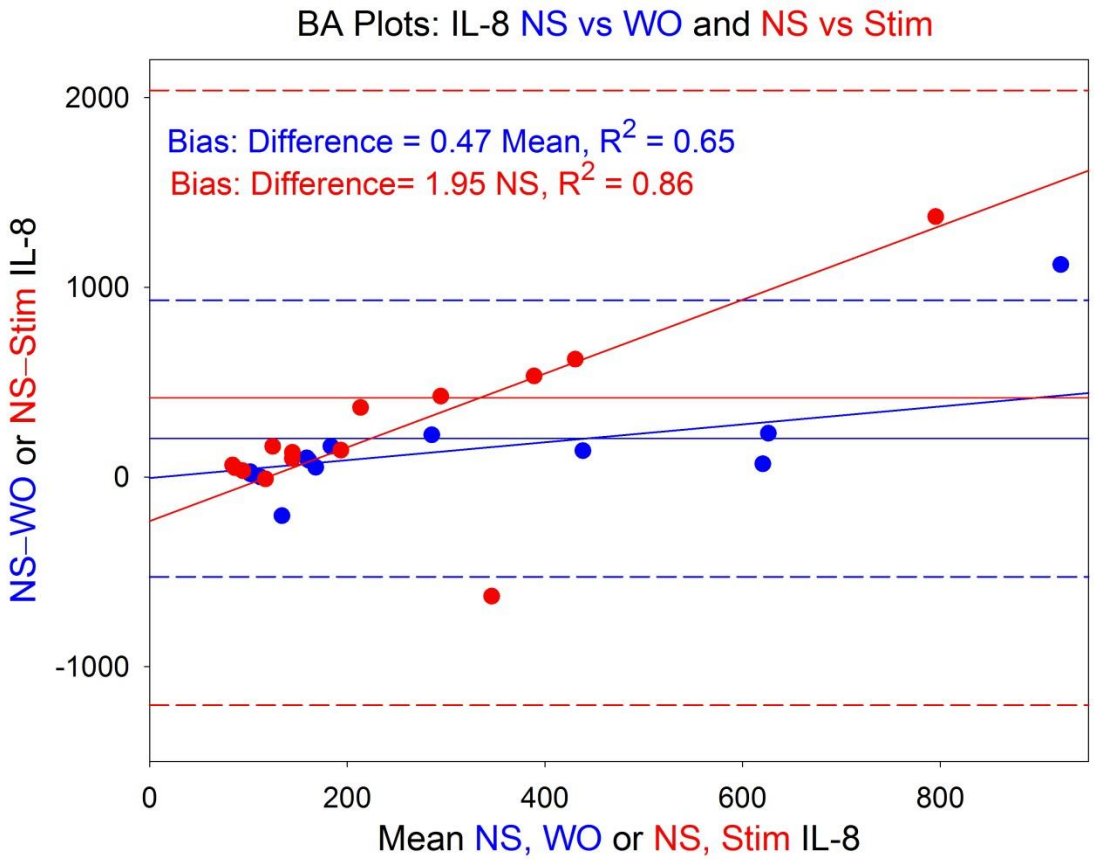

Figure 30: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-8 (blue circles) and NS versus Stim levels of IL-8 (red circles). Difference between NS and WO tear IL-8 and NS vs Stim tear IL-8 levels is plotted against mean level for paired tear samples. Slopes of both BA plots are statistically significant, but the NS vs Stim relationship shows much stronger proportional bias. Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim=stimulated.

between NS and Stim tear levels (Figure 36). In the case of NS vs WO tear IL-1RA, again the WO level averages just over 25% of the NS level, with wide scatter of data, but the positive correlation coefficient is statistically significant ($p<0.016$). IL-1RA Bland-Altman plots (Figure 37) show statistically significant ($p<0.001$) positive proportional bias for both NS vs WO and NS vs Stim relationships, but the trend is stronger for NS vs Stim tears. The bias for both NS vs WO and NS vs Stim relationships, but the trend is

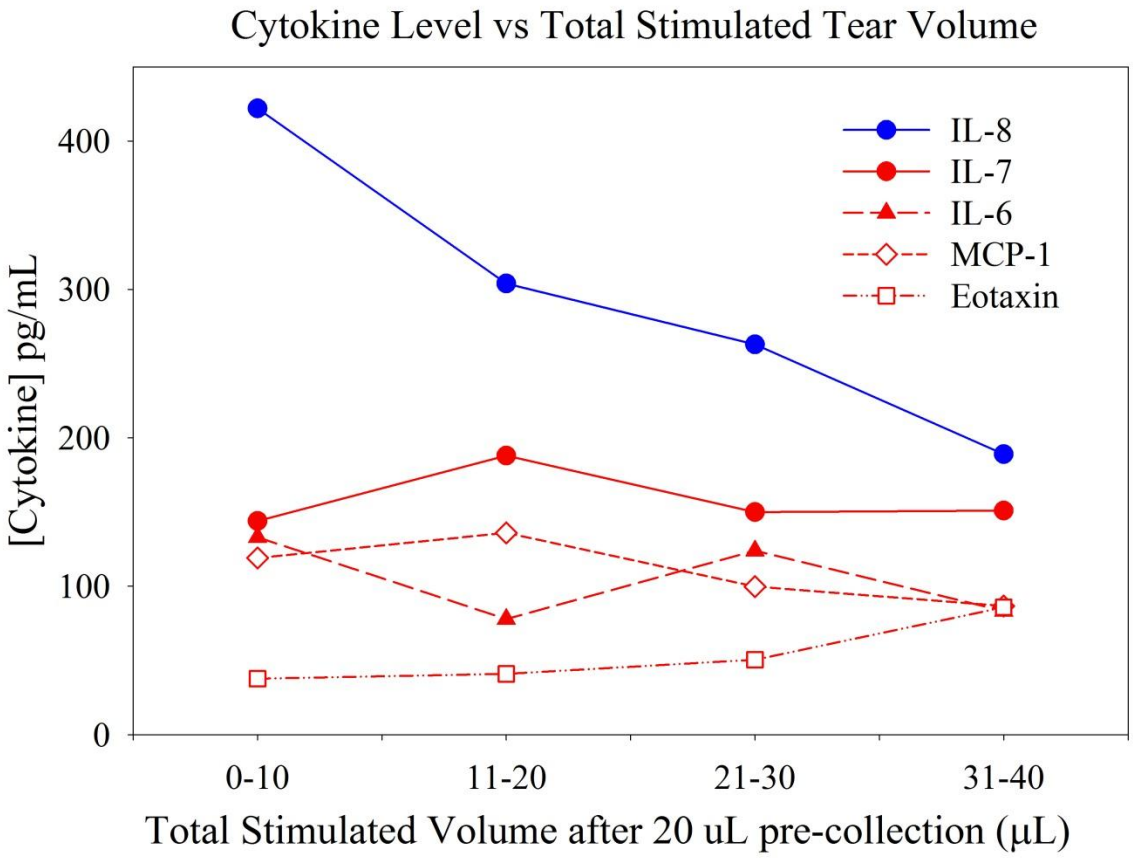

Figure 31: Change in Stim tear cytokine levels for four consecutive Stim tear samples collected after "pre-collecting" 20 µL of tears following onset of stimulus. Cytokines levels were measured with a Bio-Rad 6-Plex kit. IL-8 (blue symbols) showed a significant decrease in concentration with continued stimulus. Stim = stimulated.

stronger for NS vs Stim tears. The very high correlation coefficient in the latter case clearly reflects the fact that Stim tear IL-1RA levels do not increase at all as NS IL-1RA increases.

Stim tear G-CSF levels continue the previous pattern of very poor correlations with NS levels (Figure 38). In this case, the NS vs WO tear G-CSF level is relatively strong; WO values averaging 67% of NS tear levels. Corresponding Bland-Altman plots reveal little bias for NS vs WO tears and statistically significant ($p<0.022$), positive proportional bias for NS vs Stim tears.

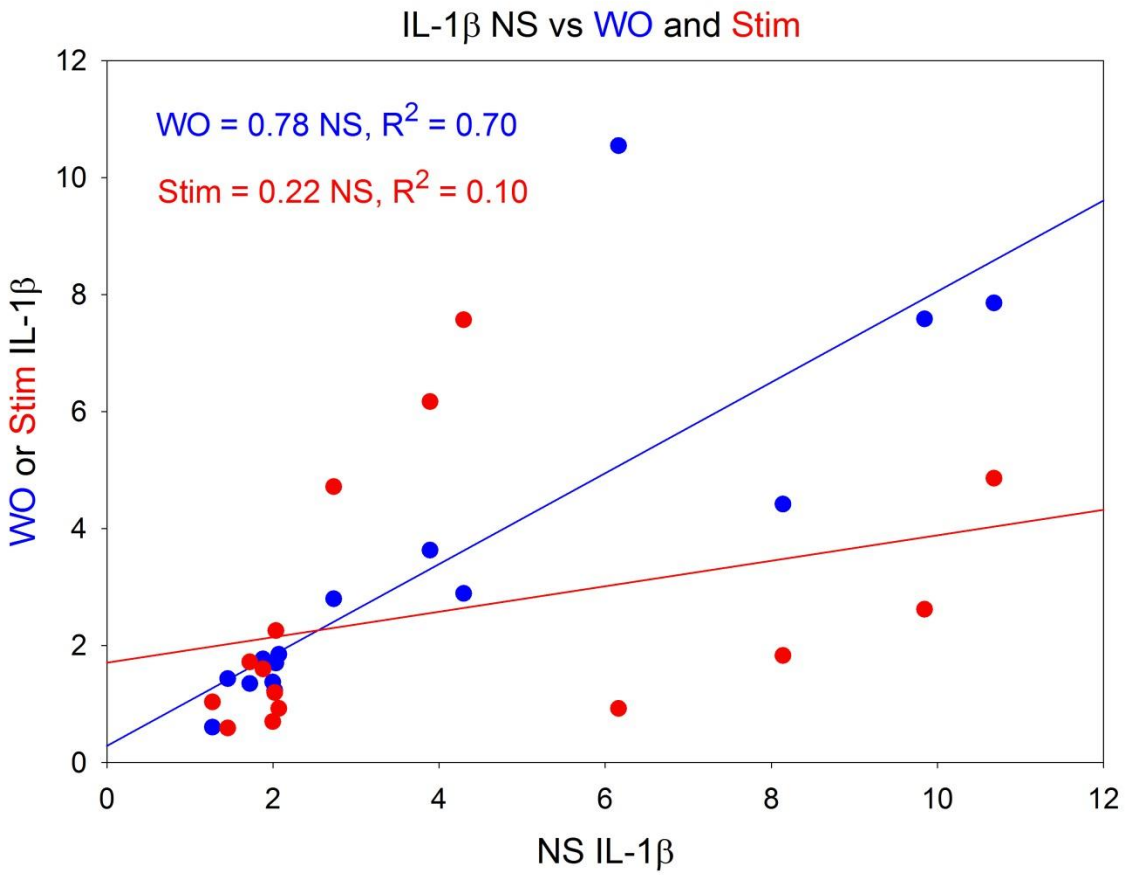

Figure 32: Scatterplot showing correlation between NS and WO tear levels of IL-1β (blue circles) and between NS and Stim levels (red circles). Slope of the linear regression line shows that WO tear IL-1β levels averaged 78% of NS. However, Stim IL-1β levels averaged 22% of NS levels. Only the NS vs WO correlation coefficient was statistically significant ($p < 0.001$). NS = non-stimulated, WO = washout, Stim=stimulated.

For the cytokines that only discriminated non-AD from AD patients in the main NS vs WO study, IL-9, Eotaxin, IL-015, GM-CSF, and IL-12p70, negative proportional bias relationships were found in NS vs Stim tear Bland-Altman plots in three cases: IL-9 (Figure 40), Eotaxin, and IL-12p70 (Figure 41). Negative proportional bias indicates that NS tear levels are underestimated to a greater extent at the lower end of the NS range and to a lesser extent for higher NS values. For four of the five cytokines, moderate positive proportional bias was found in the NS vs WO relationship. In the case of IL-15, no bias was evident.

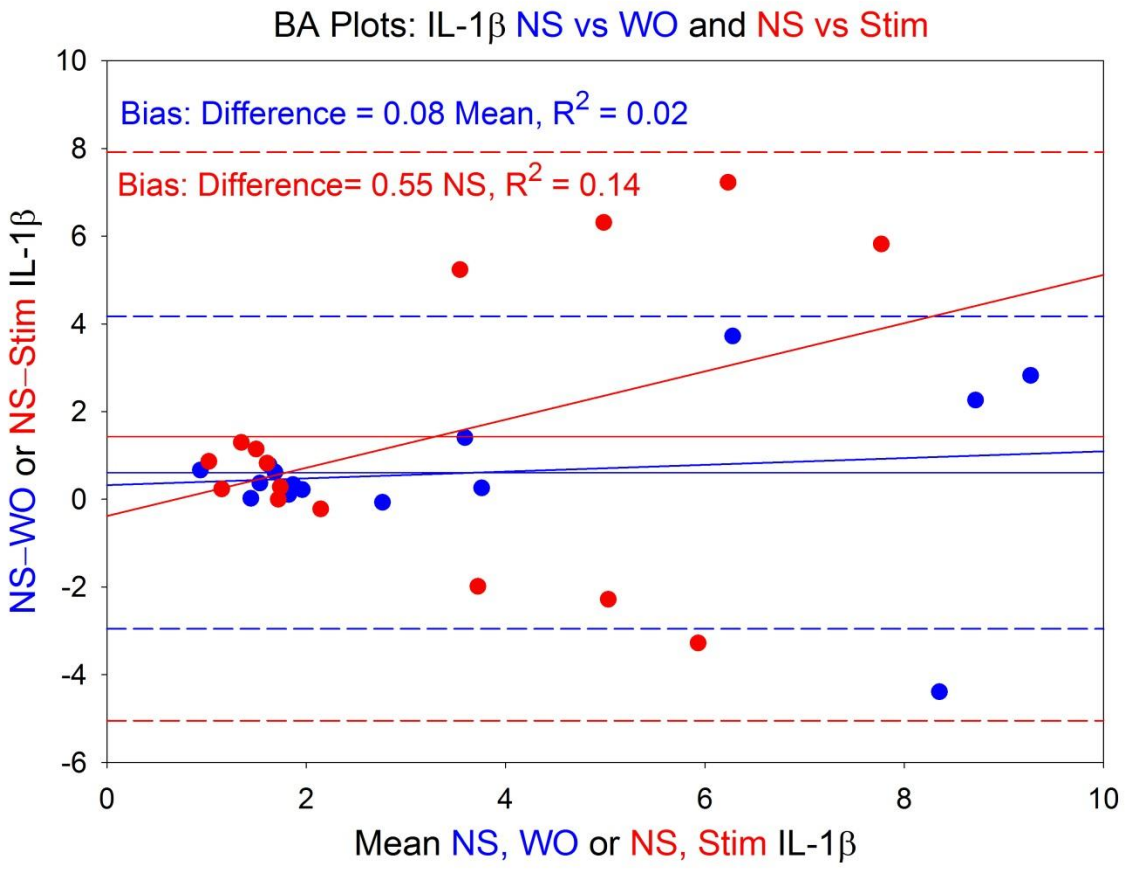

Figure 33: Bland–Altman (BA) plot showing NS versus WO tear levels of IL-1β (blue circles) and NS versus Stim levels of IL-1β (red circles). Difference between NS and WO tear IL-1β and NS vs Stim tear IL-1β levels is plotted against mean level for paired tear samples. Neither slope is statistically significant, but the NS vs Stim relationship shows wide scatter of data for the higher means and, in spite of this, still elicits greater positive proportional bias. Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim=stimulated.

In the main NS vs WO study, several cytokines commonly reported to be associated with dry eye (most notably IL-6 and TNF-α) were not found to discriminate the AD vs non-AD groups in either NS or WO tears. The relationship between NS, WO and Stim levels of these cytokines in the current study provided no evidence that Stim tears would be more likely to discriminate AD from non-AD patients than NS or WO.

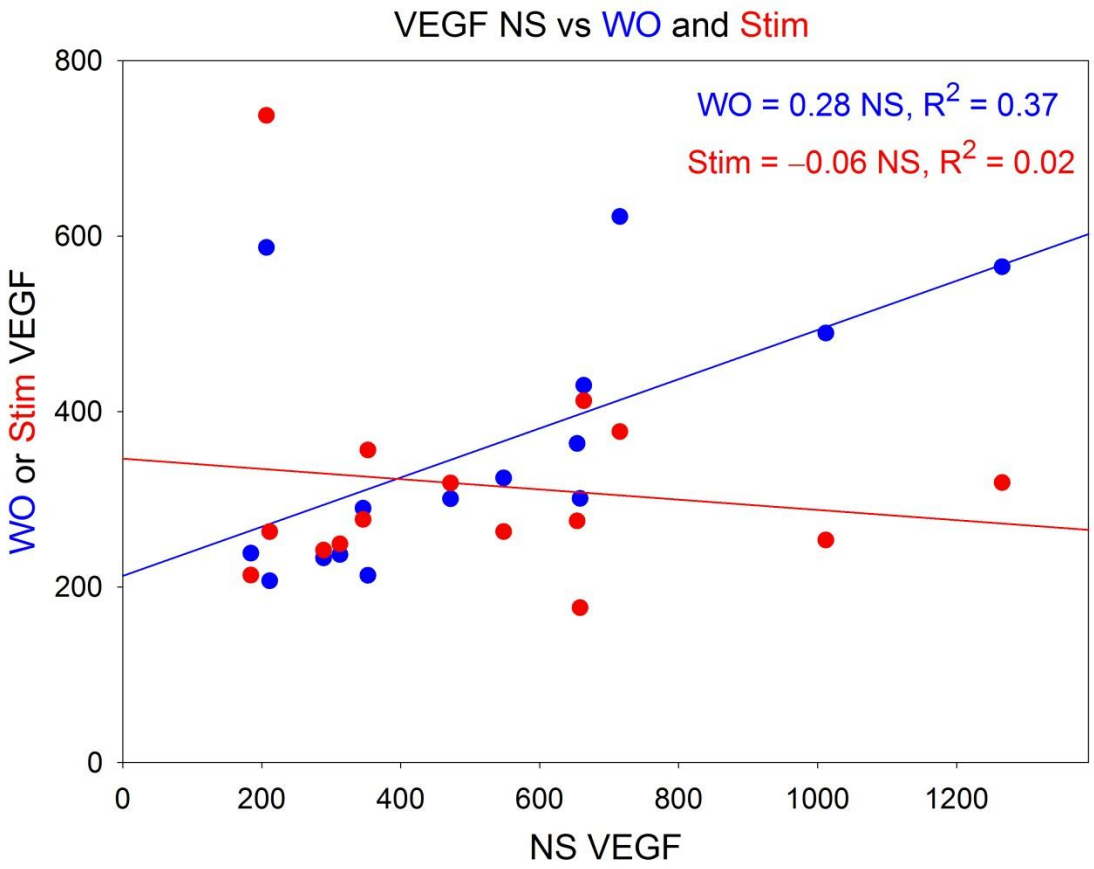

Figure 34: Scatterplot showing correlation between NS and WO tear levels of VEGF (blue circles) and between NS and Stim levels (red circles). Slope of the linear regression line shows that WO tear VEGF levels averaged 28% of NS. Stim VEGF levels showed a slight negative correlation with NS levels. Only the NS vs. WO correlation coefficient was statistically significant ($p < 0.016$). NS = non-stimulated, WO = washout, Stim = stimulated.

Figure 42 shows the relationships between NS, WO and Stim tear IL-6 levels. Slope of the statistically significant ($p<0.001$) linear regression line shows that WO tear IL-6 levels averaged 56% of NS levels, while Stim IL-6 showed a weak, not statistically significant correlation with NS levels. The IL-6 Bland-Altman plots (Figure 43) showed much stronger positive proportional bias for NS vs Stim tears than NS vs WO tears.

Trends for TNF-α revealed a weak negative correlation between NS and Stim levels (Figure 44) and a statistically significant positive correlation between NS and WO

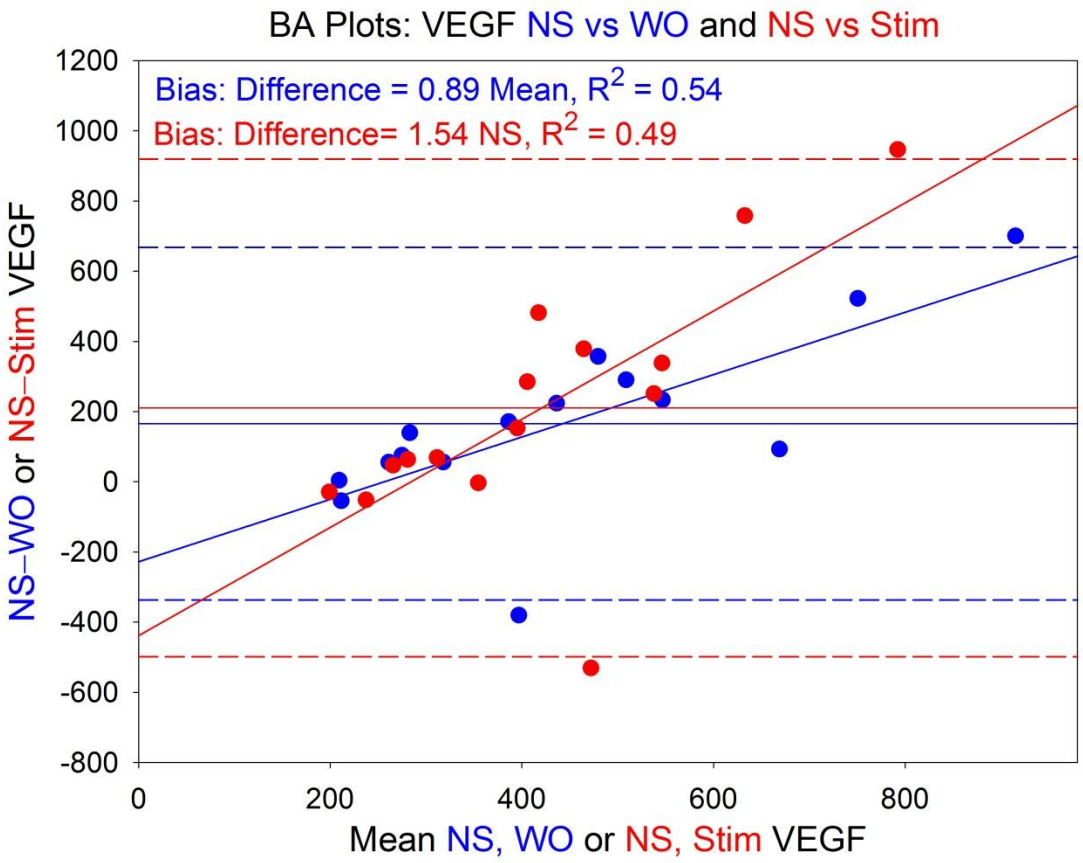

Figure 35: Bland–Altman (BA) plot showing NS vs WO tear levels of VEGF (blue circles) and NS vs Stim levels of VEGF (red circles). Difference between NS and WO tear VEGF and NS vs Stim tear VEGF levels is plotted against mean level for paired tear samples. Both slopes show strong, statistically significant, proportional bias ($p<0.001$). The bias is stronger in the NS vs Stim relationship. Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim=stimulated.

levels. Bland-Altman plots (Figure 45) showed widely scattered data for NS vs Stim TNF-α and negligible plot bias. Scatter increased with mean tear TNF-α concentration. While the NS vs WO plot show statistically significant positive proportional bias ($p<0.001$), the spread of data was considerably less than for NS vs Stim tears.

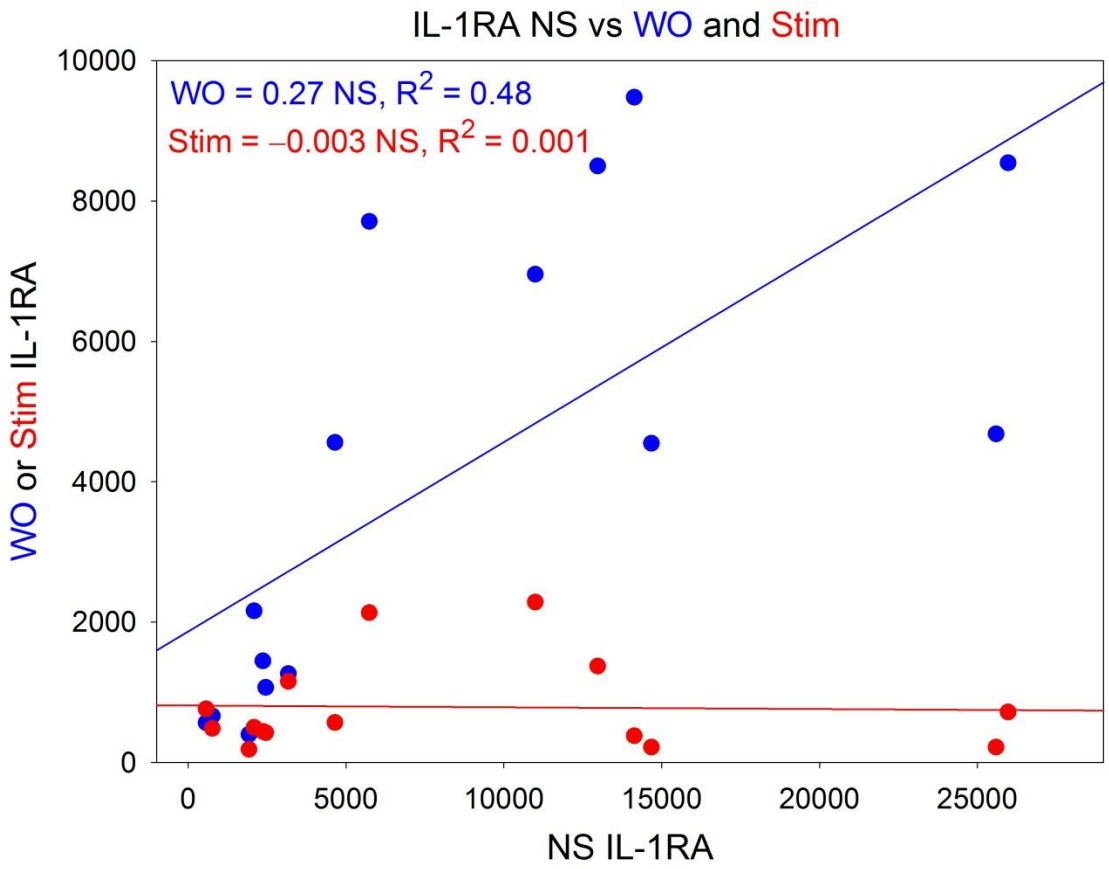

Figure 36: Scatterplot showing correlation between NS and WO tear levels of IL-1RA (blue circles) and between NS and Stim levels (red circles). Slope of the linear regression line shows that WO tear IL-1RA levels averaged 27% of NS. However, Stim IL-1β levels showed effectively zero correlation, the regression line having negligible slope. Only the Ns vs. WO correlation coefficient was statistically significant ($p < 0.004$). NS = non-stimulated, WO = washout, Stim = stimulated.

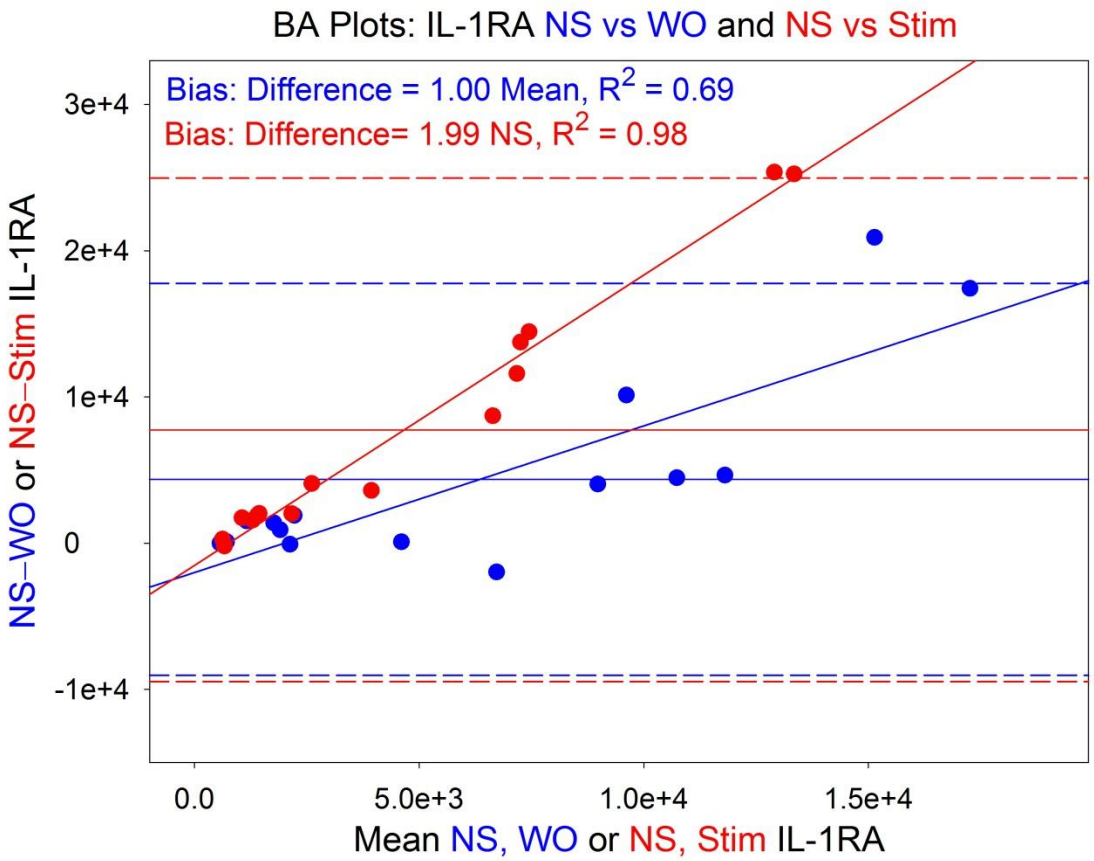

Figure 37: Bland–Altman (BA) plot showing NS vs WO tear levels of IL-1RA (blue circles) and NS vs Stim levels of IL-1RA (red circles). Difference between NS and WO tear IL-1RA and NS vs Stim tear IL-1RA levels is plotted against mean level for paired tear samples. Both slopes show strong, statistically significant, proportional bias ($p<0.001$). The bias is stronger in the NS vs Stim relationship. Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim=stimulated.

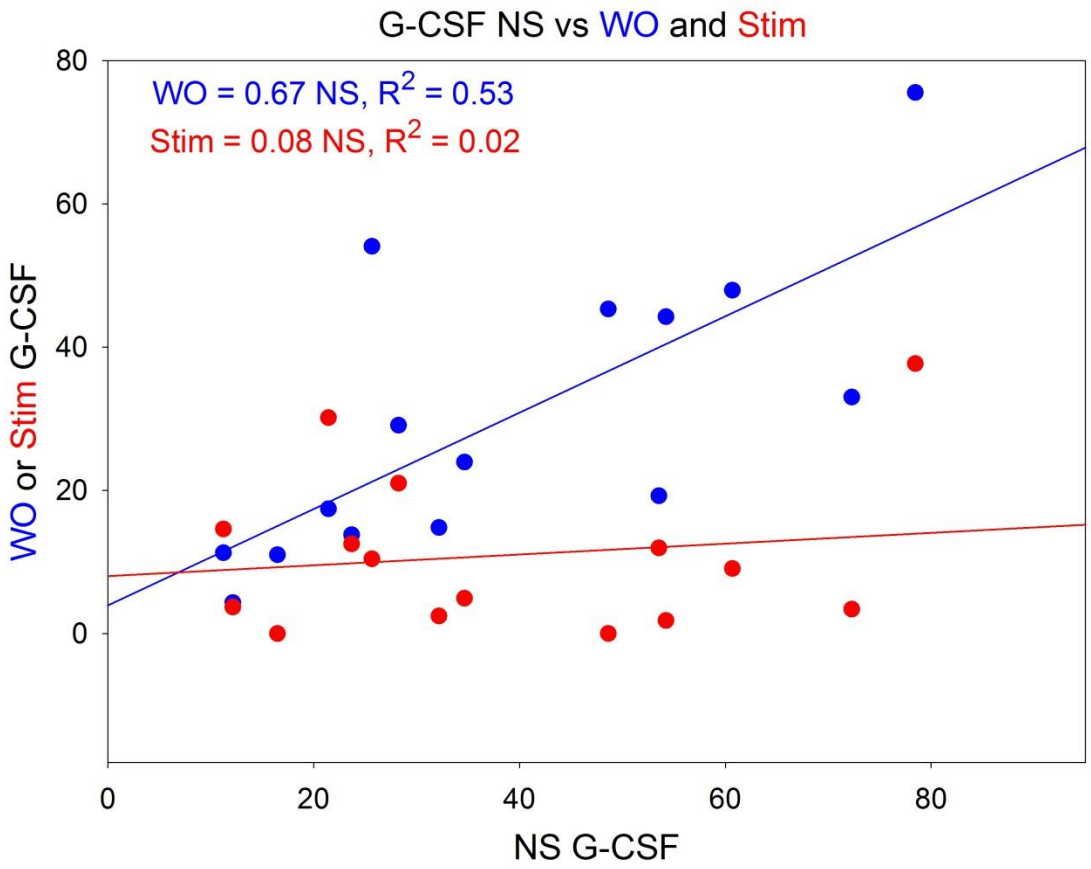

Figure 38: Scatterplot showing correlation between NS and WO tear levels of G-CSF (blue circles) and between NS and Stim levels (red circles). Slope of the linear regression line shows that WO tear G-CSF levels averaged 67% of NS. Stim G-CSF levels showed negligible correlation. Only the Ns vs. WO correlation coefficient was statistically significant ($p < 0.002$). NS = non-stimulated, WO = washout, Stim = stimulated.

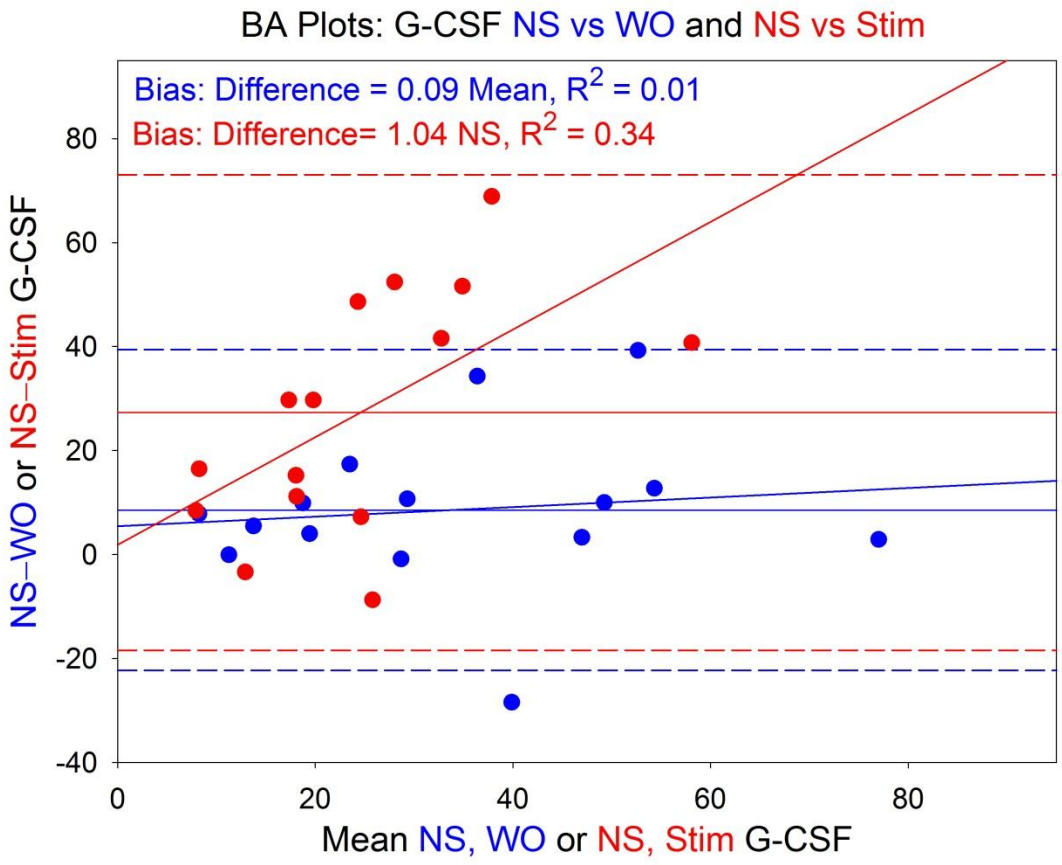

Figure 39: Bland–Altman (BA) plot showing NS vs WO tear levels of G-CSF (blue circles) and NS vs Stim levels of G-CSF (red circles). Difference between NS and WO tear G-CSF and NS vs Stim tear G-CSF levels is plotted against mean level for paired tear samples. The NS vs WO plot shows negligible bias. However, the NS vs Stim plot shows strong, statistically significant, proportional bias ($p<0.022$). Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim = stimulated.

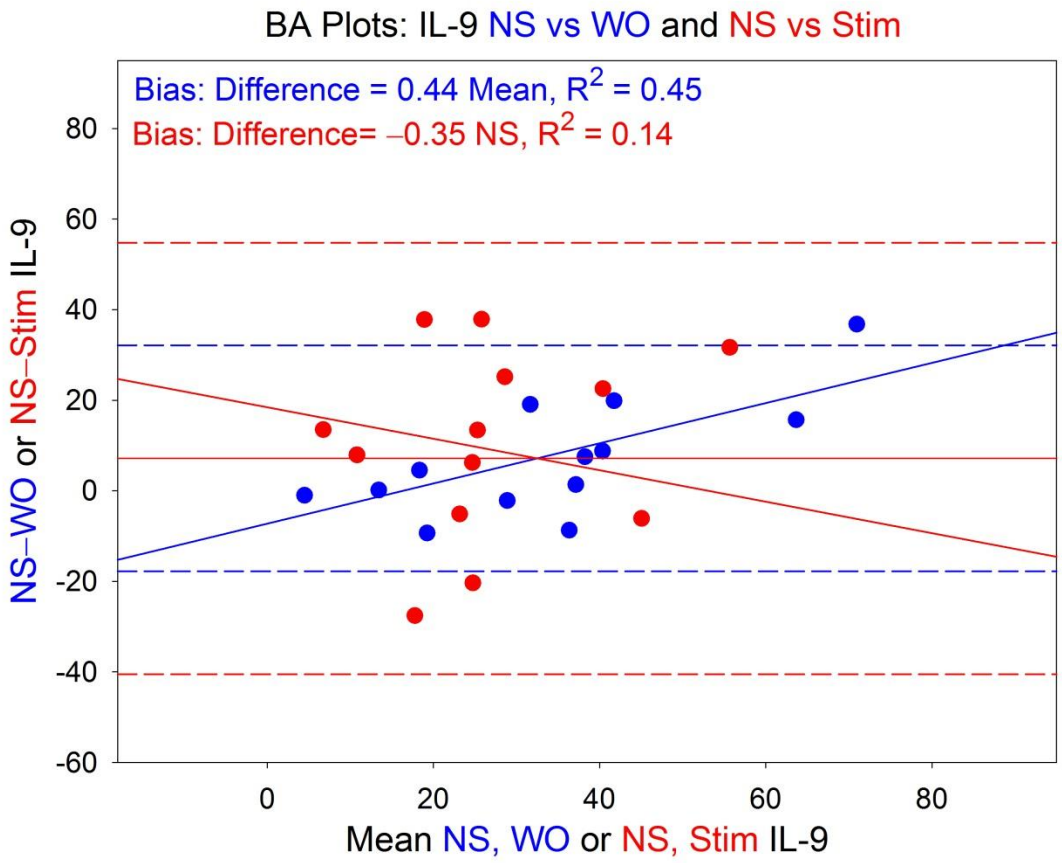

Figure 40: Bland–Altman (BA) plot showing NS vs WO tear levels of IL-9 (blue circles) and NS vs Stim levels of IL-9 (red circles). Difference between NS and WO tear IL-9 and NS vs Stim tear IL-9 levels is plotted against mean level for paired tear samples. The NS vs WO plot shows positive proportional bias, while the NS vs Stim plot shows negative proportional bias. Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim = stimulated.

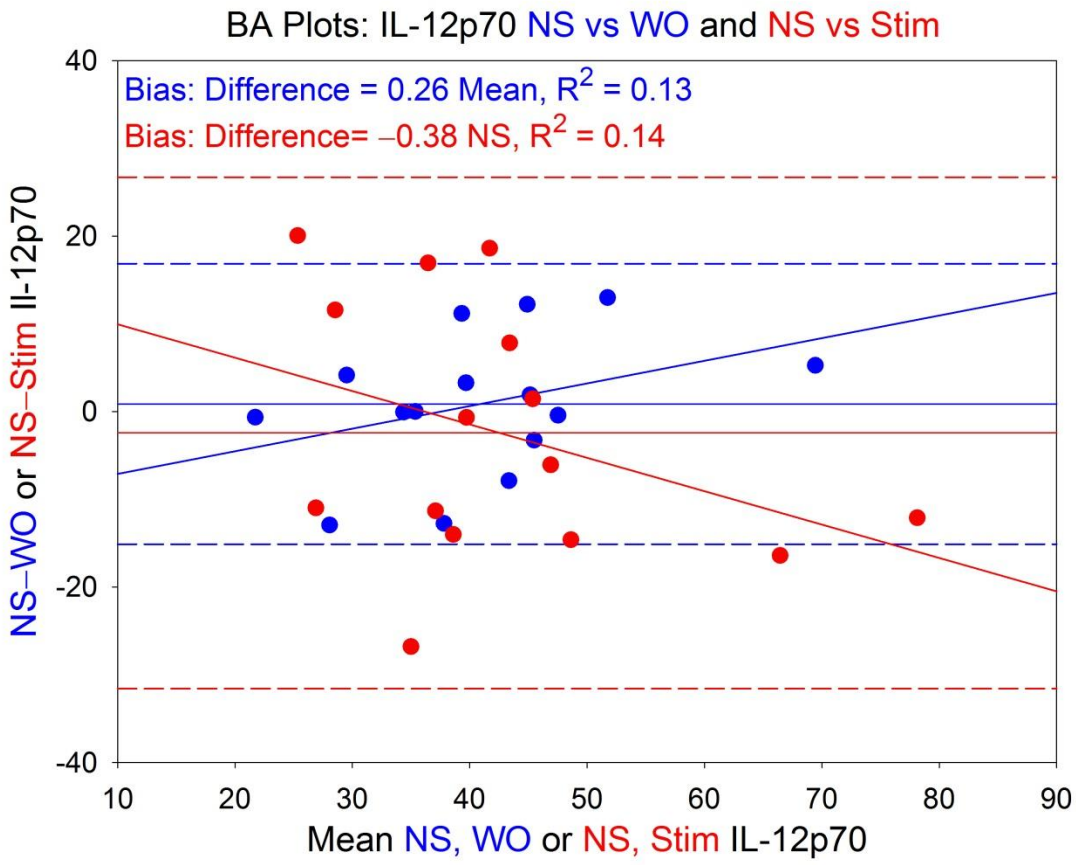

Figure 41: Bland–Altman (BA) plot showing NS vs WO tear levels of IL-12p70 (blue circles) and NS vs Stim levels of IL-12p70 (red circles). Difference between NS and WO tear IL-12p70 and NS vs Stim tear IL-12p70 levels is plotted against mean level for paired tear samples. Again, the sign of proportional bias varies between the two plots. Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim=stimulated.

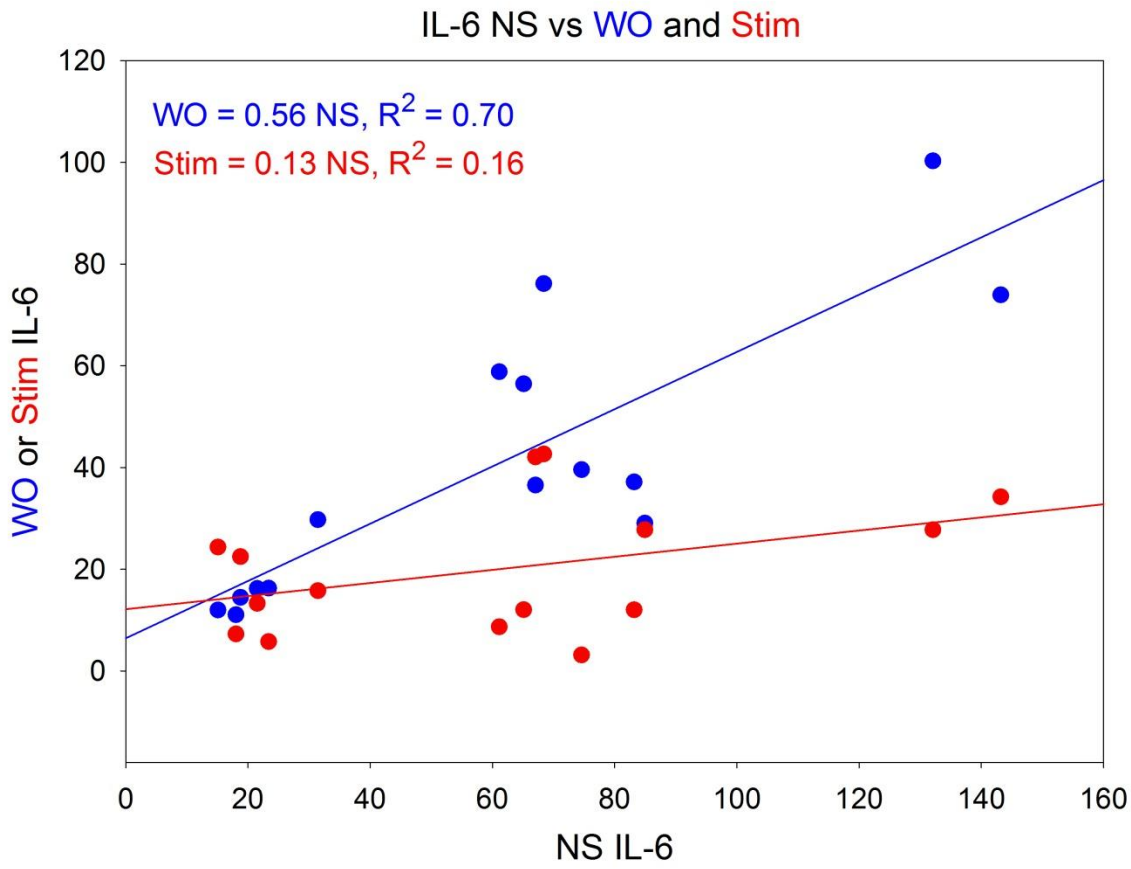

Figure 42: Scatterplot showing correlation between NS and WO tear levels of IL-6 (blue circles) and between NS and Stim levels (red circles). Slope of the linear regression line shows that WO tear IL-6 levels averaged 56% of NS. Stim IL-6 levels averaged 13% of NS levels. The Ns vs. WO correlation coefficient was statistically significant ($p < 0.001$), while the NS vs Stim correlation was not. NS = non-stimulated, WO = washout, Stim = stimulated.

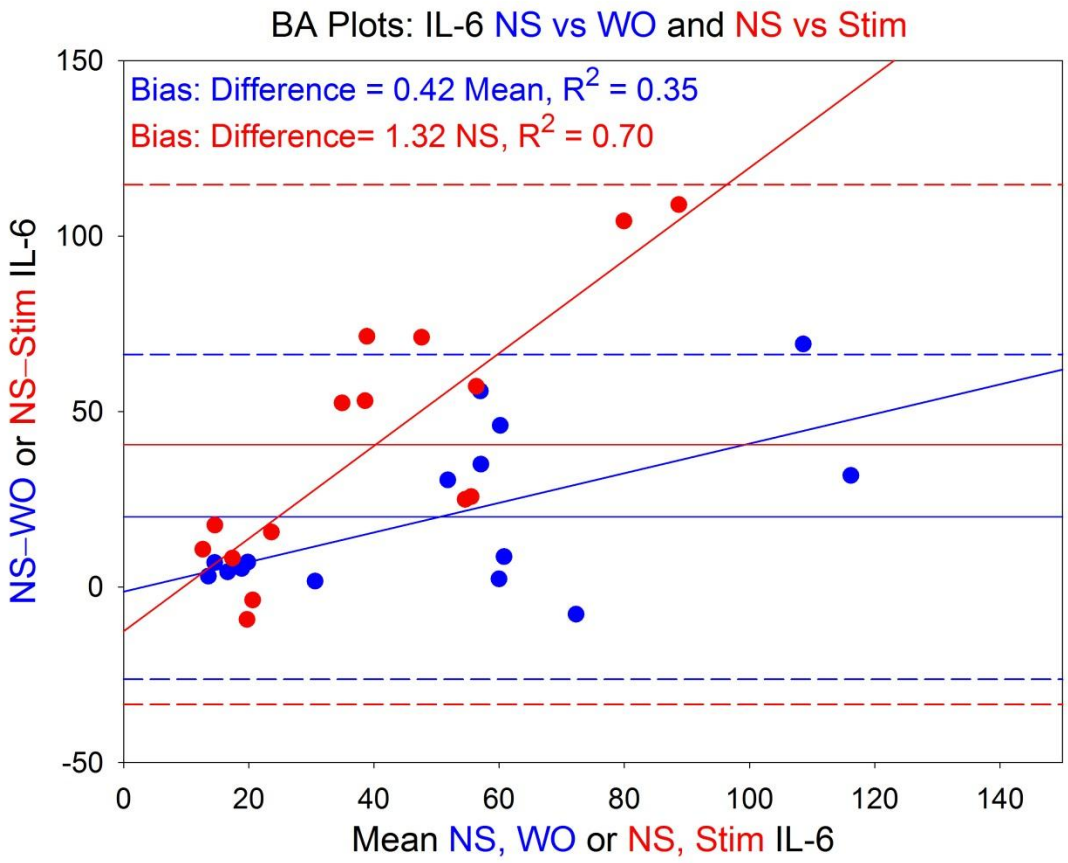

Figure 43: Bland–Altman (BA) plot showing NS vs WO tear levels of IL-6 (blue circles) and NS vs Stim levels of IL-6 (red circles). Difference between NS and WO tear IL-6 and NS vs Stim tear IL-6 levels is plotted against mean level for paired tear samples. Both plots show statistically significant positive proportional bias ($p<0.021$ and 0.001 respectively). The NS vs Stim plot shows a stronger bias. Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim=stimulated.

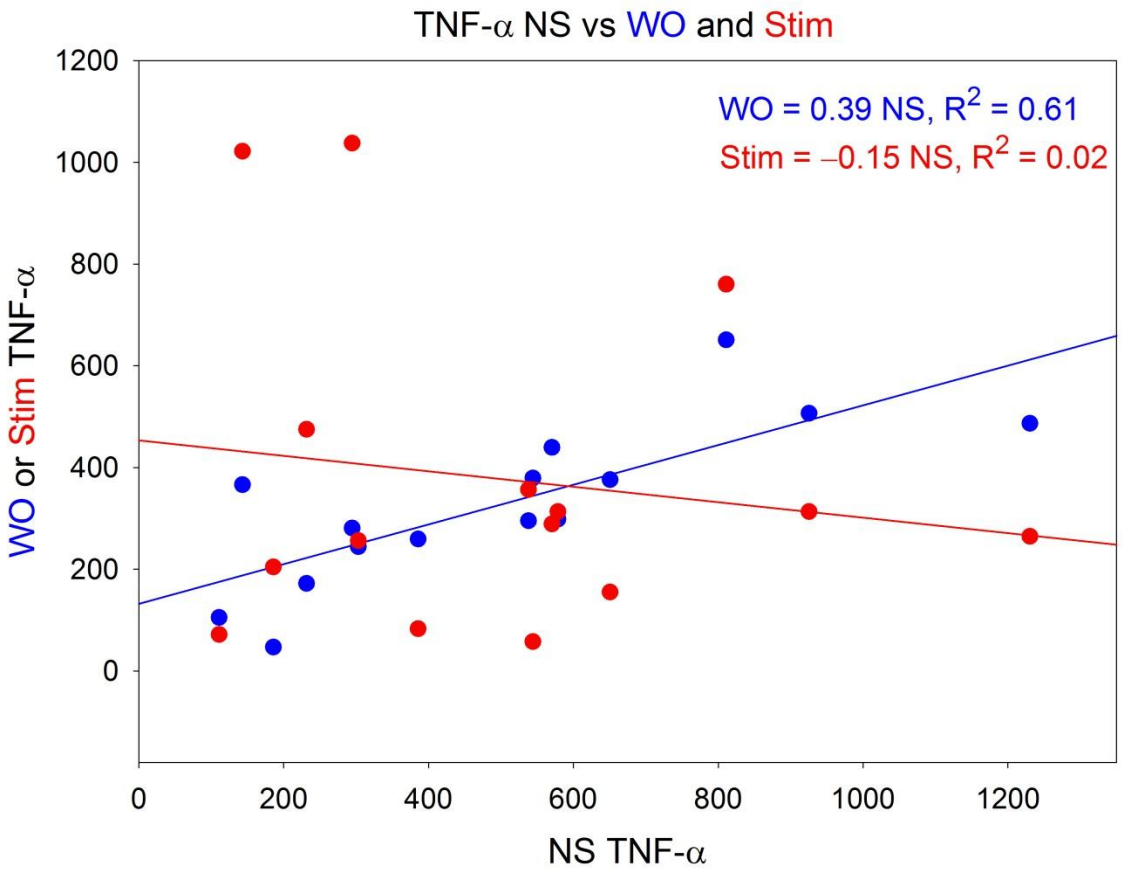

Figure 44: Scatterplot showing correlation between NS and WO tear levels of TNF-α (blue circles) and between NS and Stim levels (red circles). Slope of the linear regression line shows that WO tear TNF-α levels averaged 39% of NS ($p < 0.001$). Stim TNF-α levels correlated negatively with NS levels, although the relationship was not statistically significant. NS = non-stimulated, WO = washout, Stim=stimulated.

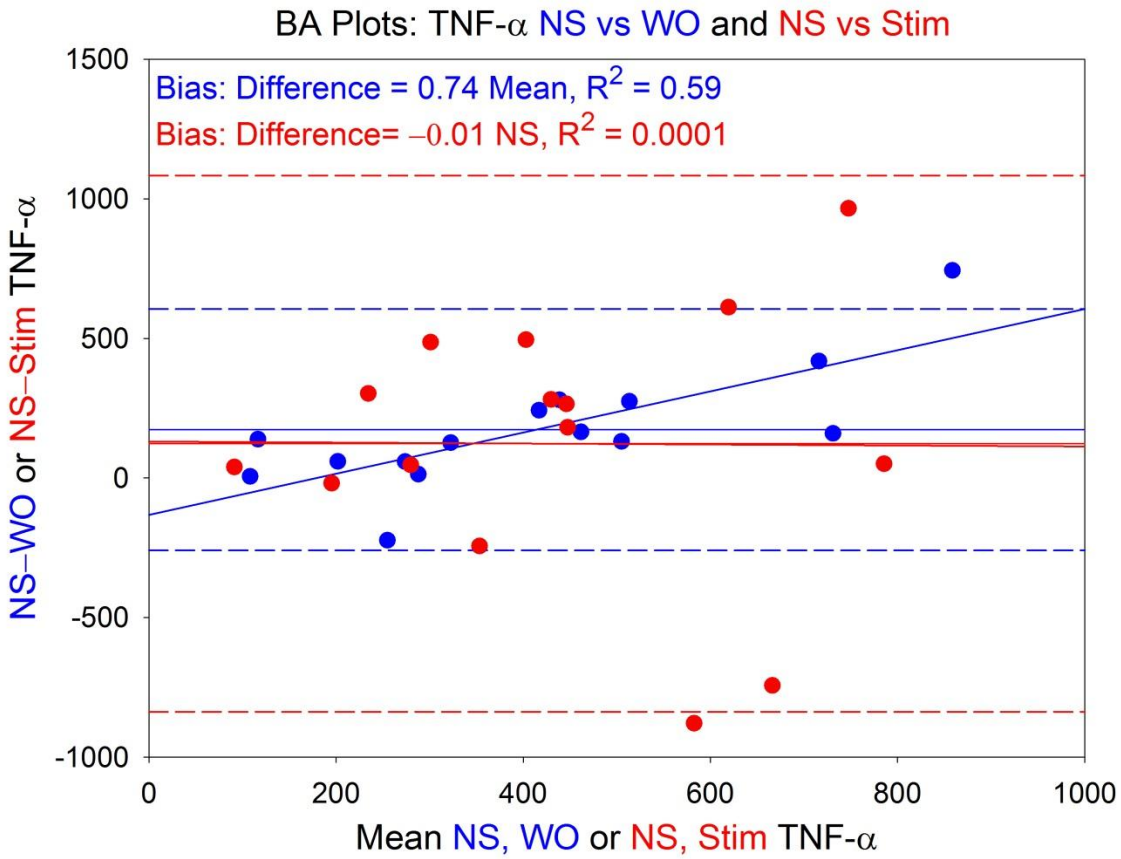

Figure 45: Bland–Altman (BA) plot showing NS versus WO tear levels of TNF-α (blue circles) and NS versus Stim levels of TNF-α (red circles). Difference between NS and WO tear TNF-α and NS vs Stim tear TNF-α levels is plotted against mean level for paired tear samples. The NS vs WO plot shows statistically significant positive proportional bias ($p<0.001$). While the NS vs Stim plot shows negligible bias, the spread of data increases with mean tear TNF-α level. Dashed lines represent 95% confidence limits. NS = non-stimulated, WO = washout, Stim=stimulated.

DISCUSSION

Main NS vs WO Study: WO Tears as an Alternative to NS Tears

Overall, 16 of the 25 consistently-detected cytokines showed strong correlations between NS and WO levels and lacked significant proportional Bland-Altman plot bias: IL-1RA, Eotaxin, GM-CSF, IL-12p70, MCP-1, TNF-α, PDGF-bb, MIP-1α, IL-13, IL-10, IL-17, RANTES, IL-4, IFN-γ, IL-2, and IL-5. On this basis, it could be argued that WO tears can be used as a substitute for NS tears for these 16 cytokines. However, other factors must also be considered. Of particular importance is the compression of cytokine concentration range in WO tears and their inability to reflect the higher NS tear cytokine levels. This showed up as lower mean cytokine values and, in many cases, lower variances in WO samples than NS tears. WO samples therefore effectively produce the same cytokine patterns as NS samples but with less statistical power. The ability of WO tears to substitute for NS tears will therefore depend in part on the application and the implications of losing the higher end of the NS cytokine concentration range. For example, more severe AD dry eye may be poorly differentiated from moderate to moderately severe cases when using WO tears. The application chosen in this project, for the practical reason that NS tear collection from AD patients is difficult and very time-consuming, was the ability to differentiate AD and non-AD patients based on tear

cytokine profile. It demonstrated that Bland-Altman plots are useful, but not definitive, in determining which cytokines remain reliable indicators of dry eye in WO tears.

Was the choice of a single clinical test outcome to classify patients, the Schirmer strip wetting length, a limiting factor in this study? The clinical profiles of patients in the AD and non-AD groups were undoubtedly heterogeneous due to the use of just one clinical test result to classify the groups and no additional inclusion/exclusion criteria such as ocular surface staining score or dry eye symptom score thresholds. This was done intentionally because NS tear collection presents the greatest challenge for patients with AD dry eye, and simple aqueous deficiency is best measured using the Schirmer test. However, the use of additional clinical criteria for group selection would have been of limited value given the clinical profiles arising from two groups. AD patients were more symptomatic for dry eye (mean OSDI of 30 compared to 17 for the non-AD group), fluorescein staining score was higher (2.1 versus 0.8), as was Lissamine green staining score (2.5 versus 1.0). Not surprisingly, tear osmolarity differed minimally between groups: mean 301 for AD and 295 for non-AD patients. While the TearLab test used in the study is currently the only viable option for clinical measurement of tear osmolarity, its discriminative value in dry eye has been questioned[27]. Sullivan et al[26] found that tear osmolarity did not increase with decreasing Schirmer score, the two tests showing the least correlation of the common clinical tests for dry eye[26].

The WO method was shown in the main study to be capable of differentiating between non-AD and AD groups based on the levels of some key dry eye biomarkers: IL-8, IL-1β, VEGF, IL-1RA and G-CSF. All showed a statistically significant effect size in differentiating AD from AD patients in both NS and WO tears. Interestingly, four of

these biomarkers showed significant Bland-Altman plot proportional bias in at least one patient group. This is simply a different way of viewing the underestimation of higher NS cytokine levels in WO tears and may be indicative of the importance of these cytokines in AD dry eye if their WO levels continue to discriminate AD from non-AD groups in spite of the positive proportional bias. Because WO cytokine levels showed less group separation in terms of means, effect size, and other factors, some of the more subtle between-group differences found with NS tears will be lost with WO tears. An apparent exception to this trend is IL-1β, which is present in low amounts (<10pg/mL) in NS tears. At NS tear levels not greatly exceeding the lower limit of quantification of the 27-Plex cytokine assay, it was somewhat surprising that WO levels could also differentiate AD from non-AD patients. This indicates that the added saline WO fluid may not simply be diluting resident tear fluid at the ocular surface. It may instead be effectively "rinsing" tears and ocular surface proteins that would otherwise not have been collected by the microcapillary during NS tear collection. WO tear levels of IL-8 averaged approximately one third of NS levels in both the non-AD and AD groups. This is the effect that would be expected with a simple saline dilution of the resident ocular surface tear fluid by WO tears. However, in the AD group, the relationship between NS and WO IL-8 levels (Figure 6) is considerably more variable. The reason that WO tears are able to differentiate non-AD from AD patients can also be deduced from Figure 6. The higher AD WO IL-8 levels clearly exceed non-AD levels just as is the case for NS tears. There is simply a greater overlap between non-AD and AD for WO IL-8 relative to NS IL-8.

The five cytokines differentiating patient groups in both tear sample types, IL-8, IL-1β, VEGF, IL-1RA, and G-CSF, have all been linked to AD dry eye in other studies[28-32]; IL-8[28-32] and IL-1β[18, 19, 33-35] in particular. Therefore, the WO method has the potential to differentiate AD dry eye from normal based on commonly accepted key biomarkers.

IL-8 has been shown to be both pro-inflammatory and pro-angiogenic[4]. Its tear levels have been reported by others to be elevated in AD dry eye. Huang et al[30] found a strong negative correlation between tear IL-8 and Schirmer score, and strong positive correlation between tear IL-8 and corneal staining score[30]. Both findings are consistent with the current study. IL-1β is associated with the early pro-inflammatory response to infection and its overproduction has been shown to be associated with many diseases that involve inflammation, including dry eye[10]. Many recent clinical studies and trials have targeted ocular surface IL-1β blockade as a treatment for dry eye[36], highlighting the accepted significance of this cytokine in the pathogenesis of dry eye. In contrast, IL-1RA, the natural inhibitor of the IL-1 response may help to slow down IL-1β effects and reduce chronic inflammation[10]. WO tear levels of IL-1RA, being able to differentiate AD dry eye from non-AD controls in the current study, could be a useful measure of dry eye treatment efficacy.

In an associated study in this lab, tear G-CSF levels in AD patients were found to correlate with conjunctival expression of 22 biomarkers, including many key pro-inflammatory cytokines[11]. G-CSF, along with IL-1β, IL-6, IL-16, and IL-33, was shown by another group to have significantly elevated levels in tears of patients with dry eye syndrome[34]. Na et. al.[33] also found that tear IL-6 and IL-1β levels were among the first to increase in dry eye.

VEGF promotes lymphangiogenesis in the cornea[37] and appears to be upregulated at the ocular surface of dry eye patients in response to other pro-inflammatory cytokines, such as IL-1β and IL-8[37]. Anti-VEGF treatments designed to decrease pro-inflammatory cytokines for dry eye syndrome have been investigated in a mouse dry eye model[8]. Another study demonstrated a decrease in dry eye related corneal epithelial disease and a decrease in sodium fluorescein staining after anti-VEGF treatment[37].

Six cytokines, IL-9, IL-12p70, IL-15, Eotaxin, GM-CSF and MCP-1, showed significant differences between AD and non-AD groups in NS tears but not in WO tears. The finding for MCP-1 is tenuous because of the small effect size. Of the remaining five cytokines, most are not recognized dry eye markers[31, 34, 37-45]. However, in a recent study, IL-12p70 levels were shown to correlate with dry eye severity[12]. In a recent study by Lee et.al[12], treatment of meibomian gland dysfunction with minocycline and artificial tears produced significant reductions of pro-inflammatory cytokines, such as IL-6, IL-1β, and IL-12p70, in the tears of these patients[25]. IL-12p70 is active in immune responses and if blocked, may slow the progression of autoimmune diseases. This effect may prove beneficial in cases of dry eye associated with Sjögren's syndrome[10].

IL-9[38, 43] and Eotaxin[42, 46] are much more commonly associated with allergy than dry eye. IL-9 induces inflammation and an allergic response by increasing IgG and IgE production[10]. Eotaxin also works to upregulate allergic responses by increasing eosinophil counts[47]. No reports could be found that describe any association between IL-15 and dry eye. However, based on its known functions, IL-15 may be involved in the pathogenesis of some dry eye types. This pleiotropic cytokine has a broad range of biological functions and is involved in modulating immune cells of both the innate and

adaptive immune systems[48]. It functions after intracellular infection to activate T-cells and natural killer cells[10]. Many cell types may produce IL-15, based on constitutive expression of IL-15 mRNA, including some that are found at the ocular surface. These include monocytes, macrophages, dendritic cells, keratinocytes, epidermal skin cells, fibroblasts, and epithelial cells, and nerve cells[48].

GM-CSF is a pro-inflammatory cytokine that acts by increasing production of a variety of immune cells[10]. In contrast to G-CSF, tear GM-CSF did not show a correlation with conjunctival gene expression of cytokines in normal or AD patients in a previous study completed in this lab[11]. MCP-1 is a pro-inflammatory cytokine that was found to be elevated in dry eye syndrome in a recent study[18]. However, Schirmer strips were used for tear collection in that study, so the results should be treated with caution. Compared to micropipette tear collection, Schirmer strips result in collection of greater amounts of ocular surface mucus, cellular debris, and other ocular surface components that may arise from the invasive effects of the sampling method.

The current study found some cytokines to be elevated in AD patient's NS tears that have not previously been documented to be elevated in dry eye. In contrast, other cytokines normally associated with dry eye were not found to differentiate non-AD from AD patients in the current study. In particular, IL-6[32, 46] and TNF-α[32, 49] have been reported to be elevated in dry eye patients but did not show differences between non-AD and AD patients in NS or WO tears in the current study. Both cytokines have been directly associated with AD dry eye in other studies[31]. Because no differences were seen in either tear sample type in the current study, limitations of the bead-based tear assay of these cytokines may be partially responsible rather than the tear collection method per se.

With the recognition that WO tears are a viable substitute for NS tears for the study of key dry eye biomarkers, the WO method may prove useful for tear collection in studies that include measures to monitor the efficacy of dry eye treatments. This would be particularly beneficial if dry eye treatments that target these key, WO-sample detectable, biomarkers were studied. Currently several treatments for dry eye target these key biomarkers either directly or indirectly. As an indirect example, omega-3 fatty acids in fish oils reduce levels of TNF-α, IL-1α, and IL-1β[41]. These cytokines have all been reported to be involved in the pathogenesis of dry eye at both the ocular surface and lacrimal gland level[41]. Restasis and TheraTears Nutrition® also work by blocking pro-inflammatory cytokines[41]. Restasis, apart from its known inhibitory effect on IFN-γ[16], has also been reported to inhibit inflammation in part by blocking IL-1[50] and its efficacy could therefore potentially be monitored in a clinical trial through WO tear IL-1β levels. Difluprednate is a steroid that is currently in clinical trials for dry eye treatment efficacy[51, 52]. It causes a general downregulation of many pro-inflammatory cytokines, meaning that WO tear assay may be a viable option for monitoring treatment efficacy. Antibiotics, like tetracyclines, also show anti-inflammatory effects by blocking matrix metalloproteinases (MMPs) and IL-1[50]. Anakinra, a recombinant form of IL-1RA inhibits IL-1α and IL-1β receptor binding, thus, preventing inflammation[53, 54]. Either treatment type could be monitored via WO tear measurement of IL-1β and IL-1RA. Lifitegrast, a small molecule integrin antagonist, inhibits T-cell mediated inflammation by blocking lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule (ICAM-1)[55]. Based on the known functions of ICAM-1, its inhibition would be expected to reduce levels of IL-1β at the ocular surface[56]. CF-101, an A3 adenosine

receptor agonist, is another relatively new drug being evaluated for dry eye treatment. CF-101 targets neutrophil-mediated tissue injury. It modulates key signaling proteins to inhibit degranulation and inflammatory cytokine/chemokine production by inducing inflammatory cell apoptosis.[57]. Adenosine receptor agonists have been shown in previous studies on other tissues to reduce the levels of TNF-α, IL-1, IL-6 and MIP-1α[58, 59]. Again, the ability to monitor IL-1β in WO tears would make WO tear collection a viable option to monitor the efficacy of CF-101 treatment through biomarker levels.

Some treatments work by promoting tear secretion, such as Diquafosol, which is currently in clinical trials[60-62]. In rabbit tissue culture models, control of MUC5AC gene expression has been linked to several pro-inflammatory cytokines, including TNF-α, IL-1β, IL-2, IL-8, and IL-6[63]. If Diquafosol is acting to modulate mucin expression at the ocular surface, including that of MUC5AC, changes may be seen in the tear levels of these cytokines. Both IL-8 and IL-1β could be monitored in WO tears according to the findings of the current NS vs WO study.

NS vs WO vs Stim Tear Comparison

Stim tear collection procedures can be highly variable and difficult to control. The tear flow rate is different for every patient and cannot be reliably controlled from person to person. Often individual patients will exhibit markedly variable Stim tear flow-rate even when using the same standardized sneeze reflex procedure to induce reflex lacrimation. Based on an earlier study in this lab[6], levels of most cytokines in Stim tears differed significantly from those in NS tears. Of the 22 regularly detected cytokines in

that study, 15 showed statistically significant differences between NS and Stim tear levels. In addition, the extent of stimulus varied considerably from patient to patient, and collection rate appeared to exert considerable influence on the levels of some cytokines, such as IL-8. While NS tear collection rate can vary within an individual, it tends to be much more consistent than Stim flow-rate. NS collection is subject to very little variation in AD patients. For all individuals involved in the two studies reported in this thesis, WO tear collection rates were more or less characteristic of each individual patient, much like NS collection rates.

Overall, WO tear cytokine profiles more closely matched the profile created by NS tears than did the Stim tear cytokine profile. Stim tear cytokine levels were significantly lower than NS and WO tears for the majority of cytokines assayed in the current study. ANOVA and global comparisons among means revealed four of the key AD dry-eye detecting cytokines, IL-8, IL-1RA, G-CSF and MCP-1 to have significantly lower levels in Stim tears relative to NS. For comparison, none of the WO samples for these key cytokines showed a significant difference from NS tears (Table 4, Figure 27). For the remaining cytokines, five showed significantly lower levels in Stim tears vs NS (IL-6, IL-17, IL-4, IFN-γ and IL-2) by ANOVA and Tukey's test and none showed a significant difference between NS and WO levels. Bland-Atman plots supported the poor ability of Stim tears to reflect NS tear cytokine values. For four of the five key dry eye-detecting cytokines, IL-8, IL-1RA, VEGF and G-CSF, the NS vs Stim regression plot showed almost no correlation, the line of best fit being close to horizontal. For the remaining cytokine, IL-1β, the slope of the NS vs WO regression line was three times steeper than the NS vs Stim slope. Similar correlation patterns were seen with the

remaining dry-eye detecting cytokines, the regression line being consistently steeper for the NS vs WO correlation than the corresponding NS vs Stim correlation. Finally, IL-6 and TNF-α, two cytokines often reported to be associated with dry eye, showed a much poorer correlation between NS and Stim tear levels than the correlation between NS and WO levels.

Of particular importance in this study, Stim tear collection resulted in a much greater loss of information about important cytokines, such as IL-8, IL-1β, and others than occurred with WO tears. This appears to be particularly evident when the lacrimal gland is only a minor source of a given cytokine. In such cases, lacrimal gland fluid is simply acting as a diluent of ocular surface-resident cytokines. IL-8 and IL-1β both fit into this category. In fact, IL-1β was previously reported to not be reliably detected in Stim tears despite being consistently quantifiable in NS tears[6]. IL-17, a cytokine important in autoimmune disease, was also not consistently detected in Stim tears in that study[6]. In the current study, IL-17 was detected in less than 50% of Stim tear samples, whereas it was undetectable in only two WO samples and one NS sample.

Because most of the Stim cytokine levels measured in the current study were significantly different from NS cytokine levels, especially for cytokines important in dry eye, Stim tears appear to be a poor alternative to NS tears for differentiating non-AD from AD patients. A drawback of the current study comparing NS, WO, and Stim tears was the small number of participants (n = 15). This made it statistically infeasible to break patients into AD and non-AD groups because of the loss of statistical power. However, the differences between NS and Stim tears showed a clear pattern for most cytokines, being were greater than those found between NS and WO tears.

Many literature reports describing "non-stimulated" or "basal" tear levels of tear cytokines described tear collection procedures that are inconsistent with lack of stimulus. A recent study referred to an average micropipette "basal" tear collection rate of 4.6 μL/ minute, which greatly exceeds the upper limit of NS tear collection rate[24]. Yoon et al[15] reported the collection of 30 μL of "basal" tears in a relatively short time period from both control and dry eye patients. This is entirely inconsistent with lack of stimulus. It also raises the important question of variability between studies in reported tear cytokine levels. With substantially lower levels of tear cytokines in Stim tears, comparisons between studies become problematic. Furthermore, for studies in which Stim tears are either deliberately or unknowingly collected, the authors may be missing important information about tear cytokine levels. If tear collection rate varies considerably between patients, the study outcome may be skewed or at least subject to an unnecessary additional source of variance.

Comparison of Main NS vs WO Study to other WO Studies

Other studies that have attempted a WO method have generally used larger WO volumes and studied more abundant tear proteins. Argueso[64] compared Sjögren's syndrome patients to normal patients using a 60 μL WO, but the collected volume did not correlate with total tear protein. Markoulli[24] also used a 60 μL WO volume and found a more than five-fold increase in WO tear collection rate over NS but only a 50% reduction in total protein. Another study showed NS and WO tears to be similar by qualitative gel electrophoresis[22]. One study looked at WO tear lipocalin compared to total protein and

found it to be decreased in Sjögren's dry eye versus non- Sjögren's dry eye and control patients but lysozyme was not decreased[65]. Because IgA/total protein did not decrease in WO tears relative to NS, it can be concluded there was little reflex tear contamination in the WO samples[2]. Secretory IgA levels are known to be very sensitive indicators of stimulus during tear collection[1-3]. The same result was seen in a study of MMP-9 in cats with 10 µL WO[23].

Yawn-induced reflex tears were compared to 18 µL WO samples and showed greater day-to-day[23] and diurnal[66] variation between the two methods. Another study used a 20 µL WO volume method to look at doxycycline effects on tear MMP in rosacea or meibomian gland disease-related dry eye[67], and found a reduction in MMP-9.

In conclusion, the WO microcapillary tear collection, using 10 µL sterile saline as washout solution, produces biomarker profiles that are consistent with NS tear collection for the majority of cytokines studied. Of particular importance, several cytokines known to be key AD dry eye biomarkers can elicit differences between non-AD and AD patients in WO tears just as they do in NS tears. The main drawback is that the more subtle differences between dry eye groups detected by NS collection will not be reflected in WO tears because of compression at the upper end of the cytokine range and accompanying loss of statistical power for WO tears. This is reflected statistically as between-group differences that are lower in WO tears than NS tears. Despite imperfect reproduction of NS tears, the WO method may be a suitable alternative to Schirmer strip and other invasive and less controlled sampling procedures when NS tear collection is impractical.

NS tear collection is a challenge for patients with AD dry eye. It takes AD patients much longer to collect sufficient NS tear volume to enable a valid quantitative assay. In this study, samples were stored in appropriate buffer at –86°C after each ten minutes of tear collection. However, for severely AD patients this resulted in multiple small sample aliquots that had to be pooled for assays, with accompanying greater risk of sample loss due to evaporation and pipetting losses. A WO tear collection method could largely circumvent these issues.

Stim tear collection is not a viable replacement for NS tears, especially in discriminating non-AD and AD patients, because it loses information about important dry eye biomarkers. While excessively diluting many of the important ocular surface-derived biomarkers, Stim tear flow-rate is difficult to control and impossible to standardize from patient to patient.

Overall, the WO method can be used to replace NS tears in discriminating non-AD from AD patients, while Stim tears would be a very poor alternative. Collecting WO tears is easier and quicker than NS tears for all patients but especially for those with AD dry eye.

Further studies would be warranted to investigate the WO method using a more rigorous set of criteria for classifying non-AD versus AD. A greater range of patient categories could also be studied, including Sjögren's versus non-Sjögren's dry eye. It is doubtful that a follow-up study of Stim tears in AD vs non-AD patient groups would provide any meaningful information given the very poor outcome seen in the current study for Stim tear biomarker profiles and their relationship to NS tear profiles.

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APPENDIX A

IRB APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on January 24, 2017. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56.

Principal Investigator:	FULLARD, RODERICK J
Co-Investigator(s):	
Protocol Number:	**X100704004**
Protocol Title:	*Assay of Multiple Inflammatory Biomarkers for Use in Predictive Modeling of Ocular Surface Disease States*

The IRB reviewed and approved the above named project on 7-3-13. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 7-3-13

Date IRB Approval Issued: 7-3-13

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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