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By

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Abstract

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Population-based studies found that approximately 60% of symptomatic dry eye patients presented with visible signs of Meibomian gland dysfunction (MGD), which is considered the most common cause of dry eye symptoms.[1,2] The prevalence of MGD varies widely between 3.5%-70%.[1–6] An estimated 85% of adolescents and young adults between 12-24 years old suffer from acne and over 13 million people, including children, have been treated with isotretinoin since its availability in 1982.[8] Isotretinoin is a vitamin A analogue used to treat severe inflammatory, nodular acne, and has been linked to MGD.[7] Lipid mediators, which are biomarkers for normal physiology or pathophysiology in many tissues including ocular surface and lacrimal gland, are among the earliest inflammatory/immune regulators released in response to stress, but their role in MGD has not been investigated.

The research aimed to elucidate the short- and long-term effects of isotretinoin therapy on Meibomian gland structure and function and meibum lipid mediator profiles via (1) a single-visit cohort study to compare past- and non-users of isotretinoin, and (2) a 12-month longitudinal study to compare changes from baseline during and after therapy among isotretinoin and control cohorts.

Among many commonly reported Meibomian gland (MG) characteristics, MG contrast was found to be a repeatable and reliable measure for MG function. Specifcally, low MG contrast was found to be a risk factor for lipid-deficient dry eye. During isotretinoin therapy, MG contrast decreased significantly but returned to baseline by 6-months post treatment. In general, there were significant differences in many meibum-related clinical measures and symptoms between study groups during treatment, and some of those differences persisted six months post-therapy, including number of expressed glands and meibum quality score. Past-users had worse symptoms, worse MG function, and lower levels of prostaglandin D_2, a potential biomarker for meibocyte function. Furthermore, elevated docosahexaenoic acid (DHA) levels were significantly correlated with worse symptoms, suggesting that its detection in meibum represents active inflammation and/or a metabolic deficiency preventing the effective and/or efficient conversion of DHA to specialized pro-resolving lipid mediators. In summary,

isotretinoin patients develop signs of MGD during isotretinoin therapy that persist long after treatment. Lipidomic results provide potential biomarkers for detecting inflammation, DHA metabolic deficiency, and/or PGD_2-related MGD.

Chapter 1 Table of Contents

Chapter 1 - Introduction

An estimated 5 million Americans, 50 years or older, have severe dry eye while tens of millions more have less severe symptoms.[1–3] Population-based studies with clinical evaluation of the ocular surface found that approximately 60% of symptomatic dry eye patients presented with visible signs of meibomian gland dysfunction (MGD), such as telangiectasia, collarettes, and plugged meibomian glands (MGs).[4,5] The prevalence of MGD, which is considered the most common cause of dry eye symptoms, varies widely between 3.5%-70%.[4–9] Several potential risk factors have been identified for MGD-induced dry eye, including isotretinoin.

Isotretinoin, a vitamin A analogue used to treat severe inflammatory, nodular acne, has been linked to MGD.[10] This is significant, since an estimated 85% of adolescents and young adults between 12-24 years old suffer from acne and over 13 million people, including children, have been treated with isotretinoin since its availability in 1982.[11] Acne is a chronic inflammatory disease of the pilosebaceous unit resulting in increased sebum production secondary to rising androgen levels, such as in puberty.[12] Isotretinoin has been shown to be a highly effective treatment by causing atrophic changes in the sebaceous gland acini with a marked long-lasting decrease in sebum production.[13]

One of the first animal studies to investigate the effects of systemic administration of isotretinoin on MGs found up to 75% reduction in the mean volume of MG acinar tissue in hamsters.[14] Histology of the treated glands showed thickening of gland ductal epithelium and a decrease in the number of mature lipid-laden acinar cells.[14] Rabbits experienced MG acini necrosis and decreased basaloid cells lining the acini walls after systemic exposure to isotretinoin and etretinate.[15]

Studies have investigated isotretinoin effects on the human ocular surface, including lacrimal glands, goblet cells, and tear film. Two studies have looked at the drug's effects on human MGs, one *in vitro* and another *in vivo*. In one study, immortalized human MG epithelial cells that were cultured with various doses of isotretinoin resulted in significant inhibition of cell proliferation with a dose-dependent decrease in cell number after just a few days of treatment.[13] There was also evidence of a dose-dependent increase in percentage of cells with late apoptosis/necrosis within 24 hours of treatment.[13] Isotretinoin also significantly altered expression of almost 7000 genes, including ones involved in cell proliferation, cell death, differentiation, keratinization, and inflammation (mediators and proteases), in human MG epithelial cells.[13] A significant rise in inflammatory mediators was noticeable as early as 2 hours after isotretinoin exposure. Although it had been shown previously that isotretinoin causes altered gene expression, inhibited cell proliferation and terminal differentiation, and apoptosis of sebocytes, this was the first study to demonstrate that similar events take place in MGs with the same exposure. Because isotretinoin is an effective treatment for severe acne by suppressing sebum production by up to 90%, its potential long-term impact on MGs can be damaging.

Mathers, et al., observed the impact of systemic isotretinoin therapy *in vivo* on MG morphology, meibum thickness and volume, osmolarity, tear production, and conjunctival staining with rose bengal in 11 patients.[16] The study found no differences in tear production or conjunctival staining between measures at baseline and during therapy.[16] They did find that tear osmolarity increased during treatment but regressed to baseline after discontinuing treatment.[16] The central 10 MGs of the lower eyelid showed signs of atrophy or decreased density with infrared transillumination biomicrography, and the meibum thickness increased while the excreted volume decreased.[16] Of the eight patients who were followed after discontinuing treatment, seven patients' MG morphology regressed to baseline, and one patient continued to show signs of atrophy at four months after treatment.[16] Although tear osmolarity and meibography images appear to regress to baseline in 7 of 8 patients after discontinuing treatment, the study did not report post-therapy quality and quantity of expressed meibum, which would have been a useful indicator of long-term isotretinoin effects on MGs. To date, no prospective studies have evaluated the long-term effects of isotretinoin use on the MGs. However, there have been several reports of persistent isotretinoin-associated keratoconjunctivitis sicca lasting several years after discontinuation of therapy.[17–19]

Because severe acne can have inflammatory and/or infectious components, it is worth noting that among the earliest inflammatory/immune/wound healing regulators that are released in response to injury, infection or stress are lipid mediators such as eicosanoids. Two families of enzymes generate these early response lipid signals, namely lipoxygenases (5-LOX, 12-LOX, 15-LOX) and cyclooxygenases (COX-1, COX-2) metabolize arachidonic acid to form eicosanoids or omega-3 PUFA to resolvins, protectins and maresins in humans and mice. These lipid mediators and their pathway markers are biomarkers for normal physiology or pathophysiology in many tissues including ocular surface and lacrimal gland. Their role in MGD has not been explored.

In summary, dry eye is a common ocular surface disorder, and a popular and highly effective treatment for severe acne, especially among adolescents and young adults, is believed to contribute to MGD-induced dry eye in the young adult population. Although there is evidence that isotretinoin adversely impacts MG structure and function, there have been very few human studies to determine the distribution and frequency of isotretinoin therapy-induced MGD. Even fewer studies have investigated the impact on adolescent ocular health. This study aimed to conduct a 12-month prospective cohort study to evaluate short-term changes due to therapy and a cross-sectional study to evaluate potential long-term implications of the treatment. The results will provide insight into the risk of developing isotretinoin-induced MGD so that patients and prescribing physicians can make informed decisions about an acne treatment plan, caregivers can establish a co-management philosophy during isotretinoin treatment, eye care practitioners can develop a more targeted ocular surface examination and MGD treatment plan for patients with a known history of isotretinoin use, and to provide knowledge that may lead to the development of innovative prevention and/or treatment plans for isotretinoin-induced MGD.

Chapter 2 - Meibomian Gland Contrast

2.1 Repeatability of Meibomian Gland Contrast, a Potential Indicator of Meibomian Gland Dysfunction

2.1.1 Introduction

Meibomian gland dysfunction (MGD) is believed to be the most common cause of ocular dryness symptoms [20]. Extensive effort has been made to understand the pathophysiology of MGD-induced evaporative dry eye, particularly the relationship between Meibomian gland dropout and meibum output *in vivo*. Meibomian gland dropout implies partial or total gland loss or atrophy, and it has been estimated by meiboscopy and meibography. Meiboscopy allows visualization of Meibomian glands by retroillumination of the eyelids, usually with a penlight or transilluminator, while meibography also includes photo-documentation. More recent meibography innovations include biomicroscopes and corneal topographers equipped with infrared cameras that produce images with better contrast between the Meibomian glands and the surrounding tarsal plate and tissues. More advanced systems integrate both retroillumination and infrared photo documentation to take advantage of both methods.

When evaluating meibography images, we make the assumptions that the glands, which appear as bright linear structures on meibography, are supposed to extend the full length of the tarsal plate and, when they do not, they are assumed to have functional gland loss, or atrophied. The degree of atrophy is most commonly assessed as a percentage of gland loss area (e.g., space unoccupied by Meibomian glands) compared to the presumed full area of the tarsal plate. The estimated percentage can then be assigned a grade using one of several ordinal scales to represent severity [21–27]. While grading atrophy is useful, especially in cases like obstructive MGD in which glands appear shorter due to hypo-reflectivity proximal to the blockage, it may not be as useful in cases of hyposecretory MGD, where a global suppression of meibum production may result in overall dimming or fading of whole glands, not just shortening. In those instances, we would expect to see decreased intensity of all Meibomian glands along their full lengths but would be unable to characterize them using the existing Meibomian gland atrophy grading system.

2.1.2 Summary of Results

The published manuscript titled “Repeatability of Meibomian Gland Contrast, a Potential Indicator of Meibomian Gland Function”, which is included in the Appendices, discusses the methods, results, and general conclusions about the study findings.

2.1.3 Conclusion

Meibomian contrast in the region of the central five Meibomian glands from meibography images captured with the OCULUS Keratograph 5M was demonstrated to be repeatable between visits and showed good agreement between different head positions and room lighting conditions.[28] In general, changes in Meibomian gland contrast greater than 11 and 18 intensity units in the upper eyelid and lower eyelid, respectively, are less likely due to normal variability. Having this objective, reliable, and repeatable grading method can be valuable for detecting subtle changes in meibography due to age, disease, or intervention, particularly when Meibomian gland length may not change.

2.2 Meibomian Gland Contrast: Sensitivity and Specificity in the Diagnosis of Hyposecretory Meibomian Gland Dysfunction

2.2.1 Introduction

Meibomian gland lipid production is important in maintaining good ocular surface health.[29] Gland function is often assessed by measuring tear-lipid thickness using interferometry and evaluating meibum quality and quantity through gland expression.[30] Meibography has long been used to evaluate gland structure, or atrophy, either subjectively using various published grading scales or objectively using software.[31–35] However, Meibomian gland atrophy has not always correlated with other clinical parameters or symptoms.[35–37]

Meibomian gland contrast was recently introduced as a repeatable, objective measure that can potentially be used to assess Meibomian gland function.[38] The method is of particular value when there is an overall suppression of oil production in the body, including in the sebaceous and Meibomian glands, which can result in fading, not shortening/atrophy, of glands on meibography. The research brought to light a case of isotretinoin intervention that resulted in a lowering of Meibomian gland contrast, but shortening/atrophy. It is possible that other risk factors for Meibomian gland dysfunction (MGD) can similarly alter Meibomian gland contrast, rather than length, at least in the early phases of intervention or disease.

In this study, we aim to determine the significance of Meibomian gland contrast among a group of hyposecretory MGD cases and controls. First, we evaluate the differences in ocular surface parameters, including Meibomian gland contrast, between cases and controls. We also determine the validity of using Meibomian gland contrast as a classifier for hyposecretory MGD by developing a receiver operating characteristic (ROC) curve and estimating the sensitivity and specificity at the optimal cut-point for Meibomian gland contrast in the diagnosis of hyposecretory MGD. Finally, we evaluate Meibomian gland contrast as a risk factor for hyposecretory MGD.

2.2.2. Methods

<u>Subjects</u>

Clinical data and meibography images were extracted from the Ocular Surface study database at the Clinical Research Center (CRC) at the University of California, Berkeley, School of Optometry. Subjects were filtered and classified based on two parameters: Standard Patient Evaluation of Eye Dryness (SPEED) score and average tear-lipid thickness (LipiView; Johnson & Johnson Vision). This was a case-control study in which hyposecretory MGD individuals (cases) were those who had severe symptoms (SPEED>=10) and thin average tear-lipid thickness (<=35 nm), and normal individuals (controls) were those with very mild or no symptoms (SPEED <=2) and thick average tear-lipid thickness (>=80 nm). The data from the database were collected after obtaining written informed consent from all study participants. The study adhered to the tenets of the Declaration of Helsinki and was approved by the University of California, Berkeley, Office for the Protection of Human Subjects.

Ocular Surface Database

The ocular surface database is a compilation of uniformly collected data on demographic variables, medical history, symptoms, and ocular surface data from 345 subjects. For the purposes of this study, the following data were extracted from the database: demographic variables, ocular and systemic medical history, SPEED scores, average tear-lipid thickness measured with the LipiView, fluorescein tear breakup time, Schirmer's tear test results, upper eyelid meibography images (OCULUS Keratograph 5M; OCULUS, Inc., Arlington, WA), and upper eyelid meibomian gland expression quality and quantity scores. Meibomian gland expression was performed with the Korb Meibomian Gland Evaluator to assess both the quantity and quality of the expressed meibum. Evaluation, scoring, and calculations for meibum quality and quantity scores were previously described.[39,40] Percent Meibomian gland atrophy and Meibomian gland contrast values of upper eyelid meibography images were also measured using a previously described method.[28,41]

Statistical Methods

Robust (clustered) logistic regression models using the Huber-White standard error estimator clustered by subject (Stata/IC 14.0; vce(cluster) option) to account for within-subject correlations between eyes were used to evaluate differences in clinical parameters between the case and control groups. With Meibomian gland contrast as a classifier, a receiver operating characteristic (ROC) curve was developed and area under the curve (AUC) was calculated while controlling for correlations between eyes using participant IDs as sampling clusters. Sensitivity, specificity, and classifier cut-point at the optimal correct classification of cases and controls was estimated with one-eye (randomized) analysis. AUC was also estimated with percent Meibomian gland atrophy and meibum quantity score as classifiers for comparison. Since higher values of Meibomian gland contrast and meibum quality scores represent better health status, the reciprocal (inverse) of these values were used to calculated AUC and plot ROC curves, which require that higher values of classifiers represent higher risk. All analyses were conducted with Stata (Stata/IC 14.0; StataCorps LLC).

2.2.3 Results

Subjects

Of the 345 subjects in the Ocular Surface Study database from 2012 to 2018, 31 eyes of 22 subjects (including both eyes for nine subjects) fulfilled the criteria to be controls and 13 eyes of 12 subjects (including both eyes for one subject) fulfilled the criteria to be cases. Their demographic data are presented in **Table 2.2.3-1**. In general, cases were more likely to be older, male, and non-users of contact lenses but were similar to controls with respect to race.

Table 2.2.3-1. Characteristics of cases and controls.

		Control (n=22; 31 eyelids)	**Case (n=12; 13 eyelids)**
Age – years		22.7 ± 5.5	43.9 ± 17.2
Gender – no. (%)			
	Male	5 (23%)	7 (58%)
	Female	17 (77%)	5 (42%)
Race – no. (%)			
	White	5 (23%)	4 (33%)
	Asian	10 (45%)	5 (42%)
	Other	7 (32%)	3 (25%)
Contact Lens – no. (%)			
	Users	11 (50%)	4 (33%)
	Non-users	11 (50%)	8 (67%)

Case vs. Control

Cases showed significantly worse ocular surface signs for all characteristics observed in this study, except for Schirmer's tear test (**Table 2.2.3-2**). Cases had, on average, 11.9 units lower Meibomian gland contrast ($p<0.001$), 18.9 higher percent Meibomian gland atrophy ($p=0.039$), 7.6 units lower meibum quality ($p=0.038$) scores, 7.7 units lower meibum quantity ($p=0.023$) scores, and 9.5 seconds shorter fluorescein tear breakup time ($p<0.001$) compared to controls. The difference in Schirmer's tear test results was not statistically significant ($p=0.768$).

Table 2.2.3-2. Comparison of ocular surface characteristics between cases and controls.

	Case (n=12; 13 eyelids)	Control (n=22; 31 eyelids)	Mean Difference (95% CI)	P Value*
MG Contrast	25.2 ± 8.8	37.1 ± 8.7	-11.9 (-17.7 to -6.1)	<0.001
Percent MG Atrophy	36.9 ± 31.8	18.0 ± 11.9	18.9 (0.98 to 36.9)	0.039
Meibum				
Quality Score	17.2 ± 10.5	24.7 ± 11.0	-7.6 (-14.7 to -0.4)	0.038
Quantity Score	11.3 ± 9.4	19.0 ± 10.3	-7.7 (-14.2 to -1.1)	0.023
Fluorescein Tear Breakup Time (seconds)	4.2 ± 2.8	13.7 ± 10.8	-9.5 (-14.3 to -4.7)	<0.001
Schirmer's I – mm	26.4 ± 10.9	25.2 ± 12.0	-1.2 (-9.2 to 6.8)	0.768

*P Value using univariable linear regression model with Huber-White standard error estimator clustered by subject

Sensitivity and Specificity of Meibomian Gland Contrast

The receiver operating characteristic (ROC) curve with the inverse of Meibomian gland contrast as a classifier for hyposecretory MGD diagnosis resulted in an area under the curve (AUC) of 0.83 (95% CI: 0.75, 0.99). **Table 2.2.3-3** and **Figure 2.2.3-1** show the superiority of Meibomian gland contrast over both percent Meibomian gland atrophy (AUC=0.66) and (inverse of) meibum quantity score (AUC=0.73) in diagnosing hyposecretory MGD. A Meibomian gland contrast cut-point of 28.3 intensity units yielded optimal correct classification of study subjects at a rate of 85.3% with a sensitivity of 0.67 and specificity of 0.95. Using a cut-off of 28.3 intensity units, individuals with low Meibomian gland contrast (≤28.3 intensity units) had 2.7 higher odds of having hyposecretory MGD (p=0.001, 95% CI: 1.04, 4.4) compared to individuals with high Meibomian gland contrast (>28.3 intensity units).

Table 2.2.3-3. Summary of receiver operating characteristic (ROC) area under the curve (AUC) for potential classifiers of hyposecretory Meibomian gland dysfunction (N=44).

	ROC AUC	95% Confidence Interval
MG Contrast*	0.83	0.70 to 0.96
Normalized MG Contrast*	0.80	0.64 to 0.95
Percent MG Atrophy	0.66	0.45 to 0.86
Meibum Quantity Score*	0.73	0.55 to 0.90

* Inverse values were used to generate ROC curves

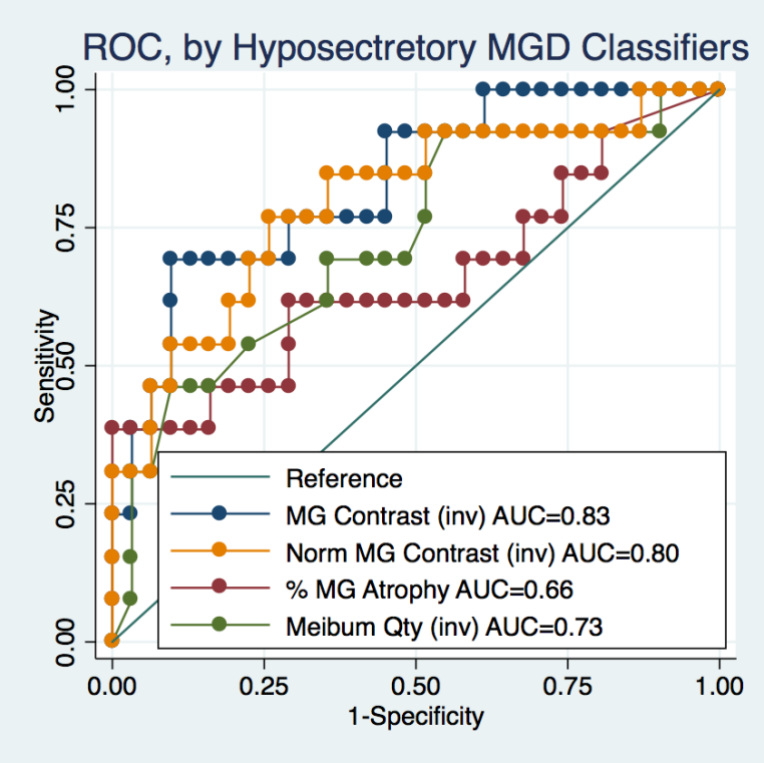

Figure 2.2.3-1. Receiver Operating Characteristic (ROC) curves for Meibomian gland contrast, normalized Meibomian gland contrast, percent Meibomian gland atrophy area, and meibum quantity scores in the diagnosis of hyposecretory Meibomian gland dysfunction.

2.2.4 Discussion

In this study, we explored the significance of Meibomian gland contrast in the diagnosis of hyposecretory MGD, which was defined in this study as presence of thin tear-lipid layer (≤35 ICU) and severe symptoms (SPEED≥10). The study found that cases had significantly worse ocular surface characteristics than those of controls in all areas evaluated in this study and that Meibomian gland contrast is a good diagnostic indicator for hyposecretory MGD with good sensitivity and excellent specificity. Furthermore, those with low Meibomian gland contrast (≤28 intensity units) had 2.7 higher odds of having hyposecretory MGD compared to those in the high Meibomian gland contrast group.

This study was prompted by a previously reported finding in which a patient undergoing isotretinoin treatment presented with a fading, not shortening, of Meibomian glands on meibography. The hypothesis was that, despite clinical and research emphasis on Meibomian gland atrophy, Meibomian gland intensity or contrast may also be a useful or better indicator of hyposecretory MGD, especially in diseases or treatments that suppress sebum or meibum production. **Figure 2.2.3-2** presents meibography of one control and three cases. Judging from the images alone, one might mistakenly guess that the image for the control subject was a case due to the moderate atrophy. However, as indicated below the images, the individuals with

same or less atrophy but lower contrast were more symptomatic and presented with worse clinical signs. Lipids contained in meibum are believed to be highly reactive to infrared light, giving it the high intensity or contrast.[42] **Figure 2.2.3-2** suggests that even moderate Meibomian gland atrophy may not affect downstream ocular surface parameters or induce symptoms. If Meibomian gland contrast is high enough, whatever remains in the Meibomian gland duct can be sufficient to maintain good ocular surface health and stave off any symptoms of discomfort or dryness.

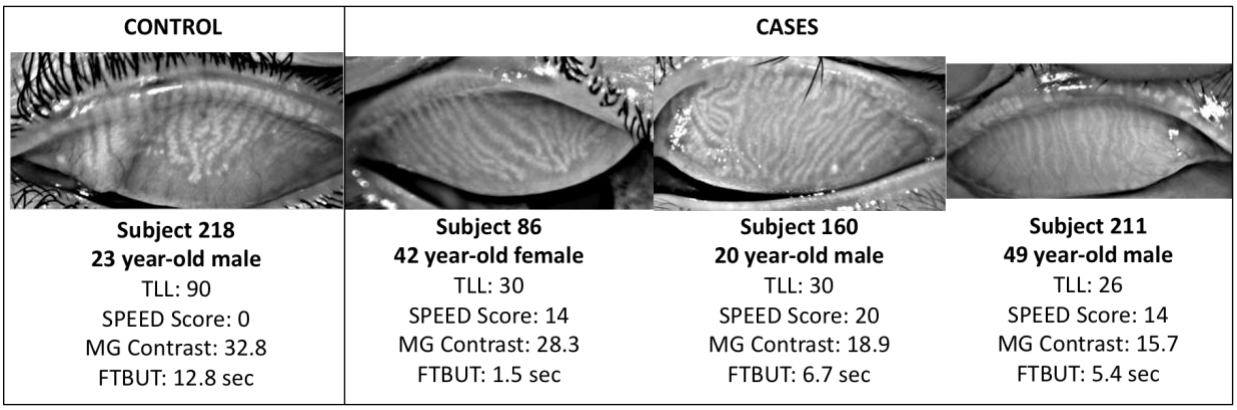

Figure 2.2.3-2. Meibography images for a control subject (leftmost) and cases. Despite the presence of meibomian gland atrophy in the control subject, tear-lipid layer (TLL) was thick provided good tear film stability (fluorescein tear breakup time (FTBUT)) and no dryness symptoms (Standard Patient Evaluation of Eye Dryness (SPEED) score). Cases on the right have minimal atrophy but have thin TLL and severe symptoms. All cases had Meibomian gland (MG) contrast less than or equal to 28.3 intensity units, the cut-off for optimal sensitivity and specificity for the diagnosis of hyposecretory Meibomian gland dysfunction in this study.

In the side-by-side comparison of hyposecretory MGD cases and controls (**Table 2.2.3-2**), all ocular surface parameters investigated in this study were worse in cases compared to controls. First, the study found that Meibomian gland contrast was approximately 12 units lower in cases than controls, which is just outside the range of normal intra-subject variability, suggesting that this is also clinically significant.[38] Furthermore, cases averaged 19% higher Meibomian gland atrophy compared to controls. These results suggest that evaluating both Meibomian gland atrophy *and* contrast are important in trying to diagnose and understand etiology of symptoms. The images from **Figure 2.2.3-2** are evidence of this need. Moderate Meibomian gland atrophy may also be accompanied by an abundance of healthy meibum remaining in the gland, sufficient enough to maintain a healthy ocular surface. However, if the meibum quantity or composition changes systemically without Meibomian gland atrophy, evaluating Meibomian gland contrast is just as important.

Cases also had poor meibum quality and lower meibum quantity upon expression with a Korb Meibomian Gland Evaluator, which applies a standard pressure. The meibum quality score is a gross evaluation of the meibum appearance with classifications of clear, cloudy, or inspissated,

while the quantity classifications were copious, moderate, or minimal.[39,40] Such inexact measures are subject to misclassification, but in this study population, there was a distinct difference between the study groups. Cases also had significantly lower fluorescein tear breakup time, further supporting the hypothesis that compromised Meibomian gland morphology (atrophy and/or low contrast) can lead to dysfunction (poor meibum quality or reduced meibum quantity) and downstream effects on tear film (shorter tear breakup time) and symptoms.[29,43]

The ROC with the inverse of Meibomian gland contrast as a classifier for hyposecretory MGD diagnosis resulted in an AUC of 0.83, which suggests that Meibomian gland contrast is a good diagnostic test for the disease (**Figure 2.2.3-1, Table 2.2.3-3**). In fact, Meibomian gland contrast yielded a better AUC than percent Meibomian gland atrophy (AUC=0.66; poor) and the inverse of expressed meibum quantity (AUC=0.73; fair). Based on **Figure 2.2.3-2**, it is not surprising to see that meibum quantity may be a better classifier for hyposecretory MGD than percent Meibomian gland atrophy, despite high variability of meibum quantity scores. In individuals treated with isotretinoin, meibum production drastically decreases, as intended by the treatment, and is apparent with gland expression.[38] However, the characteristic meibography for those undergoing isotretinoin treatment no gland shortening/atrophy despite the decreased availability of meibum upon gland expression. It is possible that lengthier treatment durations or extremely high dosages could result in gland atrophy, but the duration and dosage typical of a course of isotretinoin treatment appears to worsen Meibomian gland expression and contrast, but not length.[16,38] We suspect that similar meibography findings may also be characteristic of other systemic conditions associated with MGD or sebaceous gland atrophy, such as atopic dermatitis, psoriasis, and androgen deficiency.[44]

With the understanding that Meibomian gland contrast is a good classifier of hyposecretory MGD, we wanted to understand the odds of having the disease if Meibomian gland contrast was low. This study found that individuals with low Meibomian gland contrast ($\leq$ 28 intensity units) had 2.7 higher odds of having hyposecretory MGD compared to those with high Meibomian gland contrast (>28 intensity units). Although the odds are not overwhelming at a distinct cut-point of 28 intensity units, the results continue to support that Meibomian gland contrast is an important consideration in diagnosing hyposecretory MGD and dry eye disease.

In summary, Meibomian gland contrast is a good indicator of hyposecretory MGD, which was defined in this study as presence of thin tear-lipid layers ($\leq$35 ICU) and severe symptoms (SPEED$\geq$10). The good AUC, good sensitivity, and excellent specificity of Meibomian gland contrast for hyposecretory MGD diagnosis suggests that Meibomian gland contrast may even be a better indicator of hyposecretory MGD than Meibomian gland atrophy and meibum quality scores. Although it is recommended that meibum expression and percent Meibomian gland atrophy be considered in evaluating dry eye patients, Meibomian gland contrast should also be considered, particularly for symptomatic patients with normal aqueous production and minimal percent Meibomian gland atrophy.

Chapter 3 - Comparison of Symptoms and Meibomian Gland Structure and Functions between Past-Users and Non-Users of Isotretinoin

3.1 Introduction

A recent comprehensive review of the literature on isotretinoin effects on the Meibomian glands and the ocular surface demonstrated the need for clinicians to be aware of isotretinoin-associated MGD, especially in young adults, during treatment.[45] However, the literature also suggests that ocular surface changes exhibited during isotretinoin therapy return back to "normal" after discontinuing treatment, which is not in agreement with some anecdotal evidence.[16] Because Meibomian glands are specialized sebaceous glands, we hypothesized that isotretinoin can also permanently reduce meibum production in the Meibomian glands resulting in symptoms of dry eye, similar to the way isotretinoin can permanently reduce sebum production in the facial sebaceous glands. In this study, we aimed to determine if there are, in fact, differences in symptoms and ocular surface characteristics based on past isotretinoin exposure in a cross-sectional study. In addition to symptoms, we will evaluate various clinical parameters, including Meibomian gland contrast, which was previously established in Chapter 2 as an objective, repeatable measure of lipid-deficient dry eye.

3.2 Methods

This was a cross-sectional study consisting of two groups: past-users and non-users of isotretinoin. Study participants were recruited from the University of California, Berkeley campus and surrounding communities. Participants were required to be 18-39 years old and available for a single visit at the Clinical Research Center in the School of Optometry. Volunteers were excluded if they were using isotretinoin at the time of enrollment, had a history of ocular surgery or any active ocular inflammation or infection, or had systemic diseases, including arthritis, gout, diabetes, and hypercholesteremia. Participants were asked to discontinue contact lens wear, make-up, and facial moisturizers 24 hours prior to the scheduled visit. Written informed consent was obtained from all study participants, and the study adhered to the tenets of the Declaration of Helsinki. The study protocol was approved by the University of California, Berkeley, Office for Protection of Human Subjects.

Sample size calculations estimated the need for 20 subjects in each group to detect a 15% difference in population means for Meibomian gland contrast with 80% power and 0.05 level of significance using previously published data, where mean Meibomian gland contrast for normal is 37.1 intensity units and an approximate common variance of 8.8 intensity units.

<u>Measurements and Procedures</u>

Participants were asked to complete a comprehensive medical history questionnaire, the Standard Patient Evaluation of Eye Dryness (SPEED) questionnaire, and Visual Analogue Scale symptoms questionnaire prior to the ocular examination. Upon completing both questionnaires, participants underwent a battery of tests. Performed first were non-invasive procedures, including tear-lipid thickness (Lipiview®; Johnson & Johnson Surgical Vision) and non-invasive tear breakup time measurements (Medmont E300; Medmont Pty Ltd; Australia; averaged from 3 measurements per eye, alternating), as well as a slit lamp examination with white light. Following those were invasive measurements, which included tear breakup time with sodium fluorescein (FTBUT; seconds) using 1 μl of 2% fluorescein measured three times per eye, alternating between eyes. Corneal staining was scored immediately after FTBUT measurements on the SICCA scale [46]. Meibomian gland expression was then performed on the lateral, central, and medial regions of the lower and upper eyelid margins with a Korb Meibomian Gland Evaluator™. This procedure involved applying 10-15 seconds of gentle pressure on the skin inferior to the lower eyelid margin while the participant was in upgaze and superior to the upper eyelid margin while the participant was in downgaze. The secretions were scored based on quality and quantity [39,40]. Next, 1% lissamine green ophthalmic solution was instilled to assess conjunctival staining using the SICCA scale [46] and positioning of Marx's line with respect to Meibomian gland orifices using the scale defined by Yamaguchi, et al. [47] Five minutes later, a second instillation of the lissamine green/fluorescein combination drop was instilled to assess length and width of lid wiper epitheliopathy after everting the upper eyelid using the scale previously defined by Korb, et al. [48] Finally, meibography images of both the upper and lower lids were captured and analyzed using the OCULUS Keratograph 5M (OCULUS, Inc., Arlington, WA) and processed to measure Meibomian contrast, as previously described in Chapter 2.[28]

Statistical Methods

This study explored differences in ocular surface characteristics between past-users and non-users of isotretinoin. Main outcomes of interest included SPEED scores, Meibomian gland contrast, percent Meibomian gland atrophy, eyelid notching, lid margin redness, number of expressed glands, meibum quality and quantity, tear-lipid thickness, tear meniscus height, fluorescein tear breakup time, Schirmer's I test, and symptoms (SPEED and VAS scores).

Relationships between each outcome and several independent variables were evaluated using univariable linear and logistic regression models with a robust variance estimator. Independent variables included the following: age, sex, contact lens use, hormonal birth control use, and elapsed time since isotretinoin treatment. The final regression model for each outcome consisted of isotretinoin exposure as an independent variable and controlled for factors that were significant. Final models were selected by considering F-test p-values and testing assumptions using residual and other diagnostic plots and diagnostic tests. Multivariable linear and logistic regression models used the Huber-White standard error estimator clustered by subject (Stata/IC 14.0; vce(cluster) option) to account for within-subject correlations between eyes of subjects.

3.3 Results

Demographics

Thirty-six past-users [age (SD)=22.5 (4.0) years] and 254 non-users [age (SD)=23.6 (6.1) years] of isotretinoin completed the study (**Table 3.3-1**). Distribution of males and contact lens users were similar between groups. However, the proportion of females using hormonal birth control (HBC) was greater among the past-users compared to non-users of isotretinoin.

Table 3.3-1. Study demographics by isotretinoin exposure groups.

		Isotretinoin Unexposed n=254	Isotretinoin Exposed n=36
Age – years			
	Mean (SD)	22.5 (4.0)	23.6 (6.1)
	Range	18-38	18-39
Sex			
	Males	75 (30%)	11 (31%)
	Females	179 (70%)	25 (69%)
Contact Lens			
	Non-Users	117 (46%)	20 (56%)
	Users	137 (54%)	16 (44%)
Birth Control (Females only)			
	Non-Users	122 (68%)	9 (36%)
	Users	57 (32%)	16 (64%)

Outcomes

In univariate analyses, all outcomes were tested for significance with isotretinoin exposure, age, sex, contact lens use, hormonal birth control use, and elapsed time since isotretinoin treatment. Frequency and severity of burning/watering symptoms, eyelid margin notching, number of expressed Meibomian glands, and meibum quality scores were significantly associated with isotretinoin exposure but not with any other demographic characteristics. Frequency and severity of soreness/irritation symptoms, percent Meibomian gland atrophy, and eyelid margin redness were significantly associated with isotretinoin exposure and age. Meibomian gland contrast was significantly associated with isotretinoin exposure and sex. Meibum quantity score was significantly associated with isotretinoin exposure and hormonal birth control use. Characteristics that were not associated with isotretinoin exposure were total SPEED score, tear-lipid thickness, tear meniscus height, tear breakup time, and Schirmer's I test. These relationships were then used to formulate multivariable models discussed below. No outcomes were significantly associated with contact lens use or elapsed time since isotretinoin treatment.

Total SPEED scores were not significantly different between isotretinoin exposure groups (p=0.081), but when specific symptoms were analyzed, frequency and severity of ocular surface soreness/irritation and burning/watering eyes were significantly different between exposure groups. After controlling for age, past-users of isotretinoin were more likely to "sometimes" (OR=2.5; 95% CI: 1.1, 5.4) or "often" (OR=3.2; 95% CI: 1.004, 10.0) feel soreness/irritation compared to unexposed individuals (**Table 3.3-2**). The soreness/irritation reported by past-users were more likely to be "tolerable" compared to unexposed individuals, also after controlling for age (OR=2.3; 95% CI: 1.01, 5.4). Past-users of isotretinoin were also more likely to "often" or "constant(ly)" feel burning/watering symptoms (OR=4.5; 95% CI: 1.6, 14.5), and these symptoms were more likely to be "uncomfortable" among isotretinoin-exposed individuals than unexposed (OR=3.3; 95% CI: 1.3, 8.6).

Among the characteristics that were associated with isotretinoin exposure but not with any other demographic characteristic were eyelid margin notching, number of expressed Meibomian glands, and meibum quality. Isotretinoin-exposed individuals had 11.2 higher odds of having eyelid notching (p<0.001; 95% CI: 3.4, 37.0), approximately 3.7 fewer expressed Meibomian glands (p<0.001; 95% CI: -5.6, -1.8), and approximately 10.8 units lower meibum quality scores (p<0.001; 95% CI: -16.6, -4.9), compared to unexposed individuals.

Meibomian gland atrophy and eyelid margin redness were both significantly associated with age, but after controlling for age, neither Meibomian gland atrophy (Coeff=5.9; 95% CI:-0.01, 11.9) nor eyelid margin redness (OR=2.5; 95% CI: 0.96, 6.4) were statistically significant.

While controlling for sex, we found that Meibomian gland contrast was 4.6 intensity units higher among past-users of isotretinoin compared to non-users (p=0.025; 95% CI: 0.6, 8.7). More specifically, among females, past-users of isotretinoin had 6.2 intensity units higher in Meibomian gland contrast than non-users (p=0.015; 95% CI: 1.2, 11.1). There was no difference in Meibomian gland contrast between male past-users and non-users (p=0.810).

While controlling for hormonal birth control use, meibum quantity score was approximately 5.2 units lower for isotretinoin-exposed compared to unexposed individuals (p=0.048; 95% CI: -10.4, -0.04). This difference was driven by HBC users; isotretinoin-exposed HBC females had 8.7 lower meibum quantity score than isotretinoin-unexposed HBC females. Further, among past-users of isotretinoin, HBC females had 15.8 units lower meibum quantity score than non-HBC females (p=0.003; 95% CI: -26.2, -5.4). No difference in meibum quantity scores were found between exposure groups among non-HBC females (p=0.589) or males (p=0.115).

Tear-lipid thickness (p=0.369), fluorescein tear breakup time (p=0.097), tear meniscus height (p=0.110), and Schirmer's I test (p=0.478) were not significantly different between exposure groups.

Table 3.3-2. Summary of scores from Standard Patient Evaluation of Eye Dryness (SPEED) and Visual Analogue Scale symptoms questionnaires by isotretinoin exposure groups.

	Isotretinoin Unexposed n=254		Isotretinoin Exposed n=36		Mean Difference (95% CI)	p value
	# of Subjects		# of Subjects			
SPEED						
Total Score	254	5.8 ± 4.8	36	7.3 ± 5.1	1.5 (-0.2 to 3.2)	0.081
Frequency						
Dryness, Grittiness, Scratchiness	254	0.84 ± 0.79	36	0.86 ± 0.76	0.03 (-0.25 to 0.30)	0.850
Soreness or Irritation[A]	254	0.46 ± 0.63	36	0.78 ± 0.68	0.3 (0.08, 0.52)	***0.008***
Never	155		13		+	+
Sometimes	81		18		2.5 (1.1 to 5.4)	***0.020***
Often or Constant	18		5		3.2 (1.004 to 10.0)	***0.049***
Burning or Watering	254	0.42 ± 0.63	36	0.72 ± 0.77	0.30 (0.07 to 0.52)	***0.011***
Never	163		17		+	+
Sometimes	76		12		1.5 (0.7 to 3.3)	0.302
Often or Constant	15		7		4.5 (1.6, 12.5)	***0.004***
Fatigue	254	0.92 ± 0.80	36	0.89 ± 0.85	-0.03 (-0.31, 0.25)	0.843
Severity						
Dryness, Grittiness, Scratchiness	254	1.03 ± 1.03	36	1.0 ± 1.01	-0.04 (-0.39 to 0.32)	0.846
Soreness or Irritation[A]	253	0.58 ± 0.86	36	0.89 ± 0.92	0.27 (-0.03 to 0.58)	***0.075***
No Problems	158		15		+	+
Tolerable	51		12		2.3 (1.01 to 5.4)	***0.046***
Uncomfortable	35		7		2.0 (0.7 to 5.3)	0.171
Bothersome or Intolerable	9		2		2.0 (0.4 to 10.8)	0.423
Burning or Watering	254	0.60 ± 0.96	36	0.94 ± 1.09	0.3 (0.004 to 0.69)	***0.048***
No Problems	163		17		+	+
Tolerable	51		8		1.5 (0.6 to 3.7)	0.373
Uncomfortable	23		8		3.3 (1.3 to 8.6)	***0.013***
Bothersome or Intolerable	17		3		1.7 (0.4 to 6.4)	0.437
Fatigue	254	0.95 ± 0.96	36	1.22 ± 1.20	0.3 (-0.1 to 0.6)	0.128
Meibomian Gland Contrast[B]	235	38.5 ± 11.7	35	43.2 ± 13.8	4.6 (0.6 to 8.7)	***0.025***
Meibomian Gland Atrophy -- %[A]	216	16.9 ± 13.6	21	23.4 ± 14.4	5.9 (-0.1 to 11.9)	0.055
Eyelid Notching	254	8 (1.2)	36	11 (15.3)	11.2 (3.4 to 37.0)	***<0.001***

Eyelid Margin Redness[A]	252	49 (9.8)	36	17 (23.6)	2.5 (0.96 to 6.4)	0.061
Number Expressed Glands	252	15.3 ± 9.3	36	11.6 ± 5.3	-3.7 (-5.6 to -1.8)	***<0.001***
Meibum Quality Score	252	42.5 ± 27.9	36	31.7 ± 15.8	-10.8 (-16.6 to -4.9)	***<0.001***
Meibum Quantity Score[C]	252	31.5 ± 22.9	36	25.1 ± 14.1	-5.2 (-10.4 to -0.04)	***0.048***
Tear Lipid Layer Thickness – nm	253	64.2 ± 20.1	36	67.5 ± 22.3	3.3 (-3.9 to 10.4)	0.369
Tear Meniscus Height – mm	252	0.25 ± 0.08	36	0.23 ± 0.05	-0.02 (-0.03 to 0.003)	0.110
Tear Breakup Time – seconds	252	8.1 ± 7.8	36	11.0 ± 10.5	2.9 (-0.5 to 6.3)	0.097
Schirmer's I Test – mm	246	20.4 ± 33.6	35	18.7 ± 10.7	-1.6 (-6.2 to 2.9)	0.478

* All outcomes were tested for significance with age, sex, contact lens use, and hormonal birth control use, and final model only controlled for these factors if they were significantly associated with the outcome.

[A] Controlled for age

[B] Controlled for sex

[C] Controlled for hormonal birth control use

+ Reference parameter

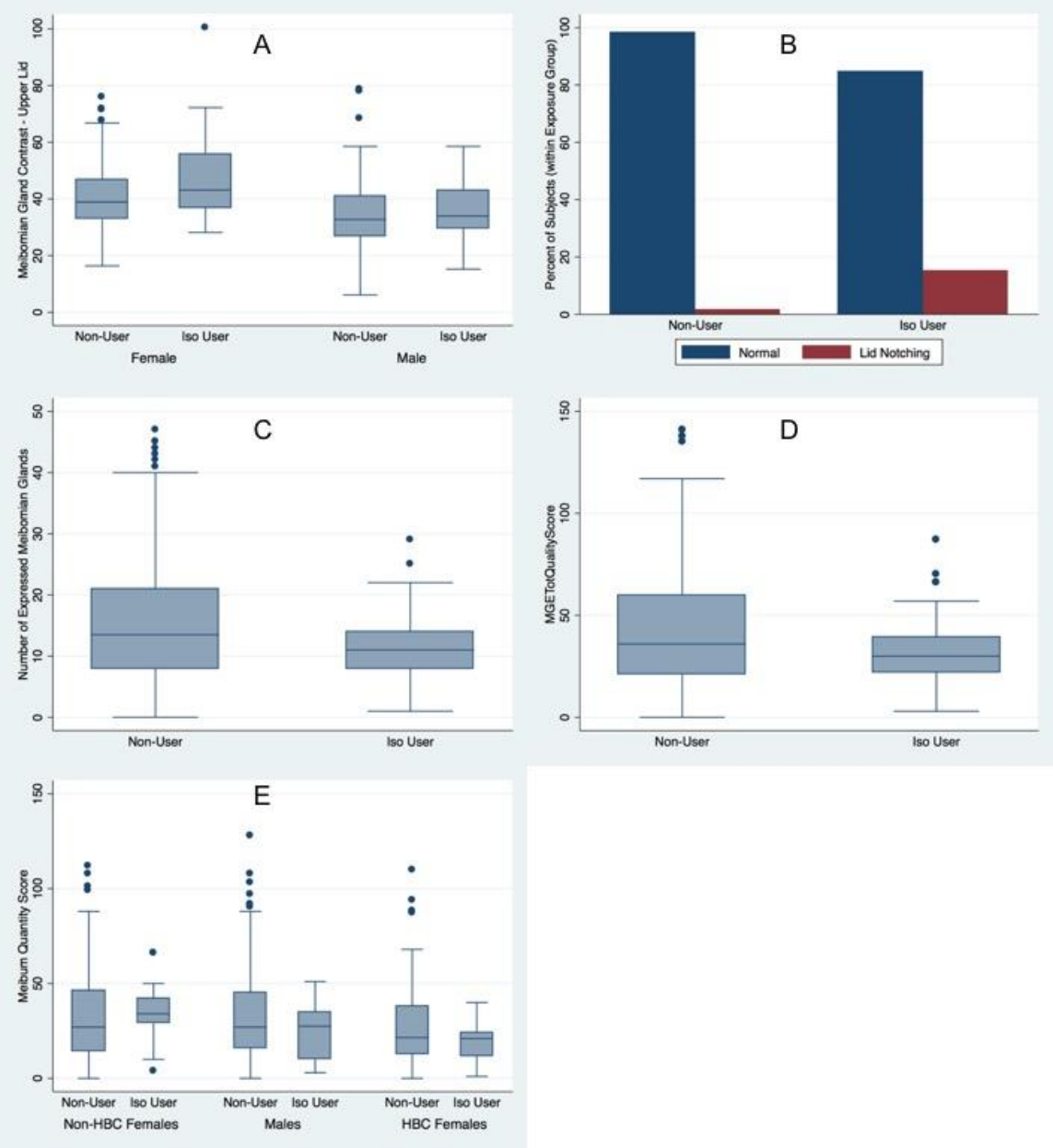

Figure 1. Plots comparing ocular surface parameters between isotretinoin exposure groups. (A) Meibomian gland contrast vs. isotretinoin exposure, by sex. (B) Lid notching vs. isotretinoin exposure. (C) Number of expressed Meibomian glands vs. isotretinoin exposure. Expressed meibum (D) quality vs. isotretinoin exposure and (E) quantity by sex and hormonal birth control (HBC) cohorts.

3.4 Discussion

This study aimed to determine if ocular dryness symptoms and ocular surface characteristics of individuals who were treated with isotretinoin (aka Accutane, 13-*cis*-retinoic acid) in the past for severe, nodulocystic acne are significantly different from those individuals who were never treated with the medication. Understanding what differences there may be can elucidate any long-term implications of the medication to the ocular surface, particularly to the structure and function of Meibomian glands, as well as how to best diagnose, treat, and manage these patients.

Total SPEED score was not significantly different between the exposure groups. Past isotretinoin users reported worse severity and frequency of specific symptoms, soreness/irritation and burning/watering eyes, but were no different from their unexposed counterparts regarding other symptoms reported on the SPEED questionnaire, such as dryness/grittiness/scratchiness and eye fatigue. The worse symptoms among isotretinoin-treated individuals can be explained by many of the ocular surface findings. In this study, isotretinoin-treated individuals had a greater odds of eyelid margin notching, fewer expressed glands and worse meibum quality and quantity scores, which we predicted given the known effects of isotretinoin on both the sebaceous and Meibomian glands.[13,49]

For meibum quantity score, isotretinoin-treated individuals had lower scores, on average, than untreated individuals, and this relationship strongly interacted with hormonal birth control use. Specifically, the difference in meibum quantity score was significantly different between isotretinoin-exposure groups only among HBC females (not among non-HBC females or males) and between HBC and non-HBC females among the isotretinoin-treated group. This suggests that hormonal birth control use, together with isotretinoin treatment, greatly increases the risk of having less meibum availability compared to both untreated HBC females and treated non-HBC females. Since Meibomian glands are believed to be androgen-regulated, the interaction of sex and HBC use with meibum quality and quantity relationships, respectively, to isotretinoin exposure is not surprising.[50–53]

Beyond the Meibomian gland functional characteristics, the study also found that eyelid notching, which is more commonly seen in an aging population, was observed in 15% of eyes in the isotretinoin-exposed group and just 1.5% of eyes in the unexposed group. A study of 185 severe acne patients found that facial scarring affected 95% of both males and females to some degree, so it is not surprising to that the eyelid margin may also be affect in this cohort.[44] However, since there are no baseline measurements in this study, it is unclear if the eyelid notching is tissue alteration resulting from the disease (acne), previous acne treatments (e.g., hormonal birth control, antibiotics), or from the isotretinoin treatment. Nonetheless, eyelid margin abnormalities can obstruct Meibomian gland orifices or alter surrounding tissue and decrease meibum availability, consistent with results in this study.

Another structural characteristic, Meibomian gland atrophy, was not statistically significantly different between isotretinoin exposure groups, after controlling for age, although there was a

strong trend of more atrophy among isotretinoin-exposed individuals (p=0.055), controlling for age. The average difference between groups was estimated to be only 5.9%, which is not clinically detectable and likely within range of normal variability, since such measurements can change slightly depending on the degree of lid eversion and how the borders are defined for atrophy area. Again, because baseline measurements are not available, presence of atrophy can be due to causes other than isotretinoin, including the acne disease and/or prior medications used to treat acne vulgaris before resorting to isotretinoin.

The results for Meibomian gland contrast, which were significantly higher among the isotretinoin-exposed group as a whole but, more specifically, among women, was surprising. We hypothesized that decreased meibum production among isotretinoin-treated individuals would be reflected as lower Meibomian gland contrast in that cohort. However, these unexpected results can be explained by what we understand about androgen levels in persons with and without acne. Androgens stimulate sebum production, and it is key in the development of acne vulgaris, especially among women.[54] It has been reported that higher serum androgen levels are associated with the presence of acne in women but not in men (i.e., although men have higher androgen levels than women, some men never develop acne).[54] As a result, we would expect that women with acne would have higher Meibomian gland contrast prior to isotretinoin treatment due to the androgen-driven increase in meibum production compared to women without acne. Additionally, since serum androgen levels are not strongly associated with acne in men, we would expect that Meibomian gland contrast be similar among men with or without acne.[54] Those assumptions match our study results perfectly for both women and men, suggesting then that Meibomian gland contrast did not change with treatment, rebounded back to baseline after treatment, or decreased approximately equally across all groups with treatment such that the relative difference between groups remained the same. In order to understand the true effects of isotretinoin on Meibomian gland contrast, and presumably meibum production, a longitudinal study with baseline measurements is warranted.

The meaning of Meibomian gland contrast is still unclear, but Meibomian gland contrast was shown to be a strong indicator of hyposecretory Meibomian gland dysfunction, showing high sensitivity and specificity for diagnosing the disease (manuscript in draft). This would imply that Meibomian gland contrast is indicative of poor meibum quality or quantity, or both. In this study, that assumption correlates well with what we know about serum androgen levels and acne/sebum production. What is not clear is why Meibomian gland contrast was either similar (males) or higher (females) among isotretinoin-treated individuals compared to untreated individuals, despite the fact that the number of expressed Meibomian glands and meibum quality and quantity scores were lower in the exposed groups. One possible explanation is that despite the quantity or volume of meibum present inside the Meibomian gland ducts, poor quality, or composition of, meibum reduces its availability at the orifice. Arguably, if meibum quantity is “normal” in the Meibomian gland ducts but the meibum quality is poorer (i.e., cloudy, inspissated), then it is more challenging for the meibum to make its way to the orifice, and subsequently to the tear film. In order to verify this theory, further research is needed to understand meibum composition differences between isotretinoin exposure groups.

Another finding that did not agree with our hypothesis was related to tear-lipid thickness, which was not significantly different between isotretinoin exposure groups, but we know from previous studies that there are substantial differences in composition, appearance, and uniformity in lipid films constituted from meibum lipids and human tear lipids.[55] Therefore, although we expected to see a reflection of meibum quality or production in tear-lipid thickness in this study, it is not surprising that a relationship does not exist in this cohort. Simply put, any compositional changes which occur to meibum in the glands might not be measurable in the tear lipid layer thickness.

This study has limitations. It was designed to determine if there are any significant differences in symptoms or ocular signs based on history of isotretinoin use. The results are based on self-reported history of isotretinoin treatment, hormonal birth control use, and contact lens use. The study's limitations include potential for misclassification due to poor participant recall, lack of information related to isotretinoin dosage and treatment duration, as well as hormonal birth control brand, dosage, and duration of use. Each of these factors, alone, can influence the outcomes explored in this study. Future studies, particularly a longitudinal study should collect and control for these factors.

In summary, isotretinoin-treated individuals, overall, had more frequent and severe symptoms of ocular soreness/irritation and burning/watering eyes compared to untreated individuals. The symptoms may be attributed to the higher prevalence of eyelid notching, fewer expressed Meibomian glands, and worse meibum quality and quantity scores among isotretinoin-treated individuals compared to untreated individuals. The higher Meibomian gland contrast (and presumably higher meibum volume in the ducts) among past-users of isotretinoin, particularly females, along with lower meibum quality and quantity scores suggest that the abundance of poor meibum composition in the ducts resulted in low meibum availability upon gland expression. Isotretinoin-treated individuals appear to be more symptomatic and susceptible to dry eye disease, but due to the nature of the study design, the true etiology for the signs and symptoms is inconclusive. A longitudinal study with baseline (pre-treatment) data would help to confirm causal relationships hypothesized and supported in this study. In the meantime, a thorough examination of the eyelid margin and Meibomian gland expressibility is critical in the diagnosis and management of past-users of isotretinoin.

Chapter 4 - Effects on Meibomian Gland Structure and Function: A 12-Month Prospective Cohort Study

4.1 Introduction

In the previous study, we found that individuals with past isotretinoin exposure presented with significant differences in ocular surface parameters, including Meibomian gland contrast, meibum quality and quantity, compared to controls who have never used the medication. Due to the non-prospective nature of the previous study, the causal effect of isotretinoin to these undesirable clinical outcomes cannot be established. Therefore, we conducted a single-center longitudinal, prospective cohort study of individuals undergoing isotretinoin treatment and their controls to test our hypotheses: (1) significant alterations to meibum secretions, Meibomian gland morphology, and symptoms will occur during the treatment period, and (2) meibum quality, quantity, and Meibomian gland morphology remain changed from baseline after discontinuing treatment. To test these hypotheses, we examined changes in clinical signs and symptoms in both the treated and untreated group prior to and at the end of the treatment period, as well as 6 months after treatment discontinuation.

4.2 Methods

Cohort Population

Subjects were recruited from the University of California, Berkeley, and surrounding communities. Eligibility criteria were age 18 years or older and willingness to attend up to nine two-hour visits over an approximately 19-month period. Volunteers with history of ocular surgery, with active ocular diseases or systemic conditions that can affect ocular surface health, and currently using systemic or ophthalmic medications were excluded from the study. Recruitment targeted subjects who had intention to begin isotretinoin treatment for severe acne, as well as individuals who were not planning to use isotretinoin but were willing to participate in the 19-month study. Written informed consent was obtained from all study participants, and the study adhered to the tenets of the Declaration of Helsinki. The study protocol was approved by the University of California, Berkeley, Office for Protection of Human Subjects.

From January 2017 to September 2018, a total of 28 subjects completed a baseline visit at the Clinical Research Center. Study participants who reported intention to begin isotretinoin treatment were enrolled into the isotretinoin cohort, while the remaining subjects were enrolled in the unexposed cohort. All subjects in the isotretinoin cohort were asked to come in for a 2-hour visit at baseline (prior to commencing treatment), monthly during treatment, and then 6 and 12 months after last dose. Subjects who were not undergoing isotretinoin treatment were asked to come in for monthly visits for seven consecutive months, and again at 13 and 19 months after the baseline visit. Isotretinoin-treated individuals were asked to

provide dosing information at each of their visits, and all subjects were asked to notify the study of any health-related changes since their last visit.

Outcomes

The primary outcome was the mean change from baseline in Meibomian gland contrast. The following measures were secondary outcomes: changes in meibum quality and quantity scores, number of expressible glands, proportion of subjects with eyelid notching, proportion of subjects with eyelid margin redness, bulbar and limbal conjunctival redness (OCULUS Keratograph 5M), Schirmer's I test results, tear meniscus height, fluorescein tear breakup time, and Standard Patient Evaluation of Eye Dryness (SPEED) questionnaire scores.

Statistical Methods

For primary and secondary outcome analyses, baseline values were those obtained at the first visit after study enrollment. The values used for assessing change from treatment were the values obtained at the last visit prior to discontinuing isotretinoin treatment for the isotretinoin cohort and at the seventh visit (six months after baseline) for the untreated individuals. The values used for assessing residual change were those obtained six months after discontinuing treatment for the isotretinoin-treated group and at the 12-month visit for the untreated group. Analyses were performed according to the intention-to-treat principle.

Comparisons of the mean change in continuous measures between treatment groups and associated 95% confidence intervals were based on mixed effects models with a robust variance estimator, clustered by subject to account for correlations between eyes in the same person. Comparisons of categorical outcomes were based on chi-square tests. Fisher's exact test was used when the number of patients in a group with a given adverse event was 5 or fewer.

We determined that a sample of nine subjects per cohort would provide the trial with 80% statistical power to detect a 12-intensity unit mean difference between trial groups in the mean change in the Meibomian gland contrast, assuming a standard deviation of 9 intensity units and missing data for 30% of subjects. This report includes data that were available by March 22, 2019. Statistical computations were performed with Stata/IC, version 14.0 (StataCorp LLC).

4.3 Results

Demographic Factors and Baseline Characteristics

A total of 12 subjects were enrolled in the isotretinoin group and 12 subjects in the untreated group (**Table 4.3-1**). Two subjects in the isotretinoin group were lost to follow-up: one subject completed only three visits and one subject completed only one visit. At baseline, the isotretinoin treatment group presented with significantly thinner tear lipid layer thickness

(p=0.007; 95% CI: -26.0 to -4.0), nasal bulbar (p=0.023; 95% CI: 0.03 to 0.45) and limbal (p=0.006; 95% CI: 0.08 to 0.48) redness, and frequency of burning/watering symptoms (p=0.003; 95% CI: 0.17 to 0.80) (**Table 4.3-2**). The number of expressed Meibomian glands was 3.5 glands fewer for the isotretinoin-treated group compared to the control group with marginal significance (p=0.057; 95% CI: -6.9 to 0.10).

Table 4.3-1. Study population demographic distribution.

Characteristic		**Isotretinoin Users (N=10)**	**Controls (N=12)**
Age – years		18.8 ± 1.3	18.8 ± 2.2
Sex – no. (%)			
	Female	8 (80%)	10 (83%)
	Male	2 (20%)	2 (17%)
Race			
	White	3 (30%)	2 (17%)
	East Asian (Chinese, Korean, Vietnamese)	2 (20%)	3 (25%)
	Mixed	2 (20%)	5 (41%)
	Other	3 (3%)	2 (17%)

Table 4.3-2. Study participant baseline clinical signs and symptoms summary.

	Isotretinoin-Treated (N=10)	**Controls (N=12)**	**p-value**
Clinical Parameters			
Meibomian Gland Contrast	42.6 ± 6.5	41.9 ± 8.4	0.743
Number of Expressed Glands	8.9 ± 3.7	11.9 ± 5.7	0.079
Meibum Quality	12.2 ± 9.7	19.1 ± 10.6	0.065
Meibum Quantity	10.3 ± 9.3	18.0 ± 11.0	0.053
Lid Margin Redness	0.06± 0.25	0.33 ± 0.48	0.076
Tear Meniscus Height	0.24 ± 0.07	0.22 ± 0.05	0.509
Tear Lipid Thickness	54.1 ± 9.3	70.2 ± 20.3	***0.007***
Conjunctival Redness (OCULUS)			
Bulbar			
Nasal	0.81 ± 0.41	0.54 ± 0.29	***0.023***
Temporal	0.47 ± 0.19	0.53 ± 0.29	0.496
Limbal			
Nasal	0.54 ± 0.45	0.26 ± 0.17	***0.006***
Temporal	0.23 ± 0.12	0.20 ± 0.18	0.354
Fluorescein Tear Breakup Time	5.9 ± 5.2	6.9 ± 4.5	0.392
Schirmer's I Test	11.8 ± 9.2	18.4 ± 9.9	0.061
SPEED Score			
Total Score	2.9 ± 2.0	3.4 ± 3.2	0.575
Soreness/Irritation			
Frequency	0.13 ± 0.34	0.25 ± 0.44	0.349
Severity	0.13 ± 0.34	0.33 ± 0.64	0.249
Burning/Watering			
Frequency	0.63 ± 0.50	0.17 ± 0.38	***0.003***
Severity	0.75 ± 0.68	0.33 ± 0.87	0.197

Outcomes

End-of-Treatment Period

At the end of the treatment period, the isotretinoin-treated group presented with significant changes from baseline for several clinical signs and symptoms: Meibomian gland contrast ($p<0.001$), eyelid margin redness ($p<0.001$), tear-lipid thickness ($p<0.001$), conjunctival redness ($p<0.050$), and total SPEED score ($p=0.049$). Frequency and severity of soreness/irritation and burning/watering eyes were either statistically or marginally significantly worse compared to baseline (**Table 4.3-3**). In the control group, a significant increase in temporal bulbar conjunctival redness ($p<0.001$) and shortening of tear meniscus height ($p=0.031$) were observed at the end of the treatment period. Many changes were immediately apparent at the 1-month visit (after starting treatment) (**Figure 4.3-1 and Figure 4.3-2**) and could be appreciated on meibography images (**Figure 4.3-3**).

Table 4.3-3. Summary of clinical signs and symptoms for each cohort at the end of treatment.

	End of Treatment Period					
	Isotretinoin-Treated Group			Control Group		
	Mean (SD)	Mean Change from Baseline*	p-value*	Mean (SD)	Mean Change from Baseline*	p-value*
Meibomian Gland Contrast	22.4 ± 9.1	-20.4 (-25.1 to -15.7)	***<0.001***	44.5 ± 8.7	3.3 (0.3 to 6.3)	***0.033***
Number of Expressed Glands	7.6 ± 4.9	-1.4 (-4.8 to 2.1)	0.433	9.1 ± 4.4	-3.4 (-6.8 to 0.1)	0.060
Meibum Quality	8.9 ± 7.9	-3.0 (-8.6 to 2.7)	0.301	16.0 ± 9.1	-3.7 (-12.1 to 4.7)	0.389
Meibum Quantity	5.8 ± 6.2	-4.1 (-9.8 to 1.6)	0.161	13.1 ± 7.9	-6.0 (-13.8 to 1.7)	0.128
Lid Margin Redness	0.89 ± 0.32	0.8 (0.6 to 1.1)	***<0.001***	0.56 ± 0.51	0.2 (-0.2 to 0.5)	0.397
Tear-Lipid Thickness	38.1 ± 14.1	-16.5 (-25.5 to -7.6)	***<0.001***	71.1 ± 20.0	0.6 (-8.7 to 9.8)	0.907
Conjunctival Redness (OCULUS)						
Bulbar						
Nasal	1.23 ± 0.40	0.45 (0.24 to 0.67)	***<0.001***	0.72 ± 0.43	0.15 (-0.04 to 0.34)	0.131
Temporal	0.67 ± 0.22	0.19 (0.05 to 0.32)	***0.006***	0.67 ± 0.35	0.11 (0.05 to 0.16)	***<0.001***
Limbal						
Nasal	0.86 ± 0.54	0.33 (0.11 to 0.56)	***0.004***	0.51 ± 0.48	0.23 (-0.10 to 0.56)	0.167
Temporal	0.38 ± 0.29	0.11 (0.03 to 0.19)	***0.006***	0.28 ± 0.24	0.07 (-0.03 to 0.17)	0.162
SPEED						
Total	6.4 ± 5.4	3.96 (0.02 to 7.9)	***0.049***	4.3 ± 4.1	0.3 (-1.1 to 1.8)	0.654
Frequency						
Soreness/Irritation	0.56 ± 0.70	0.49 (-0.007 to 0.98)	0.053	0.33 ± 0.49	0.005 (-0.01 to 0.02)	0.543
Burning/Watering	1.22 ± 0.94	0.61 (0.13 to 1.09)	***0.012***	0.33 ± 0.49	0.12 (-0.09 to 0.33)	0.245
Severity						
Soreness/Irritation	0.89 ± 1.13	0.85 (0.04 to 1.67)	***0.040***	0.44 ± 0.98	0.003 (-0.315 to 0.322)	0.984
Burning/Watering	1.44 ± 1.10	0.73 (-0.03 to 1.50)	0.059	0.44 ± 0.70	0.01 (-0.29 to 0.31)	0.943
Tear Meniscus Height	0.20 ± 0.04	-0.04 (-0.09 to 0.007)	0.090	0.19 ± 0.04	-0.03 (-0.05 to -0.003)	***0.031***
Fluorescein Tear Breakup Time	6.4 ± 4.5	0.6 (-2.9 to 4.0)	0.734	5.4 ± 4.1	-1.5 (-4.1 to 1.2)	0.275
Schirmer's I Test	11.8 ± 8.9	0.3 (-9.0 to 9.6)	0.949	16.7 ± 11.8	-3.7 (-7.8 to 0.5)	0.084

SD=Standard Deviation

*Mean Change from Baseline (95% confidence interval) and p-value represents significance of the change.

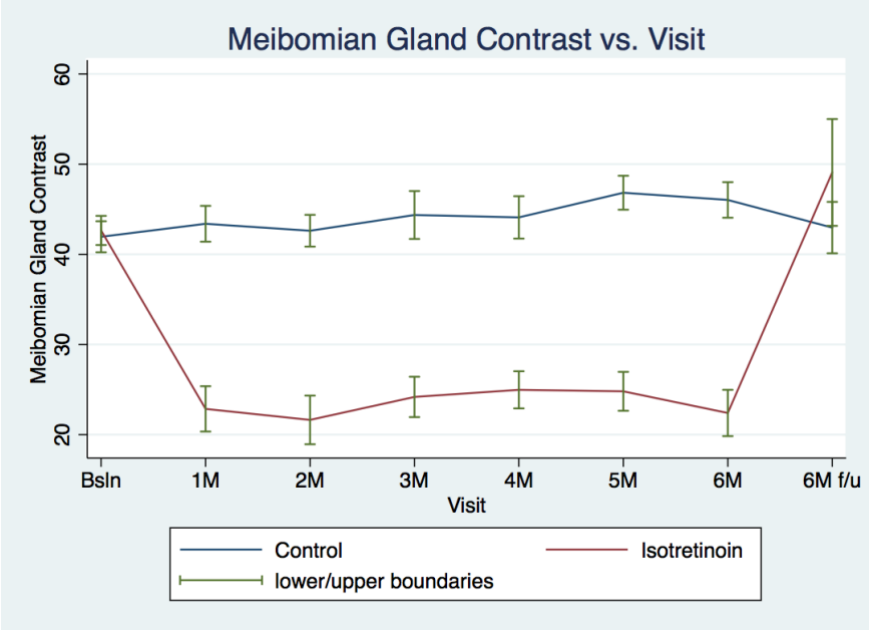

Figure 4.3-1. Mean Meibomian gland contrast at baseline, monthly during isotretinoin treatment period (1M to 6M), and 6 months after discontinuing treatment (6M f/u) for both the isotretinoin-treated and control groups.

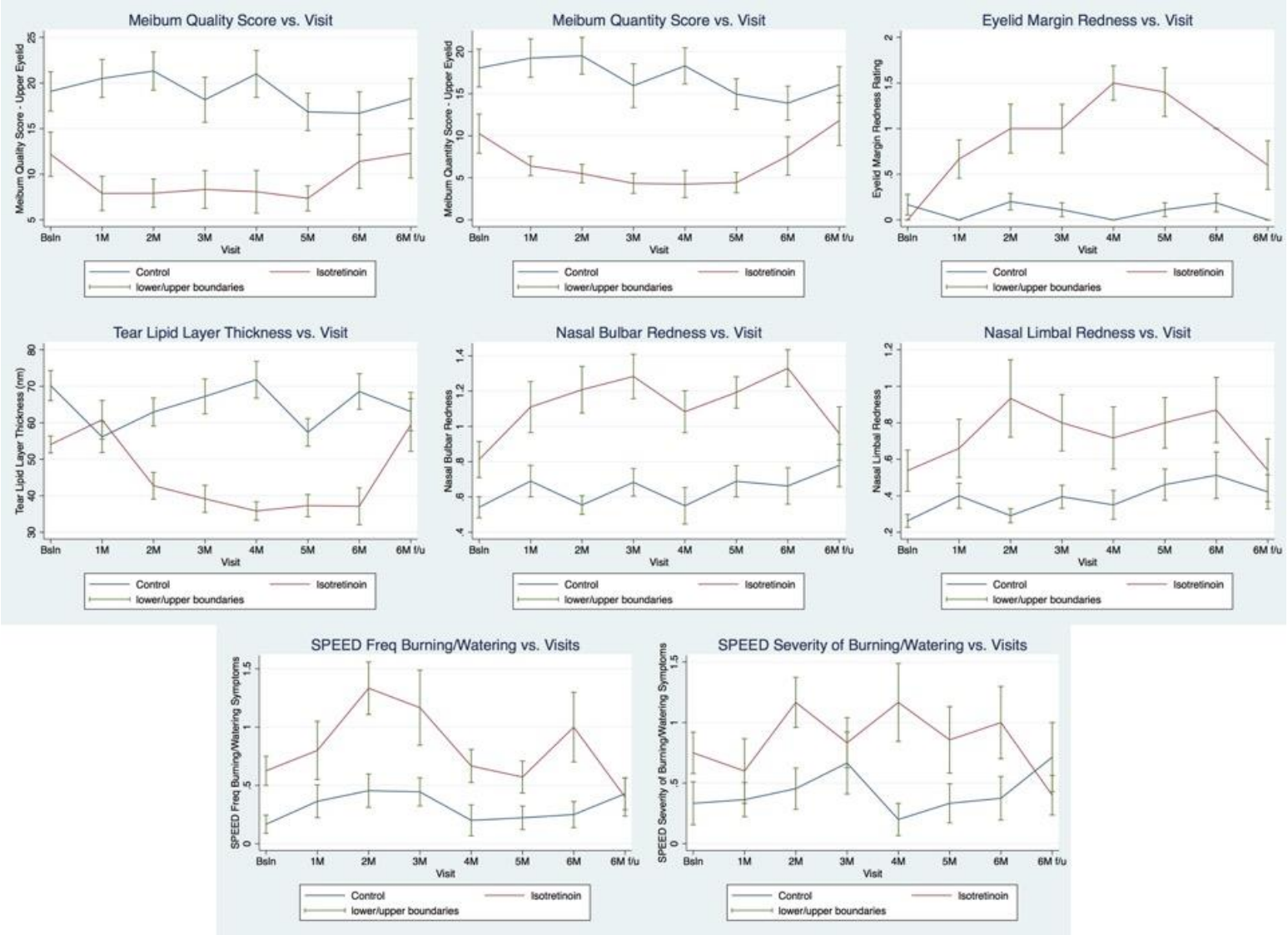

Figure 4.3-2. Changes to ocular surface parameters by visit for (a) meibum quality score, (b) meibum quantity score, (c) eyelid margin redness, (d) tear-lipid thickness, (e) nasal bulbar redness, (f) nasal limbal redness, (g) frequency of burning/watering symptoms (SPEED), and (h) severity of burning/watering symptoms (SPEED).

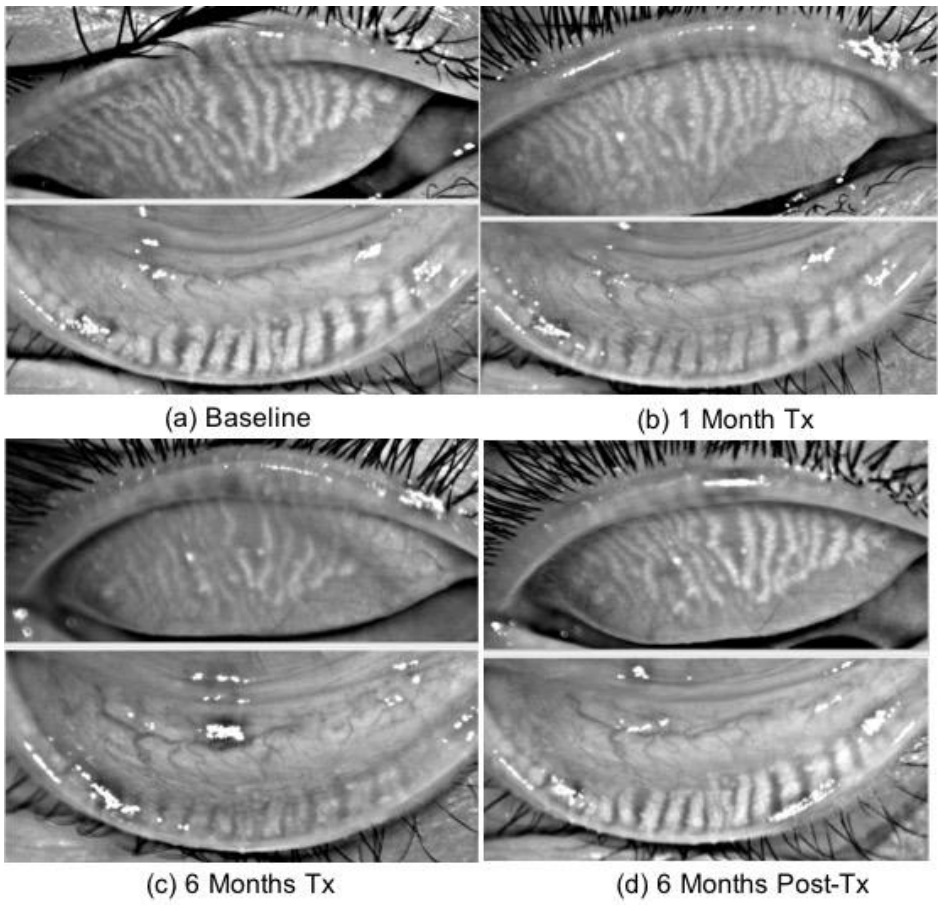

Figure 4.3-3. Meibography images for Subject 1 at (a) baseline, (b) 1 month after commencing treatment, (c) 6 months after commencing treatment, and (d) 6 months after discontinuing treatment showing decreased contrast during the treatment period and regression after discontinuing treatment.

Six Months Post-Treatment

By six months after discontinuing treatment, many ocular characteristics returned to baseline for the isotretinoin-treated group, including the primary outcome, Meibomian gland contrast (**Table 4.3-4**). At this stage, Meibomian gland contrast in the isotretinoin-treated group was similar to baseline (p=0.418; 95% CI: -8.6 to 20.7). The control group, which presented with significant changes to Meibomian gland contrast, temporal nasal bulbar conjunctiva, and tear meniscus height at the end of the treatment period, continued to have increased eyelid and bulbar conjunctival redness and shortened tear meniscus height 12 months after the baseline visit (**Table 4.3-4**).

Table 4.3-4. Summary of clinical signs and symptoms for each cohort six months after discontinuing treatment.

	Six Months Post-Treatment					
	Isotretinoin-Treated Group			Control Group		
	Mean ± SD	Mean Change from Baseline	p-value	Mean ± SD	Mean Change from Baseline	p-value
Meibomian Gland Contrast	49.1 ± 18.7	6.0 (-8.6 to 20.7)	0.418	43.0 ± 10.7	0.4 (-3.3 to 4.1)	0.843
Number of Expressed Glands	7.6 ± 4.4	-1.9 (-4.7 to 0.8)	0.171	10.9 ± 4.8	-1.5 (-4.1 to 1.1)	0.248
Meibum Quality	12.3 ± 8.6	-1.1 (-5.5 to 3.2)	0.603	18.3 ± 8.2	-1.5 (-7.8 to 4.7)	0.632
Meibum Quantity	11.8 ± 9.4	0.6 (-5.1 to 6.3)	0.834	16.1 ± 8.0	-3.9 (-9.4 to 1.6)	0.165
Lid Margin Redness	0.60 ± 0.52	0.6 (0.1 to 1.0)	***0.014***	0.71 ± 0.47	0.4 (0.1 to 0.8)	***0.010***
Tear-Lipid Thickness	59.4 ± 22.8	5.7 (-6.1 to 17.6)	0.343	63.1 ± 19.7	-9.3 (-27.0 to 8.5)	0.305
Conjunctival Redness (OCULUS)						
Bulbar						
Nasal	0.96 ± 0.48	0.09 (-0.24 to 0.42)	0.606	0.78 ± 0.45	0.23 (0.03 to 0.42)	***0.023***
Temporal	0.61 ± 0.21	0.13 (-0.04 to 0.29)	0.128	0.65 ± 0.44	0.16 (0.02 to 0.30)	***0.020***
Limbal						
Nasal	0.42 ± 0.35	0.003 (-0.30 to 0.31)	0.983	0.42 ± 0.35	0.16 (-0.10 to 0.42)	0.234
Temporal	0.21 ± 0.13	-0.01 (-0.10 to 0.08)	0.822	0.23 ± 0.17	0.08 (-0.03 to 0.19)	0.157
SPEED						
Total	2.4 ± 2.5	0.8 (-2.4 to 4.0)	0.614	5.9 ± 3.4	1.1 (-0.7 to 3.0)	0.233
Frequency						
Soreness/Irritation	0 ± 0	0.03 (-0.38 to 0.45)	0.867	0.57 ± 0.76	0.15 (-0.12 to 0.43)	0.867
Burning/Watering	0.40 ± 0.52	-0.09 (-0.54 to 0.35)	0.677	0.43 ± 0.51	0.14 (-0.10 to 0.38)	0.241
Severity						
Soreness/Irritation	0 ± 0	0.21 (-0.31 to 0.73)	0.436	0.71 ± 1.07	0.15 (-0.10 to 0.41)	0.235
Burning/Watering	0.40 ± 0.52	-0.14 (-0.73 to 0.45)	0.649	0.71 ± 1.07	0.16 (-0.08 to 0.39)	0.187
Tear Meniscus Height	0.18 ± 0.03	-0.07 (-0.13 to -0.003)	***0.039***	0.18 ± 0.04	-0.04 (-0.06 to -0.02)	***<0.001***
Fluorescein Tear Breakup Time	5.4 ± 5.0	-1.1 (-6.5 to 4.4)	0.705	4.7 ± 1.4	-1.7 (-3.7 to 0.2)	0.076
Schirmer's I Test	13.4 ± 12.2	0.2 (-11.3 to 11.7)	0.970	5.9 ± 3.4	-1.4 (-9.1 to 6.2)	0.712

SD=Standard Deviation

*Mean Change from Baseline (95% confidence interval) and p-value represents significance of the change.

4.4 Discussion

In this 18-month prospective cohort study to evaluate ocular surface changes with isotretinoin treatment for severe nodulocystic acne, symptoms of burning/watering eyes and several clinical signs, including Meibomian gland contrast, changed significantly for the isotretinoin-treated group by the end of the treatment period compared to baseline, and the difference in change between the study groups was significant at this time point. Among those characteristics that were worse in the isotretinoin-treated group were meibum quality and quantity scores, eyelid and conjunctival hyperemia, tear-lipid thickness, and frequency and severity of burning/watering symptoms. However, by six months after discontinuing treatment, many ocular characteristics returned to baseline and were no longer significantly different between the study groups, except for number of expressed Meibomian glands, meibum quality scores, and eyelid margin redness, which continued to be worse for the isotretinoin-treated group.

This study's sample size limits the generalizability of the results. The sample size was calculated to have 80% statistical power to detect a 12-intensity unit change in Meibomian gland contrast, but for measurements with inherently high variability, such as meibum quality and quantity, significance may not be detected in this study population size. Subjects were not restricted from finding ocular symptom relief during their participation; however, no subjects reported using artificial tears or other treatments that could potentially confound the observations made during the treatment period. The 1-year follow-up period minimized the effect of seasonal factors. There was no objective measure of dosage or compliance/adherence to treatment, which were both self-reported.

The results of this study are similar to those of a study conducted by Mathers, et al., which evaluated the meibum volume and viscosity, Meibomian gland atrophy on meibography, tear osmolarity, and aqueous production in 11 patients being treated with isotretinoin (60-120 mg/day) over a four-month period with examinations at baseline and after two months of treatment [cite]. This study also found decreased expressibility of meibum and poorer quality meibum upon expression with no change in aqueous production. Mathers, et al., reported evidence of Meibomian gland atrophy or decreased density. However, this study found that the glands did not shorten in appearance on meibography during treatment but, instead, decreased in contrast (**Figure 4.3-3**), perhaps suggesting that an overall reduced presence of meibum in the ducts resulted in less infrared light reflection. This study also evaluated and reported increased hyperemia on the eyelid margins and conjunctiva, especially the nasal bulbar and limbal regions, indicating a low-grade inflammatory response.

After cessation of isotretinoin treatment, patient ocular characteristics, including tear osmolarity and meibography, were reported by Mathers, et al., to return to "normal" at their follow-up visits between 1- to 4-months after treatment discontinuation. Our study found that all observed clinical signs and symptoms returned to baseline, except eyelid margin redness remained clinically significantly higher than at baseline. It is interesting to note that, although the difference in mean Meibomian gland contrast was not significantly different between the

groups six months after discontinuing treatment, there was much greater variability in Meibomian contrast among the isotretinoin group, compared both to baseline and to the control group at the same time point. This may suggest some residual effects of the isotretinoin treatment on meibum production for some individuals. Furthermore, despite Meibomian gland contrast and meibum quantity scores appearing to return to baseline six months after discontinuing treatment, the meibum quality and number of expressible glands was noticeably poorer six months after stopping treatment compared to baseline. This suggests that perhaps Meibomian gland contrast may be a good indicator of meibum quantity/volume present in the Meibomian gland ducts but not of the quality of the meibum that resides there.

In conclusion, patients who were treated for severe nodulocystic acne with isotretinoin experienced significant changes from baseline compared to the control group in ocular surface characteristics, including Meibomian gland contrast, meibum quality and quantity scores, and eyelid and conjunctival hyperemia, as well as symptoms of watering/burning eyes at the end of the treatment period. All characteristics returned to baseline 6 months after stopping treatment, except for eyelid margin redness. Additional follow-up is required to understand the long-term impact of isotretinoin on eyelid margin health and meibum quality.

Chapter 5 - Meibum Lipid Mediators: Isotretinoin-Exposed vs. Unexposed

5.1 Introduction

Sebaceous glands play an important role in the innate immune system, supplying antimicrobial peptides, neuropeptides, and antibacterial lipids. When these glands overproduce sebum, it can create problems in the skin, specifically, on the face, chest, and back are most prone to acne. Although the sequence of events in acne development is unclear, it is believed that an androgen-induced over-production of sebum causes normal *propionibacterium acnes* flora to proliferate. Through a series of inflammatory events, an accumulation of inflammatory cells and propionic and acetic acid from the bacteria causes the follicular wall to rupture and induce a non-immune foreign body reaction.

Eicosanoids are biologically active lipid mediators that play a role in regulating inflammation and its resolution. They are made by enzyme-controlled oxidation of 20-carbon, polyunsaturated fatty acids (PUFAs). The most studied of the eicosanoic fatty acids is arachidonic acid (AA), although eicosapentaenoic acid (EPA) have important physiological properties, as well. AA is involved in the initiation phase of inflammation by forming pro-inflammatory leukotrienes (LTB_4 and LTC_4) and prostaglandins (PGE_2 and PGD_2).[56] These molecules trigger the expression of inflammatory enzymes, including COX-2, chemokines, and cytokines that initiate and accelerate inflammation. Specialized pro-resolving lipid mediators include eicosanoid LXA_4 and ω-3 derived resolvins and protectins. Lipid mediators have been studied on the ocular surface in dry eye mouse models or patients. Leukotrienes and PGD_2 were found to stimulate goblet cell secretion in rat goblet cells.[57] LXA_4 was found to be produced during inflammation to help return the tissue to homeostasis.[57,58] RvE1 was reported to promote tear production, corneal epithelial integrity, decrease COX-2-induced inflammation, and increased goblet cell density.[59,60] Presence and levels of eicosanoids and specialized pro-resolving mediators in meibum has not been investigated.

In this study, we aimed to understand the presence and levels of lipid mediators in human meibum and to explore differences, if any, between past-users of isotretinoin to non-users. We also explore relationships between eicosanoid levels to clinical signs. The results will provide insight into homeostatic levels of lipid mediators in meibum, their impact on ocular surface health, and changes as a result of isotretinoin therapy.

5.2 Methods

<u>Subjects</u>

This was a single-visit, prospective cohort study consisting of two groups: past-users and non-users of isotretinoin. Study participants were recruited from the University of California, Berkeley campus and surrounding communities. Participants were required to be 18-39 years old and available for a single visit at the Clinical Research Center in the School of Optometry. Volunteers were excluded if they were using isotretinoin at the time of enrollment, had a history of ocular surgery or any active ocular inflammation or infection, or had systemic diseases, including arthritis, gout, diabetes, and hypercholesteremia. Participants were asked to discontinue contact lens wear, make-up, and facial moisturizers 24 hours prior to the scheduled visit. Written informed consent was obtained from all study participants, and the study adhered to the tenets of the Declaration of Helsinki. The study protocol was approved by the University of California, Berkeley, Office for Protection of Human Subjects.

Sample size calculations were calculated based on Meibomian gland contrast since no previous study, to our knowledge, has reported on bioactive lipids in meibum. To detect a 15% difference in population means for Meibomian gland contrast with 80% power and 0.05 level of significance using previously published data (mean Meibomian gland contrast for normal is 37.1 intensity units and an approximate common variance of 8.8 intensity units), an estimated 20 subjects is needed in each cohort.

Measurements and Procedures

Participants were asked to complete a comprehensive medical history questionnaire, the Standard Patient Evaluation of Eye Dryness (SPEED) questionnaire, and Visual Analogue Scale symptoms questionnaire prior to the ocular examination. Upon completing both questionnaires, participants underwent a battery of tests. Performed first were non-invasive procedures, including tear-lipid thickness (Lipiview®; Johnson & Johnson Surgical Vision) and non-invasive tear breakup time measurements (Medmont E300; Medmont Pty Ltd; Australia; averaged from 3 measurements per eye, alternating), as well as a slit lamp examination with white light. Following those were invasive measurements, which included tear breakup time with sodium fluorescein (FTBUT; seconds) using 1 μl of 2% fluorescein measured three times per eye, alternating between eyes. Corneal staining was scored immediately after FTBUT measurements on the SICCA scale [46]. Meibomian gland expression was then performed on the lateral, central, and medial regions of the lower and upper eyelid margins with a Korb Meibomian Gland Evaluator™. This procedure involved applying 10-15 seconds of gentle pressure on the skin inferior to the lower eyelid margin while the participant was in upgaze and superior to the upper eyelid margin while the participant was in downgaze. The secretions were scored based on quality and quantity [39,40]. Next, 1% lissamine green ophthalmic solution was instilled to assess conjunctival staining using the SICCA scale [46] and positioning of Marx's line with respect to Meibomian gland orifices using the scale defined by Yamaguchi, et al. [47] Five minutes later, a second instillation of the lissamine green/fluorescein combination drop was instilled to assess length and width of lid wiper epitheliopathy after everting the upper eyelid using the scale previously defined by Korb, et al. [48] Finally, meibography images of both the upper and lower lids were captured and analyzed using the OCULUS Keratograph 5M

(OCULUS, Inc., Arlington, WA) and processed to measure Meibomian contrast, as previously described in Chapter 2.[28]

Lipid Mediator Lipidomics

Via the Meibomian gland expression procedure, secreted meibum were collected on a dacron swab (Fisher Scientific), pooled, and stored plastic Eppendorf tube containing HLC methanol at -80°C. Prior to sample processing, an internal standard was added to the tube containing the sample. The internal standard consisted of methanol and deuterated internal standards (PGE_2-d4, lipoxin A_4 (LXA_4)-d5, leukotriene B_4 (LTB_4-d4), 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE-d8], arachidonic acid (AA)-d8, and DHA-d5 at 400 pg/each) to calculate recovery of different classes of oxygenated fatty acids and PUFAs. Eicosanoids, docosanoids, and PUFAs were identified and quantified by LC/MS/MS-based lipidomics.[61,62] In brief, samples were analyzed by a triple quadruple linear ion trap LC/MS/MS system (MDS SCIEX 4500 QTRAP) equipped with a LUNA C18-2 mini-bore column using a mobile phases A2 (water:aceonitrile:acetic acid, 72:28:0.01, v:v:v) and B2 (isopropanol; acetonitrile, 60:40, v:v) that was run as a linear gradient with a 0.45 ml/flow rate. MS/MS analysis was carried out in negative ion mode, and lipid mediators and PUFAs were quantitated using scheduled multiple reaction monitoring (MRM) using 4-6 specific transition ions for each analyte. Calibration curves (1–1000 pg) and specific LC retention times for each compound were established with synthetic standards (Cayman Chemical).

Statistical Methods

The main outcomes of interest in this study are the presence of lipid mediators arachadonic acid (AA), docosahexaenoic acid (DHA), 5-hydroxyeicosatetraenoic acids (5-HETE), 12-hydroxyeicosatetraenoic acids (12-HETE), prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), and thromboxane B_2 (TXB_2). Since the absolute sample weight is unknown, the lipid mediator-to-AA weight is calculated and compared between groups. Comparisons are conducted with chi-squared tests, Mann-Whitney tests, and Pearson correlation (Stata/IC 14.0).

5.3 Results

Demographics

Meibum samples from 26 non-users [Age(SD)=25.8(11.6) years] and 22 past-users [Age(SD)=22.0(4.8) years] of isotretinoin completed the study (**Table 5.3-1**). Nearly three-quarters of both groups were females, and a significantly larger proportion of females in the isotretinoin-exposed group (65%) are current hormonal birth control (HBC) users, compared to just 5% in the unexposed group ($p<0.001$). The proportion of contact lens users was also significantly greater among the isotretinoin-exposed group (64.7%) compared to unexposed group (35.5%) ($p=0.050$).

Table 5.3-1. Descriptive statistics.

		Non-Isotretinoin Users N=26	Past-Isotretinoin Users N=22	p-value
Age, years		25.8 (11.6)	22.0 (4.8)	
		18-62	18-39	
Females		19 (73%)	17 (77%)	1.000
HBC Use (Females only)		1 (5%)	11 (65%)	***<0.001***
Omega-3 Use (Current)				
	Yes	1	2	0.587
	No	25	20	
Meibomian Gland Dysfunction OD		10 (38%)	13 (59%)	0.128

Outcomes

LIPID MEDIATORS

The polyunsaturated fatty acids and lipid mediators identified include arachidonic acid (AA), docosahexaenoic acid (DHA), 5- and 12-hydroxyeicosatetraenoic acids (5-HETE and 12-HETE), prostaglandins E_2 and D_2 (PGE_2 and PGD_2), and thromboxane B_2 (TXB_2) (**Table 5.3-2**). Isotretinoin-exposed individuals had significantly less 5-HETE ($p=0.015$), PGE_2 ($p<0.001$), PGD_2 ($p<0.001$), and TXB_2 ($p<0.001$) compared to non-users of isotretinoin. However, mean mass of DHA detected per sample was estimated to be nearly double in the isotretinoin-exposed group compared to the unexposed ($p=0.042$). AA levels were not significantly different between the study groups ($p=0.591$). Since the absolute weights of collected meibum could not be known, the weight/sample estimates were normalized against that of their precursor, AA. The normalized PGD_2 ($p<0.001$), PGE_2 ($p<0.001$), and TXB_2 ($p=0.002$) values were less for the isotretinoin-exposed group compared to the unexposed group (**Table 5.3-3**). Significant differences in estimate PGD_2 (Mean$\pm$SEM for Non-users: 18.85 ± 2.58, Past-users 5.86 ± 1.77 pg/sample; $p<0.001$) and normalized PGD_2 (Mean$\pm$SEM for Non-users: 5.43 ± 0.80, Past-users: 1.69 ± 0.46; $p<0.001$) were particularly striking.

Table 5.3-2. Estimated mass per sample of PUFAs and lipid mediators in human meibum.

	Non-Isotretinoin Users N=26		Past-Isotretinoin Users N=22		p-value
	Mean (SEM)	**% Subjects$^{\forall}$**	**Mean (SEM)**	**% Subjects$^{\forall}$**	
Arachadonic Acid (AA) (ng)	4.29 (0.62)	100.0	3.80 (0.60)	100.0	0.591
Docosahexaenoic acid (DHA) (ng)	0.07 (0.02)	46.2	0.13 (0.03)	68.2	***0.042***
5-hydroxyeicosatetraenoic acids (5-HETE) (pg)	0.76 (0.11)	92.3	0.42(0.12)	54.5	***0.016***
12-hydroxyeicosatetraenoic acids (12-HETE) (pg)	17.63 (2.49)	84.6	14.12 (3.33)	86.4	0.199
Prostaglandin E_2 (PGE_2) (pg)	2.31 (0.50)	92.3	0.36 (0.20)	59.1	***<0.001***
Prostaglandin D_2 (PGD_2) (pg)	18.85 (2.58)	57.7	5.86 (1.77)	13.6	***<0.001***
Thromboxane B_2 (TXB_2) (pg)	32.48 (4.20)	80.8	9.40 (3.40)	31.8	***<0.001***

$^{\forall}$ Percentage of subjects for whom samples contained some level (>0 mass/sample) of respective PUFA or lipid mediator.

Table 5.3-3. Comparison of normalized levels of eicosanoids in meibum. (All values 10^{-3}; Mean(SEM))

	Non-Isotretinoin Users N=26	Past-Isotretinoin Users N=22	p-value
PGD_2/AA	5.43 (0.80)	1.69 (0.46)	***<0.001***
PGE_2/AA	0.75 (0.16)	0.06 (0.04)	***<0.001***
TBX_2/AA	10.74 (1.84)	2.61 (1.06)	***0.002***

CLINICAL CHARACTERISTICS

Symptoms were significantly worse for past-users compared to non-users of isotretinoin, measured both with the SPEED questionnaire (p=0.024) and VAS (Daily Comfort) questionnaire (Right Eye: p=0.012, Left Eye: p=0.017) (**Table 5.3-4**). Meibomian gland contrast, tear-lipid thickness, fluorescein tear breakup time, and meibum quality and quantity scores for the upper and lower eyelids were similar between groups for both eyes (p>0.05) and not associated with lipid mediator measures (p>0.05).

Table 5.3-4. Symptoms and clinical characteristics for each study group. Mean(Standard Deviation)

	Non-Isotretinoin Users N=26	Past-Isotretinoin Users N=22	p-value
CLINICAL SIGNS			
RIGHT EYE			
MG Contrast	42.1 (10.3)	39.3 (12.3)	0.285
TLL_Avg	65.0 (20.6)	71.4 (21.2)	0.357
NITBUT_Avg	18.1 (24.2)	14.7 (14.6)	0.836
MG Expression Quality Score UL	15.5 (8.3)	16.0 (11.0)	0.876
MG Expression Quantity Score UL	13.0 (7.1)	12.6 (11.7)	0.320
MG Expression Quality Score LL	14.6 (8.6)	14.0 (9.9)	0.633
MG Expression Quantity Score LL	11.1 (6.1)	12.6 (9.9)	0.901
LEFT EYE			
MG Contrast	41.1 (9.0)	45.0 (12.6)	0.234
TLL_Avg	63.3 (23.5)	72.7 (23.1)	0.143
NITBUT_Avg	19.6 (29.8)	14.2 (15.5)	0.664
MG Expression Quality Score UL	17.2 (8.8)	16.8 (9.8)	0.967
MG Expression Quantity Score UL	15.5 (9.0)	13.1 (9.8)	0.237
MG Expression Quality Score LL	14.9 (8.9)	12.7 (10.6)	0.309
MG Expression Quantity Score LL	12.1 (9.0)	10.8 (10.3)	0.468
DRY EYE QUESTIONNAIRE SCORES			
SPEED Score	4.3 (4.4)	7.5 (5.1)	***0.024***
VAS – Avg Daily Comfort (Right Eye)	83.1 (16.2)	67.0 (25.2)	***0.012***
VAS – Avg Daily Comfort (Left Eye)	81.5 (19.6)	67.2 (24.4)	***0.017***

Symptom scores, however, were strongly correlated with DHA levels. Higher DHA levels were significantly correlated with higher SPEED scores (r=0.34, p=0.017) and lower VAS scores (r=-0.34, p=0.018) (**Figure 5.3-1**).

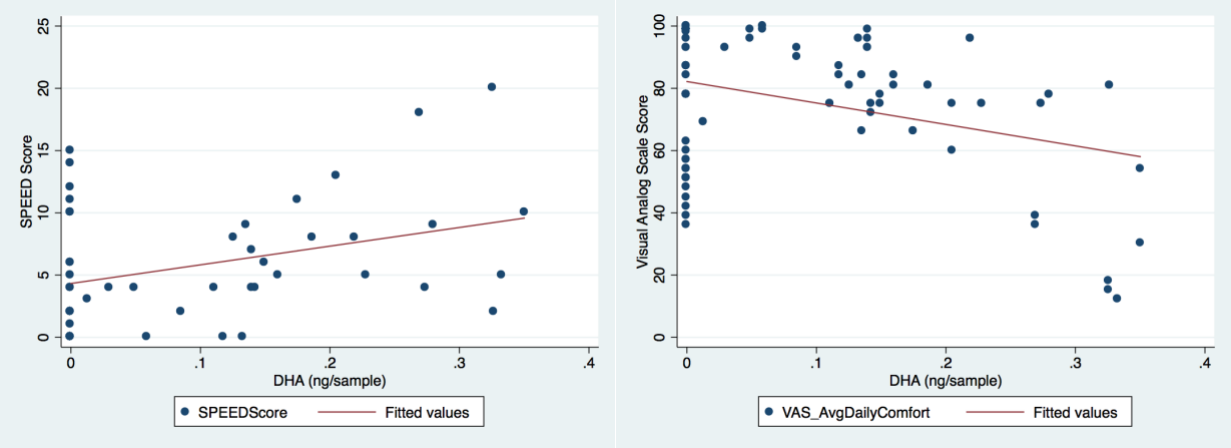

Figure 5.3-1. Higher DHA levels detected in human meibum were significantly correlated with worse symptoms, higher SPEED scores (left) and lower VAS scores (right). SPEED=Standard Patient Evaluation of Dry Eye; VAS=Visual Analog Scale.

5.4 Discussion

This single-visit cohort study was conducted to profile lipid mediators present in human meibum and to compare the profiles between isotretinoin-exposed and unexposed groups. Polyunsaturated fatty acids and lipid mediators identified in meibum included AA, DHA, 5-HETE, 12-HETE, PGE_2, PGD_2, and TXB_2. The isotretinoin-exposed group had significantly lower normalized levels of PGD_2, PGE_2, and TXB_2 compared to the unexposed group. To our knowledge, this study is the first report of bioactive lipid mediators, particularly PGE_2, PGD_2 and TXB_2, in human meibum. Their presence in meibum and their lower levels in isotretinoin-exposed individuals suggest previously unrecognized roles of prostanoids in Meibomian gland (MG) physiology and/or pathophysiology.

Although there were significant differences between study groups in absolute and normalized quantities of several lipid mediators, there were no significant differences in clinical signs in this study. However, the isotretinoin-treated group were, on average, more symptomatic than the unexposed group, and that relationship to DHA levels detected in meibum will be discussed later in this section. Results from a different study comparing clinical observations from a larger population of controls to those belonging to a similar-sized group of isotretinoin-treated individuals found that isotretinoin-treated individuals had more frequent and severe symptoms of ocular soreness/irritation and burning/watering eyes, as well as higher prevalence of eyelid notching, fewer expressed Meibomian glands, and worse meibum quality and quantity scores (Chapter 2). The sample size for the current study was calculated to detect differences in

Meibomian gland contrast, so it is possible that this study population was too small to detect differences in other clinical parameters explored in this study.

The PGD_2 findings in this study are of particular interest due to the marked difference in levels between the isotretinoin-treated and untreated groups. PGD_2 is capable of signaling a wide range of activities. PGD_2 acts through two major receptors: type D prostanoid receptor 1 (DP1) and PGD_2 receptor 2 (DP2). DP1 drives vascular allergic responses and DP2 is an active drug-development target because it activates and recruits T-effector cells (Th2) in asthma. PGD_2 is an established mediator of allergic immune responses and a major product of mast cells.[63] Of particular relevance to this study, PGD_2 was reported to be associated with dry eye symptoms, bald scalps, and hypertrophied sebaceous glands.[64–66] In a study of symptomatic dry eye patients and controls, PGD_2 levels in tears and PGD synthase mRNA expression in Meibomian glands of symptomatic dry eye patients were significantly lower compared to the control group.[64] These results are consistent with our findings and support the idea that symptoms, potentially from lipid-deficient dry eye, is associated with decreased levels of PGD_2. A study comparing bald-scalp to hair-scalp men with androgenetic alopecia found elevated levels of PGD_2 synthase and its product, PGD_2, in the bald scalp compared to the haired scalp.[65] This is significant because it has been reported that sebaceous glands, which attach to each hair follicle, hypertrophy in bald scalps and may be the reason for oily scalps commonly seen in this population.[66] Together, these results suggest a relationship between PGD_2 and sebum/meibum levels in the oil glands, but the relationship and mechanism are unclear. One clue may be from a study that found PGD_2 induces inflammation in sebocytes of patients with eosinophilic pustular folliculitis by increasing eotaxin-3 (chemoattractant of eosinophils) mRNA expression via peroxisome proliferator-activated receptor gamma (PPARγ). If PGD_2 plays a similar role with meibocytes, it is possible that elevated PGD_2 can lead to inflammation and hypersecretory Meibomian gland dysfunction (MGD) while too little PGD_2 can suppress meibum production resulting in hyposecretory MGD.

This study found a significant correlation between worse symptoms (higher SPEED scores and lower VAS score) and higher absolute levels (pg/sample) of DHA in meibum. These results seem to contradict what we know about DHA's pro-resolution activity.[67] Specifically, specialized pro-resolving mediators (SPMs), which include resolvins and (neuro)protectins, and maresins, are unique derivatives of omega-3 PUFAs (DHA and EPA) that serve to resolve host inflammation.[68–72] The correlation between elevated DHA levels and worse symptoms may be explained by the inflammatory resolution process. DHA is an essential fatty acid stored in adipose tissues and is released when there is an energy deficit.[73] It is possible that DHA is a marker of SPM induction in the presence of inflammation. Another possible explanation is the elevated DHA levels may be indicative of a metabolic deficiency in which DHA cannot be effectively or efficiently metabolized to SPMs, resulting in symptoms of discomfort. More research is needed to understand the role of PUFAs and lipid mediators in the Meibomian glands.

In conclusion, polyunsaturated fatty acids and lipid mediators identified in meibum included AA, DHA, 5-HETE, 12-HETE, PGE_2, PGD_2, and TXB_2. Compared to the control group, past-users of isotretinoin had significantly lower normalized levels of PGD_2, PGE_2, and TXB_2, and worse

symptoms, but clinical signs were similar between the groups. The results suggest a possible relationship between PGD_2 levels and meibum production/availability, but additional research is necessary to better understand their relationship.

Chapter 6 – Discussion and Conclusion

The 12-month and single-visit cohort studies aimed to gain a better understanding of the short- and long-term changes, respectively, to Meibomian gland structure and function that may be a result of or associated with isotretinoin therapy for severe nodulocystic acne. We hypothesized that isotretinoin treatment would induce decreased lipid production in the Meibomian glands. Because Meibomian gland contrast was found to be a strong risk factor for lipid-deficient dry eye and an objective, repeatable measure, it served as the primary outcome to detect changes in the glands over time and differences between past- and non-users of isotretinoin. In general, the studies found that Meibomian gland contrast decreased significantly within the first month of therapy, plateaued by the end of the treatment period, and returned back to baseline by 6-months post treatment. The mean difference in change between the treatment and control groups was significantly different at the end of the treatment period but was no longer different at 6-months post-treatment. Interestingly, Meibomian gland contrast was found to be approximately 5-intensity units higher in past-users compared to non-users of isotretinoin with statistical significance.

In addition to Meibomian gland contrast, changes to other ocular surface parameters were considered. During isotretinoin treatment, increased eyelid and conjunctival hyperemia, decreased meibum quality and quantity, thinner tear-lipid layer, and increased symptoms of burning or watering eyes were observed. By 6-months post-treatment, indicators of gland function, including meibum quality and number of expressed glands, continued to be suppressed in the isotretinoin treatment group. The single-visit cohort study found that past-users had higher percent Meibomian gland atrophy (marginal significance, $p=0.055$), greater eyelid margin irregularities, worse meibum quality and quantity, and more frequent and severe symptoms of soreness/irritation and burning/watering eyes.

Human meibum were collected from past-users and non-users of isotretinoin for the identification and quantification of polyunsaturated fatty acids and lipid mediators. Arachidonic acid (AA), docosahexaenoic acid (DHA), 5- and 12-hydroxyeicosatetraenoic acids (5-HETE and 12-HETE), prostaglandins E_2 and D_2 (PGE_2 and PGD_2), and thromboxane B_2 (TXB_2) were all present in the samples collected, and their levels were similar to those previously identified in keratinocytes and sebaceous glands isolated from hairless mice.[74] To our knowledge, this is the first report of eicosanoids in human meibum. The normalized PGD_2 ($p<0.001$), PGE_2 ($p<0.001$), and TXB_2 ($p=0.002$) values were less for the isotretinoin-exposed group compared to the unexposed group, which supports previous findings that retinoids inhibit the transcription of COX-2, the rate-limiting enzyme in the production of prostaglandins and thromboxane.[75] However, it is interesting that the impact on prostanoid levels appear to persist long after discontinuing therapy. The clinical significance of these findings is unclear. PGD_2 was previously reported to cause sebaceous gland hypertrophy in bald scalps, and these results suggest that PGD_2 may also play a role in meibum levels in the human Meibomian glands.[76] Decreased PGE_2 and PGD_2 may suggest down-regulation of meibum production (e.g., meibocyte proliferation), but the relationship and mechanism are unclear. Prostanoids are also regulators

of vascular function, epithelial ion channels, apoptosis and normal physiology.[77] Eyelid margin redness in past-users may indicate that impaired prostanoid formation disrupts homeostasis. The correlation between elevated DHA levels and worse symptoms suggests that elevated DHA may be indicative of active inflammation and/or a metabolic deficiency preventing the conversion of DHA to SPMs. Further research is warranted to understand the role of DHA and prostanoids in Meibomian gland physiology, regulation by isotretinoin, and potential role in MGD.

In conclusion, Meibomian gland contrast was a reliable objective measure to monitor changes with treatment. Although contrast decreased, as expected, with isotretinoin treatment, the elevated amount in past-isotretinoin users may be indicative of an overcompensation post-treatment or normal state of elevated lipid production in acne-prone individuals. Ocular signs and symptoms of Meibomian gland dysfunction develop during treatment and persist after stopping therapy. DHA and PGD_2 may provide more clues about short and long-term alterations to Meibomian gland function.

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