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Conjunctival Gene Expression And Tear Biomarkers In Atopic And Non-Atopic Keratoconus Patients Relative To Matching Controls

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CONJUNCTIVAL GENE EXPRESSION AND TEAR BIOMARKERS IN ATOPIC AND NON-ATOPIC KERATOCONUS PATIENTS RELATIVE TO MATCHING CONTROLS

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ABSTRACT

Purpose: The aim of this study was to adapt conjunctival impression cytology (CIC) and RNA isolation to genetic analysis of the conjunctival surface of keratoconus (KC) patients with (AKC) and without atopic disease (AD) and with normal contact lens wearers (NCL). If RNA quantity and quality was sufficient, full transcriptome analysis would be conducted using RNA-Seq, rather than the more limited microarray approach.

Methods: CIC samples were the ocular surface of 30 participants from four groups: 1) KC, 2) AKC, 3) AD, and 4) NCL. Several extraction and purification methods were investigated, including density gradient centrifugation, precipitation, conventional pelleting, and column-based kits. Minimal sample integrity requirements for RNA-Seq were based on an RNA integrity number (RIN) >7.0, where 10 indicates a perfect sample. Three RNA isolates from each study group were selected for full RNA analysis. Intergroup comparisons: KC versus NCL, AKC versus NCL, and AKC versus AD were conducted using Ingenuity® Pathway Analysis (IPA) to identify the best differentiating genes, pathways, associated diseases, and molecular and cellular functions among paired study groups.

Results: Optimization of the Qiagen column-based RNA purification procedure produced RNA with RIN adequate for RNA-Seq. Entire genome and transcriptome analysis was therefore conducted on all 12 selected samples. IPA identified several, mainly signaling,

pathways, up- and down-regulated genes with inflammatory associations, and molecular and cellular functions involving signaling, maintenance and compromise, that best differentiated KC and AKC from the other groups.

Conclusions: CIC can be used to collect sufficient cellular material from the ocular surface to extract, purify, and isolate RNA of sufficient quantity and integrity for downstream genome and transcriptome sequencing. Heterogeneity within each patient group limited the number of differences found between atopic and non-atopic KC patients. However, IPA identified gene and pathway differences between KC and AKC that provide a basis for future studies of differences in their pathogenesis. A large scale study with more homogenous groups would be required for such an investigation, but the gene and pathway differences found in this study will provide a useful starting point.

Keywords: conjunctival impression cytology, keratoconus, atopic disease, RNA-Seq, pathway analysis

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AD	atopic disease
AKC	atopic keratoconus
ALOX12B	arachidonate 12B-lipoxygenase
ALOX5AP	arachidonate 5-lipoxygenase activating protein
CD28	cluster of differentiation 28
CIC	conjunctival impression cytology
cDNA	complementary deoxyribonucleic acid
CL	contact lens
DNA	deoxyribonucleic acid
DOCK9	dedicator of cytokinesis 9
ECM	extracellular matrix
FLG	filaggrin
GWAS	genome-wide association studies
ICAM4	intercellular adhesion molecule 4
IFN-α	interferon alpha

IFN-β	interferon beta
IFN-α2	interferon alpha 2
IFNG	interferon gamma
IgE	immunoglobulin E
IL	interleukin
IL1	interleukin 1
IL1α	interleukin 1 alpha
IL1β	interleukin 1 beta
IL4	interleukin 4
IL5	Interleukin 5
IL6	interleukin 6
IL12	interleukin12
IL13	interleukin 13
IL17C	interleukin 17C
IL17	interleukin 17
IPA	Ingenuity Pathway Analysis
K	keratometer estimate of total corneal power

KC	keratoconus
KLK7	kallikrein-related peptidase 7
MAPK1	mitogen-Activated Protein Kinase 1
MMPs	metalloproteinases
MMP-9	metalloproteinase 9
mRNA	messenger ribonucleic acid
NCL	normal contact lens wearer
ncRNA	non-coding ribonucleic acid
OX40	protein encoded by tumor necrosis factor superfamily member 4
PCA	principal component analysis
RGP	rigid gas permeable
RIN	ribonucleic acid integrity number
RNA	ribonucleic acid
RNA-Seq	ribonucleic acid sequencing
rRNA	ribosomal ribonucleic acid
SFRP1	secreted Frizzled-Related Protein 1
SFRP2	secreted Frizzled-Related Protein 2

SOD1	superoxide dismutase 1
sIgA	secretory immunoglobulin A
SNPs	single nucleotide polymorphisms
SPINK5	serine peptidase inhibitor, Kazal Type 5
tRNA	transfer ribonucleic acid
TLR2	toll-like receptor 2
TNF	tumor necrosis factor
TNF- α	tumor necrosis factor alpha
TNFRSF4	tumor necrosis factor superfamily member 4
VSX 1	visual system homeobox 1
WGCNA	weighted gene co-expression network analysis
ZFHX3	zinc finger homeobox 3
ZFP42	zinc finger protein 42

CHAPTER 1

INTRODUCTION

Cornea

The cornea is the most anterior structure of the eye, consisting of five main layers and the recently identified Dua's layer. The most superficial layer of the cornea is the epithelial layer, which consists of six to eight cell layers stacked on top of one another, and averages around 50 μm in thickness.[1] Adjacent to the basement membrane of the epithelium lies Bowman's layer, which is the most anterior aspect of the stromal layer. It is distinguished from the epithelium and the rest of the stroma by its lack of structure and the absence of fibroblasts.[1] Bowman's layer provides strength and protection for the rest of the cornea due to its dense, fibrous sheet of interwoven collagen fibrils.

Beneath Bowman's layer is the corneal stroma, the thickest layer of the cornea. It is measures 500-700 μm thick and makes up about 90% of the corneal thickness.[2] The stroma is anchored to Bowman's layer anteriorly and lies between two cellular layers; the epithelium and the endothelium. The stroma is mainly composed of type I collagen fibrils are highly organized in flat bundles called lamellae that run parallel to the corneal surface and extend the length of the cornea. In the cornea, as well as the sclera, the collagen is associated with proteoglycans and glycoproteins that form the extracellular matrix that surrounds the fibrils. Throughout the matrix are flattened fibroblasts, referred to as keratocytes in the cornea, that are responsible for synthesizing stromal collagen and extracellular matrix, serve play an important role in corneal transparency. Transparency

of the stroma is dependent on the integrity and intactness of both the epithelium and endothelium on either side of it.

The most posterior layer of the cornea is the endothelium which is the most metabolically active layer of the cornea. The endothelium is a single layer of cells that continuously secret Descemet's membrane, which is a basement membrane that separates the stroma from the endothelium. Descemet's membrane is approximately 5 μm in children and thickens with age to approximately 15 μm. Descemet's membrane is composed of type IV and type VIII collagen allowing it to be a strong resilient layer[1]. The endothelial cell layer is approximately 5 μm thick and is in direct contact with the aqueous humor in the anterior chamber.

The endothelium also has an important role in corneal transparency because it continuously pumps ions across cell membranes. This results in water movement from the corneal stroma to the aqueous humor, counteracting the leaky tight junctions. Mitochondria are found in greatest concentration in the endothelial cell layer reflecting the high metabolic activity of the endothelial cells. The cell density of the endothelium decreases with age, and unlike any other cellular layer of the cornea, the endothelium does not regenerate as cells die. Instead surrounding endothelial cells increase in size (polymegathism) and change shape (pleomorphism) which can be detrimental to the active pump function, and therefore affect transparency.

Dua's layer is only 15 μM thick, but it is a very strong layer that can withstand high pressure.[3] It is located at the posterior boundary of the corneal stroma, and appears to be highly resistant to rupture. It was recently suggested that Dua's layer may have a

role in acute hydrops of keratoconus, as suturing of the layer caused rapid resolution of the hydrops.[3]

The most important property of the cornea is its transparency. Corneal transparency depends on the structural and functional integrity of each corneal layer. Vital to the maintenance of transparency are the corneal epithelial and endothelial layers. They serve as both physical and osmotic barriers. In addition, the corneal endothelial pump prevents deturgescence, or swelling of the cornea. Collagen organization in the stroma is much more precisely organized compared to that of the sclera, which is also a contributing factor to corneal transparency. The cornea is avascular and the absence of blood vessels is another important contributor to corneal transparency.[3]

The primary functions of the cornea are to refract and transmit light as well as to protect and provide structural integrity. The cornea provides approximately two thirds of the refractive power of the eye due to its shape, curvature of the anterior and posterior surfaces, and the change in refractive index from air to the tear film, and cornea to aqueous humor[2]. The cornea is able to transmit light with minimal scattering due to the organized structure of each layer, the transparency as a result of regular arrangement, and the smooth anterior surface due to the tear film.

Another important function of the cornea is protection and structural integrity. There are several types of junctional complexes in the cornea that vary depending on the corneal layer and tight junctions, specifically in the anterior cell layers, contribute to the corneas ability to serve as a selective barrier to prevent free movement of water, debris, or pathogens to the posterior parts of the cornea.

Conjunctiva

The conjunctiva is a thin, translucent, well-vascularized mucous membrane composed of an outer epithelial layer and sub-surface connective tissue layers. As the external mucous membrane covering the exposed scleral surface, it serves important protective functions. The palpebral conjunctiva lines the posterior surface of the eyelids, while the bulbar conjunctiva covers the anterior sclera. These conjunctival regions are continuous and the junctions at which they meet in the superior and inferior cul-de-sac are called the fornices. The bulbar conjunctival epithelium is continuous with the corneal epithelium. Interspersed among epithelial across the entire conjunctival surface are numerous mucus-secreting goblet cells. While goblet cells are found across the palpebral and bulbar conjunctiva, their density is greatest at the fornices.[2] Mucus secreted by the goblet cells forms a thin layer that becomes intermixed with a large part of the aqueous tear component. It also interacts with glycocalyx-secreted long chain mucins. These mucins form a scaffolding to anchor the tear film to the anterior ocular surface.

While the sclera constitutes five sixths of the connective tissue coat of the globe, the cornea forms the remaining one sixth.[2] The sclera is a thick, dense, connective tissue layer composed of collagen fibrils that vary in diameter from 25-230 nm. These fibrils are arranged in irregular lamellae that contribute significantly to scleral strength.[2] In contrast to the regular arrangement of corneal collagen, the variability in scleral collagen fibril size and irregular arrangement results in an opaque structure. The sclera has a minimal blood supply because it is relatively metabolically inactive. In addition to its protective functions, the sclera provides the important attachment sites for extraocular muscles.

Preocular Tear Film

Both the conjunctiva and cornea are bathed anteriorly by the preocular tear film. By smoothing out the highly irregular surface of corneal epithelial cells, the tear film effectively constitutes the most anterior, and most powerful refracting element of the eye's optical system. The tear film is composed of three layers; 1) an outer lipid layer, 2) a middle aqueous layer, and 3) an inner mucin layer.[4] The most anterior layer, the lipid layer, is produced primarily by the meibomian glands, which are located in the tarsal plate of the upper and lower eyelids. Both polar and non-polar lipids function together to form a relatively inert physiological barrier to the external environment, while also being able to spread rapidly and uniformly across the middle aqueous tear layer with each blink.[5, 6] Two accessory lipid glands, the glands of Zeiss and Moll, also contribute to the lipid barrier function of the corneal surface. Their functions are to lubricate eyelashes lashes and to prevent aqueous tear spillover at the lid margins[6].

The middle aqueous layer is produced mainly by the lacrimal gland, while the glands of Krause and Wolfring contribute on a much smaller scale. In addition to aqueous (water), the aqueous layer contains a variety of proteins including lactoferrin, lipocalin, and lysozyme which protect the ocular surface from potentially pathogenic microbes. Additionally, the aqueous layer is responsible for supplying oxygen and nutrients to the anterior corneal structures, and washing away debris, toxins, and foreign bodies.[7]

The mucus layer is the most posterior layer of the tear film and consists of transmembrane proteins, gel forming mucins, and soluble mucins that are produced by the lacrimal gland, ocular surface epithelium and goblet cells.[8] As previously mentioned, mucins provide the scaffolding to which the anterior tear layers to adhere.

Keratoconus

Keratoconus (KC) is a progressive degenerative disease of the cornea that results in corneal stromal thinning, and disruption of Bowman's layer and stromal lamellae. These processes change corneal shape from regular prolate aspheric to conical, the cone apex typically lying inferior to the visual axis. Bowman's layer is thought to be the initial site of damage, but every corneal layer can be involved in the disease process. Stromal thinning occurs over a long period of time, and studies have demonstrated that keratocyte apoptosis contributes to the stromal thinning in KC.[9] Because corneal structure is altered by the disease process, the tear film is also disrupted, both changes contributing to a decrease in visual acuity.[10]

Clinically, KC typically presents as a bilateral condition, but often is asymmetric between eyes and progresses at variable rates. Signs and symptoms include a decrease in vision, halos around lights and "ghost" images, irregular astigmatism, and significant amounts of myopia as a result of corneal ectasia. Major clinical features on biomicroscopy include; 1) Fleischer's Ring: iron deposits surrounding the base of the cone, 2) Munson's sign: protrusion of the lower lid in down-gaze as it conforms to the cone-shaped corneal apex, 3) Vogt's striae: vertical stress lines in Descemet's membrane, 4) corneal hydrops: breaks in Descemet's, and 5) prominent corneal nerves.[10]

KC is one of the most common corneal dystrophies in America according to the National Eye Institute, which states that KC affects one in every 2000 Americans. Although the epidemiological figures on KC have changed greatly with new advances in research, it is known that KC is most prevalent in teenagers and young adults.[11] Both males and females are affected by KC, but some studies show that the prevalence is

equal, others studies suggests males are more affected, while others found females to be more affected.

The cause of KC is not well understood, but studies suggest a variety of potential causes, including ocular rubbing, long term contact lens (CL) wear, inflammatory processes, genetic predisposition, and several systemic and ocular diseases. While theories abound, no one theory adequately explains the pathogenesis of KC, suggesting that KC is a complex condition of multifactorial etiology.

Some studies report a correlation between KC and trauma as a result of ocular rubbing associated with atopy (a genetic tendency to develop allergic disease), ocular allergies, and symptoms of itching[11]. In fact, some studies have found that more than 80% of KC patients reported that they rubbed their eyes compared to 51 to 58% of controls.[12,13] It is thought that eye rubbing results in traumatic epithelial damage to the cornea, leading to stromal thinning due to keratocyte apoptosis, and eventually resulting in ectasia. There are many documented cases of unilateral KC due to vigorous eye rubbing that support this theory, and patients should be counseled to avoid eye rubbing as much as possible.[14]

Another aspect of asymmetric or unilateral keratoconus is hand dominance and eye rubbing. Theories suggest that asymmetry of KC may be explained by hand dominance, the more advanced eye being on the side of the dominant hand. Currently, there is little research and therefore no conclusive evidence associating both eye rubbing and hand dominance in KC patients.[15],[16] Similarly to eye rubbing as a form of chronic trauma to the cornea, long term CL wear has also been shown to contribute to the pathogenesis of KC.

A study conducted by Bawazeer, Hodge, and Lorimer concluded that the most important risk factor for the development of KC is eye rubbing.[17] They found that KC patients with atopy developed the corneal disease due to the vigorous eye rubbing in response to severe itching. In addition to eye rubbing, research suggests that there may be an inflammatory component that potentially causes a more progressive form of KC in atopic patients, because of the inflammatory nature of AD.[15]

Another potential contributor to KC is the role of inflammation in non-atopic KC patients. This has garnered considerable attention in recent years, studies suggesting that KC does have an inflammatory component. Historically, KC has been thought of exclusively as a non-inflammatory disease. While KC does not present with all the typical signs of inflammation, including redness, heat, swelling, and pain, there is ample evidence that inflammation can exacerbate the pathogenic processes of KC. This includes evidence of degradative enzyme activity, wound healing, and reduced anti-inflammatory capacity[15].

Chronic mechanical trauma due to eye rubbing and CL wear elicits an inflammatory response in those KC patients with significantly increased tear expression of several pro-inflammatory biomarkers. These include matrix metalloproteinases, interleukins, and tumor necrosis factor (TNF) α and β.[15,18] Matrix metalloproteinases (MMPs) are important contributors to the KC disease process because they are responsible for the degradation of extracellular matrix proteins (ECM) and are secreted in response to pro-inflammatory mediators.[17] In addition, chronic mechanical trauma can lead to chronic keratocyte apoptosis in the anterior stroma in patients with KC mediated by cytokines, such as including interleukin-1 (IL-1).[19]

Inflammatory mediators have been found in tears of KC patients in higher quantities than NCL patients, supporting the theory that KC may have an inflammatory component. Recent studies have demonstrated that pro-inflammatory mediators, including MMP-9, TNF-α and IL-13, are increased, and inflammatory inhibitors, such as lactoferrin and secretory immunoglobulin A (sIgA), are proportionately decreased.[15,20]

Apart from traumatic and inflammatory contributions to KC, reports of hereditary associations with KC range from 7% according to the National Eye Institute to 13.5% from the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) study.[11, 21] This indicates that genetics may play a role in a subset of KC cases. Genetic studies suggest that KC is not the result of a single gene mutation, but shows heterogeneity. Some of the genes frequently reported to be linked to KC are Secreted Frizzled-Related Protein 1 (SFRP1)[22], superoxide dismutase 1 (SOD1), visual system homeobox 1 (VSX1), and dedicator of cytokinesis 9 (DOCK9)[23] (Table 1).

There are several hereditary systemic conditions that are often associated with the development of KC including, Marfan's syndrome, Down's syndrome, Ehlers-Danlos syndrome, and mitral valve prolapse.[11,17] There are also ocular conditions that have reported associations with KC including vernal keratoconjunctivitis, floppy eyelid syndrome, Leber's congenital amaurosis, and retinitis pigmentosa.[24]

Atopic Disease

Atopic diseases (AD) include inflammatory skin condition, referred to as atopic dermatitis, asthma, and allergic rhinoconjunctivitis.[25] The clinical picture usually presents

Table 1

Target genes identified through literature review to show significant changes in keratoconus, atopic disease, or both.

Gene	Atopic Disease	Keratoconus
18S	×	
ACTB	×	
BAX	×	
C3	×	
CCL11	×	×
A2M		×
ACTB		×
ADRB2	×	
ALOX5	×	
ASAH2C	×	
AZGP1		×
B2M*		
BAG3	×	
CCL11	×	
CCL5	×	×
CD14	×	
CD244	×	
CD28	×	
CD4	×	
CMA1	×	
CREB3L2	×	
DEFB1	×	
DOCK9		×
DYNC2LI1	×	
ENPP3	×	
FCER1A	×	
FLG	×	×
GAPDH*		
GATA3	×	
GEMIN6	×	
GJB2	×	
GSTP1	×	
HPRT1*		
HRH1	×	×
HRH2	×	×

Gene	Atopic Disease	Keratoconus
ICAM1	×	×
IFNG	×	
IGFBP3		×
IGFBP5		×
IGKC		×
IL10	×	
IL12A	×	×
IL12B	×	×
IL12B	×	×
IL13	×	
IL17A		×
IL18	×	
IL1B		×
IL1RA	×	
IL22	×	
IL23A	×	
IL25	×	
IL33	×	
IL4	×	×
IL4R	×	
IL5		×
IL6		×
IL8	×	×
IRF2	×	
KLK7	×	
LCN1		×
LTF		×
MMP1		×
MMP13		×
MMP3		×
MMP7		×
MMP9		×
NGFRAP1	×	
PDGFRB	×	
PGRMC1	×	
PLAC8	×	
PPF1BP1	×	
RAB3GAP1		×
RCAN3	×	

Gene	Atopic Disease	Keratoconus
RGS1	×	
RGS16	×	
RPRD1B	×	
RPS9*		
SETD7		×
SFRP1		×
SLC30A6	×	
SOD1		×
SPINK5	×	
STAT6	×	
TBP*		
TBXAS1	×	
TGFB1		×
TIMP1		×
TIMP2		×
TLR2	×	
TMTC3	×	
TNF	×	×
TNFAIP6		×
TNFRSF1A	×	
TNFSF10	×	
TNFSF11	×	
TNFSF8	×	
TRIB1	×	
VEGFA		×
VSX1		×
ZFHX3	×	

Note: *Housekeeping genes were included in the list in preparation for micro-array assays if RNA-Seq is not successful

as a triad with eczema dermatitis, allergic rhinitis, and asthma with symptoms of excessive pruritus. Atopy is the term for hypersensitivity reactions directed against common environmental allergens in persons with hereditary background of allergic disease.[26] In younger infants, the skin condition is usually an acute inflammation of the skin involving the cheeks, scalp, and extensor aspects of the arms and legs, whereas in

adults it shifts to a chronic inflammation that has a predilection for flexural aspects of the arms and legs. Atopy is a characterized as a chronic condition that can have a chronically relapsing course throughout life.[25] AD is a condition that effects the entire body, including the eyes.

AD can affect the superficial layers of the cornea and conjunctiva as well as the adnexa. Ocular features of AD include intense pruritus resulting in eye rubbing, conjunctival hyperemia, Dennie-Morgan infraorbital crease or fold, anterior subcapsular cataracts, periorbital skin darkening, and KC. Children with chronic ocular complications of AD tend to develop vernal keratoconjunctivitis that features large, cobblestone-like papillae on the superior palpebral conjunctiva, and adults manifest allergic keratoconjunctivitis, which is the most common clinical ocular presentation.[25, 26]

AD is the most common chronic inflammatory skin disease, with prevalence increasing over the last 30 years.[27, 28] According to the American Academy of Dermatology, 10-20% of children around the world have AD with an onset typically between infancy and early childhood, with 90% of people developing disease before age 5. Females are slightly more likely to develop AD more than males, with an equal incidence in all races, but higher incidence in more affluent populations.[29]

Atopy is a complex genetic disease with environmental influences. According to the World Allergy Organization, atopy is tightly linked to the presence of allergen-specific immunoglobulin E (IgE) antibodies in the serum. While IgE is not a prerequisite in all patients with AD, it has been found to be increased in approximately 80% of patients with AD.[25] Atopy is the genetic predisposition to make IgE antibodies in response to allergen exposure. An important risk factor for the development of AD, is

parental history of atopy, suggesting genetic inheritance of AD. Research has identified several genes that fall into two different categories based on their role in the allergic reaction: 1) genes encoding epidermal proteins; and 2) genes encoding immunologic proteins.[25] Some of the genes that are implicated in AD include but are not limited to filaggrin (FLG), serine peptidase inhibitor, Kazal type 5 (SPINK5), kallikrein-related peptidase 7 (KLK7), toll-like receptor 2 (TLR2), interleukin4 (IL4), interleukin-5 (IL-5), interleukin-12 (IL-12), and interleukin-13(IL-13)[25], all of which were identified as part of the initial literature review (Table 1).

Because atopic dermatitis is considered to be an inflammatory condition, many inflammatory mediators are either up-regulated or down-regulated. Many cytokines, including ILs, MMPs, and TNF-α, are reported to be altered in both AD and KC.[30]

Target Genes Identified by Literature Review

Table 1 summarizes target genes that were selected based on literature review. Genes that were implicated in other reports describing KC and/or AD were included in the Table 1 list. Genes that have been reported by others to be highly expressed or under-expressed in KC or at least demonstrated the potential to differentiate between KC and non-KC groups were also included. Table 1 indicates whether the gene was associated with KC, AD, or both. Most of the 96 genes were identified in studies of AD rather than KC. This is expected because the cellular and molecular mechanisms of KC are not as well understood as those of AD.

Gene Expression

In multicellular organisms, nearly every cell contains the same genome and thus the same genes. However, not every gene is transcriptionally active in every cell, which is why different cells show different patterns of gene expression. These variations underlie the wide range of physical, biochemical, and developmental differences seen among various cells and tissues and may play a role in the difference between health and disease. Thus, by collecting and comparing transcriptomes of different types of cells or tissues, researchers can gain a deeper understanding of what constitutes a specific cell type and how changes in transcriptional activity may reflect or contribute to disease.

Many important clues about gene function come from determining when and where the genes are expressed, which is the basis of genomics. Gene expression can be investigated in done several ways. A common method involved creating complementary deoxyribonucleic acid (cDNA) libraries using messenger ribonucleic acid (mRNA) from collected samples. In eukaryotes, mRNA is then transcribed into deoxyribonucleic acid (DNA) as a series of repetitive sequences that are not transcribed, and are therefore not represented in cDNA libraries. This is beneficial because the cDNA library is enriched with fragments from actively transcribed genes, while non-coding RNA (ncRNA) information does not interrupt the process of creating and scanning the cDNA library. Because cDNA contains only sequences that are present in mRNA, the non-coding protein information is not included and therefore does not contribute to the resulting DNA library.[31] Recent research shows that the ncRNA may play a role in disease processes and progression. Quantifying ncRNA may therefore facilitate a more thorough investigation of the pathogenesis of KC in patients with and without AD.

Genome vs. Transcriptome

Two types of genetic information are traditionally sought, each providing different insights into tissues in normal or disease states; 1) Genome information and 2) transcriptome information. Full genome sequencing is a DNA level application that directly sequences the whole genome of a species to enumerate complete genome sequences using a bioinformatics approach. Genome sequencing has played a very important role in our understanding of the molecular evolution of a species, its genetic components, and their regulation.[32] It looks at an array of genes, up to the entire genome using genome-wide association studies (GWAS) and other methods. Full genome sequencing has been applied extensively in many areas of genetic research for years.

On the other hand, to look at the range of genes that are undergoing active transcription at any given point in time in a given cell or tissue, a different approach is required: transcriptome analysis or sequencing. Transcriptome sequencing is the foundation and starting point for studying gene function and structure, which is particularly important when studying pathogenesis of progressive diseases including KC and AD. With reference genome sequence, scientists can obtain much more information, such as gene expression, alternative splicing, optimizing-gene structure, and new genes by comparing transcriptome sequencing with genomic DNA sequences.[32] The transcriptome is essentially a snapshot into what is actively happening within the cell.

Many eukaryotic genes contain coding regions called exons and non-coding regions called introns. All the introns and exons are initially transcribed into ribonucleic acid (RNA) but, after transcription, the introns are removed by splicing and the exons are joined to form mature mRNA, which ultimately leads to the production of proteins.

Transcriptome analysis creates a snapshot of the range of intracellular DNA that is being transcribed to RNA at any given time. Many of these transcribed sequences are "non-protein coding" (ncRNA) in more complex organisms, including humans. This is different from mRNA, which carries the genetic code from DNA to form amino acids and ultimately protein. ncRNA includes transfer ribonucleic acid (tRNA), ribosomal ribonucleic acid (rRNA) and other RNA forms. tRNA forms an "adaptor molecule" between mRNA and protein to facilitate protein synthesis. It is effectively the deciphering code to allow mRNA to produce amino acids. rRNA associates with ribosomal proteins and actively catalyzes the translation of nucleotide sequences into protein.

Beyond the genome and ncRNAs, each gene may produce more than one variant of mRNA for a variety of reasons, including alternative splicing, RNA editing, or alternative transcription initiation and termination sites. These variants may directly contribute to many disease processes. The transcriptome therefore represents a far greater level of complexity than the simple genome sequence.[33]

Conjunctival Impression Cytology

Conjunctival Impression Cytology (CIC) is a non-invasive technique that has been used extensively to collect epithelial and goblet cells from the conjunctival surface. It is preferred over more invasive full-thickness biopsies because CIC rarely produces patient discomfort or side effects.[34] CIC has been used extensively to investigate ocular surface pathology using both histological and immunohistochemical approaches. It has

also been used to obtain conjunctival surface cell RNA for extraction, processing, and analysis for subsequent application to gene expression studies. Ocular surface cells, being the source of genetic material, can provide essential information to identify key biomarkers and essentially inflammatory pathways that can help lead to further information about the pathogenesis of KC.

Research using CIC to study patients with KC and AD demonstrated conjunctival cell differences between those with disease and matching controls. In KC patients, goblet cell loss and squamous metaplasia correlated with the extent of KC progression.[35] KC patient CIC samples also elicited higher levels of lysosomal enzyme compared to controls.[36] In atopic patients CIC revealed goblet cell loss and conjunctival squamous metaplasia that correlated with the number of flare-ups.[26] Increased understanding of the changes actually occurring at the ocular surface, including cellular level changes, may help to explain the pathogenesis and the subsequent clinical appearance of these potentially blinding disorders.

Other studies have demonstrated that both the conjunctiva and cornea are affected by KC and AD. This is to be expected because the conjunctival epithelium is continuous with the corneal epithelium. Correlating conjunctival changes with clinical test results for KC and/or AD would be the preferred approach because CIC is considerably less invasive. The in situ cornea is not a viable option for epithelial cell collection because doing so would produce an epithelial defect and considerable pain for the patient. In addition, the tears bathe the cornea and the conjunctiva. They act as collection medium for biomarkers expressed on the ocular surface, and can also be used to gather biomarkers and genetic material for analysis.

Processing of CIC Samples

RNA extraction and purification methods vary considerably, some being more suitable for certain tissue or sample types than others. It is important to optimize an extraction and purification technique for conjunctival surface samples that will be most likely to result in quantitative genetic data.

When using CIC samples to generate gene expression profiles, both epithelial and goblet cells are collected from the ocular surface, with density of the two cell types varying across the ocular surface. CIC is also limited in terms of the total number of cells that can be obtained from a patient.[37] Limited source tissue means limited RNA collection. This makes a high-yield RNA processing procedure essential to ensure that both the quantity and quality of RNA are sufficient for subsequent processing. All steps from the RNA extraction method to the final gene expression analysis procedure must therefore be optimized for the limited available source tissue.[38]

Gene Expression Assay

There are several options for gene assay, all of which have specific minimum RNA quantity and quality requirements. Assays that employ more efficient enhancement techniques than real-time reverse transcription PCR require less starting material per assayed gene. This enables the study of a larger number of genes in a given sample. Newer approaches include microarrays, which can typically target between 96 and 1,000 genes per "chip". Newer and higher density microarray chips are regularly increasing the gene limit. The most efficient method currently available, RNA-Sequencing (RNA-Seq®) is frequently used to sequence the entire genome.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Assay

RT-PCR is based on conversion of source RNA to DNA followed by the amplification of single copies of this DNA over several orders of magnitude. The DNA used for amplification is produced by reverse transcribing the sample-derived RNA into its complementary DNA (cDNA) under the catalytic action of the enzyme, reverse transcriptase. After this, the newly synthesized cDNA is amplified by traditional PCR.[38]

To catalyze production and amplification of each DNA molecule of interest, a coding "primer" must be present in the PCR container or plate – one for each DNA molecule sequence to be quantified. This is because, while the polymerase enzyme can catalyze a single strand of DNA to make a copy, it is dependent on the primer to initiate this process. The primer is a small region of single-stranded DNA that binds specifically to a particular location on the single strand DNA molecule. This binding, or annealing, is achieved by cooling the PCR mixture. The DNA polymerase enzyme then copies the single strand molecule (hybridization), starting at the bound primer region. During the final step in each PCR cycle, there is extension of the DNA from the annealed primer to make a complementary copy of the single strand. From this point, amplification proceeds by a sequence of heating steps to break the double-stranded DNA into single strand then annealing again to the primer. The more abundant a particular RNA sequence in the starting sample, the more efficiently the amplification proceeds and the earlier the "threshold" cycle at which amplification of that DNA commences during PCR.[38]

Microarray Assay

Microarrays can provide information about the expression of many genes, enabling the study of the genes that are active in a particular tissue. This approach has been used to investigate gene expression changes in the course of biological processes, including disease progression.[39]

The microarray substrate consists of DNA probes fixed to a solid support, such as a chip, nylon membrane, or glass slide, each well "spot" containing a specific DNA probe. RNA extracted from study cells is reverse transcribed in the presence of labeled nucleotides to produce single stranded complementary (cDNA) molecules with a fluorescent tag attached. The cDNA's are then mixed and hybridized to DNA probes on the microarray substrate, and are then scanned spot by spot. Fluorescent intensity is a measure of gene expression for each gene being probed on the microarray.[31]

The primary limiting factor for microarray technology is that it is not practical to investigate the entire genome in a given study because of the prohibitive sample quantity and large number of Microarray chips that would be required. While very high density tiling arrays have reduced this limitation for simpler organisms, it remains an obstacle for larger, more complex, eukaryotic genomes.[40]

For a typical microarray study: 1) the most appropriate array of target genes (or other genetic indicators) is determined, and, 2) the chip capacity is matched with the most appropriate source sample processing method. Target genes for each study groups would be based on their potential to differentiate disease from control. If more than one disease and/or control group is included, differentiators across all groups would be sought. Once

suitable genes are identified, gene expression patterns could be used as the basis for differentiating patient groups.

Microarrays are a popular choice when a limited range of target genes is of interest. For example, in the context of the current study, genes known or suspected to be modified in KC or AD would be logical choices.

Transcriptome Assay/Analysis

Gene expression studies were historically restricted to small-scale quantitative PCR analyses of candidate genes, but improved the level of cross-species hybridization on Microarrays, as discussed above.[41] With advances in gene sequencing technology, also called massively parallel sequencing, analysis of gene expression has greatly expanded and is more inclusive of different RNA types. RNA-Seq® is the current state of the art in next generation sequencing. The important advantage of this technique is that, in addition to providing an entire genome, transcriptome level change can also be elucidated. RNA-Seq generates gene expression information equal to that Microarrays, with the added benefit that the entire transcriptome is surveyed. Choices as to which transcribed regions are affected are unnecessary because all transcripts and their variants are assayed[42].

For transcriptome analysis, high quality source material (RNA) remains essential, but the methods of analysis are fundamentally different. An ultra-high throughput sequencing approach, next generation sequencing, is used. As stated above, RNA-Seq is the current state of the art for obtaining the entire genome and transcriptome, yielding absolute resolution down to a single base. RNA-Seq is capable of distinguishing different isoforms and allelic expression in addition to the greater than 8,000-fold gene expression

level, all with a minimal amount of isolated, but high quality, highly purified RNA. It is the minimal amount of isolated RNA that makes RNA-Seq attractive in terms of CIC sample analysis with limited source tissue.

Comparison of RNA-Seq and Microarrays

In contrast to microarray technology, next generation sequencing allows identification of novel transcripts. It does not require a sequenced genome and is not subject to the technical problems of background noise associated with fluorescence-based methods to quantify amplification. In addition, unlike hybridization-based detection, such as PCR, RNA-Seq allows genome-wide analysis of transcription at single nucleotide resolution, including identification of variants, such as alternative splicing events and post-transcriptional RNA editing events.[43]

The microarray technique requires a larger amount of isolated RNA and is limited in terms of its ability to distinguish isoforms and/or allelic expression. RNA-Seq is currently more expensive than microarray, but yields an entire transcriptome with additional detection of novel sequences and splice variants. It includes detection of non-coding RNA, single nucleotide polymorphisms, and fusion genes all from the same source.[33] In terms of cost per gene or transcript, RNA-Seq is very economical.

Many studies have verified that RNA-Seq is a much more powerful and sensitive assay technique information than microarrays. For example, in a psoriasis study, 42 skin samples were examined by both RNA-Seq and microarrays with the following findings[44]. There were marked differences in sensitivity, with transcripts identified only by RNA-Seq, and at much lower expression than the detection threshold for microarrays. RNA-

Seq identified many more differentially expressed transcripts enriched in immune system processes. Weighted gene co-expression network analysis (WGCNA) revealed multiple modules of coordinately expressed epidermal differentiation genes from RNA-Seq data.[44]

Overview of Major Biological Pathways

A considerable body of evidence demonstrates that genes and their protein products are organized into functional groups according to cellular processes and pathways.[45] Pathway analysis is used to identify these closely related proteins. This approach is helpful when studying differential gene expression in a disease such as KC or AD. Pathway analysis will find clusters of highly correlated genes, place them in their corresponding pathways, and quantify the significance of pathway differences between study groups.

Pathway analysis therefore facilitates network based gene screening to identify candidate biomarkers or therapeutic targets. These methods have been successfully applied in various biological contexts, e.g. cancer, mouse genetics, yeast genetics, and analysis of brain imaging data.[46] Gene expression data obtained by the optimized method determined by the current study will be analyzed using pathway analysis to look for key genes and pathways that differentiate between atopic and non-atopic KC patients.

Major pathways relevant to the current study include transmembrane transport, immune system, signal transduction, disease, and others. These major pathways can be broken down to numerous sub-levels all the way down to the single gene or single protein level. Changes to a specific pathway, for example the immune system pathway, may lead to the identification of an important inflammatory mediator in KC, and potentially a genetic connection to AD. Examining changes in gene expression, such as up and down

regulation within groups, may suggest where a breakdown is occurring in a specific pathway or multiple pathways.

When investigating specific genes, it is important to include their pathway associations. To differentiate study groups, the most useful comparisons occur when members of each group are closely related. For instance, in carefully controlled animal models, using knock-in or knock-out mice, will maximize the chance of finding significant differences between groups. In human studies of gene expression and pathway analysis, the greatest differentiation is found when studying an entire family. This is the ideal approach to study heritable diseases.

Disease processes are not well understood in many conditions, especially at the molecular level. Many human disease pathways involve three main mechanisms: infection, altered structure and function of human proteins, and change in gene expression. Altered structure and function, and changes in gene expression are of particular importance when using transcriptome analysis to identify connections within and differences between study groups.

Transmembrane transport of small molecules pathway is the biological process in which a solute is transported across the lipid bilayer of the cell. Transmembrane transport can occur by different means, depending on the size, shape, and charge of the molecule or compound. There are three broad categories into which proteins with transport functions can be classified: ATP-powered pumps, channels, and transporters. In active transport, the substrate is moved against a concentration gradient, and so ATP-hydrolysis provides the energy required for the ATP-powered pump. The active transporters are generally large and span the entire membrane. They are involved in moving a variety of substrates,

including amino acids, lipids, peptides, proteins, metals, and drugs, into and out of the cell. They are also involved in intracellular compartmental transport.[47] Channels act as open passageway for diffusion of solutes across the membrane. Most channels are gated, to regulate the movement of solutes. Transporter carrier proteins undergo a conformational change once they bind their solute to create a hydrophilic pocket surrounding the solute until it crossed the membrane and switches conformation to expose the solute to the other side of the membrane.[47]

Cell-to-cell communication pathways are essential to all multicellular organisms because they coordinate cellular responses to signals from their environment and from other cells. Cell-to-cell communication depends on the distance between the cells, the presence of cellular junctions between cells and receptors present on the cell surface. There are five common modes of communication between cells: direct intercellular signaling, contact-dependent signaling, autocrine signaling, paracrine signaling, and endocrine signaling.[47] Cells respond to signals in three stages: receptor activation, signal transduction, and cellular response.

The signal transduction pathway is stimulated by an activated transmembrane receptor in response to an extracellular signal or ligand. Once the transmembrane receptor is activated, it causes a conformational change in the transmembrane protein, which results in the production of different intracellular signaling cascades. The downstream signaling cascade may impact cell proliferation, differentiation, and cell survival, but signal transduction ultimately regulates growth and behavior of an organism. Specific signal transduction molecules are responsible for changes in gene

expression and cellular metabolism, which makes this pathway specifically important when investigating pathogenesis of a disease.[47, 48]

The pathway responsible for extracellular matrix organization is of particular importance because the cornea's transparency is dependent on precise organization of the extracellular matrix (ECM), collagen, and other structural components. The ECM is a dynamic network of glycosaminoglycans and fibrous proteins, including collagen, elastin, and fibronectin, embedded in a viscoelastic gel.[49] In addition to its structural role, the ECM influences cellular proliferation, adhesion, migration, differentiation, and cell death. The ECM also plays an important role in inflammation. The biochemical and structural properties of the ECM are responsible for modulating early steps of the inflammatory cascade, including immune cell migration into inflamed tissues and immune cell differentiation.[50] The ECM is constantly being remodeled by matrix metalloproteinases and growth factors that influence organization, synthesis, and degradation of the ECM and components.[51] Disruption or changes to this heterogeneous and highly dynamic pathway can lead to cellular dysfunction and ultimately disease, because of its essential role in major cellular processes and inflammatory pathways.

The metabolic pathway is responsible for generating energy in cells through a series of biochemical processes. Each metabolic pathway has unique chemical reactions that are connected via specific intermediates. These reactions are catalyzed by enzymes that require other important molecules to ensure the reaction is complete. Any disruption in this pathway can lead to a build-up of toxic byproducts, resulting in cell death and a reduction in the energy necessary to support the cellular functions.

Another important group of pathways are those that are responsible for the metabolism of proteins, including; translation, post-translational modification, and protein folding. This group of pathways is often targeted when studying disease pathogenesis, because the associated protein modifications are considered to be the difference between health and disease. Transcriptome analysis targets this particular group as it analyzes changes at all three levels.

CHAPTER 2

AIMS AND RATIONALE

Specific Aims

AIM 1: Optimize a CIC and RNA extraction, isolation, and purification procedure for gene expression analysis. To be selected as the method of choice, the RNA yield and quality should be sufficient to enable reliable gene expression analysis. The two methods that will be compared are: 1) RNA transcriptome analysis using RNA-Seq and 2) gene microarray analysis.

AIM 2: Apply the gene expression analysis method from Aim 1 to a clinical/genetic study of KC and non-KC patients divided into the following four groups: 1) KC only; 2) AD only; 3) AKC; and 4) NCL wearer. Given the expected heterogeneity of these four patient groups, this will be a preliminary study to investigate gene expression differences, in particular between atopic and non-atopic KC patients.

Null Hypothesis: improvements to CIC sample collection and processing will not result in RNA of sufficient quantity and integrity to enable genome or transcriptome-level comparisons between atopic and non-atopic presentations of KC by RNA-Seq.

Research Hypothesis: improvements to CIC sample collection and processing will result in RNA of sufficient quantity and integrity to enable genome or transcriptome-level comparisons between atopic and non-atopic presentations of KC by RNA-Seq.

Rationale

Because the cause of KC is not well defined, investigating correlations between KC patients with AD and those without, may lead to a better understanding of the disease process and differences in progression patterns between the two disease groups. It is known that patients with AD have an increased risk of developing a more progressive form of KC. The correlations that are identified in the study will help guide early intervention and treatment options for KC and AKC patients.

Many inflammatory mediators may be up- or down-regulated in each KC and AKC. Of particular importance will be differences in these mediator profiles between KC and AKC; for example MMP and cytokine differences. Because cytokines are a family of secreted proteins involved in immunoregulatory and inflammatory processes, it is logical that they would be most altered in the group with both conditions.

For this study, it is hypothesized that patients with AKC will have a more progressive form of KC due to a fundamental difference in disease pathogenesis compared to the other study groups. The genes that are identified in both the KC and AD groups may lead to more insight about the pathogenesis and progression of AKC[20].

A fully optimized cell collection and RNA processing procedure may provide the choice of either transcriptome analysis or at least the broadest possible selection of genes

from which to classify KC groups. With either approach, our understanding of the different types of KC and the changes that occur in each type should be improved.

Tear collection was part of the study experimental design and tear collection was conducted at the same study visit as CIC. However, tear analysis will be part of another project comparing the levels of biomarkers present on the ocular surface with gene expression for each study group. Key biomarkers may be identified for patients with KC, AD, and AKC. While gene expression patterns and/or transcriptome variants from CIC samples can be used to compare differences among the four groups, these variants can also be correlated with the levels of biomarkers at the ocular surface based on tear cytokine assays.[38]

This approach may ultimately provide a better understanding of treatment, potentially highlighting the risks and benefits of different treatment options for each group. Of course, larger follow-up studies would be needed with greater patient numbers to be able to quantify more gene expression patterns. At most, the current study may provide a clearer picture of the feasibility of using an optimized RNA extraction and purification method that would be sufficient to do large scale gene expression studies of using CIC in KC patients.

CHAPTER 3

EXPERIMENTAL DESIGN

AIM 1

Sample Collection and Sample Processing

For RNA extraction, isolation, and purification, the primary method used was the Qiagen RNeasy Plus Mini Kit® (Qiagen Group, CA). This is a spin column-based method that utilizes a guanidine-isothiocyanate buffer to inactivate RNases and ensure isolation of intact RNA. This method was included because it allows parallel processing of multiple samples in a relatively short timeframe. In addition, it produces purified RNA in suitable form for downstream applications, such as RNA-Seq and microarrays. Based on previous dry eye research conducted in this laboratory, the Qiagen RNeasy Plus Mini Kit® column-based extraction method was considered to have the potential to be the optimal method for RNA isolation and purification.[38]

Because salt contamination commonly occurs with guanidine-isothiocyanate based extraction methods, and ethanol carry-over is a common problem that can decrease the RNA quantity and quality, modifications to reduce their effect were incorporated as alternative procedures if NanoDrop 1000 (Thermo Scientific Inc., Wilmington, DE) results indicated suboptimal RNA quality. These alternative procedures would be utilized on an "as needed" basis.

Entirely different RNA extraction, purification, and isolation protocols were also investigated, including the GE Illustra RNAspin Mini Isolation method (GE Healthcare,

Pittsburgh, PA) with 5 Prime phase lock gel for optimal density gradient phase separation. A traditional density gradient precipitation and RNA pelleting method was also investigated. These methods were considered second choice alternatives because they incorporate the more toxic substances, phenol and chloroform.

Sample Quality and Quantity

When evaluating quantity and quality of purified RNA using the NanoDrop 1000, two key indicators were monitored: (1) protein yield defined by the A260/280 value (RNA/protein ratio), the goal being a value of no less than 2.0, and (2) lack of contaminating extraction salts in the final RNA extract, as indicated by the A260/230 value. In this case, the ideal outcome is a low A230 value and therefore high A260/230 ratio. These two parameters formed the basis for identifying the optimal RNA purification protocol and were key indicators of the need for modifications to be incorporated into each base method. After determining the optimal RNA purification protocol, this method would be used to prepare samples for all downstream applications, whether they involved RNA-Seq or microarray gene expression analysis.

Following RNA isolation, samples were evaluated for A260/280 and A260/230 to determine initial suitability for downstream analysis. Samples showing greatest potential were then more thoroughly analyzed by capillary electrophoresis using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). This device assessed both RNA purity and degree of breakdown, to produce an "RNA Integrity Number" (RIN). The RIN is based on analysis of total RNA and the components contributing to the total. rRNA comprises more than 80% of cellular RNA, while mRNA makes up less than 3%.[52]

Because rRNA far exceeds mRNA in cellular extracts, RNA integrity is usually evaluated based on the rRNA transcripts. A single RNA transcript cleaves into a 28S (5,070 nucleotides) and an 18S (1,869 nucleotides) product. The difference in number of nucleotides between 28S and 18S means that electrophoretic separation for perfectly intact rRNA should yield a 28S:18S nucleotide ratio of approximately 2.7.[53] In practical terms, a 28S:18S ratio >2.1 indicates very high quality, pure RNA.[54] rRNA integrity in a given sample is generally a reliable indicator of mRNA integrity, the RNA of interest for downstream quantification. tRNA is also sequenced by RNA-Seq, but not by microarray.

The Agilent Bioanalyzer performs a virtual electrophoretic separation on all purified RNA samples to provide a RIN value. The system software assigns an RIN from 1 to 10, with one being the most degraded and 10 being the most intact, or highest quality.[52] For quantitative analysis of the isolated RNA using RNA-Seq in Aim 2, the consistent finding of a RIN value ≥7 was the goal. Failing this goal, the alternative downstream method, microarrays, would be chosen.

When analyzing the quality of a sample using the Bioanalyzer electropherograms, high quality RNA will have clear, well defined 18S and 28S peaks that are of equal height or a 28S peak that is higher than the 18S peak and there should be low noise on the baseline between the two peaks. As the RNA quality decreases, the 28S peak decreases in height and broadens at either side of the base due to noise. The RIN value is lower as a result. When the RNA is significantly degraded, the 18S and 28S peaks will be absent and the electropherogram will appear primarily as noise. Examples of each scenario are depicted in Figure 1.

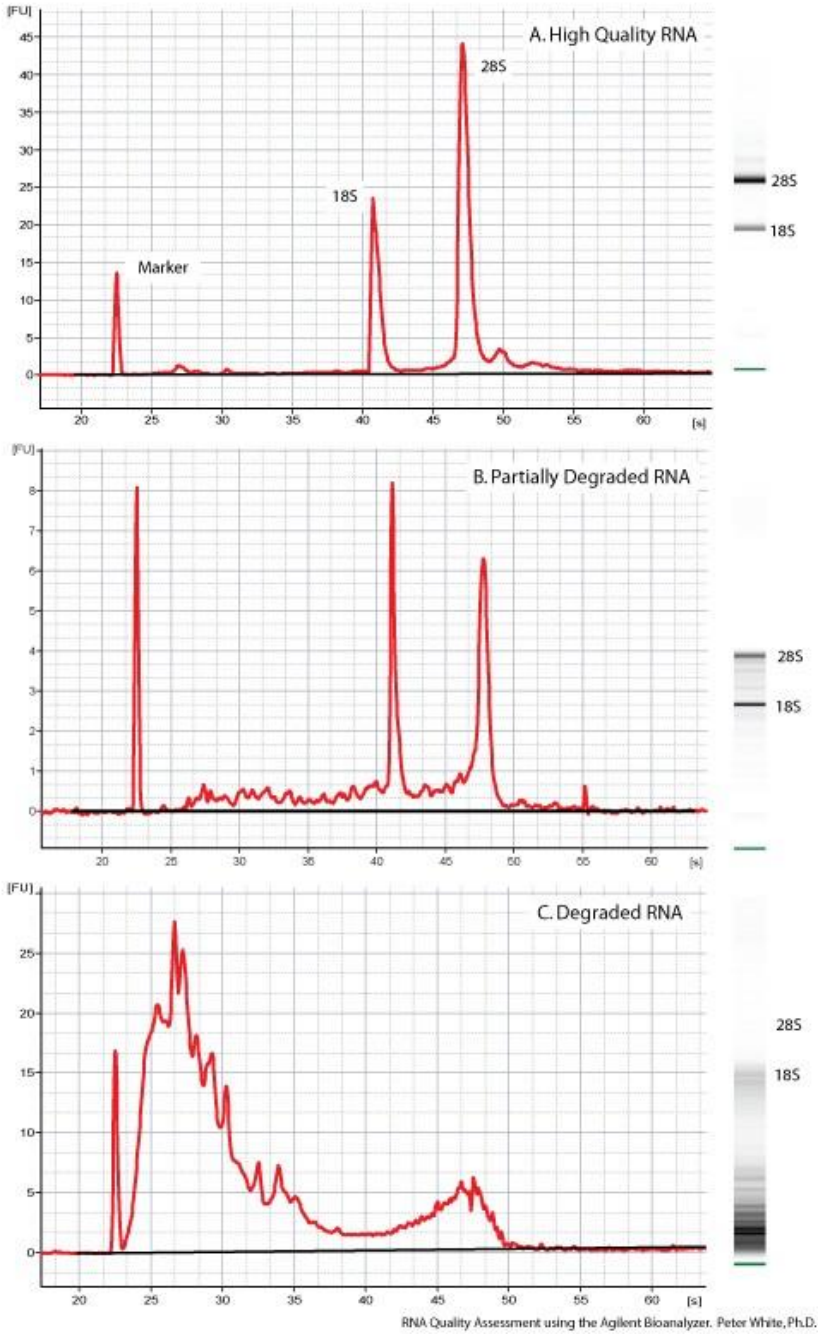

Figure 1. Agilent Bioanalyzer electropherograms for a) high quality RNA, B) partially degraded RNA, and C) degraded RNA.

AIM 2

Identifying and Classifying Study Participants

Study patients were UAB School of Optometry clinic patients who were either normal contact lens wearers (NCL), or had previously been diagnosed with KC, AD. Based on a suitable previous diagnosis, patients were invited to participate in the current study. Entering participants were classified based on their previous diagnosis, telephone interview outcome, and results of clinical diagnostic tests performed during study Visit 1. While it was not possible to ensure many shared traits within each target group beyond KC and AD diagnosis, it was considered essential to exclude any candidates for which the KC and AD classification was uncertain.

Group classifications were based on the following inclusion-exclusion criteria:

1. KC: To be classified as a KC patient, the study participant must have been previously diagnosed with KC by an optometrist or ophthalmologist. The diagnosis was confirmed by corneal topography on Visit 1.
2. AD: To be classified as an AD patient, the participant must have chronic allergic conditions and have been diagnosed with or confirmed to have allergic conjunctivitis at UAB Eye Care. They must have experienced one or more of the following; asthma, rhinitis, hay fever, eczema, dry or itchy skin, and/or allergies.
3. AKC: To be classified as an AKC patient the participant had to meet the requirements for both the KC and AD group.
4. NCL: to be classified as a normal contact lens wearer, the participant had to have healthy eyes with no previous diagnosis of KC or other corneal or conjunctival

abnormality. In addition, they could not have a history of allergies, rhinitis, hay fever, allergic conjunctivitis, or any skin conditions such as eczema.

In addition to the grouping criteria, all participants were required to be habitual contact lens wearers, ≥3 days per week. For all four groups, participants being excluded if they had previous ocular surgery or Intacs®.

Pathway Analysis of Genetic Data

Gene expression was investigated to determine differences between atopic and non-atopic KC patients. To investigate these differences, the four patient groups were paired as follows: 1) KC versus NCL, 2) AKC versus NCL, and 3) AKC versus AD. Initially, the samples were processed with RNA-Seq to identify genes and transcripts that differentiated those with the disease versus the controls. Gene and transcriptome data were then filtered to analyze the genes and transcripts eliciting a fold change ≥2.

Many methods have been proposed for genome and transcriptome analysis.[55] Two of the most cited accepted approaches were chosen for the current study: Reactome® pathway analysis (www.reactome.org) and Ingenuity pathway analysis® (Qiagen, Redwood City, www.qiagen.com/ingenuity).

Following RNA sequencing, the genes and transcripts were analyzed by Reactome®, a curated pathway database, to identify pathways patterns and their associated genes and transcripts.[56, 57] Reactome® provides information about the primary pathways involved based on the individual gene and transcripts that are entered, but it is unable to differentiate the paired groups based on the identified pathways. Data was then

further analyzed using Qiagen Ingenuity® Pathway Analysis (IPA). IPA of gene expression data is much more comprehensive than Reactome®. It provides a more comprehensive analysis of pathway involvement for the genes and transcripts identified by RNA-Seq. In addition to providing "top" pathways; that is pathways that best differentiate between a disease group and control group, IPA identifies upstream regulators, gives insight into molecular and chemical interactions, and identifies diseases and conditions expected to differ significantly between these pathways.

Pathways expected to differentiate KC and AKC are those involving connective tissue, tissue breakdown, and inflammation. Upon identification of affected pathways, more detail of specific pathway involvement was sought, along with individual genes or gene subsets that may be involved in causing or maintaining the disease. This information can then be used as a basis for future research. For example, "knockout" animal studies could be used to determine the effects of specific gene deletions on the disease to determine if the disease still occurs, or if the severity changes. This could lead down the road to the design of treatments for humans, or prophylactic/early intervention protocols for at risk patients or early stage affected patients.

CHAPTER 4

METHODS

All aspects of this study adhered to the guidelines of the Declaration of Helsinki and were conducted with the approval of the University of Alabama at Birmingham Institutional Review Board.

AIM 1

Sample Collection: Conjunctival Impression Cytology Technique

Sample collection was performed on all candidates during development of the optimal RNA purification method (Aim 1). It was also utilized in Study Aim 2 for all patients at Study Visit 2. CIC is a minimally invasive sample collection technique that causes minimal discomfort for the patient. CIC was used to obtain cell samples from each patient's bulbar conjunctiva. The CIC procedure used for this study was adopted and modified as described by Bradley et al.[38]

CIC sample collection discs of 6 mm diameter were punched out from a 47-mm diameter filter disc (Millipore 0.45 μm HAWP047A0, Millipore Corporation, Temecula, CA) using a sterile stainless steel hole punch (Fisher, Pittsburgh, PA). Eighteen to twenty CIC discs were punched from each large filter. The 6 mm CIC filter discs were placed between two blotting pads provided with the original filters, then inserted into a 9 x 13 cm sterilization pouch (Fisher, Pittsburgh, PA). The CIC technique required curved jeweler's forceps, straight jeweler's forceps, and a borosilicate glass rod to perform the

technique. One set of instruments was placed in a 9 × 13 cm sterilization pouch. The sterilization pouch containing the CIC filter discs and the required instruments was autoclaved (Hirayama Hiclave HV25, Hirayama Corporation, Minato-Ku Japan) for 20 minutes at 121°C. The sterilization pouches were then allowed to fully dry once removed from the autoclave.

Extraction buffer was prepared prior to sample collection. Eight 2-mL collection tubes were labeled 1 - 4 OD and 1 - 4 OS. A mixture of 350 µL of Buffer RLT Plus from the Qiagen RNeasy Plus Mini Kit® plus 3.5 µL β-mercaptoethanol was added to each tube. Contents of all tubes were thoroughly mixed using a pipette.

Topical anesthetic (0.5% proparacaine, Akorn, Lake Forest, IL) was instilled into both eyes of the patient by the study clinician. The process of sample collection from the conjunctival surface proceeded as follows. A sterile 6-mm CIC disc was placed on the conjunctival surface, starting with the right eye using curved jeweler's forceps. To ensure adequate adhesion to the bulbar conjunctiva a borosilicate glass rod was used to exert pressure on the disc once it was placed on the conjunctiva. This filter was then removed using curved forceps and transferred to a second pair of straight jeweler's forceps, ensuring no contact between the two forceps.

Each CIC collection disc was then placed into a 2-mL collection tube containing extraction buffer, extra precaution being taken to ensure that the sample collection forceps did not touch the extraction buffer. A total of four collection tubes per eye were used, with samples from collection sites I and II being placed into tube one, and sites III and IV into tube 2, sites V and VI into tube 3, and sites VII and VIII into tube 4. Extra precaution was taken during the transfer of the disc from the curved forceps to the

straight forceps, and again when then disc was placed into the extraction buffer, to ensure no extraction buffer came into contact with the sample collection curved forceps. The same collection and disc storage procedures were used for all eight samples collected from each eye. CIC samples were collected in the following sequence: right eye superior-temporal, superior-nasal, inferotemporal, inferonasal, temporal-slightly superior, temporal-slightly inferior, nasal-slightly superior, and nasal-slightly inferior. The same sequence was then used for the left eye.

During collection, to ensure continued patient comfort, the ocular surface of both eyes was re-anesthetized after the fourth disc was collected or sooner if the patient felt any discomfort. The same process was repeated for the left eye. Once all samples were collected, collection tubes were vortexed at maximum speed for 60 seconds, then centrifuged at 10,000g for 20 seconds. The tubes were then stored at −80°C until processing.

CIC Sample Processing

Procedure 1 and Modifications: Qiagen RNeasy Plus Mini Kit®

Using the Qiagen RNeasy Plus Mini Kit®, RNA from the collected samples was extracted, isolated, and purified. Processing of collected, lysed, conjunctival cells involved further lysis, homogenization, genomic DNA removal, washing of total RNA washed, and isolated of the final purified RNA extract.

Prior to processing, samples were removed from the −80°C freezer and thawed at −30°C for 1 hour, then 4°C for 1 hour. While samples thawed, four RNeasy Plus Mini Kit gDNA eliminator columns (two per eye) were labeled, in addition to two RNeasy spin

column eluate collection tubes (one per eye), two 2-mL collection tubes with unattached caps (one per eye), and two 1.5-mL tubes (one per eye) for storing the final RNA extract. A volume of 4.67 mL of 70% ethanol in water was prepared in a 50-mL tube.

Thawed samples were lysed and homogenized by vortexing all eight tubes at maximum speed for 60 seconds, then centrifuging at 10,000g for 20 seconds. A 1-mL pipette was used to transfer 400 μL of cell lysate solution from two of the OD extraction tubes (containing four filters) to a gDNA Eliminator spin column. The same steps were repeated for the other two OD extraction tubes, the solution being placed in the second OD gDNA Eliminator column. The entire process was repeated for both pairs of OS extraction tubes.

Genomic DNA was removed by centrifuging all four gDNA Eliminator columns for 30 seconds at 10,000g. Collection tubes containing eluate were retained and the columns discarded. A volume of 600 μL of 70% ethanol solution was added to the first eluate-containing collection tube and the solution was mixed by pipetting up and down four times. 600 μL of this mixture (extract + ethanol) was transferred to the first OD RNeasy spin column, whose function was to bind nucleic acid. This process was repeated for the first OS tube. Tubes were then centrifuged for 20 seconds at 10,000g and the eluate discarded. The top rims of tubes were blotted on a fresh, large Kim Wipe to prevent subsequent column wicking. The remaining ethanol-extract mix from the first OD eluate column was added to the OD RNeasy spin column. The process was repeated for OS. Tubes were then centrifuged for 20 seconds at 10,000g. Eluate was discarded and the upper rims of the open tubes were blotted. The process was repeated for the additional 600 μL and the remainder for the other OD and OS tubes, the same RNeasy spin columns

being re-used. At this stage, all the retained RNA was bound to the single OD RNeasy spin column and single OS RNeasy spin column.

Two additional washes were then performed, each time adding 600 µL of 70% ethanol to each spin column (OD and OS), then centrifuging for 20 seconds at 10,000g. Eluate was discarded and the upper rims of the tubes were again blotted.

For the next step, samples were washed by adding 700 µL Buffer RW1 to each RNA spin column and centrifuging at 10,000g for 20 seconds. The eluate was discarded and the tubes were blotted. The RNeasy spin column was then removed from the collection tube so that the column did not contact the flow-through to avoid contamination, and the collection tubes were emptied completely.

The first pre-elution wash was performed by adding 500 µL Buffer RPE to each column and centrifuging at 10,000g for 20 seconds. Eluate was then discarded and the upper rims of each tubes were blotted.

For the final washing step, another 500 µL of Buffer RPE was added to the tube followed by centrifugation at 10,000g for 2 minutes to ensure the column was completely dry. The eluate was discarded and the upper rims of the tubes were blotted.

To ensure the spin column membrane was completely dry, two drying steps involving no buffer addition were performed. During the first drying step, the spin-column was transferred to a new 2-mL tube and centrifuged at 20,000g for 1 minute to dry the column. This was followed by drying step 2, for which the RNeasy Spin column lid was opened in the laminar flow hood and allowed to air dry for five minutes. During drying step 2, the NanoDrop spectrophotometer was prepared for use by rinsing the two

liquid contact pedestals with RNase-free water from the RNeasy kit, wiping off the water then blanking the instrument with a freshly applied drop of water.

Final elution of RNA involved inserting the Spin column into a 1.5 mL clean collection tube and adding 31 µL of RNase-free water was added. RNase free water was added directly to the center of the column membrane, avoiding direct membrane contact by the pipette tip. The Spin column was centrifuged at 10,000g for 1 minute to elute RNA. The eluate was re-applied to the Spin column in the center of the membrane and the column again centrifuged at 10,000 for 1 minute. This second step theoretically increased RNA yield.

Procedure 1 Modifications

Modifications were designed to be incorporated only as necessary. A low A260/230 value measured with the NanoDrop spectrophotometer, suggesting salt contamination, would indicate the need for some or all of the following modifications:

- Additional washing and drying steps to remove as much of the salt as possible.
- Storage of the Buffer RPE at room temperature instead of +4°C.
- When reusing collection tubes between washing steps, greater care would be taken to remove residual flow-through from the rim by blotting on clean Kim Wipes each time instead of reusing them between all washes for a sample.
- To minimize ethanol carry-over during the final washing step with Buffer RPE, a long centrifugation at ≥10,000g for 2 min at 20–25ºC would be added to ensure the RNeasy spin column membrane was completely dry. After the

long centrifugation, the spin column is carefully removed from the collection tube so that the column did not contact the eluate, to prevent any ethanol carryover.

Procedure 2: GE Illustra™ RNAspin Mini RNA Isolation Kit

The GE Illustra™ kit (GE Healthcare, Pittsburgh, PA) procedure was performed using the modifications described by Frost[58]. Thawed samples were homogenized in the sample collection tube with 200 µl TRIzol® solution using a disposable mini-pestle for 4 minutes. 800 µl of TRIzol® solution was then added to rinse the mini-pestle following homogenization. The sample was allowed to sit at room temperature for 5 minutes, after which it was centrifuged at 21,000g for 20 minutes at 4°C to pellet cellular debris.

The recovered volume, typically 900 µl, was then added to a phase-lock gel (PLG) tube containing 100 µL 1-bromo-3-chloropropane (BCP) + 100 µl RNase-free water. The sample was vigorously mixed by hand, then incubated at room temperature for 5 minutes. Following the mixing step the sample was centrifuged at 14,000g at 4°C for 10 minutes to separate the organic and aqueous phases. The aqueous phase was collected (typically 650 – 700 µl) and ethanol was added to the aqueous phase in two half-volume steps. To avoid RNA precipitation, the tube was vortexed immediately and then loaded on to a Desalting spin column in two parts, to ensure that maximum volume did not exceed 750 µl. The column was then spun at 8,000g for 30 seconds.

The collection tube was replaced and 350 µl of membrane desalting buffer (MDB) was added followed by centrifugation at 11,000g for 1 minute to completely dry the membrane. A mixture of 10 µl reconstituted DNase (40 µl aliquots) and 90 µl DNase

buffer was prepared and 95 µl of the mixture was applied to each membrane. The sample was incubated at room temperature for 15 minutes.

The membranes then underwent a series of washing steps. They were first washed with 200 µl Buffer RA2 (GE Healthcare Bio-Sciences, Pittsburgh, PA), a proprietary buffer containing guanidine thiocyanate, at 11,000g for 1 minutes after which the collection tube was replaced. This was followed by a wash with 600 µl of Buffer RA3 (proprietary wash buffer) at 11,000g for 1 minute. Finally, the membranes were washed with 250 µl of Buffer RA3 at 11,000g for 2 minutes. Each column was then placed into a fresh 1.5ml tube and the membrane was washed and eluted by centrifugation at 11,000g for 1 minute twice with the same 40 µl H_2O. The samples was then quantified using the NanoDrop spectrophotometer.

Procedure 3: Non-column Based RNA Isolation with Density Gradient Separation and Sample Pelleting

Procedure 3 was modified from a method described by Joseph et al[59]. CIC-membrane bound cells were homogenized in 300 µL of TRIzol in an Eppendorf microcentrifuge tube for 5 minutes at room temperature (15-30ºC). To help detach cells from the membrane, the TRIzol solution was mixed by pipetting. Turbulence created by pipetting was also sufficient to separate membranes. Next, 60 µL of chloroform was added to the sample in each Eppendorf tube and the tube was shaken vigorously by hand for 15-30 seconds. The samples were subsequently incubated at room temperature for 5 minutes. After incubation, samples were centrifuged at 12,000g for 15 minutes at 4ºC to separate the phases in to a lower red phenol-chloroform phase containing cellular

proteins, an interphase containing the DNA, and a colorless upper aqueous phase containing the RNA.

The upper aqueous phase was transferred to a clean Eppendorf tube and 150 µL of isopropyl alcohol was added and mixed well by hand for 15 seconds. Samples were then incubated overnight at –20ºC. Following overnight incubation, sample tubes were centrifuged at 12,000g for 15 minutes at 4ºC.

Supernatant was removed and the RNA pellet was washed with 700-1,000 µL of 70% ethanol. The sample was mixed well by gentle vortexing and then centrifuged at 7,5000g for 7.5 minutes at 4ºC. The supernatant was removed, and the RNA pellet was air dried for approximately 2 to 3 minutes (but always less than 5 minutes), ensuring the walls were fluid free. 15 µL of RNase-free water was used to dissolve RNA, or 30 µL if the samples were combined.

Sample Quality and Quantity

The NanoDrop spectrophotometer was used to determine the quantity and quality of the sample RNA immediately following the last centrifugation step. A 1 µL drop of the purified RNA was pipetted onto the lower pedestal of the NanoDrop and a measurement taken 3 times to get an average value. The remaining 20 µL was then stored at −80°C.

An Agilent Bioanalyzer 2100, located at the UAB Heflin Center, was used to analyze the integrity of the highest quality RNA samples obtained during the project. Sample choice was based both on patient clinical profile, to populate the four patient groups defined for Aim 2 of the study (below), and NanoDrop results. The Agilent

Bioanalyzer measured the extent of RNA breakdown as previously described based on the ratio of RNA chain lengths. An RNA Integrity Number (RIN) was assigned based on the outcome. RIN values were crucial to driving the choice between microarrays and full RNA-Seq genome/transcriptome downstream analysis. If RNA-Seq was to be performed, this would be conducted by the Heflin Genomics Core facility and the data analyzed in this lab.

AIM 2

Identifying and Classifying Study Participants

UAB School of Optometry clinic patient records were used to screen for potential study participants for each of the four patient categories. Suitable candidates were then contacted by their last UABSO clinician of record by letter. Those agreeing to participate were contacted by telephone and further screening questions were asked in a pre-scripted, IRB-approved telephone interview. Patients considered to be suitable study candidates based on the interview were sent two surveys, details of which are described below.

Keratoconus Questionnaires (Appendices A and B)

The Keratoconus Symptom & Severity Questionnaire (KSSQ) was designed to determine the degree of discomfort experienced by the patient (Appendix A). This was followed by a more comprehensive "Keratoconus Survey": Phenotypic and Genotypic Analysis of Keratoconus (Appendix B). Results were used to help classify each patient into one of the four study groups. In addition to demographic information, the

questionnaires asked about general ocular history, and ocular comfort based on perceived redness, watering, burning, dryness, discharge, and itching. Contact lens (CL) type and wearing patterns, KC diagnosis and severity, and eye rubbing habits were also explored in the questionnaires. Medical information was sought to elicit more information about severity of AD including skin conditions, asthma, and allergies. Family history was used to determine any possible hereditary links to KC or AD.

Each qualifying patient was invited for a total of two study visits.

Study Visit 1: Clinical Testing

At Visit 1, preliminary clinical testing was performed (Appendix 3), including visual acuity with and without correction, and CL type and wearing schedule. Handedness was determined both subjectively and objectively. Slit lamp exam (SLE) was performed and each of the following were graded using the Efron grading scale: 1) limbal injection, 2) bulbar injection, 3) papillary conjunctivitis, and 4) corneal edema. Other clinical signs including lens-induced epithelial imprint, neovascularization (≥2mm), Vogt's striae, Fleischer's Ring (≥2mm Arc), and Munson's sign were assessed. The cornea was examined carefully for signs of scarring and each scar was measured for size and density. Staining was then performed using sodium fluorescein and Lissamine Green, and both were graded using the Oxford grading method (Appendix 3) for corneal and conjunctival staining. Auto-refraction and corneal topography were obtained using a Nidek OPD Scan II Instrument to assess presence or absence of irregular astigmatism and corneal irregularity.

Study Visit 2: Conjunctival Impression Cytology (CIC)

Study Visit 2 involved CIC only. Eight samples were collected per eye using the same CIC procedure as described in Aim 1 for optimization of the RNA extraction and purification methods.

Pathway Analysis of Genetic Data

All samples given a RIN value of 7 or greater with the Agilent Bioanalyzer were considered to have met the threshold for RNA-Seq transcriptome analysis, to be performed at the UAB Heflin Genomics Core Facility. To analyze the genetic data, samples from each of the four patient groups were compared in three paired analyses: KC versus NCL, AKC versus NCL, and AKC versus AD. Candidate genes for analysis were those eliciting a fold difference $\geq \pm 2$ (and p value<0.05) between the two groups being compared in each case. A p value <0.05 indicated a significant difference between samples, but was less statistically robust than a q value of <0.05. Pathway analysis with these genes allowed data analysis in a more global format, increasing the potential to find differences between the various KC and non-KC groups.

Reactome analysis and IPA were chosen to investigate differences between each of the three paired groups. Once again, analysis included all of the genes with a fold difference $\geq \pm 2$ between paired groups and a p value of <0.05. It was expected in this small scale study that inherent heterogeneity within each group would limit the possibility of eliciting differences at the transcriptome level. Direct and indirect genetic relationships were selected for investigation in the pathway analysis, but endogenous

chemical associations were excluded. Relationships were only considered if confidence level was equal to that experimentally observed for this study.

Once IPA was completed, the "top" (best differentiating) canonical pathways, top upstream regulators, top disease associations and biological functions, network functions, and associated molecules were used to differentiate the paired groups and as a basis for determining the significance to the study.

CHAPTER 5

RESULTS

AIM 1

Sample Collection: Conjunctival Impression Cytology (CIC) Technique

CIC consistently provided sufficient cellular material for either RNA-Seq or microarrays to be performed downstream, followed by Reactome and IPA analysis. However, RNA quality was yet to be proven adequate.

CIC Sample Processing

Procedure 1 and Modifications: Qiagen RNeasy Plus Mini Kit®

Samples were monitored for RNA quantity and purity using the NanoDrop 1000 spectrophotometer and the results are listed in Table 2. Total RNA was very consistent for all but three samples, exceeding 2.0 in all cases, thus indicating a high nucleic acid to protein ratio. A260/230 values were considerably lower than the optimum range of 1.0 or higher, indicating that salt contamination was significant and may interfere with downstream applications. For this reason, the modifications of the RNeasy method described in Procedure 1 were implemented and the two alternative processing methods were also investigated.

Due to the low A260/230 value in the initial phases of sample processing, modifications were investigated in an effort to decrease salt contamination without compromising A260/280 or total RNA. This led to several attempts to improve salt

removal including extra alcohol wash steps, longer final centrifugation, using new tubes for every step; trying double runs through the genomic DNA tube/filter as described in the methods section (Procedure 1 with modifications).

Table 2

NanoDrop 1000 results for the twelve patient samples selected for downstream applications

#	Sample ID	Group Classification	Total RNA [ng]	A260/280	A260/230
1	01-SK OD	AD	1,410	2.18	0.19
2	02-TR OS	AD	2,130	2.23	0.14
3	03-CM OD	NCL	2,130	2.24	0.21
4	04-KT OS	NCL	1,800	2.14	0.99
5	05-KC OS	KC	2,070	2.15	0.30
6	06-TF OS	KC	1,500	2.18	0.61
7	09-DC OD	AKC	1,440	2.17	0.29
8	11-JJ OD	KC	1,230	2.18	0.11
9	12-BF OS	NCL	1,260	2.15	0.13
10	14-SL OD	AKC	1,350	2.23	0.10
11	21-DF OD	AKC	750	2.11	0.26
12	23-EC OD	AD	1,560	2.12	0.10

Table 3 shows the improvement in A260/230 value, while total RNA and purity (A260/280) remained unchanged. Because there was an improvement in A260/230, the modifications were subsequently implemented. Three study participant samples were processed by the UAB Heflin Genomics Core Facility. Results indicated that RNA integrity may be sufficient for RNA-Seq and that further modifications to decrease salt contamination may be unnecessary. Ultimately, the RNA in all four samples was found to be of sufficient quality, as summarized in Figure 2. More detailed analyses for each

Table 3

NanoDrop 1000 results using test participant cellular material to perform pilot study using Procedure 1 modifications

#	Sample ID	Group	Total RNA [ng]	A260/280	A260/230
1	RF OD X 12 #1	AD	1,388	2.17	1.25
2	RF OD X 12 #2	AD	1,360	2.16	1.17
3	RF OS X 12 #1	AD	1,484	1.90	1.13
4	RF OS X 12 #2	AD	1,423	1.90	1.10

Sample are shown in Figures 5 to 8, including comparisons of 28S and 18S peak areas, key values used to assign the RIN value. Modifications were nevertheless pursued for the processing of the remaining samples, anticipating that they would serve as useful guidelines for RNA processing in future studies. Higher A260/230 values, ideally >1.0, indicate more thorough salt removal. This would be particularly beneficial in cases where RNA quantities were low and may allow smaller amounts of RNA to be successfully processed.

Procedure 2: GE Illustra™ RNAspin Mini RNA Isolation Kit: Ambion pure placental RNA

To ensure that the quality of starting RNA was not a contributing factor, the sample was a reference pure placental RNA produced by Ambion (Life Technologies Corp, Grand Island, NY). Briefly, the reference RNA is DNase-treated and certified to represent pure, intact human RNA in all respects. Each tube contains 100 µg of pure RNA at a concentration of 1 mg/mL, making it amenable to processing at very high concentration or to dilution to levels closer to the expected yields of CIC. Table 4 shows the results of the GE Illustra™ RNA isolation and purification process of placental RNA.

Samples 1 and 2 were processed in liquid form with no CIC filter in the extraction tube. Samples 3 and 4 were applied to CIC filters (to more closely mimic the CIC procedure), then added to extraction tubes. All four samples were subjected to the entire extraction, isolation and purification procedure, despite starting off in pure form. Samples 5 and 6 contained the original unprocessed RNA at full strength for comparison.

Starting with purified RNA (Samples 1 to 4) followed by processing with the RNAspin kit did not increase RNA yield or quality (A260/280), nor did it decrease the effect of salt contamination. In fact, A260/230 values were lower relative to the CIC samples processed by the modified version of Procedure 1. The presence of CIC filters in Samples 3 and 4 resulted in higher salt contamination, suggesting that a reaction between extraction buffer and the filter material were the cause. Samples 5 and 6, which were unprocessed, showed ideal A260/230 values as expected because they had not been exposed to extraction buffer salts, such as guanidinium thiocyanate, or to CIC filters.

Applying a larger amount of conjunctival starting material was not possible in the current study due to the limitations of the quantity of RNA that can be obtained by CIC from the ocular surface. However, the placental RNA study suggests that this would not have improved the sample quality.

Procedure 3: Non-column Based RNA Isolation with Density Gradient Separation and Sample Pelleting

Procedure 3 differed from Procedure 2 by adding an RNA pelleting step as the endpoint. As shown in Table 5, the A260/280 and A260/230 values showed similar

Table 4

NanoDrop 1000 Results for placental RNA samples processed with GE Illustra™ RNAspin Mini RNA Isolation Kit

#	Sample ID	Total RNA [ng]	A260/280	A260/230
1	Placental RNA #1 No filter	1,929	1.78	0.55
2	Placental RNA #2 No filter	1,965	1.87	0.55
3	Placental RNA #1 Filter	1,293	1.74	0.36
4	Placental RNA #2 Filter	1,318	1.76	0.36
5	Placental RNA #1 Original	32,827	2.13	2.13
6	Placental RNA #2 Original	33,884	2.15	2.12

patterns to those obtained with Procedure 2. Samples #5 and #6 representing unprocessed pure RNA showed ideal RNA purity and no salt contamination, A260/230 exceeding 2.0.

Table 5

NanoDrop 1000 Results for Samples Processed with Procedure 3

#	Sample ID	Total RNA [ng]	A260/280	A260/230
1	Placental RNA #1 No filter	2,019	1.64	0.51
2	Placental RNA #2 No filter	2,082	1.65	0.50
3	Placental RNA #1 Filter	1,353	1.53	0.32
4	Placental RNA #2 Filter	1,391	1.56	0.31
5	Placental RNA #1 Original	31,137	2.06	2.15
6	Placental RNA #2 Original	31,280	2.06	2.15

Sample Quality and Quantity Assessment and RIN Values

After completing the above sample processing comparisons, Procedures 2 and 3 showed no improvement over the modified version of Procedure 1. For this reason, Procedure 1 with modifications was chosen as the standard approach to purify all samples targeted for downstream applications. The most promising samples from each study patient group were subjected to RIN assay using the Agilent Bioanalyzer. Results for all 12 samples are shown in Table 6. Greater detail for the first four samples, including electropherograms, is shown in Figures 4 to 8. For all samples, RIN values exceeded the minimum threshold of 7. In fact, most samples yielded RINs greater than 9. Therefore, all twelve samples were appropriate for RNA-Seq processing. RNA-Seq was therefore chosen as the downstream application for Study Aim 2, because all 12 samples exceeded RNA quantity and RIN quality requirements. While A260/230 value suggested persistent salt contamination, this was apparently not sufficient to compromise RNA integrity, indicating that the initial RNA optimization for Aim 1 were unnecessarily rigorous.

The batch run and A260/230 values were included in Table 6 to illustrate that, as the sample processing procedure was optimized, the A260/230 did not increase dramatically for most samples. Samples #4 and #6 had a significantly higher A260/230 value but when compared to the other samples, their RIN values did not exceed the others.

Table 6

Agilent Bioanalyzer RNA integrity numbers for all twelve samples and A260/230 value found by NanoDrop 1000.

#	Sample ID	RIN value	Batch Run	A260/230
1	01-SK OD	9.4	3	0.19
2	02-TR OS	9.9	2	0.14
3	03-CM OD	10.0	2	0.21
4	04-KT OS	9.0	3	0.99
5	05-KC OS	10.0	2	0.30
6	06-TF OS	8.7	3	0.61
7	09-DC OD	10.0	2	0.29
8	11-JJ OD	N/A*	3	0.11
9	12-BF OS	9.0	1	0.13
10	14-SL OD	N/A*	3	0.10
11	21-DF OD	9.5	1	0.26
12	23-EC OD	9.7	1	0.10

* Samples run on a card that used a smaller starting amount of RNA. As a result of this approach, quantitative RIN values were not determined. However, the Bioanalyzer electropherograms suggest that RIN value would have been between 8.5 and 9.5.

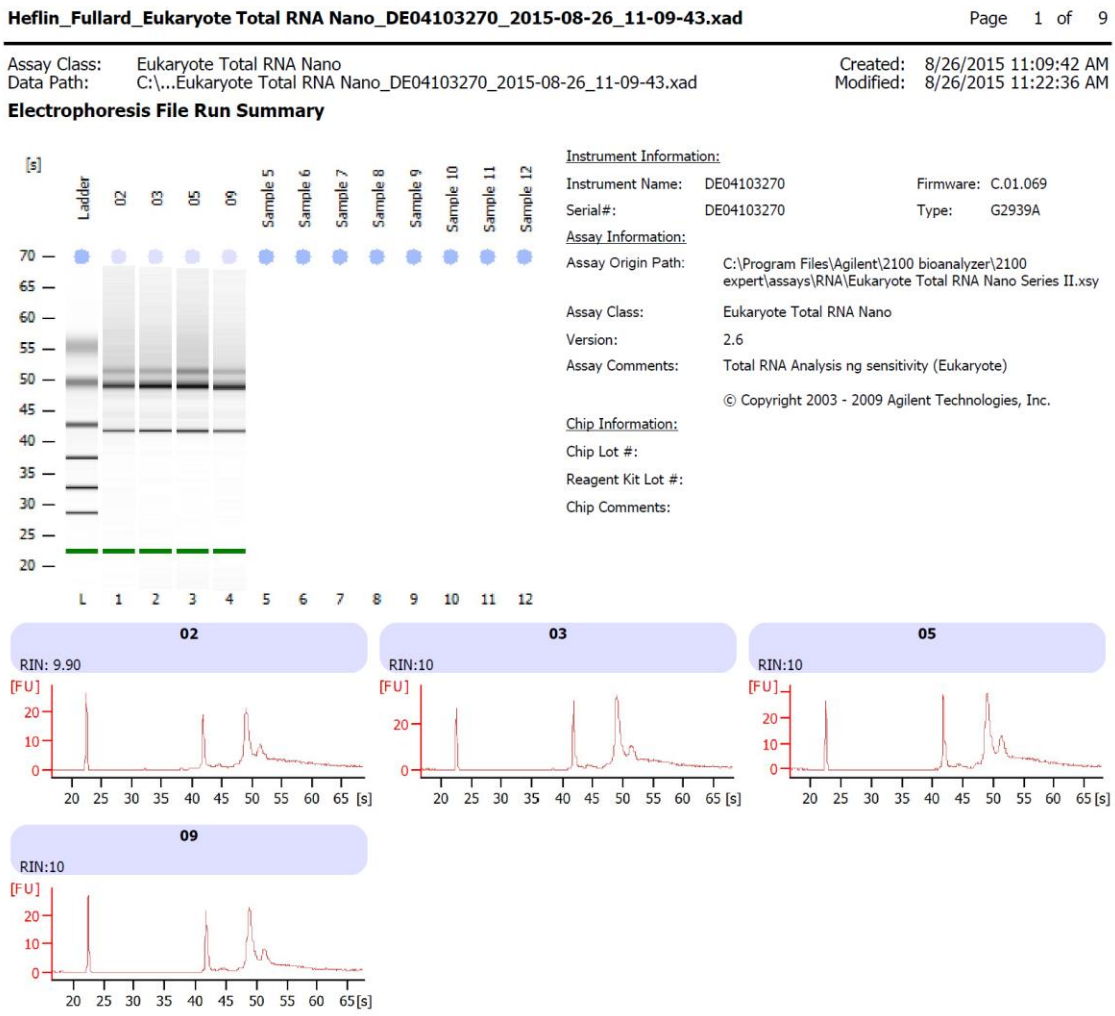

Figure 2. Summary of Agilent Bioanalyzer results for the four participant RNA samples in Batch 1. Three of the four samples had RIN values of 10, and the fourth 9.9. This indicated very high RNA integrity for all samples.

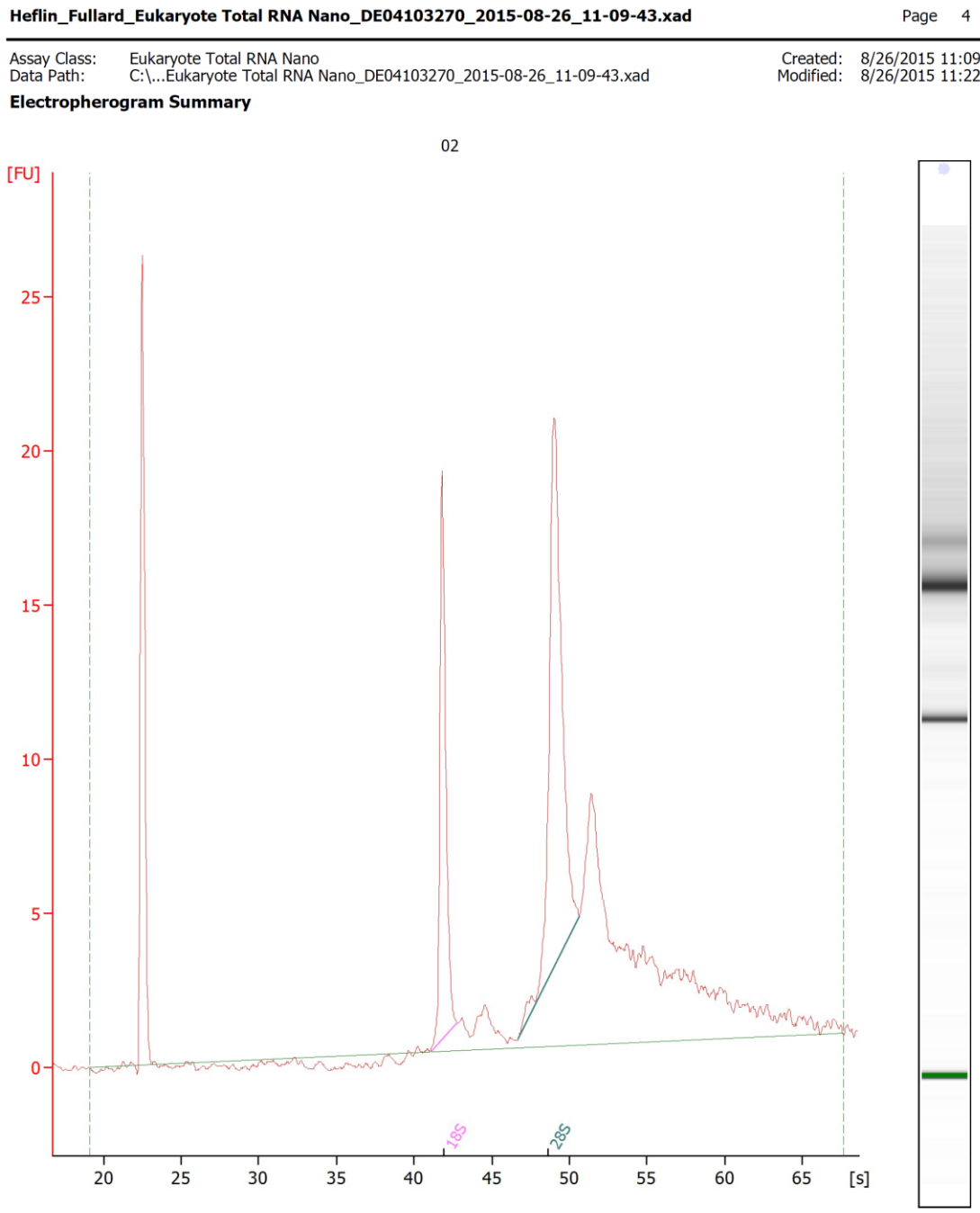

Figure 3. Detailed Agilent Bioanalyzer analysis RNA sample from Batch 1 Participant 1, showing a 28S peak area > 2 times 18S and RIN value of 9.9. The simulated capillary electrophoresis electropherogram is shown to the right.

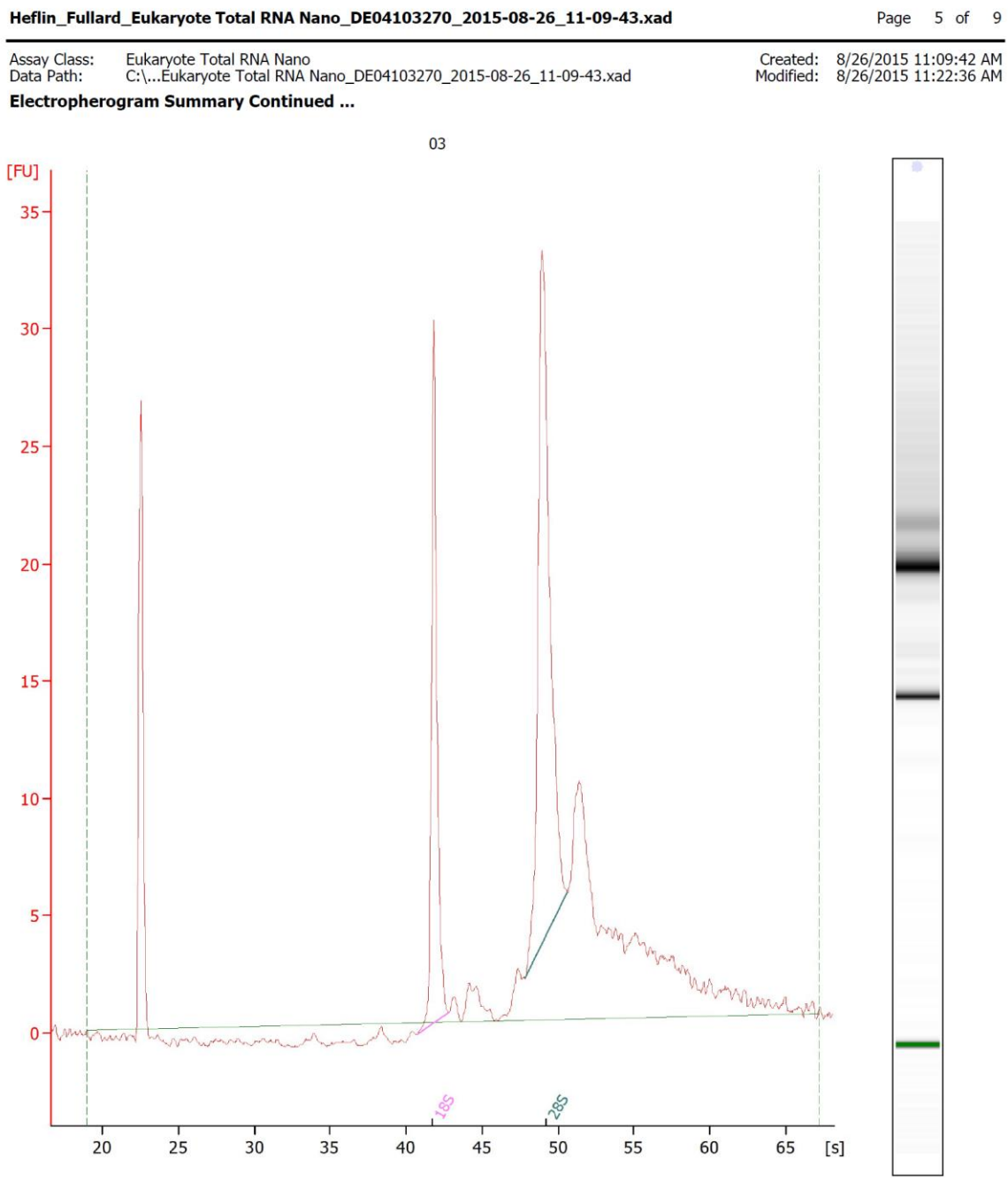

Figure 4. Detailed Agilent Bioanalyzer analysis RNA sample from Batch 1 Participant 2, again showing a 28S peak area > 2 times 18S and RIN value of 10. The simulated capillary electrophoresis electropherogram is shown to the right.

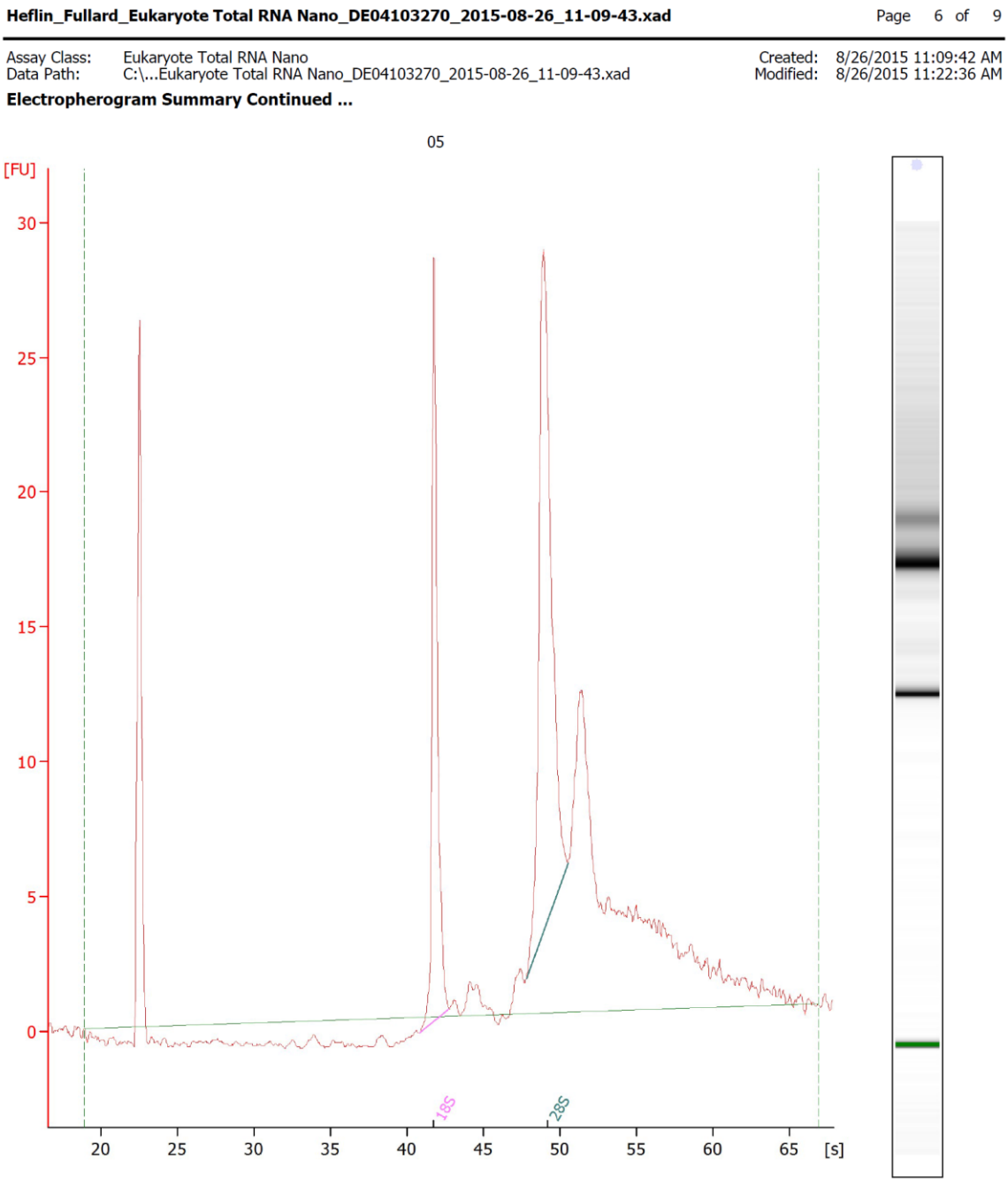

Figure 5. Detailed Agilent Bioanalyzer analysis RNA sample from Batch 1 Participant 3, again showing a 28S peak area > 2 times 18S and RIN value of 10. The simulated capillary electrophoresis electropherogram is shown to the right.

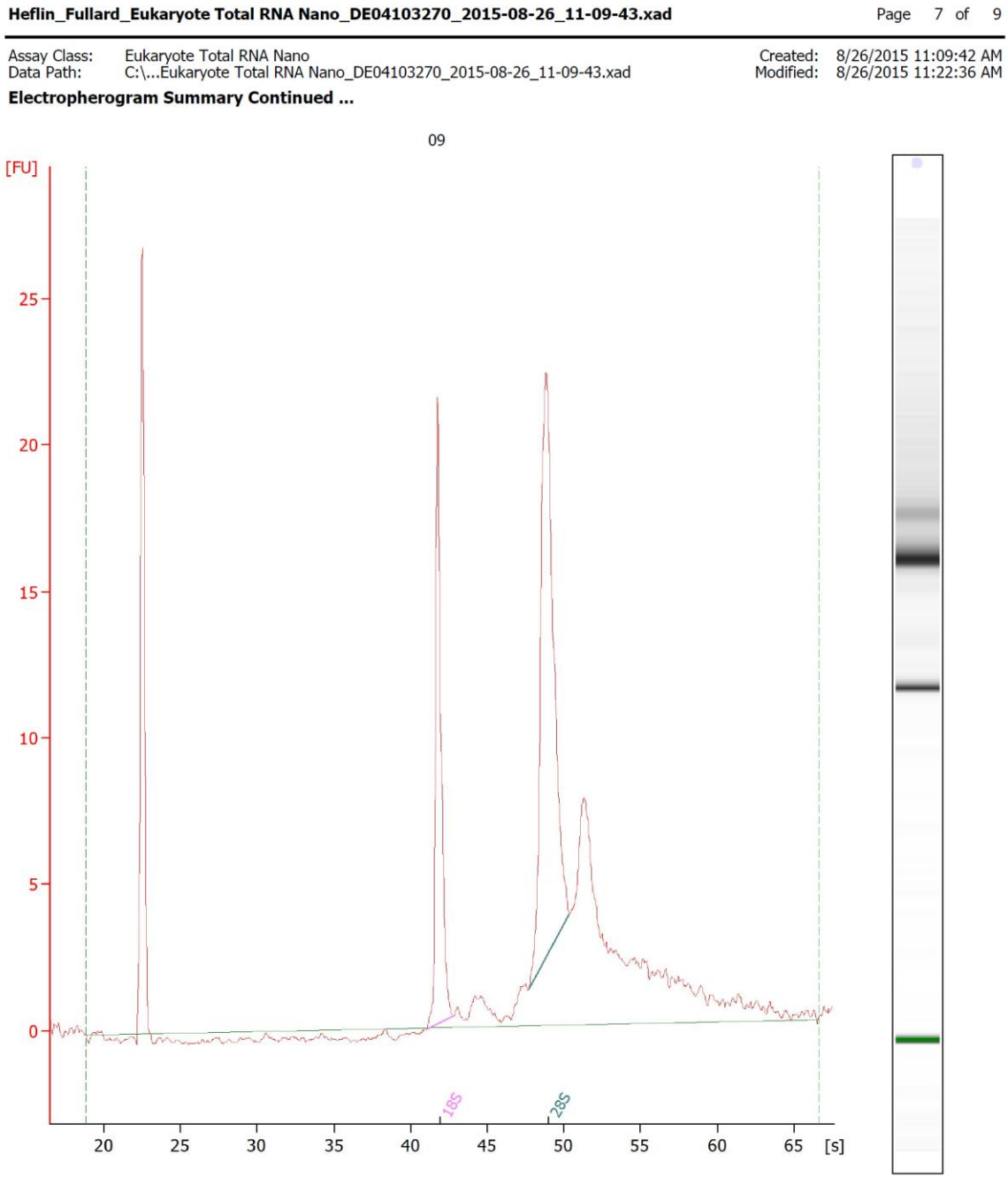

Figure 6. Detailed Agilent Bioanalyzer analysis RNA sample from Batch 1 Participant 4, again showing a 28S peak area > 2 times 18S and RIN value of 10. The simulated capillary electrophoresis electropherogram is shown to the right.

AIM 2

Identifying and Classifying Study Patients

Questionnaires

Two questionnaires were used to obtain information on several aspects of ocular history and symptoms. Table 7 shows the patient responses to questions regarding ocular itching and habits of eye rubbing.

Table 7

Study participant responses to questions regarding ocular itching and eye rubbing.

#	Sample ID	Do You Experience Itching	Do You Rub Your Eyes	How Often	How Vigorously	Group Classifications
1	01-SK OD	Yes	Yes	Often	Vigorously	AD
2	02-TR OS	Yes	Yes	1-2×/day	Gently	AD
3	03-CM OD	Yes	Yes	<1×/day	Gently	NCL
4	04-KT OS	Yes	Yes	<1×/day	Gently	NCL
5	05-KC OS	Yes	Yes	Often	Firmly	KC
6	06-TF OS	Yes	Yes	1-2×/day	Gently	KC
7	09-DC OD	Yes	Yes	Almost never	Firmly	AKC
8	11-JJ OD	Yes	Yes	<1×/day	Gently	KC
9	12-BF OS	Yes	Yes	1-2×/day	Gently	NCL
10	14-SL OD	No	No	Never	N/A	AKC
11	21-DF OD	Yes	Yes	Almost never	Gently	AKC
12	23-EC OD	Yes	Yes	Often	Firmly	AD

Ocular itching was common among all but one of the twelve participants who were classified as AKC. Because several past reports suggest that eye rubbing can increase the risk of developing KC, it was further investigated in the current study. Patient "handedness" to investigate if there was any correlation between eye rubbing habits (Table 8) and the dominant hand of the participant in terms of severity of KC. The more severe eye was determined by corneal topography and magnitude of irregular astigmatism.

Table 8

Relationship between the eye with more severe KC with handedness in the six study KC participants

#	Sample ID	Dominant Hand	More Severe Eye	Group Classification
1	01-SK OD	OS	N/A*	AD
2	02-TR OS	OD	N/A*	AD
3	03-CM OD	OD	N/A*	NCL
4	04-KT OS	OD	N/A*	NCL
5	05-KC OS	OD	OD	KC
6	06-TF OS	OD	OS	KC
7	09-DC OD	OD	OD	AKC
8	11-JJ OD	OD	OD	KC
9	12-BF OS	OD	N/A*	NCL
10	14-SL OD	OD	OD	AKC
11	21-DF OD	OD	OS	AKC
12	23-EC OD	OD	N/A*	AD

* AD and NCL wearers did not have corneal irregularities.

Of the six keratoconus participants, four had more severe disease in the eye corresponding to their handedness, equally split across the two types: KC and AKC. Due to the small number, this was clearly not a significant trend.

Clinical Tests

Corneal topography assessment was performed at Visit 1 on each study participant. Topography for the participant's test sample eye was analyzed and the average flat and steep keratometry (K) measurements on the opposing sides of the same meridian were calculated (Table 9). The K measurements for study patients with KC had large differences between principal meridians and/or have Ks that significantly exceed 43.0 D, the average normal K value.

Table 9

Corneal topography averages of the flat and steep keratometry measurements in opposing position on the same meridian and the change in keratometry readings.

#	Sample ID	Avg Flat K	Avg Steep K	Change in K
1	01-SK OD	44.74	45.16	0.42
2	02-TR OS	43.06	43.44	0.38
3	03-CM OD	39.77	40.46	0.69
4	04-KT OS	45.32	45.35	0.02
5	05-KC OS	42.07	50.29	8.22
6	06-TF OS	54.04	55.04	1.00
7	09-DC OD	40.25	48.93	8.68
8	11-JJ OD	30.77	58.24	27.47
9	12-BF OS	41.97	42.50	0.53
10	14-SL OD	42.08	48.19	6.11
11	21-DF OD	45.29	47.68	2.39
12	23-EC OD	44.50	45.28	0.78

Pathway Analysis of Genetic Data

Because RIN values for all twelve study samples exceeded the minimum requirement of 7.0, RNA-Seq was performed. This eliminated the less powerful choice of

microarray analysis and allowed full genomes and transcriptomes to be generated for each sample.

The RNA-Seq data analysis program provided information ranging from initial gene identification in each sample to identification of genes and transcripts that differentiated patient samples between study groups. Initial analysis consisted of a review of the raw genome and transcriptome data identifying all the genes aligning with each of the study samples. The second step was to evaluate consistency of the gene identities based on flagging only genes that exhibited the appropriate number of "aligned reads" or "read depth." Samples were also evaluated for genes whose fold-change was at least 2 times higher (up-regulated in a sample from one study group versus another) and the difference was statistically significant ($p < 0.05$). An additional analysis screened out genes for which the fold change exceeded the false discovery rate based on number of genes and samples. This left genes identified by a "q" value: ($q < 0.05$), which means that the difference between samples for that gene remained significant after allowing for the false discovery rate. Finally, transcripts that were novel or contained novel isoforms were identified.

All of the identified genes were subsequently analyzed using the much more comprehensive IPA approach, with the added benefit that it localizes genes to all pathways that differentiate between patient groups. IPA was used to identify the major pathways, up-regulated and down-regulated genes, diseases, and molecular and cellular functions that best differentiated among the paired study groups: KC vs NCL; AKC vs. NCL, and AKC vs AD.

Comparison of KC and NCL participants

Table 10 shows the top canonical pathways that differentiated KC from NCL patients. All five top canonical pathways were signaling pathways and/or involved in cellular signaling.

Table 10

Top canonical pathways identified by Ingenuity Pathway Analysis differentiating keratoconus patients from normal contact lens wearers.

Name	p-value	Overlap
Interferon Signaling	8.91E-09	16.7%
Antigen Presenting Pathway	1.06E-08	16.2%
Autoimmune Thyroid Disease Signaling	4.73E-08	12.8%
Graft-versus-Host Disease Signaling	5.39E-08	12.5%
Type I Diabetes Mellitus Signaling	4.50E-07	6.4%

The top upstream regulators that differentiated KC from RNA-SEQ are depicted in Table 11. Several of the regulators have shown significance in literature review to be involved in AD or KC, or potentially both as discussed in the discussion.

Table 11

Top upstream regulators identified by Ingenuity Pathway Analysis differentiating keratoconus patients from normal contact lens wearers

Name	p-value of overlap	Predicted Activation
IFN beta	1.68E-13	Activated
TNF	2.62E-13	Activated
MAPK1	4.58E-11	Inhibited
IFNA1	6.01E-11	Activated
Interferon alpha	7.04E-11	Activated

Table 12 shows the top diseases and biological functions that differentiated KC from NCL. Dermatological disease and conditions, immunological disease, and inflammatory disease are all important because both KC and AD are thought to have association with all three.

Table 12

Top diseases and disorders identified by Ingenuity Pathway Analysis differentiating keratoconus patients from normal contact lens wearers

Diseases and Disorders	p-value	#Molecules
Dermatological Diseases and Conditions	1.70E-02 – 6.53E-13	34
Immunological Disease	1.89E-02 – 1.65E-11	29
Inflammatory Disease	1.89E-02 – 1.65E-11	29
Infectious Diseases	1.70E-02 – 4.62E-09	27
Neurological Disease	1.70E-02 – 5.86E-09	24

Table 13 shows the top molecular and cellular functions that differentiated KC from NCL. Cellular signaling is a prominent differentiating factor between groups. Cell death and survival, cellular compromise, and cellular functions and maintenance are all significant because all are known to be involved in both KC and AD.

Table 13

Top molecular and cellular functions identified by Ingenuity Pathway Analysis differentiating keratoconus patients from normal contact lens wearers

Top Molecular and Cellular Functions	p-value	#Molecules
Cell Signaling	1.37E-02 – 1.55E-07	13
Cell Death and Survival	1.70E-02 – 1.01E-04	33
Cellular Compromise	1.27E-02 – 1.01E-04	10
Cell-to-Cell Signaling and Interaction	1.86E-02 – 1.09E-04	22
Cellular Functions and Maintenance	1.52E-02 – 1.09E-04	31

Table 14 shows the top network functions differentiating KC from NCL. Many networks are relevant to the current study including: dermatological disease and conditions, immunological disease, inflammatory disease, and many cellular networks.

Table 14

Top Networks identified by Ingenuity Pathway Analysis differentiating keratoconus patients from normal contact lens wearers

Associated Network Functions	Score
Dermatological Diseases and Conditions, Immunological Disease, Inflammatory Disease	39
Cardiovascular System Development and Function, Lymphoid Tissue Structure and Development, Organismal Development	39
Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance	26
Dermatological Diseases and Conditions, Cellular Movement, DNA Replications, Recombination and Repair	21
Molecular Transport, Gene Expression, Cell Death and Survival	19

Table 15 shows the top analysis ready molecules that were up-regulated and found to have an exponential fold change which differentiated KC from NCL and Table 16 shows the top analysis ready molecules that were down-regulated and found to have an exponential fold change which differentiated KC from NCL.

Table 15

Top analysis-ready molecules identified by Ingenuity Pathway Analysis differentiating keratoconus patients from normal contact lens wearers

Exp Fold Change Up-regulated Molecules	Exp Value
B3GALT4	↑INF
HLA-DRA	↑INF
HLA-G	↑INF
IL17C	↑INF
RNF5	↑INF
FCGBP	↑37.327
DDR1	↑23.800
SIRPB1	↑17.027
RNF183	↑9.999

Table 16

Top Analysis-Ready Molecules identified by Ingenuity Pathway Analysis differentiating keratoconus patients from normal contact lens wearers

Exp Fold Change Down-regulated Molecules	Exp Value
HLA-B	↓-38.711
GLDC	↓-6.816
ELOVL4	↓-5.202
IGF2	↓-4.866
Mlr-34	↓-3.930
KANK4	↓-3.655
PIK3C2A	↓-3.387
NT5E	↓-3.336
MGST1	↓-3.315

Comparison of AKC with NCL Participants

Table 17 shows the top canonical pathways that differentiated AKC from NCL. All five pathways identified were signaling pathways. Table 18 shows the top upstream regulators that differentiated AKC versus control. Several of the regulators have been

shown in various reports to differ significantly from controls in AD or KC, or potentially both.

Table 17

Top Canonical Pathways identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from normal contact lens wearers.

Name	p-value	Overlap
Dendritic Cell Maturation	3.27E-09	5.1%
OX40 Signaling Pathway	9.96E-09	7.9%
Autoimmune Thyroid Disease Signaling	6.19E-09	12.8%
Graft-versus-Host Disease Signaling	1.17E-10	14.6%
Type I Diabetes Mellitus Signaling	4.38E-08	6.4%

Table 18

Top Upstream Regulators identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from normal contact lens wearers.

Name	p-value of overlap	Predicted Activation
TNF	2.31E-12	Activated
Lipopolysaccharide	2.66E-12	Activated
IL1B	6.93E-12	Activated
IL1A	1.45E-11	
IFNG	7.30E-11	

Table 19 shows the top diseases and disorders that differentiated AKC from NCL. Connective tissue disorder, inflammatory disease, and dermatological diseases and conditions may be associated with KC, AD, or both.

Table 19

Top Diseases and Disorders identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from normal contact lens wearers.

Diseases and Disorders	p-value	#Molecules
Connective Tissue Disorders	3.05E-03 – 3.72E-09	21
Inflammatory Disease	6.10E-03 – 3.72E-09	24
Skeletal and Muscular Disorders	6.10E-03 – 3.72E-09	28
Dermatological Diseases and Conditions	6.10E-03 – 6.42E-09	26
Endocrine System Disorders	3.90E-03 – 1.49E-07	24

Table 20 shows the top molecular and cellular functions that differentiated AKC from NCL. All five functions were cellular functions. Table 21 shows the top network functions that differentiated AKC from NCL wearers. Dermatological disease and conditions, immunological disease, and inflammatory disease are all associated with KC, AD, or both.

Table 20

Top molecular and cellular functions identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from normal contact lens wearers.

Molecular and Cellular Functions	p-value	#Molecules
Cell Death and Survival	6.10E-03 – 2.65E-07	29
Cellular Compromise	6.10E-03 – 2.65E-07	6
Cellular Movement	6.10E-03 – 1.22E-06	20
Cell-to-Cell Signaling and Interaction	6.10E-03 – 2.35E-06	20
Cellular Functions and Maintenance	5.12E-03 – 7.04E-06	25

Table 21

Top networks *functions identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from normal contact lens wearers*.

Associated Network Functions	Score
Small Molecule Biochemistry, Lipid Metabolism, Metabolic Disease	36
Dermatological Diseases and Conditions, Immunological Disease, Inflammatory Disease	26
Dermatological Diseases and Conditions, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function	23
Cell Death and Survival, Infectious Diseases, Cell Morphology	16
Infectious Diseases, Cellular Movement, Cardiovascular System Development and Function	12

Table 22 shows the top analysis ready molecules that were up-regulated and found to have an exponential fold change which differentiated AKC from NCL. Table 23 shows the top analysis ready molecules that were down-regulated and found to have an exponential fold change which differentiated between AKC versus control.

Table 22

Top analysis-ready molecules identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from normal contact lens wearers.

Exp Fold Change Up-regulated Molecules	Exp Value
IL17C	↑INF
WDR72	↑30.030
DDR1	↑25.173
BCL2A1	↑23.772
CYP1A1	↑15.457
SFRP2	↑13.507
HSPA1A/HSPA1B	↑9.584
ALOX	↑9.388

Table 23

Top analysis-ready molecules that were down regulated as *identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from normal contact lens wearers*.

Exp Fold Change Down-regulated Molecules	Exp Value
HLA-DRB5	↓-85.133
HLA-DRB1	↓-35.033
KANSL1	↓-23.345
LSP1	↓-19.846
LY6D	↓-9.084
HLA-DQB1	↓-5.681
IL6	↓-4.338
COL17A1	↓-3.596
CDK9	↓-2.532
MXD4	↓-2.176

Comparison of AKC with AD Participants

Table 24 shows the top canonical pathways that differentiated AKC from AD control patients. All five of the top canonical pathways are signaling pathways.

Table 24

Top canonical pathways identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from those with only atopic disease

Name	p-value	Overlap
Autoimmune Thyroid Disease Signaling	4.62E-06	10.6%
Graft-versus-Host Disease Signaling	5.13E-06	10.4%
OX40 Signaling Pathway	7.32E-06	6.7%
Type1 Diabetes Mellitus Signaling	2.47E-05	5.5%
Cdc42 Signaling	2.82E-05	4.2%

Table 25 shows the top upstream regulators that differentiated AKC from AD control patients. Several of the regulators have shown significance in literature review to be involved in AD or KC, or potentially both as discussed in the discussion.

Table 25

Top Upstream Regulators identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from those with only atopic disease

Name	p-value of overlap	Predicted Activation
Lipopolysaccharide	1.12E-16	Activated
TNF	1.52E-13	Activated
IFNG	2.12E-13	
NGkB (complex)	7.47E-12	

Table 26 shows the top diseases and disorders that differentiated AKC from AD control patients. All of the pathways listed, excluding skeletal and muscular disorders and thought to be associated with KC, AD, or both. Table 27 shows the top molecular and cellular functions that differentiated AKC from AD control patients. All five identified were cellular functions.

Table 26

Top diseases and disorders identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from those with only atopic disease

Diseases and Disorders	p-value	#Molecules
Dermatological Diseases and Conditions	1.04E-02 – 1.65E-16	69
Immunological Disease	1.04E-02 – 1.31E-10	36
Inflammatory Disease	1.04E-02 – 1.31E-10	34
Connective Tissue Disorders	8.07E-03 – 1.91E-06	29
Skeletal and Muscular Disorders	9.22E-03 – 1.91E-06	37

Table 27

Top molecular and cellular functions identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from those with only atopic disease

Molecular and Cellular Functions	p-value	#Molecules
Cellular Function and Maintenance	7.49E-03 – 1.14E-07	29
Cellular Movement	1.04E-02 - 1.93E-06	27
Cell-To-Cell Signaling and Interaction	1.04E-02 – 1.59E-05	29
Cell Death and Survival	1.04E-02 – 2.72E-05	41
Cellular Development	8.57E-03 – 8.12E-05	23

Table 28 shows the top network functions that differentiated AKC versus AD. Several of the associated network functions included dermatological disease and disorders, cellular functions, and immunological and inflammatory response. Table 29 shows the top analysis ready molecules that were up-regulated and found to have an exponential fold change which differentiated AKC from AD control patients.

Table 28

Top associated network functions identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from those with only atopic disease

Associated Network Functions	Score
Inflammatory Response, Lymphoid Tissue Structure and Development, Cellular Function and Maintenance	37
Dermatological Diseases and Conditions, Developmental Disorder, Hereditary Disorder	34
Dermatological Diseases and Conditions, Immunological Disease, Inflammatory Disease	25
Drug Metabolism, Molecular Transport, Small Molecule Biochemistry	25
Gene Expression, Cellular Development, Cellular Movement	20

Table 29

Top analysis-ready up-regulated molecules identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from those with only atopic disease

Exp Fold Change Up-regulated Molecules	Exp Value
IHLA-DRA	↑INF
WDR72	↑30.497
BCL2A1	↑16.329
ICAM4	↑15.106
CCL20	↑12.932
SPRR2D	↑12.155
MYEOv	↑11.313
IL17C	↑10.989
SLC15A1	↑10.712
PLAC8	↑9.810

Table 30 shows the top analysis ready molecules that were down-regulated and found to have an exponential fold change which differentiated AKC from AD control patients.

Table 30

Top analysis-ready down-regulated molecules identified by Ingenuity Pathway Analysis differentiating keratoconus patients with atopic disease from those with only atopic disease

Exp Fold Change Down-regulated Molecules	Exp Value
HLA-C	↓-33.604
FDCSP	↓-20.430
LSP1	↓-19.316
MUC7	↓-14.534
HLA-B	↓-12.905
ZFP42	↓-11.843
GABRB2	↓-8.077
MS4A8	↓-7.211
HLA-A	↓-7.208

CHAPTER 6

DISCUSSION

AIM 1

Sample Collection: Conjunctival Impression Cytology Technique

Because CIC is a relatively non-invasive technique to collect cells from the ocular surface, it was used as the cell collection method for the entire study. The CIC technique described in the current study proved to be successful in that each sample yield was sufficient to run transcriptome level downstream analysis on each of the samples.

CIC Sample Processing

Procedure 1 with Modifications: Qiagen RNeasy Plus Mini Kit®

The main focus of the initial stage of the study was to prepare samples of isolated RNA, using the previously successful Qiagen RNeasy Plus Mini Kit®[38], that were of both sufficient quality and quantity to perform downstream full RNA sequencing.

In the current study, the Qiagen RNeasy Plus Mini Kit® method of extraction, isolation, and purification, with modifications, consistently produced RNA of sufficient quantity and quality for RNA-Seq Analysis. Again, RNA extract salt contamination concerns proved to be unfounded, the samples enabling successful genome and transcriptome-wide analysis by RNA-Seq.

RNA-Seq provided entire genomes and transcriptomes for all twelve Aim 2 Study patient samples, meaning that the substantially less comprehensive 96-gene microarray

approach would not be necessary. Because microarrays provide much more limited gene expression information due to the small number of analyzed target genes, important genes not currently recognized as important in KC would almost certainly have been missed. There was therefore no reason to pursue microarrays.

Procedure 2: GE Illustra™ RNAspin Mini RNA Isolation Kit Ambion pure placental RNA

The GE Illustra™ RNAspin Mini RNA Isolation Kit incorporated several key differences from the Qiagen RNeasy Plus Mini Kit®. A more toxic storage/extraction agent, TRIzol® (containing phenol), was used. This required a modified CIC collection procedure with stringent safety controls and numerous changes of forceps to eliminate even the remotest possibility of TRIzol® contact with the patient's eye. The isolation and purification method replaces several column-based isolation steps with density gradient centrifugations. The density gradient DNA/RNA separation step is facilitated by the incorporation of a sterile gel that separates into a layer between the DNA and RNA, facilitating removal of purified RNA without DNA contamination.

Procedure 3: TRIzol® and Chloroform Technique using placental RNA

Procedure 3 was similar in many respects to Procedure 2, but chloroform was used and pellet formation was the endpoint. As depicted in Table 5, the A260/280 (nucleic acid purity) and A260/230 (residual salt contamination) values showed minimal improvement despite total RNA being higher for Samples #1-4.

Sample Quality and Quantity

Despite concerns about salt contamination of the RNA samples (low A260/230 ratio), the high RIN values verified that the salt contaminates did not compromise integrity of RNA, and this method of extraction, purification, and isolation produces sufficient RNA for downstream analysis. RIN values were higher than anticipated for all twelve samples. The goal was RIN values of 7 or higher, with 10 being the highest. All twelve samples surpassed the minimum requirement for downstream genetic analysis.

AIM 2

Identifying and Classifying Study Participants

For the current study, it was predicted that each group would be heterogeneous, with significant sources of intergroup variation unrelated to KC or AD fully. Systemic conditions other than AD and medications were not exclusion criteria in the study because of limited participant numbers, which was a clear source of intergroup heterogeneity. When studying heterogeneous study groups, non-KC and non-AD related variation was expected to dilute the number of significant differences among the study groups. Despite these limitations, demographics were controlled to the extent possible when selecting the samples for downstream genetic analysis.

Pathway Analysis of Genetic Data

Study limitations precluded the use of power analysis-based patient numbers. However, a total of 12 CIC samples were processed and analyzed, representing the KC

and AKC patient groups, as well as AD and NCL. Three patient samples from each group, were used to perform RNA-Seq. Again, it is expected that if a power analysis was performed, it would indicate a much larger sample size to identify significant differences between study groups.

The 96 genes initially determined by literature review to be key genes in KC or AD were expected to be among the most likely to differentiate among groups (Table 1). That is, they were expected to be among the genes that were significantly up- or down-regulated in KC, AD, or both. More of the 96 genes were identified in studies of AD than KC, which is expected because the cellular and the molecular mechanisms of KC are not as well understood.

In the current study, RNA-Seq was successfully applied and genetic data obtained for three patients from each of the 4 main participant groups: KC, AD, AKC, and NCL. Pathway analysis was then used to differentiate among these groups. It is important to note that the level of success with sample preparation and suitability of samples for transcriptome analysis exceeded initial study expectations. In fact, it greatly exceeded expectations. As a result of the initial skepticism, the design of the Aim 2 Study with three patients per group and minimal exclusion criteria was relatively modest. It was therefore entirely expected that the heterogeneity of patients in the current study would greatly limit the number of significant differences found at the genome level. Beyond this, the ability to go further and compare intergroup single nucleotide polymorphisms (SNPs) and other variations at the transcriptome level was considered unrealistic. Additional analytical procedures, including Principal Component Analysis (PCA), revealed that the heterogeneity within each group was a major limiting factor for this

study. However, application of paired t-tests to the RNA-Seq genomic data revealed intergroup differences. These analyses identified a number of genes that differentiated among the paired groups ($p \leq 0.05$).

Once identified, these genes, were analyzed using the much more comprehensive IPA approach, with the added benefit that it localizes genes to all pathways that best differentiate among the paired study groups: KC vs NCL; AKC vs. NCL, and AKC vs AD. IPA was performed to enable the most comprehensive possible comparison of differences among the four patient groups using the gene expression data resulting from RNA-Seq. Pathway analysis supplied important markers that will serve as a reference point for future studies that investigate the pathogenesis of KC and the differences between the atopic and non-atopic variants of the disease.

KC versus NCL and AD

Those with KC were compared to NCL and AD. Pathway analysis found that signaling pathways played an important role with four of the five canonical pathways differentiating KC from non-KC patients being signaling pathways. The major differentiating pathways in this paired group were: 1) Interferon Signaling, 2) Autoimmune Thyroid Disease Signaling, 3) Graft-versus-Host Signaling, and 4) Type 1 Diabetes Mellitus Signaling.

At the individual gene level, paired comparisons between groups revealed several key differentiating upstream regulators: Mitogen-Activated Protein Kinase 1 (MAPK1) was found to be inhibited in the KC group, whereas tumor necrosis factor (TNF), interferon beta (IFN-β), interferon alpha (IFN-α), and interferon alpha 2 (IFN-α2) were

all activated. All of the upstream regulators found to differentiate the two groups, are proteins that play an essential role in cell signaling, which is consistent with the finding that the top canonical pathways were signaling pathways.

Interleukin 17C (IL17C) was found to be substantially up-regulated in the KC versus NCL group. Several studies have shown that IL17 plays an important in role in the pathogenesis of KC and an increase in IL17 suggests tissue degenerative processes that contribute to thinning and weakening of corneal connective tissue in KC[20]. For this reason, IL17 had been selected as one of the 96 target genes in the initial stages of the experiment and IPA confirmed that it is a differentiating gene. Therefore, IL17 is a candidate for further investigation as we try to learn more about KC.

The five diseases and disorders whose pathway associations were the most significant differentiators between KC and NCL according to pathway analysis were: 1) dermatological disease and conditions, 2) immunological disease, 3) inflammatory disease; 4) neurological disease and 5) infectious disease. Each of these disease pathway findings is significant. Many recent studies, including the current study, support the idea that KC incorporates inflammatory, autoimmune, and neuronal components. This pattern was also verified in the key associated network functions that differentiated KC and AKC patient groups.

The top molecular and biological functions that were identified in the analysis included cell signaling, cell death and survival, cell-to-cell signaling and interaction, and cellular function and maintenance. It is important to note that these molecular functions were also identified in the networks discussed above. In other studies, cell signaling and

apoptosis have both been found to be strongly associated with the pathogenesis of KC and AD,[9, 19, 26] making them important targets of further research.

AKC versus NCL

When those with AKC were compared to NCL, there were five canonical pathways identified; four of which were signaling pathways. The top canonical pathways that differentiated these groups were: 1) Autoimmune Thyroid Disease Signaling, 2) Type 1 Diabetes Mellitus Signaling, 3) OX40 Signaling Pathway, 4) Graft-versus-Host Disease Signaling, and 5) Dendritic Cell Maturation. As previously mentioned, signaling pathways appear to be very important differentiators between those with and without KC, and should be investigated further.

Because this comparison is between those with AKC versus NCL, it is important to note that the OX40 Signaling Pathway is dependent on the activation of cluster of differentiation 28 (CD28), which is important for T-cell activation in inflammatory pathways of atopic patients and has been found to be up-regulated in other studies[60]. CD28 was selected as one the 96 genes, specifically associated with AD that would differentiate those with AD from those without. Again, IPA has confirmed that this pathway differentiates the two. Therefore, OX40 Signaling Pathway along with the molecules it is dependent upon including CD28 should be investigated in future research.

Interleukins are essential signaling cytokines that facilitate communication between cells. Because signaling pathways were identified as differentiating disease from non-disease in this comparison it is expected that there were interleukins identified as key up-stream regulators as well as other signaling molecules including: IL1α and IL1β, and

TNF. IL1 has been identified in previous research as a mediator in the acute phase of inflammation and the release of IL1 from the epidermis promotes inflammatory skin conditions, including AD[61]. This is the reason it was included among the 96 target genes. IL1β has been found to be associated with KC as a mediator in cell proliferation, differentiation, and apoptosis[62] and because of its role in the pathogenesis of KC it was also included among the 96 target genes. Wojick et al.[63] demonstrated that a loss of beta-actin (β-actin) or upregulation of IL1 and IL6 in KC eyes increases oxidative and nitrative stress in corneal stromal cells, ultimately disrupting the lipid and nitric oxide pathways. Pathway disturbance leads to an accumulation of toxic by-products resulting in apoptosis.[63] Because IL1 has associations with both KC and AD, and was identified to be a differentiator with IPA, it also warrants further research.

IPA found that the expression of Arachidonate 12B-Lipoxygenase (ALOX12B) was up-regulated in AKC with versus NCL. This family of genes plays an essential role in the lipoxygenase pathway, which is a key component of the inflammatory pathway. Arachidonate 5-lipoxygenase activating protein (ALOX5AP) was selected for the current study as one of the 96 target genes because it was found to be responsible for synthesis of leukotrienes, important mediators for a number of inflammatory and allergic conditions[64]. Pathway analysis also found ALOX to differentiate between patient groups. While it is not the same gene as ALOX5AP, it is a member of the same protein family and both involved in inflammatory and allergic conditions. This gene family should be investigated further in future research.

Another important differentiating molecule on the list of 96 target genes, was SFRP1. The SFRP1 gene was selected as a target gene because You et al. found it to be

significantly decreased in the tears of KC patients compared to controls and is directly involved in the Wnt signaling pathway.[22] Another recent study found an association between KC and the Wnt/beta-catenin signaling pathway, although the gene that was directly indicated in this particular study was an up-regulator of ANGPTL7 in KC patients.[65] This relationship suggests that the Wnt signaling pathway plays an important role in the KC disease process. IPA found an SFRP family member that differentiated KC from controls. Secreted Frizzled-Related Protein 2 (SFRP2) was up-regulated in the KC with AD group compared to controls. The SFRP protein family and Wnt signaling pathway should be included in future research because of the potential to differentiate AKC from normal control patients.

IL6 is produced primarily at sites of acute and chronic inflammation and was selected among the 96 target genes based on studies demonstrating that it was up-regulated in KC patients.[20, 66] Surprisingly, IPA found that IL6 was significantly down-regulated in atopic KC patients compared to controls. Despite these conflicting findings, IL6 should be further investigated as a potential differentiating gene in AKC.

Inflammatory disease and dermatological diseases and conditions were considered differentiating diseases and/or disorders in the comparison of AKC versus control, which was the case when comparing KC versus control as well. However, connective tissue disorders are a differentiating criterion that showed up in the analysis of those with AKC, but not in the KC versus NCL comparison. It has been well documented in the literature that both KC and AD have an inflammatory component which is strongly supported by findings of this study. Interestingly, immunological disease showed up in the KC versus

control comparison, but not in the comparison of AKC versus control. There is a large body of research that confirming the autoimmune nature of AD.

Analysis of key molecular functions analysis showed similarities between the two disease groups (KC with and without AD) versus control (non-KC), overlap being found for signaling, cell death and survival, and cellular compromise. All showed significant intergroup differences. All of the top molecular functions were also identified in top associated networks. Taken together, the evidence points strongly towards a connection with pathway disruption.

The paired group comparisons discussed above shared many common findings. In addition to broad similarities, there were also specific top upstream regulators that showed significant differences. In particular, IL1α and IL1β appeared in the AKC versus control pairing, but not in the KC versus control comparison. Because IL1 resides at a key trigger point for the entire inflammatory pathway, this gene family should be investigated further to learn more about the possible differences in those with KC compared to those with AKC.

AKC versus AD

This analysis compared AKC to the AD only "control" group. The atopy control group had no signs of KC so the results that were also indicative of differences between KC and non-KC patients.

The top canonical pathways for this comparison shared two of the same signaling pathways with the other two paired groups; 1) Autoimmune Thyroid Disease Signaling and 2) Graft-Versus-Host Disease Signaling. There were patterns elucidated in the

analysis of the two paired groups with patients that had AD including: 1) every canonical pathway was a signaling pathway; 2) OX40 appeared in both AD paired groups, but not in the comparison between KC versus control; 3) lipopolysaccharide was a top upstream regulator. This information illustrates the importance of signaling in the disease process and signaling pathway analysis, constituting a key area for further KC research.

Another important finding was the significance of IFNG, which appeared in both comparisons with AD patients. IFNG is an important immune response cytokine coded by the IFNG gene. It plays a role in the synthesis of collagen and prostaglandin E2 by fibroblasts.[67] Studies have shown that IFNG levels are reduced in patients with AD, which made this a gene of interest when initially selecting the key 96 genes to investigate.

IPA and pre-study literature review found that the zinc finger family may be associated with AD. Zinc finger homeobox 3 (ZFHX3) was reported to be down-regulated in AD patients according to Li et. al. when they looked at differential gene expression in those with skin conditions, including AD.[60] IPA confirmed that the zinc finger family may be associated with KC in those with both KC and AKC because it zinc finger protein 42 (ZFP42) was found to be significantly down-regulated in both groups. Although they are not identical, the zinc finger family plays an important role in regulating many cellular functions and they have been identified in the pathophysiology of AD.

Another important class of molecules identified by initial literature review as a significant differentiator and confirmed with IPA is the intercellular adhesion molecules (ICAMs). ICAM proteins are ligands for leukocyte adhesion proteins. Lema et al.[68]

showed that ICAM1 plays a role in both KC and AD, which is the reason it was selected as a target gene in the current study. ICAM1 and several other proinflammatory cytokines were seen in increased levels in the tears of KC patients that wore rigid gas permeable (RGP) contact lenses,[68] suggesting that there is an inflammatory component to the pathogenesis of KC. In atopic patients, ICAM1 was found to be significantly up-regulated in general, but even more so in response to allergens.[69] Although, ICAM1 was not specifically identified in the current study, ICAM4 was found to be down-regulated in those with AKC when compared to AD controls. This class of molecules would be an area for further research.

The top diseases and disorders in this paired group analysis included; 1) Dermatological Diseases and Disorders; 2) Immunological Disease; 3) Inflammatory Disease; 4) Connective Tissue Disease; and 4) Skeletal and Muscular Disorders. Both of the patient groups that were compared in this analysis have AD, but the differentiating findings are really between those with KC and those without KC. The IPA would suggest that there is an immunological and inflammatory component to the pathogenesis of KC.

Overall, a very small Aim 2 study group comprising 12 participants produced a surprising amount of information relating to differences between those with and those without keratoconus. While not as extensive, differences between non-atopic and atopic keratoconus were also found. Together these findings provide ample evidence that a larger scale follow-up study, based on power analysis numbers, and incorporating more stringent inclusion/exclusion criteria should produce greater insight into KC and differences from AKC.

CHAPTER 7

CONCLUSION

CIC is widely used to obtain cellular content from the ocular surface of human participants for gene expression analysis. This project successfully demonstrated that it is possible to isolate and purify RNA from conjunctival impression cytology specimens of sufficient quality and quantity to enable full genome sequencing and transcriptome analysis using state-of-the-art sequencing technology, RNA-Seq. In addition, it showed that the resulting genomic data could differentiate between non-KC and control patients.

What was neither fully realized nor expected in the Aim 2 study was that a systematic comparison of transcriptome level differences between KC, AKC, and control patients would provide useful results. This would require a much high level of homogeneity within study groups to eliminate as many non-KC sources of transcriptome heterogeneity as possible. Such an undertaking was well beyond the scope of the current project. However, genome level results provided definite stepping stones towards future studies.

KC was the main contributing factor to virtually all of the significant intergroup difference found in the current study. While demonstrating that the patients groups were clearly heterogeneous, pathway analysis using IPA revealed several significant patterns that will provide direction for further research. Some factors that would help group homogeneity would be to include siblings or other relatives, and to better control for associated diseases and medications. Based on previous reports, even familial studies

have revealed heterogeneity between siblings that made it difficult to identify key KC genes. This is strong evidence that the disease is indeed multifactorial, and could potentially have a different progression when the patient has inflammatory diseases such as AD. It further highlights the importance of starting with the most homogeneous possible grouping criteria if AKC versus KC differences are to be elucidated.

The ultimate goal of this research was to optimize a CIC sample collection and RNA processing technique to facilitate genome level analysis by RNA-Seq. This goal was exceeded. Despite Study 2 limitations, it revealed important differences between non-KC and KC patients, but less differences between KC and AKC and that pathway analysis is obtainable. However, with larger group numbers and more homogenous study group allocation criteria, genes, and even transcripts could be investigated further. Continued research into gene expression patterns may ultimately lead to the development of improved KC treatments.

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APPENDIX A

KERATOCONUS SYMPTOM & SEVERITY QUESTIONNAIRE (KSSQ)

KERATOCONUS SYMPTOM & SEVERITY QUESTIONNAIRE (KSSQ)

For the following questions, please circle the **most appropriate** choice. All responses should be based on your average evaluation over the past week:

1. When NOT wearing contact lenses, how often did your eyes feel dry?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

2. When NOT wearing contact lenses, how often did you experience redness in your eyes?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

3. When NOT wearing contact lenses, how often did you experience tearing or discharge from your eyes?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

4. When NOT wearing contact lenses, how often did your eyes feel uncomfortable, irritated, or painful?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

5. How severe would you rate the level of discomfort, irritation, or pain experienced?

(not noticeable)	(mild)		(moderate)			(severe)			(unbearable)
1	2	3	4	5	6	7	8	9	10

6. When wearing contact lenses, how often did your eyes feel dry?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

7. When wearing contact lenses, how often did you experience redness in your eyes?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

8. When wearing contact lenses, how often did you experience tearing or discharge from your eyes?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

9. When wearing contact lenses, how often did your eyes feel uncomfortable, irritated, or painful?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

10. How often did this discomfort/irritation/pain prevent you from wearing your contacts, either at all or for a shorter period of time?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

11. How often did this discomfort/irritation/pain affect the quality of your vision?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

12. How often did this discomfort/irritation/pain cause you to rub your eyes?

(never)		(occasionally)	(1/2 the week)			(frequently)			(every day)
1	2	3	4	5	6	7	8	9	10

APPENDIX B

KERATOCONUS SURVEY:
PHENOTYPIC AND GENOTYPIC ANALYSIS OF KERATOCONUS

KERATOCONUS SURVEY
Phenotypic and Genotypic Analysis of Keratoconus

This questionnaire is designed to obtain personal, medical, and family history information from participants who do or do not have keratoconus. **Please do not leave any questions blank!** If you do not know the answer to a question, please write "unknown." Thank you very much!

Patient ID_____

Gender (circle one) *Male* *Female*

Ethnicity (circle one):

American Indian or Alaskan Native
Asian or Pacific Islander
Black, not of Hispanic origin
Hispanic
White, not of Hispanic origin

What is your highest level of education (circle one):

No high school degree
High school degree
Some college
Two year college degree
Four-year college degree
Graduate degree

What is your occupation?_____

EYE INFORMATION

How old were you when diagnosed with keratoconus? _____

At first diagnosis, how advanced was the keratoconus in your left eye? (suspect, mild, moderate, severe, unknown, or n/a) _____

At first diagnosis, how advanced was the keratoconus in your right eye? (suspect, mild, moderate, severe, unknown, or n/a) _____

What was the qualification of the doctor who diagnosed your keratoconus (Optometrist (OD), Ophthalmologist (MD), or other (please specify)?

_____

Have you undergone refractive surgery? Yes No

If yes, which eye? (left, right, or both) _____

What year did you undergo surgery? _____

Have you had a corneal transplant? Yes No

If yes, please list the date of transplant, or dates of multiple transplants in the case of graft failure

	Date	Date	Date
Right eye			
Left eye			

Have you sustained ocular trauma or injury? Yes No

If yes, to which eye? _____

Please describe incident

_____

_____

_____

What year did it occur? _____

Do you wear contacts? Yes No

If yes, please list the type of contact worn in each eye (if you wear 2, please list), how many years you have worn them, how many days per week and hours per day that you wear the contacts.

	Contact type	Length (in years) of use	Days per week	Hours per day
Right eye				
Left eye				

Do your eyes itch? Yes No

If yes, How often do your eyes itch?

Rarely
Once a month
About once a week
A couple days a week
Daily

Do you rub your eyes? Yes No

If yes, how often do you rub them?

Almost never
Less than once a day
Once or twice a day
Often during the day

How vigorously do you rub them?

Gently
Firmly
Vigorously

Do you have dry eyes? Yes No

How often are your eyes dry?

Rarely
Once a month
Once a week
A couple days a week
Daily

Do you have red eyes? Yes No

*If yes, which eye (left, right or both)?*_____

How often are your eyes red?

Rarely
Once a month
Once a week
A couple days a week
Daily

Do your eyes burn? Yes No

How often do your eyes burn?

Rarely
Once a month
Once a week
A couple days a week
Daily

Do your eyes make discharge? Yes No

If yes, which eye (L, R, or both)? _____

How often do your eyes discharge?

Rarely
Once a month
Once a week
A couple days a week
Daily

Do your eyes water? Yes No

*If yes, which eye L, R, or both)?*_____

How often do your eyes water?

Rarely
Once a month
Once a week
A couple days a week
Daily

MEDICAL INFORMATION

Have you been diagnosed with eczema? Yes No

If yes, what year was the diagnosis? _____

What areas of your skin have eczema?

_____

Do you have a history of dry, itchy skin? Yes No

If yes, since what age? _____

What areas of your skin are dry?

_____

Has your skin been dry throughout the past year? Yes No

Have you been diagnosed with asthma? Yes No

If yes, what year was the diagnosis? _____

Do you have hay fever? Yes No

*If yes, since what age?*_____

Do you have allergies? Yes No

*If yes, since what age?*_____

Please indicate if **you** or any other **blood relative** [this includes brothers, sisters (full or half), parents, children, grandparents, uncles, aunts, nephews, cousins, etc.] have had any of the following diagnoses or conditions. Please indicate which relative(s) in the space provided:

Cardiovascular disease You Relative(s)_____

Marfans syndrome You Relative(s)_____

Down Syndrome You Relative(s)_____

Systemic collagen vascular disease (lupus, rheumatoid arthritis, scleroderma, dermatomyositis)

You Relative(s)_____

Pigmentary retinopathy You Relative(s)_____

Is there any other relevant medical information you feel may be helpful to us?

_____

_____

_____

_____

Please list all prescribed and non-prescribed medications you are currently taking, including eye medications.

Medication	Dosage	Doses/Day	Date Started	Taken on day of visit with study coordinator?
				Yes/No
				Yes/No
				Yes/No
				Yes/No
				Yes/No
				Yes/No
				Yes/No
				Yes/No
				Yes/No

FAMILY INFORMATION

GRANDPARENTS: please fill in as much information as you can

FATHER'S SIDE

Grandfather

Grandfather's date of birth (approximate) _____

Deceased? Yes No
(If yes, please indicate the age and cause of death:

_____).

Country of birth_____

Ancestry_____

Grandmother

Grandmother's date of birth (approximate)_____

Deceased? Yes No
(If yes, please indicate the age and cause of death:

_____).

Country of Birth_____

Ancestry_____

Does either grandparent have keratoconus? If so, please list:

_____

MOTHER'S SIDE

Grandfather

Grandfather's date of birth (approximate) _____

Deceased? Yes No
(If yes, please indicate the age and cause of death:

_____).

Country of birth_____

Ancestry_____

Grandmother

Grandmother's date of birth (approximate)_____

Deceased? Yes No
(If yes, please indicate the age and cause of death:

_____).

Country of Birth_____

Ancestry_____

Does either grandparent have keratoconus? If so, please list:

_____

PARENTS

Father

Father's date of birth _____

Deceased? Yes No

If yes, please indicate the age and cause of death:

_____

Presence of keratoconus (if yes: left, right, or both) _____

Mother

Mother's date of birth _____

Deceased? Yes No

If yes, please indicate the age and cause of death:

_____

Presence of keratoconus (if yes: left, right, or both) _____

SIBLINGS

Please list all siblings related to you by blood. List from oldest to youngest.

	Sex	DOB	Keratoconus? (if yes: left, right, or both)
1			
2			
3			
4			
5			
6			
7			

AUNTS AND UNCLES

Please list all aunts and uncles from both sides of your family.

FATHER'S SIDE

	Sex	Date of Birth	Presence of keratoconus (if yes: left, right, or both)
1			
2			
3			
4			
5			
6			
7			

MOTHER'S SIDE

	Sex	Date of Birth	Presence of keratoconus (if yes: left, right, or both)
1			
2			
3			
4			
5			
6			
7			
8			

CHILDREN

Please list all children related to you by blood. If a child has keratoconus, please indicate which eye (left, right, or both).

	Sex	Date of birth	Presence of keratoconus (if yes: left, right or both)
1			
2			
3			
4			
5			
6			
7			

If there is any other information you feel is relevant or helpful to the study, please write below:

_____

_____

_____

_____

Signature_____

Today's date_____

Thank you very much for your help!

Adapted from Johns Hopkins Keratoconus Study Questionnaire (v5, Huang and Albert, 2010)

APPENDIX C

CLINICAL EVALUATION FORM USED IN KERATOCONUS STUDY (AIM 2)

Keratoconus Study Visit 1 Clinical Examination

Pt. ID: ☐☐☐ - ☐☐ (# / Init) Date: ☐☐☐☐14 (M M D D Y Y)

Handedness R ____ L ____

Contact Lenses

Type OD _____ OS _____

Wears Contact Lenses regularly _____

Wears Contacts but did not bring _____

Does not wear Contacts _____

Visual Acuity

OD 20 / OS 20 /

Contacts _____

Spectacles _____

Unaided _____

3D Wave Exam

Database Record # _____

Sim K OD ☐☐.☐☐@☐☐☐ (flat) OS ☐☐.☐☐@☐☐☐ (flat)

OD ☐☐.☐☐@☐☐☐ (steep) OS ☐☐.☐☐@☐☐☐ (steep)

Irregular Mires OD Yes ☐ No ☐ OS Yes ☐ No ☐

Pt. ID ____-____

Slit Lamp Examination

Limbal Injection (Efron Scale)

	OD	OS
Normal (0)	___	___
Trace (1)	___	___
Mild (2)	___	___
Moderate (3)	___	___
Severe (4)	___	___

Bulbar Injection (Efron Scale)

	OD	OS
Normal (0)	___	___
Trace (1)	___	___
Mild (2)	___	___
Moderate (3)	___	___
Severe (4)	___	___

Papillary Conjunctivitis (Efron Scale)

	OD		OS	
	Inf	Sup	Inf	Sup
Normal (0)	___	___	___	___
Trace (1)	___	___	___	___
Mild (2)	___	___	___	___
Moderate (3)	___	___	___	___
Severe (4)	___	___	___	___

Other Conjunctival Findings

OD	OS
Yes ___ No ___	Yes ___ No ___

Describe: _____

_____

Pt. ID ____-____

Slit Lamp Examination (cont.)

Corneal Edema (Efron Scale)	OD	OS
Normal (0)	___	___
Trace (1)	___	___
Mild (2)	___	___
Moderate (3)	___	___
Severe (4)	___	___

	OD	OS
Lens Induced Epithelial Imprint	Yes ___ No ___	Yes ___ No ___
Neovascularization (≥ 2mm)	Yes ___ No ___	Yes ___ No ___
Vogt's Striae	Yes ___ No ___	Yes ___ No ___
Fleischer's Ring (≥ 2mm Arc)	Yes ___ No ___	Yes ___ No ___
Other Corneal Findings	Yes ___ No ___	Yes ___ No ___

Describe: _____

_____

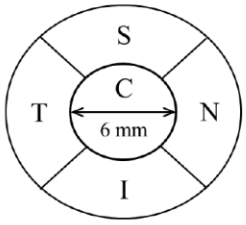
Pt. ID ____-____

Scarring and Staining

Scarring

OD Yes ___ No ___ **OS** Yes ___ No ___

Circle the CINTS location(s) that apply in the table below.
If there is more than one scar in the same zone, report the more central one first.



OD
Scarring Density (Circle one number 1.0 – 4.0 for each scar)

	Location(s)	Just Noticeable		Easily Noticeable		Dense		Opaque
Scar 1	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Scar 2	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Scar 3	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Scar 4	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Scar 5	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0

OS
Scarring Density (Circle one number 1.0 – 4.0 for each scar)

	Location(s)	Just Noticeable		Easily Noticeable		Dense		Opaque
Scar 1	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Scar 2	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Scar 3	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Scar 4	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Scar 5	C I N T S	1.0	1.5	2.0	2.5	3.0	3.5	4.0

Pt. ID ____-____

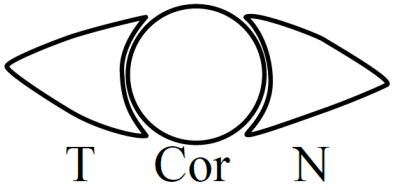
Staining

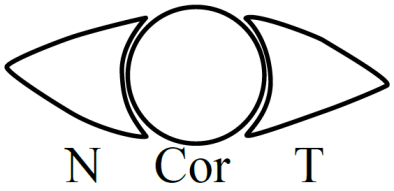
Na Fluorescein

Staining Method:

- NaFl strip, apply 1 drop PF saline to each side of strip (separate strip for each eye).
- Instill in lower fornix.
- OK to wipe corners of patient's eyes
- Evaluate staining using Wratten #12 barrier filter (patient can hold in front of objective)

Oxford Grade 0 I II III IV V Assign separate grades 0 – V to temp conj, cornea, and nasal conj.





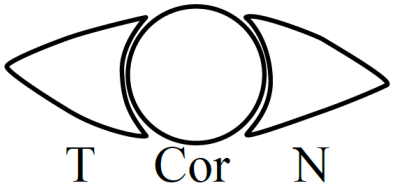
OD Total Score ____ / 15 OS Total Score ____ / 15

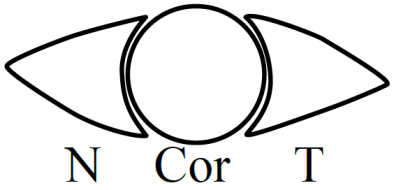
Lissamine Green

Staining Method

- Lissamine green strip, apply 1 drop PF saline to each side of strip (separate strip each eye).
- Instill in lower fornix.
- OK to wipe corners of patient's eyes
- Evaluate staining using Tiffen #25 red barrier filter (patient can hold in front of objective)

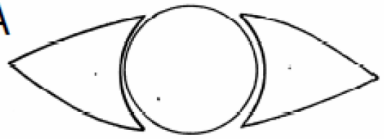
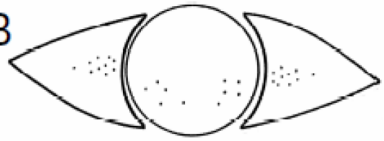
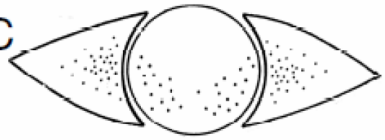
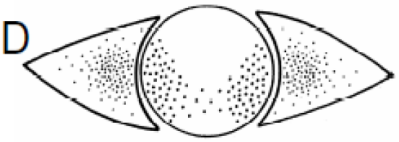
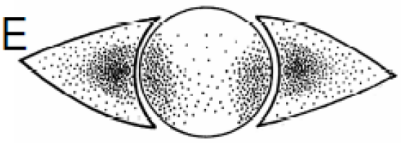
Oxford Grade 0 I II III IV V Assign separate grades 0 – V to temp conj, cornea, and nasal conj.





OD Total Score _____ / 15 OS Total Score _____ / 15

Oxford Grading Method

PANEL	GRADE	CRITERIA
A 	**0**	Equal to or less than panel A
B 	**I**	Equal to or less than panel B, greater than A
C 	**II**	Equal to or less than panel C, greater than B
D 	**III**	Equal to or less than panel D, greater than C
E 	**IV**	Equal to or less than panel E, greater than D
>E	**V**	Greater than panel E

APPENDIX D

INFORMED CONSENT IRB APPROVAL FOR PROTOCOL X131216009

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

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

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

Principal Investigator:	FULLARD, RODERICK J
Co-Investigator(s):	GORDON, ADAM LAURENT, JOHN MORROW, ERIKA MARIE
Protocol Number:	**X131216009**
Protocol Title:	*Conjunctival gene expression and tear biomarkers in atopic and non-atopic keratoconus patients relative to matching controls*

The IRB reviewed and approved the above named project on 12/23/15. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 12/23/15

Date IRB Approval Issued: 12/23/15

IRB Approval No Longer Valid On: December 23, 2016

HIPAA Waiver Approved?: No

Partial HIPAA Waiver Approved?: No

Expedited Reviewer
Member - Institutional Review Board
for Human Use (IRB)

Investigators please note:

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

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

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

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

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