Under an Institutional Review Board (IRB) approved protocol, surgically discarded and/or de-identified normal donor and other keratocoric corneas, keratoconic and normal donor corneas were obtained from the department of Ophthalmology, Loyola University Health System. A total of 12 normal and 12 keratoconic corneas were selected for this study. The tissues samples were fixed in formalin/PBS buffer and stored at 4°C until paraffin embedding and sectioning at 4 microns. Deconvolution immunofluorescence (IF) was performed on the unstained slides. After imaging, relative expression (normal vs keratoconus) of TNF-α, TGFβ2, and NF-κB was quantified in immunostained sections. For TNF-α, TGFβ2, and NF-κB, Center channel measurements were obtained per 10 µm cubes (see figures 1 and 2) in the epithelial and stromal layers of both keratoconic and normal donor corneas. Samples not containing intact epithelium were not quantified. Statistical analysis was performed using Graph Pad Prism one-way ANOVA with multiple comparisons. To determine the intensity of NF-κB in the nucleus, surfaces were created around immunostained in the 456 nm channel (dop stained nucleus) and the mean of Cy3 intensity was quantified within those volumes (see figures 3 and 4). Student’s unpaired t-test with Welch’s correction was performed in graph pad prism. See below images for examples of how Imais was used to quantify data.

Results

![Graph showing quantification of relative fluorescence intensity](image)

**Figure 1:** Quantification of relative fluorescence intensity of NF-κB in Nucleus compared to background control. The presence of NF-κB is significantly increased in keratoconus compared to normal corneas.

**Figure 2:** Quantification of relative fluorescence intensity of TNF-α in Keratoconus compared to background control. The presence of TNF-α is significantly increased in keratoconus compared to normal corneas.

**Figure 3:** Quantification of relative fluorescence intensity of TGFβ2 in Keratoconus compared to background control. The presence of TGFβ2 is significantly increased in keratoconus compared to normal corneas.

**Figure 4:** Quantification of relative fluorescence intensity of NF-κB in Nucleus compared to background control. The presence of NF-κB is significantly increased in keratoconus compared to normal corneas.

Analysis, Conclusion, and Future Experimentation

- TGFβ2 and TNF-α both demonstrated higher expression in the epithelial layer of keratoconic corneas when compared to the normal corneas (p<0.0001). NF-κB had similar expression levels in the epithelial and stromal layers of keratoconic corneas when compared to normal corneas (p=0.64 and p=0.99 respectively).

However, there was higher intensity staining of NF-κB in the nucleus of keratoconic corneal samples when compared to normal corneal samples (p=0.002). These data indicate a paradigm shift in the understanding of keratoconus from non-inflammation to an inflammatory state.

- Immunofluorescence staining for IL-33 was also performed and there was a very low level of antibody binding (data not shown). This may be due to the short half life of IL-33 and the lag time between surgical resection and tissue fixation. We will consider using fluorescent in situ hybridization with molecular probes with complimentary sequences to IL-33 mRNA.

- Future sections: Results from an assay that will measure TGF-β2 expression by cultured human corneal endothelial cells (HCEC) after challenge with TNF-α and/or mechanical disruption in the presence or absence of Smad3 small molecule, SIS3, inhibitor are pending.

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