·Clinical Research·

# *In vivo* corneal confocal microscopic analysis in patients with keratoconus

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## Abstract

• **AIM: To quantify corneal ultrastructure using laser scanning *in vivo* confocal microscopy (IVCM) in patients with keratoconus and control subjects.**

• **METHODS: Unscarred corneas of 78 keratoconic subjects without a history of contact lens use and 36 age-matched control subjects were evaluated with slit-lamp examination (SLE), corneal topography and laser scanning IVCM. One eye was randomly chosen for analysis. Anterior and posterior stromal keratocyte, endothelial cell and basal epithelial cell densities and sub-basal nerve structure were evaluated.**

• **RESULTS: IVCM qualitatively demonstrated enlarged basal epithelial cells, structural changes in sub-basal and stromal nerve fibers, abnormal stromal keratocytes and keratocyte nuclei, and pleomorphism and enlargement of endothelial cells. Compared with control subjects, significant reductions in basal epithelial cell density (5817±306 cells/mm² *vs* 4802±508 cells/mm², *P* <0.001), anterior stromal keratocyte density (800±111 cells/mm² *vs* 555±115 cells/mm², *P*<0.001), posterior stromal keratocyte density (333±34 cells/mm² *vs* 270±47 cells/mm², *P*<0.001), endothelial cell density (2875±223 cells/mm² *vs* 2686±265 cells/mm², *P*<0.001), sub-basal nerve fiber density (31.2±8.4 nerves/mm² *vs* 18.1±9.2 nerves/mm², *P*<0.001), sub-basal nerve fiber length (21.4±3.4 mm/mm² *vs* 16.1±5.1 mm/mm², *P*<0.001), and sub-basal nerve branch density (median 50.0 (first quartile 31.2 – third quartile 68.7) nerve branches/mm² *vs* median 25.0 (first quartile 6.2 – third quartile 45.3) nerve branches/mm², *P*<0.001) were observed in patients with keratoconus.**

• **CONCLUSION: Significant microstructural abnormalities were identified in all corneal layers in the eyes of subjects with keratoconus using IVCM. This non-invasive *in vivo* technique provides an important means to define and follow progress of microstructural changes in patients with keratoconus.**

• **KEYWORDS:** keratoconus; *in vivo* confocal microscopy; corneal nerves
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## INTRODUCTION

Keratoconus is classically considered as a progressive, non-inflammatory disorder causing an axial corneal ectasia. It is characterized by stromal thinning and corneal steepening, leading to irregular astigmatism and myopia, which cause a marked distortion in vision[1]. Classically, onset is at puberty, with progression until the third or fourth decade of life, when it usually stabilizes [1]. The exact pathophysiologic process of keratoconus is still unknown. The abnormalities in keratoconus include the degeneration of epithelial basal cells and breaks in Bowman's layer, as well as the release of catabolic enzymes and cytokines that cause thinning of collagen matrix lamella and apoptosis of keratocytes[2,3].

*In vivo* confocal microscopy (IVCM) is a relatively new technique that enables real-time, *in vivo*, high-resolution microscopic imaging of the cornea and has been increasingly utilized to evaluate the corneal changes in keratoconus [4-8]. The purpose of this study was to quantitatively analyse laser scanning IVCM images of all corneal layers in a large cohort of patients with keratoconus and control subjects.

## SUBJECTS AND METHODS

**Subjects** Seventy-eight eyes of 78 patients (46 females, 32 males) with an established diagnosis of keratoconus and 36 eyes of 36 healthy subjects (17 females, 19 males) were enrolled in this cross-sectional study undertaken at a single university hospital. For keratoconic subjects in whom both eyes were eligible for the study, one eye was randomly selected. Exclusion criteria were as follows: any previous ocular trauma or ocular surgery, any coexisting corneal

pathology, a history of contact lens use, a history of corneal hydrops and clinical evidence of corneal scarring. This study received local ethics committee approval and written informed consent was obtained from all subjects after a detailed explanation of the nature of the study.

All patients underwent complete ophthalmologic evaluation, including retinoscopy, slit lamp examination (SLE), and computerized topography (Pentacam, Oculus Optikgerate GmBH, Wetzlar, Germany). An eye was diagnosed as having keratoconus if there was scissoring reflex on retinoscopy, with central or paracentral thinning, anterior bulging or conicity, hemosiderin deposition (Fleischer ring), stromal striae (Vogt striae), on SLE; and central or paracentral steepening of the cornea on computerized topography. Corneal curvature readings were classified using the same system as used by researchers in the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) study [9]. According to this system, disease severity is classified with respect to the curvature of the steepest corneal meridian as follows: mild: <45 diopters (D); moderate: 45-52 D; severe: >52 D.

The subjects in the control group had no history of ocular surgery, no previous or active ocular disease, other than refractive error, no prior contact lens wear and no systemic disease that might affect the cornea.

**Methods** Laser scanning IVCM was performed on all subjects using the Rostock Corneal Module/Heidelberg Retina Tomograph lll (RCM/HRT lll; Heidelberg Engineering GmBH, Dossenheim, Germany). The full thickness of the central cornea was scanned by using the device's "section" mode, enabling instantaneous imaging of a single area of the cornea at a desired depth. The total duration of IVCM examination was approximately 2min per eye, and none of the subjects experienced any visual symptoms or corneal complications as a result of examination.

For each IVCM examination, two frames per location that contained the clearest images were selected from each of the following levels: basal epithelium, anterior stroma, posterior stroma, and endothelium. Anterior stroma was defined as the first two clear images (without motion blur or compression lines) immediately posterior to Bowman's layer, and the posterior stroma was defined as the first two clear images immediately anterior to Descemet's membrane. A standard central counting frame size of $200\times200$-$\mu m^2$ was used for all epithelial and endothelial images and a frame size of $300\times300$-$\mu m^2$ was used for the stromal images. Two frames were analysed for each corneal layer and an average was taken. The number of cells/$mm^2$ was calculated by the proprietary software within the RCM/HRT lll. Three to five high-quality images of the sub-basal nerve plexus from the center of the cornea were assessed from each subject. For all sub-basal nerve plexus images, the full $400\times400$-$\mu m^2$ frame was used. Automatic CCMetrics software, Ver. 1.0 (University of Manchester, UK) was used for the quantitative analysis of the nerve fibers [10]. Three parameters were quantified: corneal nerve fiber density (NFD), the total number of major nerves per square millimeter; nerve fiber length (NFL), the total length of all nerve fibers and branches (millimeters per square millimeter); and nerve branch density (NBD), the number of branches emanating from major nerve trunks per square millimeter [11]. A nerve branching index (number of branches per main nerve fibers) was derived from the NBD/NFD ratio.

**Statistical Analysis** Statistical analyses were performed using SPSS Ver. 17.0 (Chicago, IL, USA) software. Basic descriptive statistics were calculated on all the data gathered and are reported as the mean±SD, median (first quartile-third quartile) or $n$ (%), as appropriate. The Pearson $\chi^2$ test was used to compare categorical parameters. Normal distribution of continuous variables was confirmed with the Kolmogorov-Smirnov test. Independent samples $t$-test or Mann-Whitney $U$ test was used to compare the parameters between the keratoconus and control groups. Kruskal-Wallis test followed by Mann-Whitney $U$ test with Bonferroni adjustment or analysis of variance (One-way ANOVA) test followed by Tukey-HSD multiple comparison test was used to compare the parameters within the three subgroups of keratoconus subjects. For all evaluations, a $P$ value of less than 0.05 was considered statistically significant.

**RESULTS**

The mean age of the subjects was 25.7±5.4y (range 18-41y) in the keratoconus group and 27.3±4.3y (range 18-37y) in the control group. There was no statistically significant difference between the two groups in terms of age ($P$=0.126) and gender ($P$=0.241).

Of the 78 corneas with keratoconus, on the basis of Pentacam topography, 9 (11.54%) were classified as mild, 37 (47.44%) as moderate, and 32 (41.03%) as severe keratoconus. On SLE, Vogt's striae were clinically evident in 15 eyes (19.2%), and Fleischer ring was clinically evident in 28 (35.9%) eyes.

Qualitative analysis of the laser scanning IVCM images at the epithelial layer revealed structurally abnormal and elongated superficial epithelial cells and enlarged basal epithelial cells in eyes with keratoconus. Five (6.4%) eyes in the keratoconus group had deposition of brightly reflective material within the basal epithelial cells (Figure 1). This material was thought to represent hemosiderin accumulation corresponding to the Fleischer ring in these eyes.

Table 1 shows the summary of quantitative IVCM findings in patients with keratoconus and control subjects.

The mean basal epithelial cell density in the keratoconus group was significantly lower than the control group($P$<0.001). Furthermore, the mean basal epithelial cell density showed a

**Table 1 Comparison of *in vivo* confocal microscopic parameters between patients with keratoconus and control subjects**

| Parameters | Keratoconus ($n$=78) | Control ($n$=36) | $P$ |
|---|---|---|---|
| Basal epithelial cell density (cells/mm$^2$) | 4802±508 | 5817±306 | <0.001 |
| Anterior stromal keratocyte density (cells/mm$^2$) | 555±115 | 800±111 | <0.001 |
| Posterior stromal keratocyte density (cells/mm$^2$) | 270±47 | 333±34 | <0.001 |
| Endothelial cell density (cells/mm$^2$) | 2686±265 | 2875±223 | <0.001 |
| NFD (mean±SD) (number of major nerves/mm$^2$) | 18.1±9.2 | 31.2±8.4 | <0.001 |
| NFL (mean±SD) (mm/mm$^2$) | 16.1±5.1 | 21.4±3.4 | <0.001 |
| NBD [median (first-third quartiles)] (number of branches/mm$^2$) | 25.0 (6.2-45.3) | 50.0 (31.2-68.7) | <0.001 |
| Nerve branching index [median (first-third quartiles)] (number of branches/number of major nerves) | 1.50 (0.65-2.37) | 1.33 (1.17-2.00) | 0.269 |

NFD: Nerve fiber density; NFL: Nerve fiber length; NBD: Nerve branch density.

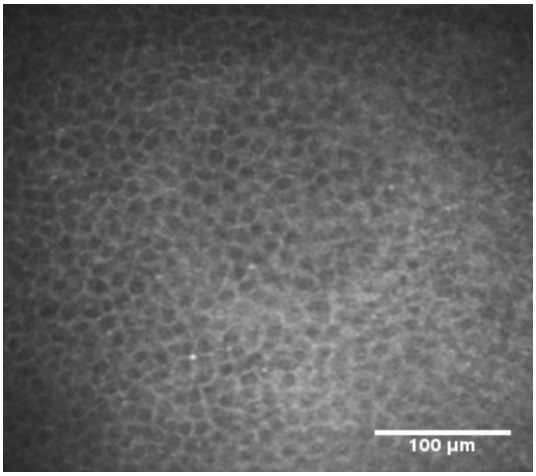


**Figure 1 Brightly reflective material deposition within the basal epithelial cells representing Fleischer ring.**

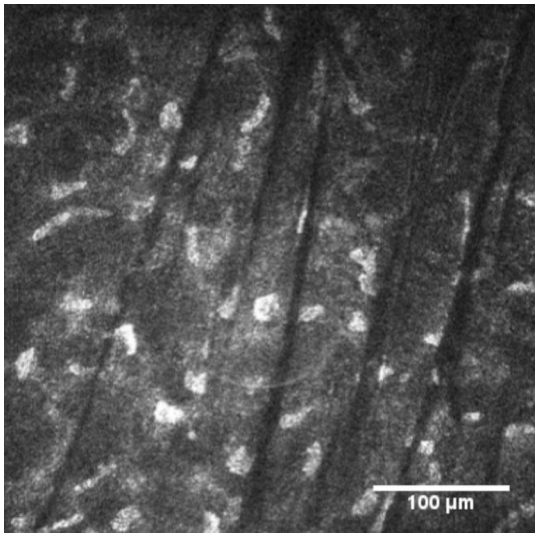


**Figure 2 Dark bands at the posterior stroma representing Vogt´s striae in an eye with keratoconus.**

significant reduction with increasing severity of the disease (mild: 5399±541 cells/mm$^2$; moderate: 4862±460 cells/mm$^2$; severe: 4563±391 cells/mm$^2$) ($P$=0.018). There was also a statistically significant difference between the mild keratoconus group and the control group ($P$=0.003).

The mean anterior stromal keratocyte density in the keratoconus group was significantly lower than in the control group($P$<0.001). Subgroup analysis in the keratoconus group showed that the mean anterior stromal keratocyte density was 751 ± 64 cells/mm$^2$ in the mild keratoconus group, 542 ±95 cells/mm$^2$ in the moderate keratoconus group, and 516 ±92 cells/mm$^2$ in the severe keratoconus group with a significant difference between mild and moderate keratoconus groups ($P$ <0.001), but no difference between moderate and severe keratoconus groups ($P$ =0.468). No significant difference was observed in anterior stromal keratocyte density between the mild keratoconus group and the control group ($P$=0.211).

The mean posterior stromal keratocyte density in the keratoconus group was significantly lower than in the control group ($P$<0.001). The mean posterior stromal keratocyte density showed a progressive reduction (mild: 338 ±51 cells/mm$^2$; moderate: 269 ±34 cells/mm$^2$; severe: 252 ±42 cells/mm$^2$) which was significant between mild and moderate keratoconus groups ($P$<0.001), but not between moderate and severe groups ($P$=0.198). No significant difference was observed in posterior stromal keratocyte density between the mild keratoconus group and the control group ($P$=0.703). Folds in the posterior stroma that were thought to represent Vogt's striae were observed in 19 (24.4%) eyes (Figure 2).

Qualitative image analysis revealed pleomorphism and polymegathism in endothelial cells in eyes with keratoconus. The mean endothelial cell density in the keratoconus group was significantly lower than that of the control group ($P$<0.001). When subgroup analysis was performed in the keratoconus group, the mean endothelial cell density showed a progressive decrease with increasing severity of disease (mild: 2965±94 cells/mm$^2$; moderate: 2747±220 cells/mm$^2$; severe: 2537 ±256 cells/mm$^2$, $P$=0.031). There was no significant difference in endothelial cell density between the mild keratoconus group and the control group ($P$=0.242).

Sub-basal nerve fibers were seen at the level of the acellular Bowman's layer. Eyes with keratoconus exhibited abnormal sub-basal nerve architecture and reduced nerve fiber density, compared with control subjects (Figure 3).

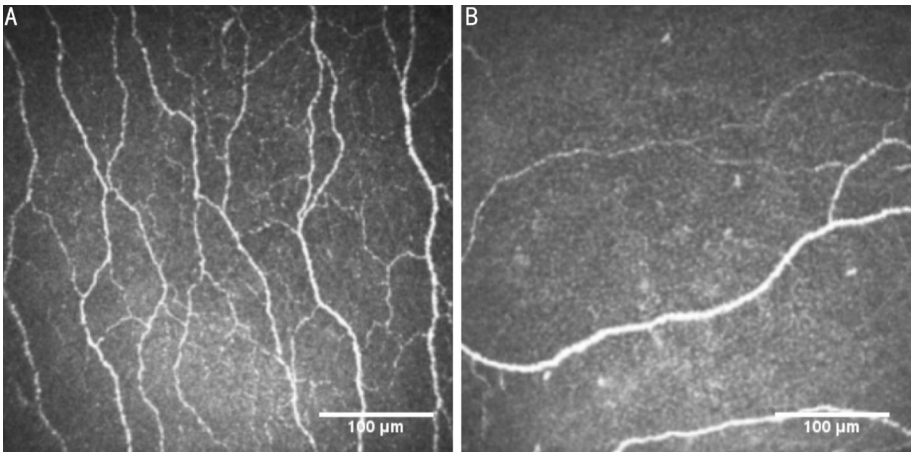


**Figure 3 Sub-basal nerve fiber layer images** A: Nerve fibers of a healthy subject; B: Nerve fibers showing reduced density, thickening and abnormal orientation in a keratoconic cornea.

The mean NFD (number of major nerve fibers/mm$^2$) in the keratoconus group was significantly lower than that of the control group ($P$<0.001). In subgroup analysis, there was a significant and progressive reduction in mean NFD (mild: 27.8±7.7; moderate: 19.8±8.5; severe: 13.5±7.6, $P$=0.025). The mean NFL [total length (mm) of all nerve fibers and branches/mm$^2$] in the keratoconus group was significantly lower than that of the control group ($P$<0.001). In subgroup analysis, the mean NFL was 19.7 ±4.9 in the mild keratoconus group, 17.2 ±4.5 in the moderate keratoconus group, and 13.9 ±4.8 in the severe keratoconus group. The difference in mean NFL between mild and moderate keratoconus groups showed no statistical significance ($P$=0.326), while there was significant difference between moderate and severe keratoconus groups ($P$=0.013). The median value of NBD (number of branches/mm$^2$) was significantly lower in the keratoconus group compared with the control group ($P$<0.001). When subgroup analysis was performed in the keratoconus group, the median value of NBD was 31.2 (first quartile 15.6 to third quartile 50.0) in the mild keratoconus group, 31.3 (first quartile 12.5 to third quartile 56.3) in the moderate keratoconus group, and 18.8 (first quartile 0 to third quartile 31.2) in the severe keratoconus group. There was no significant difference among the three subgroups ($P$=0.094). The median value of the nerve branching index of the keratoconic corneas was higher but did not differ significantly from control corneas ($P$=0.269).

## DISCUSSION

The reported prevalence of keratoconus varies from 8.8 to 54.4 per 100 000 in the general population [12]. Although recent developments in corneal topography and pachymetry systems have provided useful information for the diagnosis and follow-up of this condition, the etiopathogenesis of the disorder remains obscure. Keratocyte loss is considered to be a very important change in keratoconus and is believed to be associated with corneal thinning and loss of normal corneal architecture [4]. Patients with keratoconus generally use contact lenses, which in itself may result in structural changes in the cornea [13,14]. We used laser scanning IVCM to evaluate microstructural changes in keratoconus in comparison with healthy control eyes. The exclusion of subjects with corneal scarring and contact lens wear provided clear corneal images and reduced possible confounding variables.

In this study an enlargement and irregular arrangement of the basal epithelial cells was observed with a 17.4% reduction in basal epithelial cell density in subjects with keratoconus compared with the control group. This reduction has also been reported in previous studies[7,15-17]. Hollingsworth *et al* [3] demonstrated that in the early stages of keratoconus, degenerative changes in the basal epithelium cells precede loss of basal cells. On the other hand, Ucakhan *et al*[5] reported a significant increase in basal epithelial cell density which was thought to represent increased epithelial turnover rate, secondary to the degenerative processes in eyes with keratoconus.

Among the patients with clinically evident Fleischer ring on SLE, hemosiderin deposition in the basal epithelial cell layer was observed in 17.9% (5 of 28) of the eyes by IVCM of the central cornea. Ucakhan *et al* [5] also reported these brightly reflective deposits on IVCM images in 70% of the eyes with Fleischer ring on slit lamp examination and attributed this result to the more peripheral localization of the hemosiderin ring in those 30%.

In this study, both anterior and posterior stromal keratocyte densities were found to be significantly lower in the keratoconus group compared with the control group (30.6% and 18.9% lower anterior and posterior stromal keratocyte densities, respectively). Erie *et al* [18] reported a decrease in keratocyte density in all layers of the stroma in keratoconus patients who wore contact lenses and attributed this to epithelial injury with release of apoptotic cytokines leading to

a decrease in cell density. In another study by Hollingsworth *et al* [4] a 19% reduction in the anterior and 10% reduction in the posterior keratocyte densities were reported in 29 eyes with keratoconus, 50% of whom were wearing rigid gas-permeable contact lenses. On the other hand, Weed *et al* [7] showed a significant increase in both anterior and posterior stromal keratocyte densities in their study, in which the corneal apex of the contact lens wearing keratoconus subjects was evaluated. They attributed their findings to the corneal protrusion and thinning, leading to compression of the keratocytes at the corneal apex and increased density for the same confocal slice thickness. Four other studies performed with IVCM have demonstrated that anterior and posterior stromal keratocyte densities decrease in patients with keratoconus not using contact lenses[5,15,17,19]. The current study supports the findings of these previous studies and shows anterior and posterior stromal keratocyte loss in patients without a history of contact lens use. Furthermore, anterior stromal keratocytes seem to be affected more than the posterior stromal keratocytes, probably because of the underlying pathogenetic mechanisms.

According to the results of the CLEK study group, nearly half of the patients with keratoconus have clinically evident Vogt's striae on slit-lamp examination [9]. In a previous study, alternating dark and light bands referred to as Vogt's striae, were observed with IVCM in the stromal images of 45% of keratoconic eyes[20]. Although there were 15 (19.2%) patients with keratoconus who had Vogt's striae on slit-lamp examination, the number of eyes having dark and light band appearance on IVCM were 19 (24.4%) in the current study. Mocan *et al* [21] noted that 50% of the patients without clinically apparent Vogt's striae had bands in the stromal images of IVCM and attributed this result to the presence of the striae at a microscopic level which could not be observed on slit-lamp examination. These dark and light bands representing Vogt's striae were observed not only in the posterior stroma, but also in the anterior and mid-stroma [5]. Further studies are required to explore whether the localization or banding pattern could be associated with the severity of the disease.

In this study, a statistically significant reduction in endothelial cell density was observed in keratoconic corneas compared with the controls. Additionally, variation in the size and the shape of the endothelial cells in eyes with keratoconus was apparent. There are conflicting results among the studies analyzing the endothelial layer of keratoconic corneas. Hollingsworth *et al* [4] reported that the mean endothelial cell density was significantly higher in eyes with keratoconus and attributed this finding to the coexistence of myopia, rigid gas-permeable lens wear, and cellular redistribution in these eyes. Whilst Ucakhan *et al*[5] demonstrated a non-significant reduction in endothelial cell density in patients with keratoconus compared with the control group, patients with severe keratoconus had a significant decrease in the endothelial cell density compared with mild and moderate cases. In another study by Mocan *et al* [15] the decrease in endothelial cell density was reported to be statistically significant not only in severe cases, but also in those with mild and moderate keratoconus. In the study of Weed *et al* [7] which was performed on subjects using contact lenses, there was no significant difference in endothelial cell density between the patients with keratoconus and control subjects. Niederer *et al* [17] demonstrated a significant decrease in endothelial cell density in subjects with keratoconus and explained it by the distortion of corneal shape, resulting in an increased posterior surface area and a relative decrease in density. In conclusion, different studies have indicated either increased [4], decreased [5,15,17] or unchanged [7] endothelial cell density in keratoconus. It therefore appears that the endothelium of keratoconic corneas is unstable and prone to damage, but to fully understand the natural history of these changes, it would be necessary to perform longitudinal studies, which are of course easily possible using IVCM.

A striking result of this study was that, compared with control group, patients with mild keratoconus had a significantly lower basal epithelial cell density, while there was no significant difference in anterior and posterior stromal keratocyte densities and endothelial cell density. This finding shows that the degenerative process begins at the level of the basal epithelium, supporting a prior histopathological study[22].

Many studies have demonstrated abnormal corneal nerve morphology in keratoconus [5,6,15,23]. Based on our qualitative analysis nerve fibers showed abnormal branching patterns, reduction in density, increased tortuosity and thickening in keratoconic corneas. The orientation of nerve fibers in these eyes appeared to be altered from the predominantly vertical orientation observed in control subjects. This observation is explained with the theory suggesting that the nerve fiber bundles follow the contour of the base of the cone and form a tortuous network at the apex of the cone [23]. In the current study, quantitative analysis revealed a significant reduction in NFD, NBD and NFL, yet the nerve branching index was higher in keratoconic corneas. The presence of these changes suggests the direct involvement of the corneal nerve plexus, although whether these alterations play a causative role or are secondary manifestations of the underlying disease remains unknown.

According to a Pubmed search, this is the largest quantitative study to date investigating the structural changes in keratoconus using IVCM. A number of other investigators have employed IVCM in patients with keratoconus and have shown the changes, primarily in corneal cell parameters, and

some of them reported only the qualitative changes in the corneal nerve fibers. A unique aspect of this study is that we provide quantitative analysis of all cellular structures, in particular of the sub-basal nerve fibers. We also relate these changes to the severity of keratoconus.

The main limitation of this study is that the quantitative analysis of the IVCM images were performed by a single observer who was unmasked as to whether the images belonged to a keratoconus or a control subject, but was masked about the severity of the keratoconus. Another limitation is the small sample size of subjects with mild keratoconus, as mentioned in prior studies, but this simply reflects the lower number of patients referred to clinics with mild disease[5,21].

In conclusion, we show that IVCM represents a rapid non-invasive means to quantify all layers of the central cornea to demonstrate significant pathology of the basal epithelial cells, keratocytes, endothelial cells, and nerve fibers in patients with keratoconus. This lends itself to undertaking longitudinal studies which will be essential to define the natural history of this disease.

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