

Laser Scanning In Vivo Confocal Microscopy Reveals Reduced Innervation and Reduction in Cell Density in All Layers of the Keratoconic Cornea

Rachael L. Niederer, Divya Perumal, Trevor Sherwin, and Charles N. J. McGhee

PURPOSE. The exact pathophysiological processes underlying keratoconus remain an enigma. In this study, laser scanning in vivo confocal microscopy (IVCM) was used to define further the microstructural abnormalities in the keratoconic cornea and to establish the relationship with disease severity.

METHODS. This was a prospective, cross-sectional study comparing 52 subjects with keratoconus and 52 age-matched control subjects. Assessment included demographics, history, slit lamp biomicroscopy, computerized corneal tomography, and laser scanning IVCM.

RESULTS. Significantly lower cell densities (in cells per square millimeter, mean \pm SD) were observed in keratoconus corneas than in normal ones: basal epithelial cells, 4340.6 ± 595.2 vs. 5777.6 ± 958.2 ($P < 0.001$), anterior keratocytes, 523.6 ± 206.4 vs. 859.7 ± 219.1 ($P < 0.001$), posterior keratocytes, 240.4 ± 64.5 vs. 330.6 ± 52.3 ($P < 0.001$), and endothelial cells 2412.2 ± 339.5 vs. 2845.6 ± 313.0 ($P < 0.001$). Subbasal nerve fiber density was 52.7% lower in keratoconus corneas than in the control ($P < 0.001$). Basal epithelial cell density ($P = 0.001$), subbasal nerve fiber density ($P = 0.015$), and anterior keratocyte density ($P < 0.001$) correlated with severity of disease. Lower subbasal nerve density also correlated with younger age at diagnosis ($r = 0.397$, $P = 0.004$). Severe disease was associated with diagnosis at a younger age ($P = 0.023$), a history of eye rubbing ($P = 0.025$), and Maori or Pacific Island ethnicity ($P = 0.001$).

CONCLUSIONS. Significant microstructural abnormalities were identified at every level of the keratoconic cornea and were related to disease severity. IVCM offers a potential insight into the pathophysiology of the microstructural changes in keratoconus. (*Invest Ophthalmol Vis Sci.* 2008;49:2964–2970) DOI:10.1167/iovs.07-0968

Keratoconus is a noninflammatory ectasia of the cornea in which the cornea assumes a conical shape due to thinning and protrusion.¹ The etiology of the disease is complex: Most likely, it arises from a combination of genetic and environmental factors, and both keratocyte and epithelial changes have been implicated in disease development and progression.^{2,3} No satisfactory animal model exists for the disease, and ex vivo studies of keratoconus are largely limited to corneal buttons removed for penetrating keratoplasty in subjects with advanced disease.

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In vivo confocal microscopy (IVCM) represents a relatively new technology with the facility to image, en face, all layers of the cornea, in more physiological conditions than previously possible.⁴ We have demonstrated good repeatability and reproducibility of cell and innervation density, by using a laser scanning in vivo confocal microscope (HRT II Rostock Corneal Module [RCM]; Heidelberg Engineering, Heidelberg, Germany),⁵ and the RCM has been used to document age-related cell changes, corneal healing, and recovery of innervation after corneal transplantation.^{6,7} Using laser scanning IVCM in a cohort of keratoconus subjects, we have recently reported variably reduced keratocyte density compared with normal corneas.⁸ In the present study, we sought to use this technique to define further the microstructural abnormalities in the keratoconic cornea in subjects with no contact lens wear and to establish the associations with the clinical manifestations of disease severity.

MATERIALS AND METHODS

Subject Recruitment and Assessment

Fifty-two subjects with an established diagnosis of keratoconus were recruited from subspecialist corneal clinics in the Department of Ophthalmology, Auckland City Hospital, and were examined in the Department of Ophthalmology, University of Auckland. Clinical history included age at diagnosis of keratoconus (clinical diagnosis by an optometrist or ophthalmologist) and self-reported history of atopy, including asthma, eczema/dermatitis, and allergic conjunctivitis. Subjects were asked whether they rubbed their eyes frequently, or a great deal, and an affirmative answer was recorded as a positive history of eye rubbing. Slit lamp biomicroscopy was performed on all eyes, and each subject exhibited two or more of the following clinical signs: computed topography suggesting keratoconus, central or paracentral corneal stromal thinning, Fleischer's ring, Vogt's striae, or Munson's sign.

Subjects with corneal hydrops or scarring were excluded from further analysis. Subjects with a history of ocular trauma, ocular surgery, other corneal disease, contact lens wear (in the preceding year), or systemic disease that may affect the cornea were also excluded. In subjects in whom both eyes were eligible for the study, one eye was chosen at random for inclusion.

Fifty-two normal volunteers were recruited as a control group. These subjects 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. Systemic medications were permitted unless they were known to affect the anterior segment of the eye. All control subjects were examined by slit lamp biomicroscopy, and their corneas were confirmed to be clinically normal. Only right eyes were included for analysis in the control group.

Corneal topography and pachymetry were performed in all subjects with a combined Placido/slit-scanning elevation tomography system (Orbscan II; Bausch & Lomb Surgical, Rochester, NY). In subjects with keratoconus, the modified Rabinowitz-McDonnell test was used to confirm the diagnosis of keratoconus, and the severity of keratoconus was classified according to the steepest simulated keratometry

reading on the keratometric map (mild, <45 D; moderate, 45–52 D; severe, >52 D).⁹

This study received approval from the Auckland Ethics Committee and adhered to the Declaration of Helsinki. Written informed consent was obtained from all subjects after a detailed explanation of the nature of the study.

In Vivo Confocal Microscopy

Laser scanning IVCM was performed on all subjects with the HRT II RCM (Heidelberg Engineering GmbH). This microscope uses a 670-nm red wavelength diode laser source. A 60× objective water-immersion lens with a numerical aperture of 0.9 (Olympus, Tokyo, Japan) and a working distance, relative to the applanating cap, of 0.0 to 3.0 mm, was used for all assessments. The images produced using this lens are 400 × 400 μm, and the manufacturer quotes transverse resolution and optical section thickness as 2 and 4 μm, respectively. The RCM uses an entirely digital capture system.

Eyes were anesthetized with 1 drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Surrey, UK). Carbomer 980 (Viscotears 0.2%; Novartis, North Ryde, NSW, Australia) was used as a coupling agent between the applanating lens cap and the cornea. During the IVCM examination, all subjects were asked to fixate on a distance target aligned to enable examination of the central cornea. 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 2 minutes per eye, and no subjects experienced any visual or corneal complications after examination.

Image Analysis

For each IVCM examination, three images were taken from each of the following levels: basal epithelium, subbasal nerve plexus, anterior stroma, posterior stroma, and endothelium. Anterior stroma was defined as the first three clear images (without motion blur or compression lines) immediately posterior to Bowman's layer, and the posterior stroma was defined as the first three clear images immediately anterior to Descemet's membrane. Images were selected for analysis by an experienced observer (RLN).

Measurements were performed with a caliper tool (analySIS 3.1; Soft Imaging System, Münster, Germany). For all epithelial and endothelial images a standard central counting frame size of 200 × 200 μm was used. For all subbasal nerve plexus and stromal images, the full 400 × 400-μm frame was used. Nerve fiber density was calculated by measuring the total length of all nerve fibers and branches per square millimeter. IVCM is unable to resolve individual nerve fibers, and therefore the subbasal nerve density represents the density of nerve fiber bundles rather than individual nerves. Ten percent of all images were recounted by one of the examiners (DP) to determine the inter-examiner limit of agreement.

Statistical Analysis

All values were entered into a database (Excel; Microsoft, Redmond, WA) and subsequently imported into statistical software for analysis (SPSS, ver. 15 for Windows; Chicago, IL), with the assistance of a professional biomedical statistician. Basic descriptive statistics were calculated on all data gathered and are reported as the mean ± SD or *n* (%), as appropriate. Normal distribution of continuous variables was confirmed with the Kolmogorov-Smirnov test.¹⁰ Correlations between continuous variables were examined by calculating Pearson's correlation coefficient, Spearman's ρ , or Kendall's τ , as appropriate. The Student's independent *t*-test, Mann-Whitney *U* test, or Fisher exact test was used to compare results between groups. Multiple linear regression was performed to determine the independent predictors of visual acuity by using a variety of iterative methods (forward, backward, and stepwise selection). All tests were two-tailed, and *P* < 0.05 was considered statistically significant.

TABLE 1. Subject Characteristics

	Control <i>n</i> = 52	Keratoconus <i>n</i> = 52	<i>P</i>
Sex, <i>n</i> (%)			
Male	23 (44.2)	27 (51.9)	0.556
Female	29 (55.8)	25 (48.1)	
Age (y)	25.5 ± 7.0	27.9 ± 11.1	0.587
Range (y)	(18–54)	(13–54)	
Ethnicity, <i>n</i> (%)			
Caucasian	28 (53.8)	15 (28.8)	<0.001
Asian	14 (26.9)	7 (13.5)	
Maori/Pacific Island	10 (19.2)	30 (57.7)	
Corneal astigmatism	0.8 ± 0.5	5.7 ± 3.8	<0.001
Range (D)	(0.1–2.1)	(0.4–16.5)	
Max K	43.9 ± 1.6	53.3 ± 9.6	<0.001
Range (D)	(40.9–47.0)	(33.7–87.8)	
Min K	43.1 ± 1.5	47.6 ± 7.2	<0.001
Range (D)	(39.9–46.7)	(27.4–71.2)	
CCT	552.5 ± 35.6	431.2 ± 67.3	<0.001
Range (μm)	(455–633)	(285–549)	

No difference was observed in age or sex between the two groups. Significant differences were observed in central corneal thickness (CCT) and simulated keratometry (Max K and Min K; Orbscan II; Bausch & Lomb, Tampa, FL). Sex was compared between groups with the χ^2 test. Age, keratometry, and CCT were compared between groups with the Mann-Whitney *U* test.

RESULTS

Fifty-two subjects (52 eyes) with keratoconus were included for analysis and compared with 52 normal volunteers (52 eyes). The subjects' characteristics are reported in Table 1. Significantly more Maori and Pacific Island subjects were present in the keratoconus group than in the control group.

Mean best corrected visual acuity (BCVA) in the keratoconus group was 20/64 (mean logMAR 0.51 ± 0.51). Age at diagnosis was 21.6 ± 10.0 years (mean ± SD). No significant difference was noted in age at diagnosis between male and female patients. A history of atopy was reported in 34 (65.4%) keratoconic subjects, including asthma in 24 (46.2%), eczema/allergic dermatitis in 14 (26.9%), and allergic conjunctivitis in 18 (34.6%). Keratoconus was diagnosed in subjects with a history of atopy at a younger mean age (18.7 ± 7.3 years vs. 27.0 ± 12.3 years, *P* = 0.004). No difference was observed in the history of atopy in relation to sex or ethnicity. Eye rubbing was reported in 12 (23.1%) subjects with keratoconus. No association was observed between a history of atopy and eye rubbing (*P* = 0.179), but allergic conjunctivitis was strongly associated with a history of eye rubbing (*P* < 0.001). No difference in history of eye rubbing was observed with age, sex, or ethnicity. A family history of keratoconus was reported in nine (17.3%) keratoconic subjects. No difference in reported family history was observed with age, sex, or ethnicity.

The quality of the IVCM images was good, and epithelial, keratocyte, and endothelial cell densities could be calculated in all eyes (Fig. 1). Interexaminer 95% limits of agreement were ±7.9%.

Statistically significant lower cell and innervation densities were observed at every level of the keratoconic cornea compared with the control corneas (Table 2). Relative to control values, subjects with keratoconus exhibited 24.9% lower epithelial cell density, 39.2% lower anterior keratocyte density, 27.5% lower posterior keratocyte density, and 15.2% lower endothelial cell density (all *P* < 0.001; Fig. 1). Subbasal nerve fiber density in subjects with keratoconus was 52.7% lower than in the control (*P* < 0.001; Fig. 2).

As the control group and keratoconus group were not matched for ethnicity, the cell and innervation densities for

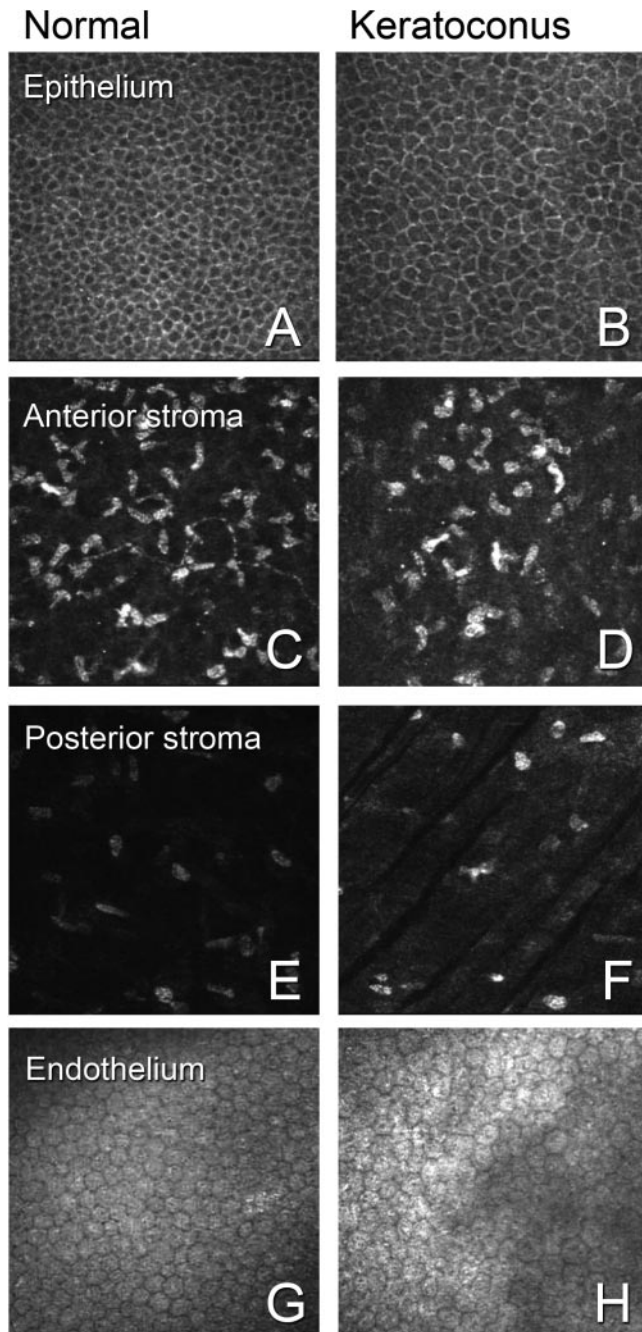


FIGURE 1. Laser scanning IVCM of the human cornea with the RCM (Heidelberg Engineering, GmbH, Heidelberg, Germany). Images cropped to $300 \times 300 \mu\text{m}$ illustrating the epithelium, anterior and posterior stroma, and endothelium in control eyes (A, C, E, and G, respectively), and the epithelium, anterior and posterior stroma, and endothelium in keratoconic eyes (B, D, F, and H, respectively). Dark bands (presumed Vogt's striae) were observed in the posterior stroma in keratoconus (F).

Maori and Pacific Island subjects with and without keratoconus were compared. Despite the lower sample number, significantly lower epithelial cell ($P < 0.001$), anterior and posterior keratocyte (both $P < 0.001$), endothelial cell ($P = 0.001$), and subbasal nerve fiber ($P < 0.001$) density was still observed in the keratoconus group.

In keratoconus subjects, subbasal nerve density correlated with age at diagnosis and with lower nerve density in subjects

in whom the disease was diagnosed at a younger age ($r = 0.397$, $P = 0.004$). No correlation was observed between cell density at any level and age at diagnosis. No difference was observed in cell or innervation density between males and females with keratoconus. No difference was observed in cell or innervation density in keratoconus subjects, with or without a history of atopy or eye rubbing.

Subbasal nerve fiber density in keratoconus eyes correlated positively with anterior keratocyte cell density ($r = 0.362$, $P = 0.008$). Endothelial cell density correlated with anterior ($r = 0.418$, $P = 0.002$) and posterior ($r = 0.304$, $P = 0.028$) keratocyte density. No correlation was observed between epithelial cell density and other cell layers, or between epithelial cell and subbasal nerve densities.

Topographic maps were generated for all control eyes and for 44 keratoconic eyes (85%; $P = 0.006$). The maps could not be produced in eight eyes with keratoconus because of advanced disease and severe corneal irregularity. Subjects with keratoconus were subcategorized as follows: mild disease ($n = 7$, 13.4%), moderate disease ($n = 14$, 26.9%), and severe disease ($n = 31$, 59.6%), according to the steepest simulated keratometry on axial keratometric maps. Subjects in whom topography was not possible were classified, on the basis of clinical examination and irregular computerized topography, as severe. For the purpose of statistical analysis, results for subjects with mild or moderate keratoconus were pooled ($n = 21$). Subject characteristics and cell and innervation density are reported for mild to moderate and severe disease in Table 3. Subjects with more severe disease were younger, both at the time of inclusion in the study ($P = 0.025$) and at the time of diagnosis ($P = 0.003$). No difference in duration of disease (taken as time from diagnosis to inclusion in the study) was observed between the two groups (mild/moderate keratoconus 5.8 ± 6.7 years vs. keratoconus 6.8 ± 7.1 years, $P = 0.59$). Maori and Pacific Island ethnicity was associated with more severe disease ($P = 0.001$), and a younger mean age at diagnosis than Caucasian and Asian subjects (17.1 ± 5.6 years vs. 27.6 ± 11.6 years, $P < 0.001$). No difference in history of atopy was observed with stage of disease, but more subjects with severe disease gave a history of eye rubbing ($P = 0.025$).

In subjects with keratoconus, logMAR BCVA correlated with CCT ($r = -0.442$, $P < 0.001$), corneal astigmatism ($r = 0.420$, $P < 0.001$), simulated maximum keratometry reading ($r = 0.467$, $P < 0.001$), and simulated minimum keratometry reading ($r = 0.374$, $P = 0.001$). A small correlation was also observed between logMAR BCVA and age at diagnosis ($r = -0.217$, $P = 0.035$), epithelial cell density ($r = -0.414$, $P = 0.012$), and anterior keratocyte density ($r = -0.412$, $P = 0.003$); however, these correlations did not prove significant in multiple linear regression when controlled for severity of disease. No association was observed between posterior keratocyte density or endothelial cell density and logMAR BCVA.

Subbasal nerve fibers exhibited increased tortuosity in subjects with keratoconus in comparison with control subjects (Fig. 2). In some subjects, abruptly terminating subbasal nerve fibers were observed in the central cornea. Stromal nerve fibers showed normal morphology, but nerve fibers bundles appeared thicker (Fig. 2).

Discontinuities were observed in Bowman's membrane in subjects with keratoconus, associated with presumed invagination of epithelial cells (Fig. 3). Vogt's striae were observed on confocal microscopy in 42 subjects with keratoconus (80.8%; Fig. 1F). The observation of Vogt's striae on IVCM was associated with lower CCT ($420.2 \pm 64.1 \mu\text{m}$ vs. $475.4 \pm 64.8 \mu\text{m}$) and lower endothelial cell count (2343.3 ± 332.2 cells/ mm^2 vs. 2701.5 ± 188.2 cells/ mm^2). No association was observed between Vogt's striae on IVCM and age, sex, ethnicity,

TABLE 2. Cell Density and Innervation Density in Keratoconus and Control Eyes

	Control <i>n</i> = 52	Keratoconus <i>n</i> = 52	<i>P</i>
Epithelium (cells/mm ²)	5777.6 ± 958.2	4340.6 ± 595.2	<0.001
Subbasal nerve density (mm nerve/mm ²)	22.4 ± 5.96	10.6 ± 5.23	<0.001
Anterior stroma (cells/mm ²)	859.7 ± 219.1	523.6 ± 206.4	<0.001
Posterior stroma (cells/mm ²)	330.6 ± 52.3	240.4 ± 64.5	<0.001
Endothelium (cells/mm ²)	2845.6 ± 313.0	2412.2 ± 339.5	<0.001

Statistically significant differences were observed in all cell densities and innervation density between the keratoconus and control groups (Mann-Whitney *U* test). Lower values were observed for all five parameters in the keratoconus group. Data are expressed as the mean ± SD.

keratometry, or other measurements of cell and innervation density.

DISCUSSION

We used laser scanning IVCN to examine microstructural changes in keratoconus in comparison with healthy control eyes. Ours is the largest IVCN study of the keratoconic cornea, and the exclusion of subjects with corneal scarring and contact lens wear provided clear corneal images and reduced possible confounding variables. The use of coherent light has enabled the production of laser scanning IVCN with improved contrast and higher image quality, particularly for the corneal stroma,¹¹ and good repeatability and reproducibility of this device have been demonstrated.⁵

Investigators in prior studies have examined the role of ethnicity in keratoconus, and whereas one group has observed

an increased incidence of keratoconus in Asian subjects and a younger age at presentation,¹² others have failed to observe a difference in keratoconus between ethnic groups.^{1,13,14} Our study is the first to report a more severe spectrum of keratoconus in Maori and those of Pacific Island ethnicity.

An important limitation of the present study is that we were unable to match control subjects for ethnicity, due to the large proportion of Maori and Pacific Island subjects with keratoconus; however, significant differences in cell and innervation density were still observed when the groups were subanalyzed for ethnicity.

Atopy and eye rubbing have both been identified as risk factors for keratoconus.^{1,13,15} We observed an association between allergic eye disease and eye rubbing, younger age at onset in atopic individuals, and a correlation between eye rubbing and disease severity. A history of atopy was observed in 65% and a history of eye rubbing in 23% of subjects with keratoconus. The rate of atopy is similar to that reported in the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) study; however, fewer patients reported a history of eye rubbing (23% vs. 48% in CLEK study), possibly due to a lower incidence of allergic conjunctivitis in our keratoconus subjects (35% compared with 53% in the CLEK study).⁹ A family history of keratoconus was reported by 17% of keratoconic subjects in this study, similar to that previously reported,^{9,15} though lower than that previously self-reported in the New Zealand population (23.5%).¹³

Enlargement and irregular arrangement of the basal epithelial cells was observed in this study, with basal epithelial cell density 25% lower in keratoconic than in control eyes and lower basal epithelial cell density in severe disease. Keratoconus has been associated with central epithelial thinning,^{16,17} and a combination of specular microscopy and a contact lens have shown the superficial epithelial cells to be enlarged and elongated in keratoconus. Keratoconic epithelium has been examined in three IVCN studies, with two reporting an increase in cell area and whorl-like arrangement of the epithelial cells and the other, conversely, reporting a decrease in cell area and a 90% increase in basal epithelial cell density.^{18–20} We observed a decrease in epithelial cell density in keratoconus, conflicting with the results of Uçakhan et al.,¹⁸ but agreeing with previous observations of increased epithelial cell area in keratoconus.^{17,19,20}

While early electron microscopy studies suggest that the primary abnormality in keratoconus may reside in the corneal epithelium,²¹ subsequent studies have documented alterations of the subbasal nerve plexus,^{22,23} stromal nerves,^{23,24} keratocytes,^{18,19,25,26} and endothelium.^{27,28} Electron microscopy studies have revealed both activated and degenerating keratocytes in the keratoconic cornea,^{26,29,30} with activated keratocytes exhibiting high levels of rough endoplasmic reticulum and discrete incursion of fine cellular processes into Bowman's membrane.^{30,31} Increased apoptosis has been noted in kerato-

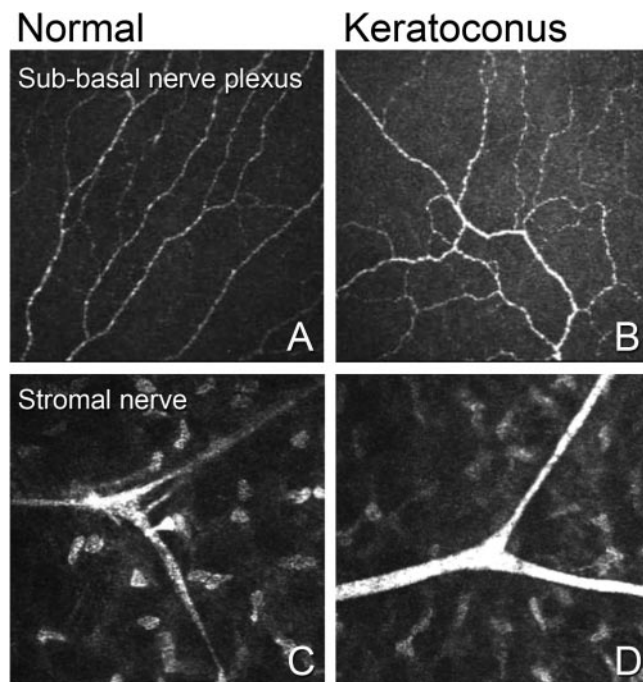


FIGURE 2. Laser scanning IVCN of subbasal and stromal nerves in keratoconus and control eyes. Laser scanning IVCN of the human cornea with the RCM (Heidelberg Engineering, GmbH, Heidelberg, Germany). Images cropped to 300 μ m \times 300 μ m illustrating the subbasal nerve plexus and stromal nerves in control eyes (A, C, respectively) and the subbasal nerve plexus and stromal nerves in keratoconus (B, D, respectively). Increased nerve tortuosity and decreased nerve density were noted in the keratoconic subbasal nerve plexus (B) and the keratoconic stromal nerve appeared thickened (D).

TABLE 3. Subject Characteristics and Cell Density with Stage of Disease

	Mild/Moderate <i>n</i> = 21	Severe <i>n</i> = 31	<i>P</i>
Age (y)	31.7 ± 10.3	25.4 ± 11.1	0.025
Age at diagnosis (y)	26.0 ± 11.1	18.6 ± 8.1	0.003
Sex, <i>n</i> (%)			
Male	11 (52.4)	16 (51.6)	
Female	10 (47.6)	15 (48.4)	1.000
Ethnicity, <i>n</i> (%)			
Maori/Pacific Island	6 (28.6)	24 (77.4)	
Caucasian/Asian	15 (71.4)	7 (22.6)	0.001
History of atopy, <i>n</i> (%)	14 (66.7)	20 (64.5)	1.000
History of eye rubbing, <i>n</i> (%)	1 (4.7)	11 (35.5)	0.025
BCVA (logMAR)	20/29 (0.16 ± 0.29)	20/110 (0.74 ± 0.50)	<0.001
CCT (μm)	480.1 ± 53.5	388.5 ± 45.7	<0.001
Astigmatism (D)	3.3 ± 2.1	7.9 ± 3.7	<0.001
Epithelium (cells/mm ²)	4654.0 ± 429.4	4128.3 ± 603.4	0.001
Subbasal nerve plexus (mm nerve/mm ²)	12.9 ± 6.1	9.1 ± 4.0	0.015
Anterior stroma (cells/mm ²)	645.4 ± 201.0	441.1 ± 167.6	<0.001
Posterior stroma (cells/mm ²)	239.1 ± 57.2	241.3 ± 69.9	0.904
Endothelium (cells/mm ²)	2510.6 ± 334.4	2345.5 ± 331.8	0.087

Severe disease was associated with younger age at inclusion in the study, younger age at diagnosis, Maori/Pacific Island ethnicity, history of eye rubbing, poorer BCVA, CCT, and astigmatism and lower epithelial cell density, subbasal nerve fiber density, and anterior keratocyte cell density. Age, BCVA, CCT, and keratometry were compared between groups with the Mann-Whitney *U* test. Cell and innervation density were compared between groups with the independent-samples *t*-test.

conic corneas,^{28,32} frequently associated with breaks in Bowman's membrane, and degenerative changes have been observed by electron microscopy, with loss of the keratocyte cell membrane and large cytoplasmic vesicles and lysosomes.²⁹

In this study, we observed a lower keratocyte density in subjects with keratoconus in comparison with that in age-matched control subjects (39.2% lower anterior keratocyte density, 27.5% lower posterior keratocyte density). In a light and electron microscopy study, no change was observed in keratocyte cell density in keratoconus in comparison with

control corneas³³; however, four subsequent IVCM studies have all documented lower keratocyte density in keratoconus.^{8,18,19,25} Notably, Erie et al.²⁵ reported both lower anterior and posterior keratocyte density in contact lens-wearing subjects with keratoconus (31.3% and 40.6% lower than in control subjects, respectively), but no significant reduction in keratocyte density was noted in subjects with keratoconus without a history of contact lens wear.

Hollingsworth et al.¹⁹ used IVCM and reported a 19% lower anterior and 10% lower posterior keratocyte density in keratoconus in comparison with control subjects, although we did not control for contact lens wear or corneal scarring. More recently, Uçakhan et al.¹⁸ published a study demonstrating anterior and posterior keratocyte density 19% and 22% lower than control subjects, respectively. However, images of the stroma were hazy and hyperreflective in 29.2% of keratoconus subjects, correlating with the degree of corneal scarring.

We have previously undertaken a laser scanning IVCM study of keratocyte density in keratoconus⁸ and a significantly lower anterior and posterior keratocyte density was observed in keratoconic eyes with contact lens wear—41.1% and 29% lower than in control subjects, respectively. Although posterior keratocyte density was also significantly lower in keratoconic eyes with no contact lens wear (19.5% lower than controls), the corresponding 15.8% reduction in keratocyte density in the anterior stroma failed to reach statistical significance, possibly due to the small sample size. Since the effect of long-term contact lens wear on the cornea has yet to reach a consensus in the literature, and may confound results, subjects who wore contact lenses were specifically excluded from the present study.^{34–37} Therefore, in the absence of contact lens wear, the present study strongly supports the observation that anterior and posterior keratocyte density is lower in keratoconus, *per se*, and provides evidence that anterior keratocyte density correlates with severity of disease.

Increased visibility of the nerve fibers on clinical examination is one of the characteristic signs of keratoconus,^{1,38} and prominent corneal nerve fibers have been reported in 34% to 91% of subjects.^{18,38,39} It was initially postulated that nerves were more visible in keratoconus, due to thinning of the

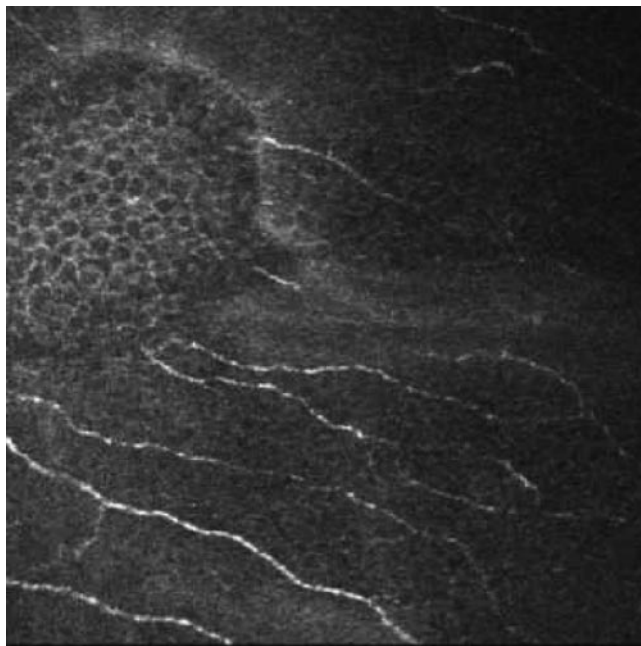


FIGURE 3. Laser scanning IVCM of discontinuity of Bowman's membrane (and subbasal nerve plexus) in keratoconus with presumed invagination of epithelial cells in the top left quadrant of the image (400 × 400 μm).

cornea⁴⁰; however, in a subsequent ex vivo study, our research group has observed localized nerve thickenings in close proximity to breaks in Bowman's membrane with wrapping of anterior keratocytes around the nerve,^{21,24,41} and a small IVCM study of 13 subjects showed an increase in stromal nerve fiber diameter in keratoconus.²³ The subbasal nerve plexus architecture is altered in keratoconus, with fragmentation of the plexus, reduced central nerve fiber density,^{22,23} and wrapping of the corneal nerves around the contour of the base of the cone.²² In the present study, we examined only the central cornea, and thus we cannot comment on the spatiotemporal relationship of nerve changes to corneal thinning outside this area; however, previous mapping of the subbasal nerve plexus in keratoconus has noted lower subbasal nerve density at the site of corneal thinning. Two other groups^{22,24} observed thickening of corneal nerve fibers at the site of destructive changes of the cornea. It has been suggested that corneal nerves may play a role in development and progression of keratoconus,²⁴ with support for this hypothesis coming from the close proximity of stromal nerve changes to breaks in Bowman's membrane^{24,41} and progression of keratoconus observed in a patient with unilateral CNV palsy.⁴² In the present study, we did not assess corneal sensation; however, previous work from our research group demonstrated a reduction in corneal sensation in keratoconus correlated to the severity of keratoconus (Patel DV, unpublished data, 2005). In the present study, we quantitatively documented significant alteration in corneal nerves in keratoconus, with subbasal nerve fiber density 52.7% lower than in control eyes, increased subbasal nerve tortuosity, increased thickness of stromal nerves, and a correlation between nerve density and severity of disease, although whether these alterations play a causative role or are secondary manifestations of the underlying disease remains unknown.

We observed a small but statistically significant lower endothelial cell density in keratoconic eyes than in control eyes. Limited attention has been given to the endothelium in keratoconus, but previous studies have mostly reported that the endothelium is normal in keratoconus (with the exception of corneal hydrops).^{18,43} However, increased apoptosis has been noted at the level of the endothelium in keratoconus,²⁸ and Chi et al.²⁷ noted that although in the early stages of the disease the endothelium appears normal, in advanced disease it exhibited pyknosis and more widely spaced nuclei. However, since the morphology of the corneal endothelium in keratoconus generally appears normal, it is possible that very small differences in endothelial cell density have been overlooked. Certainly, distortion of corneal shape in keratoconus, with a resulting increased posterior surface area, could also explain a relative decrease in density with the original number of endothelial cells covering a greater area.

Despite numerous clinical and laboratory studies, the pathogenesis of keratoconus remains an enigma. In the present study, significant alterations in corneal microstructure and cellular density were observed at every level of the keratoconic cornea in both mild to moderate and severe disease. In particular, alterations in the epithelium, subbasal nerve plexus and anterior stroma correlated with severity of disease. IVCM offers the exciting possibility of imaging corneal changes in early disease and of dynamically monitoring disease progression, thus providing scientists and clinicians with a novel and highly practical bridge between clinical and laboratory observations.

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