

# Corneal Innervation and Cellular Changes after Corneal Transplantation: An In Vivo Confocal Microscopy Study

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**PURPOSE.** Although penetrating keratoplasty is generally considered a successful procedure, transplanted corneal tissue may exhibit abnormal epithelium, decreased sensation, and declining endothelial cell counts after surgery. This study aimed to use in vivo confocal microscopy to correlate corneal microstructure and recovery of the subbasal nerve plexus of the transplanted cornea with indications for, and time from, surgery.

**METHODS.** This was a cross-sectional study comparing corneas from 42 patients after penetrating keratoplasty with those of 30 controls. Subjects were assessed by ophthalmic history and clinical examination, computerized corneal topography, and laser scanning in vivo confocal microscopy.

**RESULTS.** Time from surgery ranged from 1 month to 40 years (mean,  $85 \pm 105$  months). Significant reductions in epithelial ( $P < 0.001$ ), keratocyte ( $P < 0.001$ ), and endothelial ( $P < 0.001$ ) cell densities were noted in comparison with control corneas. Significant reductions in subbasal nerve fiber density ( $P < 0.001$ ) and nerve branching ( $P < 0.001$ ) were also noted. Endothelial cell density decreased with time after surgery ( $r = -0.472$ ;  $P = 0.003$ ), and nerve fiber density ( $r = .328$ ;  $P = 0.034$ ) increased. Keratoconus as an indication for transplantation was associated with higher subbasal nerve fiber densities ( $P = 0.003$ ) than other indications for corneal transplantation. Neither nerve fiber nor cell density was correlated with best-corrected visual acuity.

**CONCLUSIONS.** Laser scanning in vivo confocal microscopy highlights profound reductions in cell density at every level of the transplanted cornea and alterations to the subbasal plexus that are still apparent up to 40 years after penetrating keratoplasty. (*Invest Ophthalmol Vis Sci.* 2007;48:621–626) DOI:10.1167/iovs.06-0538

Corneal transplantation represents the oldest, most common, and most successful form of tissue transplantation worldwide.<sup>1</sup> In New Zealand, approximately 200 corneal transplantations are performed each year for a wide range of indications, with a 1-year survival rate of 87%.<sup>2</sup> Despite these high survival rates, the posttransplantation cornea appears to exhibit changes in cellular structure and function for many years after surgery. Corneal epithelium shows altered morphology and function,<sup>3,4</sup> and endothelial cell density progressively de-

creases at an accelerated rate for up to 20 years after corneal transplantation, with associated changes in endothelial morphology and function.<sup>5,6</sup> Penetrating keratoplasty requires a full-thickness 360° corneal incision that cuts corneal nerves and results in complete denervation of the transplanted cornea. Unsurprisingly, several studies have observed a marked reduction in corneal sensation after corneal transplantation.<sup>7–11</sup> The return of sensation to the donor tissue is highly variable, and in many cases hypoesthesia persists for many years after initial surgery.

Specular microscopy has enabled prospective longitudinal studies of endothelial cell counts to be conducted on the transplanted cornea in vivo,<sup>5,6</sup> but, until recently, studies of other cell layers and innervation have been limited to animal studies<sup>12</sup> and a small number of human ex vivo histologic studies, in the form of examination of failed corneal buttons and postmortem investigations.<sup>13,14</sup> In the past decade, the development of in vivo confocal microscopy has provided a new method for corneal examination, allowing high-resolution images to be obtained in living eyes. With this technique, it is possible to assess the cornea under more physiological conditions than previously possible, enabling the possibility of longitudinal studies of corneal reinnervation and cellular changes after surgery.<sup>15</sup>

The purpose of this study was to elucidate alterations in epithelial, keratocyte, and endothelial cell density and corneal innervation after corneal transplantation with the use of in vivo confocal microscopy.

## MATERIALS AND METHODS

### Subject Recruitment

Thirty-two patients who had undergone penetrating keratoplasty (42 corneas) were recruited from subspecialist corneal clinics in the Department of Ophthalmology, Auckland City Hospital. Patients who wore contact lenses in the preceding year, had a history systemic disease or prescription medication use with known ocular associations, or underwent corneal transplantation less than 1 month earlier were excluded from the study.

Thirty volunteers were recruited as a control group. Subjects in the control group had no ocular abnormality, did not wear contact lenses, were free of systemic disease, and did not take medication known to affect the eye. All control subjects were examined by slit lamp biomicroscopy, and their corneas were confirmed to be clinically normal.

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

### Subject Assessment

All subjects were assessed through clinical history and slit lamp biomicroscopy. Central corneal thickness was measured with a slit-scanning elevation topography system (Orbscan II; Bausch & Lomb Surgical, Rochester, NY). Patient clinical notes were reviewed to determine indications for corneal graft, date of operation, current medication, and postoperative complications. For the purpose of this study, graft rejection was defined as the development of a rejection line (epithelial or

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endothelial) or spreading corneal edema in a previously thin, clear graft with either corneal infiltrates or a unilateral anterior chamber reaction.<sup>16</sup> Increased intraocular pressure (IOP) was defined as a single IOP reading  $\geq 22$  mm Hg after penetrating keratoplasty.

### In Vivo Confocal Microscopy

Laser scanning in vivo confocal microscopy was performed on all subjects with a corneal module (HRT II; Heidelberg Retina Tomograph II Rostock Corneal Module [RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany). This microscope uses a 670-nm red wavelength diode laser source. A 60 $\times$  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. Images produced with this lens measure 400  $\mu\text{m} \times 400 \mu\text{m}$ , and the manufacturer quotes the transverse resolution and the optical section thickness as 2  $\mu\text{m}$  and 4  $\mu\text{m}$ , respectively. The module used (RCM; Heidelberg Engineering GmbH) uses an entirely digital capture system.

The central cornea of the eye that had undergone penetrating keratoplasty was examined in each patient. In the control subjects, one eye was selected at random for examination. Each eye was anesthetized with one drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Surrey, UK), and a coupling agent (Viscotears; Carbomer 980, 0.2%; Novartis, North Ryde, NSW, Australia) was used between the applanating lens and the cornea. 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 using the device's "section" mode. Total duration of in vivo confocal examination was approximately 2 minutes per eye. None of the subjects experienced any visual symptoms or corneal complications as a result of the examination.

### Image Analysis

For each cornea, three images were taken from each of the following levels: basal epithelium, subbasal nerve plexus, anterior stroma, mid 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 layer, posterior stroma was defined as the first three clear images immediately anterior to Descemet membrane, and mid stroma was defined as three images equidistant from Bowman layer and Descemet membrane in the full-thickness section. All images were subsequently randomized and encoded by a single independent observer (DP).

Measurements were performed using a caliper tool (analySIS 3.1; Soft Imaging System, Münster, Germany). For all epithelial and endothelial pictures, a standard central counting frame measuring 200  $\times$  200  $\mu\text{m}$  was used. For all subbasal nerve plexus and stromal images, the full 400  $\times$  400- $\mu\text{m}$  frame was used. Nerve fiber density (NFD) was assessed by measuring the total length of all nerve fibers and branches per square millimeter. Nerve branch density (NBD) was calculated as the number of nerve branches or anastomoses per square millimeter, and nerve branches per millimeter nerve (NBN) was calculated as the number of nerve branches or anastomoses per millimeter nerve. Interobserver error was estimated by reassessing 10% of the images captured by an independent, blinded, second investigator (DP).

### Statistical Analysis

All values were entered into a database (Excel; Microsoft, Redmond, WA) and were subsequently imported into statistical software for analysis. Snellen visual acuities were converted to equivalent LogMAR values for analysis, and statistical analysis was performed (SPSS Version 12 for Windows; SPSS, Chicago, IL). Basic descriptive statistics were calculated on all data gathered, and values are reported as mean  $\pm$  SD. Correlations between continuous variables were examined by calculating Pearson correlation coefficient ( $r$ ). Student independent  $t$  test was used to compare values between two groups. The 95% limits of agreement (LOA) for interobserver repeatability were calculated using

the method of Bland and Altman.<sup>17</sup> All tests were two-tailed, and  $P < 0.05$  was considered statistically significant.

### RESULTS

Results from in vivo confocal microscopy of 42 eyes that had undergone penetrating keratoplasty in 32 subjects were included in the study and compared with a control group of 30 eyes from 30 volunteers. Mean ages were 45.3  $\pm$  17.3 years in the posttransplantation group and were slightly younger, 40.9  $\pm$  10.5 years, in the control group, though this was not statistically significant ( $P = 0.225$ ). No significant difference based on sex was observed between the posttransplantation and the control groups (38% male in the posttransplantation group; 33% male in the control group;  $P = 0.682$ ).

The most common indication for penetrating keratoplasty in the posttransplantation group was keratoconus in 32 corneas (76%), followed by Fuchs' endothelial dystrophy (7%), pseudophakic bullous keratopathy (5%), and infectious keratitis (5%). Posttransplantation patient characteristics are reported in Table 1. Mean time from surgery was 7.1 years (85  $\pm$  105 months; range, 1–480 months). Mean best-corrected visual acuity (BCVA) was 20/50, mean corneal astigmatism was 5.7  $\pm$  3.7 D, and mean keratometry was 45.7  $\pm$  4.8 D.

The 95% limits of agreement for interobserver repeatability were epithelium ( $\pm 7\%$ ), NFD ( $\pm 16\%$ ), anterior stroma ( $\pm 10\%$ ), mid stroma ( $\pm 7\%$ ), posterior stroma ( $\pm 12\%$ ), and endothelium ( $\pm 11\%$ ).

Significant reductions in cell density were observed at all levels of the posttransplantation cornea compared with controls. Significant reductions in subbasal NFD, NBD, and NBN were observed in the transplanted corneas (Table 2). Reduction in endothelial cell density was associated with an increase in central corneal thickness in the posttransplantation cornea ( $r = -0.569$ ;  $P < 0.001$ ). No correlation was observed between NFD and epithelial ( $P > 0.05$ ), endothelial ( $P > 0.05$ ), or keratocyte ( $P > 0.05$ ) cell density in the posttransplantation cornea. No association was observed between age or sex and cell density or nerve fiber parameters at any level of the posttransplantation cornea ( $P > 0.05$ ).

No statistical association was observed between BCVA and endothelial, keratocyte, or epithelial cell density, or innervation density at the time of examination. In addition, no statistical associations were observed between mean keratometry or corneal astigmatism and cell or innervation density. A modest correlation was observed between BCVA and corneal astigmatism ( $r = .380$ ;  $P = 0.038$ ), and corneal astigmatism increased with time after surgery ( $r = .564$ ;  $P = 0.001$ ).

Endothelial cell density in the posttransplantation group decreased with time after penetrating keratoplasty ( $r =$

TABLE 1. Posttransplantation Complications and Topical Medication

	Posttransplantation Corneas	
	No.	Percentage
Postoperative complications		
Previous rejection episode	15	35.7
Previous high IOP	3	7.1
Topical medication		
No topical medication	27	64.3
Topical corticosteroid	15	35.7
Topical chloramphenicol	4	9.5
Topical ocular antihypertensive	3	7.1

$n = 42$ .

**TABLE 2.** Comparison between Control and Posttransplantation Corneal Parameters

Density	Control	Posttransplantation	<i>P</i> *
Basal epithelial cell	6397 ± 1107	5379 ± 1142	<0.001
Subbasal nerve fiber	21.6 ± 5.98	1.83 ± 3.42	<0.001
Nerve branch	86.3 ± 56.2	4.66 ± 10.3	<0.001
Nerve branch/mm nerve	3.68 ± 1.44	0.48 ± 1.12	<0.001
Anterior keratocyte	715 ± 271	372 ± 193	<0.001
Mid stroma keratocyte	340 ± 49.3	194 ± 69.5	<0.001
Posterior keratocyte	297 ± 60.4	198 ± 62.5	<0.001
Endothelial cell	2699 ± 313	1222 ± 682	<0.001
Central corneal thickness	547 ± 27.9	595 ± 47.8	<0.001

*n* = 30 controls; *n* = 42 patients. All values reported as mean ± SD (to 3 s.f.).

\* Student independent *t* test.

-0.472; *P* = 0.003). NFD (*r* = .328; *P* = 0.034) and NBN (*r* = .322; *P* = 0.038) increased with increasing time from surgery. No correlation was observed between epithelial and keratocyte cell density in the posttransplantation patients and the time after keratoplasty (*P* > 0.05).

Endothelial density was reduced in posttransplantation patients with a history of previous graft rejection (1396 ± 697 cells/mm<sup>2</sup> vs. 902 ± 539 cells/mm<sup>2</sup>; *P* = 0.023) and in patients with a history of elevated IOP (1260 ± 697 cells/mm<sup>2</sup> vs. 786 ± 192 cells/mm<sup>2</sup>; *P* = 0.018). However, no significant difference in epithelial or keratocyte cell density or nerve fiber parameters was observed in posttransplantation patients with a history of previous graft rejection or high IOP (*P* > 0.05). No significant association was observed between use of topical medication and any of the corneal parameters measured (*P* > 0.05).

Patients who underwent the initial surgery for keratoconus had higher NFD (2.36 ± 3.77 mm/mm<sup>2</sup> vs. 0.16 ± 0.39 mm/mm<sup>2</sup>; *P* = 0.003), higher NBD (6.12 ± 11.48 branches/mm<sup>2</sup> vs. 0 ± 0 branches/mm<sup>2</sup>; *P* = 0.005), and greater NBN (0.63 ± 1.25 branches/mm vs. 0.00 ± 0 branches/mm; *P* = 0.008) than patients who underwent penetrating keratoplasty for other indications. No association was observed between epithelial, keratocyte, or endothelial cell density and the indication for corneal transplantation (*P* > 0.05).

Qualitatively, subbasal nerve fibers appeared shorter and more tortuous in the posttransplantation corneas than in control corneas (Fig. 1). Particularly abnormal regeneration of the stromal nerves was noted in one patient (Fig. 2), a 37-year-old woman with keratoconus who had undergone bilateral penetrating keratoplasty. Transplantation was performed in the left eye 14 years earlier, and the stromal nerves showed prolific regeneration after a coiled course with an abnormal branching pattern. Stromal nerves in the opposite eye, 17 years after corneal transplantation, were normal.

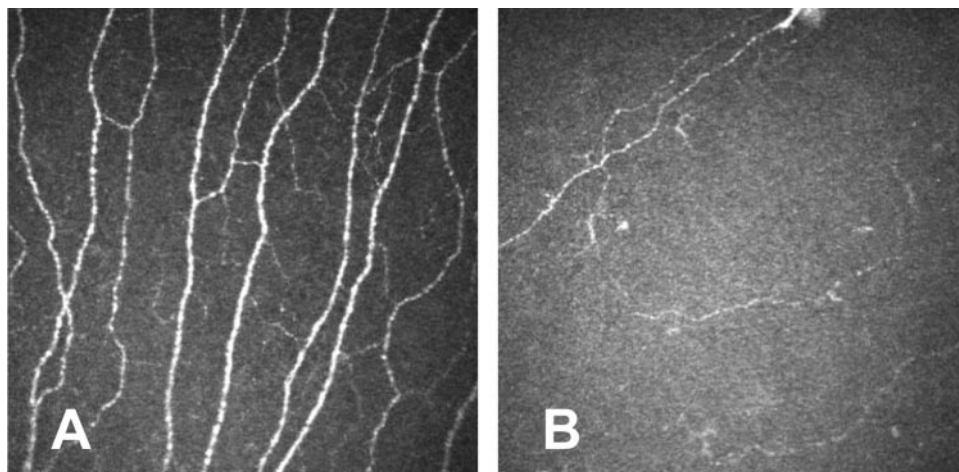
## DISCUSSION

Penetrating keratoplasty is the most frequently performed transplantation procedure, and, though success rates are high, long-term graft survival remains limited by graft rejection, late endothelial failure, and recurrence of the original disease.<sup>1,18,19</sup> This study used in vivo confocal microscopy examination of a cross-section of eyes 1 month to 40 years after corneal transplantation to assess cell density and innervation in the post-keratoplasty cornea.

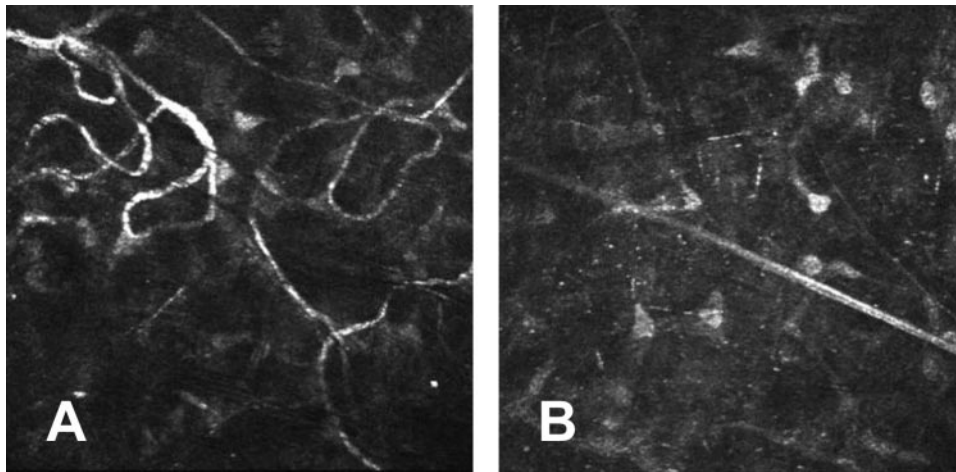
Corneal nerves provide important protective and trophic functions, and interruption of corneal innervation may result in altered epithelial morphology and function, poor tear film, and delayed wound healing.<sup>20-23</sup> Penetrating keratoplasty causes complete sensory denervation of the donor cornea; the nerves of the donor cornea undergo rapid degeneration after transplantation, though Schwann cell sheaths remain empty and intact.<sup>12</sup> In the present study, reduced subbasal NFD was observed in the cornea up to 40 years after penetrating keratoplasty. To our knowledge, this represents the first quantitative study of corneal NFD after corneal transplantation and is consistent with previous observations of highly variable corneal sensation after transplantation, with abnormal sensation observed up to 32 years after corneal surgery.<sup>7-11</sup>

Regeneration of the subbasal nerve plexus occurs at a far slower rate after penetrating keratoplasty than after cataract or refractive surgery.<sup>24,25</sup> The rate of nerve fiber regeneration after surgery is dependent on depth and circumference of incision. An incision smaller than approximately 50% corneal thickness or smaller than 360° circumference spares some of the stromal nerves, allowing neural regeneration from adjacent stromal nerves.<sup>26</sup> Impaired sensory innervation after penetrating keratoplasty may contribute to the relatively high frequency of epithelial complications observed after the procedure.<sup>4</sup>

No correlation between recipient age and reinnervation was observed in this study. A similar lack of correlation be-



**FIGURE 1.** Subbasal nerve fibers after penetrating keratoplasty. (A) Normal subbasal nerve fibers in a control subject. (B) Decreased subbasal nerve fiber density after penetrating keratoplasty.



**FIGURE 2.** Abnormal stromal nerve regeneration after keratoplasty. (A) Prolific regeneration of corneal stromal nerves in the left eye 14 years after corneal transplantation. (B) Normal appearance of a stromal nerve in right eye 17 years after transplantation.

tween return of sensation and recipient age after penetrating keratoplasty was noted in an earlier study of 71 corneas.<sup>11</sup> Others, however, have observed a correlation between age and return of sensation up to 3 years after surgery. Faster return of sensation and nerve regeneration is reported in young rabbits after corneal transplantation,<sup>12,27</sup> and studies have demonstrated quicker peripheral reinnervation and better nerve morphology in younger animals,<sup>28</sup> possibly because of age-related impairment in Schwann cell-axon interactions. No regeneration of the Schwann cells has been observed in transplanted corneas, and regenerating nerves occupy the empty Schwann cells of the donor cornea.<sup>10,12</sup> Theoretically, therefore, donor age may influence reinnervation.

If the subbasal plexus was regenerated, nerve fiber morphology was markedly altered, resulting in increased nerve tortuosity, reduced branching pattern, and shorter nerve length. Qualitative changes in corneal innervation after corneal transplantation, with slow reinnervation and increased nerve tortuosity, have been reported and have also been noted in regenerated corneal nerve fibers after refractive surgery and in the diabetic cornea.<sup>29,30</sup>

Patients who underwent penetrating keratoplasty for keratoconus experienced greater regeneration of the subbasal nerve plexus than patients who underwent surgery for other indications. Although a faster return of corneal sensation after corneal transplantation in patients with preoperative diagnoses of keratoconus has been reported,<sup>27</sup> other studies have observed no significant difference in recovery of sensation.<sup>7,9,11</sup> After corneal transplantation, corneal nerve regeneration proceeds from the peripheral recipient cornea, through the scar tissue, and into the donor cornea.<sup>10,12</sup> Hence, it may be that corneal nerve regeneration is dependent on peripheral corneal innervation. Many diseases requiring corneal transplantation, including keratoconus, are associated with abnormal corneal innervation and reduced corneal sensation.<sup>31-35</sup> However, unlike other indications for corneal transplantation in this study, keratoconus is a disease primarily of the central cornea, with relative sparing of the peripheral cornea.<sup>35</sup> In a recent study by Patel and McGhee,<sup>36</sup> *in vivo* confocal microscopy mapping of the subbasal nerve plexus in keratoconus revealed that though the corneal nerves at the keratoconus cone were markedly disrupted, peripheral innervation remained intact. Perhaps retention of healthy peripheral innervation in keratoconus is responsible for greater regeneration of corneal nerves, as demonstrated in this study. Alternatively, the keratoconus group might have been younger than patients who underwent penetrating keratoplasty for other indications and might have experienced faster corneal nerve regeneration. This study failed,

however, to observe an association between recipient age and nerve regeneration in the transplanted cornea.

The density of the basal corneal epithelium in postkeratoplasty corneas was reduced compared with those in the control group in the present study. Changes in the basal epithelial cell morphology and increased cell area have previously been noted by specular microscopy with the use of a contact lens to view the epithelium.<sup>37</sup> The high frequency of epithelial complications, particularly in the first year after keratoplasty, suggests abnormal epithelial function, and studies have identified altered epithelial metabolism and barrier function for several years after corneal transplantation.<sup>3,4</sup> No correlation was observed in the present study between time after penetrating keratoplasty and basal epithelial cell density, in contrast to the results of Imre et al.<sup>38</sup> who found a reduction in epithelial cell density between 15 months and 66 months after keratoplasty.

In the present study, keratocyte density was observed to be significantly lower in the posttransplantation corneas than in corneas in the control group. *In vivo* confocal microscopy has enabled the imaging of keratocytes in living patients, but to date only three published studies have examined keratocyte density after penetrating keratoplasty, with conflicting results.<sup>5,38,39</sup> Bourne et al.<sup>5</sup> showed a reduction in keratocyte density after transplantation at every level of the stroma. Keratocyte density was not reduced with duration of time after transplantation. Conversely, Mikek et al.<sup>39</sup> found no difference in keratocyte density between normal corneas and posttransplantation corneas. Why keratocyte density is reduced in the posttransplantation cornea is unclear. Increased apoptosis has been noted in transplanted corneas, particularly at the wound edge.<sup>40</sup> Donor cells initially persist in the donor cornea but are gradually replaced by host cells, though small subpopulations of donor keratocytes may persist in the cornea up to 5 years after transplantation.<sup>41-43</sup> It is also possible that reduced keratocyte density in the post keratoplasty cornea does not represent true loss of keratocytes but is instead the consequence of either binomial expansion caused by edema (keratocytes are conserved but are distributed in a larger volume) or optical artifact (image quality degraded by edema).

Reduced endothelial cell density in the posttransplantation cornea was identified in this study in correlation with time after keratoplasty, previous rejection episodes, and previous high IOP. *In vivo* confocal microscopy allows noninvasive imaging of changes in endothelial morphology after transplantation and may confer an advantage over specular microscopy in the imaging of patients with significant corneal edema.<sup>44</sup> The present study tends to support the observations of Bourne et al.,<sup>5,45-47</sup> who illustrated accelerated endothelial cell loss

over time in postgraft corneas (4.2% per year compared with 0.6% in healthy subjects and 2.5% in patients after cataract surgery). The cause of accelerated endothelial cell loss after penetrating keratoplasty was uncertain. An immunologic mechanism has been suggested, and a study of endothelial cell loss in autologous keratoplasty compared with homologous keratoplasty showed lower endothelial cell loss in the autologous transplants.<sup>48</sup> Furthermore, long-term increases in aqueous flare have been noted after keratoplasty, suggesting chronic, subclinical inflammation.<sup>49</sup> However, light and electron microscopy study of late endothelial failure provides no evidence of immunologic reaction,<sup>50</sup> and immunologic reaction alone does not explain why accelerated loss of endothelial cells is also observed, albeit to a lesser extent, in eyes after cataract surgery.<sup>5</sup>

This study noted a correlation between endothelial cell density and central corneal thickness. Despite this association, however, many grafts with profoundly reduced endothelial cell densities retain graft clarity and function, possibly because of the concurrent reduction in intercellular spaces associated with larger cell areas.<sup>6</sup> Patients who experienced previous corneal graft rejection had lower endothelial counts than those with no history of graft rejection. Corneal graft rejection has been associated with loss of endothelial cells,<sup>43,51</sup> but the degree of endothelial cell loss after graft rejection may be increased in older recipients and with delay in diagnosis.<sup>51</sup>

BCVA was associated with astigmatism, but no correlation was observed between BCVA and cell or innervation density. However, this was a cross-sectional study representing a wide range of visual acuity and time after penetrating keratoplasty. The role, if any, of cell density and cell reflectivity on BCVA might be further explored in a longitudinal study of corneal transplantation.

In conclusion, in vivo confocal microscopy offers exciting insight into microstructural changes in the posttransplantation cornea. This study highlights a profound reduction in cell density at every level of the transplanted cornea and alterations to the subbasal plexus that are still apparent up to 40 years after penetrating keratoplasty, which may have important implications for corneal wound healing and the health of the transplanted cornea. Further longitudinal studies are necessary to investigate the role of these alterations in graft survival, visual acuity, and postoperative complications.

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