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CLINICAL STUDY

Quantitative analysis of corneal microstructure in keratoconus utilising *in vivo* confocal microscopy

KH Weed[1], CJ MacEwen[1], A Cox[1] and CNJ McGhee[2]

Abstract

Purpose **To establish and quantify the *in vivo* confocal microscopic features of moderate to advanced keratoconus.**

Methods **Nineteen keratoconus subjects were catergorised using Orbscan-derived corneal apex power and pachymetry as exhibiting moderate ($n=7$) and advanced ($n=12$) keratoconus. Control subjects included 23 noncontact lens wearers (Group A) and 15 contact lens wearers (Group B). All subjects underwent Confoscan slit scanning *in vivo* confocal microscopy.**

Results **Compared with Group A (4912 ± 434 cells/mm^2), basal epithelial density was significantly lower in both moderate (4592 ± 414 cells/mm^2, $P<0.05$) and advanced keratoconus (4530 ± 596 cells/mm^2, $P=0.01$). In comparison to Group A (761 ± 118 cells/mm^2), anterior stroma keratocyte density was significantly greater in both moderate keratoconus (883 ± 111 cells/mm^2, $P=0.001$) and advanced keratoconus (952 ± 122 cells/mm^2, $P<0.001$). Compared to Group A (504 ± 80 cells/mm^2) posterior stroma keratocyte density was also significantly greater in advanced keratoconus (599 ± 97 cells/mm^2, $P<0.001$) and posterior stromal keratocyte density appeared to increase with increasing severity of keratoconus ($P<0.05$). However, comparing control Groups A and B, contact lens wear *per se*, was associated with significantly reduced ($P=0.000$) keratocyte density in the anterior stroma (609 ± 66 cells/mm^2) and demonstrated a trend ($P=0.056$) in the posterior stroma (470 ± 63 cells/mm^2). Keratoconic corneas (429 ± 72 μm) were significantly thinner than control Groups A (508 ± 77 mm) and B (495 ± 80 μm). The presence of keratoconus did not affect the endothelial cell density ($P=0.54$).**

Conclusion ***In vivo* confocal microscopy can provide insight into the microstructural changes that occur in keratoconus.**

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Keywords: in vivo confocal microscopy; keratoconus; cornea; contact lens

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Introduction

Keratoconus is a noninflammatory corneal ectasia which is usually bilateral and progressive.[1] The incidence of this ocular disease is approximately 1 per 2000 in the general population[2]; however, the precise aetiology of keratoconus is still unknown.[3] Keratoconus manifests itself from a 'forme fruste' or subclinical condition, only detectable by computer video-keratoscopy[4] (CVK) to severe eye disease with the typical biomicroscopy signs: apical protrusion with corneal thinning and scarring; Vogts striae and Fleishers ring. Initially, the treatment of choice for visual rehabilitation is rigid gas permeable contact lens wear,[5] however, when this becomes unsuitable penetrating keratoplasty can be performed.[6] This study's aim was to establish and quantify the *in vivo* confocal microscopic of moderate to advanced keratoconus and compare these data to a healthy control group.

Materials and methods

The Dundee University Scottish Keratoconus Study (DUSKS) is a long-term prospective study of 200 subjects with keratoconus resident in Tayside region of Scotland. This study has

received local ethics committee approval and written informed consent was obtained from all participants in the current study, before investigations. All subjects were assessed in regard to prior medical, ocular and contact lens wearing history. All corneas were assessed using the Orbscan™ (Bausch & Lomb, UK) slit scanning system to determine the area of least corneal thickness and to establish apical power.

A subset of 19 keratoconic subjects (nine female and 10 male, mean age 40.53 ± 2.12 years, range 20–56 years old) were recruited from patients attending the Ophthalmology Department, Ninewells Hospital and Medical School, Dundee. All subjects had clinical as well as videokeratographic signs of keratoconus. The severity of keratoconus was classified on the basis of corneal thickness ($<400\,\mu m>$) and apical power ($<55\,D>$). The mean number of contact lens wearing years for the keratoconic subjects (all RGP wearers) was 11.44 ± 10.52 (2–35) years.

A control group of noncontact lens wearers (Group A, nine female and 14 male, mean age 38.09 ± 2.19 years old) was recruited from staff working within Ninewells Hospital, as was a second control group of contact lens wearers (Group B, 11 female and four male, mean age 28.73 ± 1.96 years old). Group B was used only to investigate the effect of contact lens wear *per se* on normal corneal stromal keratocytes. In Group B, the mean number of years of RGP ($n = 6$) wear was between 10.7 ± 3.3 (range 6–15 years) and 4.2 ± 2.1 years for the soft ($n = 9$) contact lens wearers (range 1–6 years).

Thereafter, following topical anaesthesia (Amethocaine HCL 1%) the cornea was assessed by confocal microscopy (Confoscan™ Bausch & Lomb) using a pea-sized drop of immersion gel (Viscotears, CIBA Vision) on the objective lens (Achroplan 40/0.75 WO) to couple the instrument to the cornea in a noncontact fashion. The right eye of all subjects underwent confocal microscopy. Each subject was asked to fixate on the internal flickering yellow light during the 14 s assessment. The objective lens was brought forward slowly towards the corneal apex, until corneal epithelial cells were seen on the video monitor. The objective lens was manually focused on the corneal endothelium and then slowly refocused on the epithelium. This allowed scanning of all the corneal layers. A $40\times$ objective lens provided a field of view of 330×240 um. Owing to the immersion fluid, the corneal epithelium was not touched during any part of the assessment.

The corneal images were detected by the light sensitive LCD camera, which were then digitised in a PC-based imaging system and stored on videotape (S-VHS). One unmasked observer then identified (1) basal epithelial cells (2) anterior and posterior keratocytes and (3) endothelial cells. The number of cells/mm^2 was then calculated by the proprietary software within the confocal microscope.

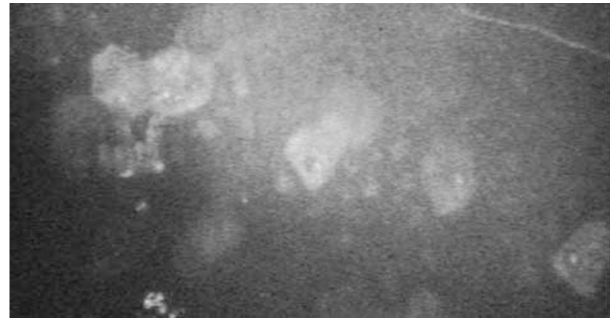

Figure 1 Desquamated superficial cells with bright cell boundaries.

Figure 2 Intermediate basal epithelium with reflective cell bodies.

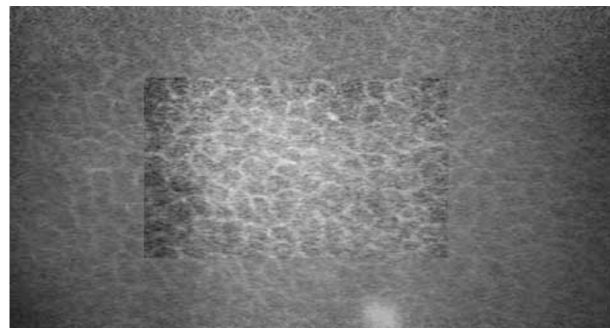

Figure 3 Basal epithelium cells in keratoconus subjects were easily visible with reflective cell borders and dark nonreflective cell bodies.

Although superficial cells and intermediate epithelial cells (Figures 1 and 2) were visible, consistency was not achieved in these layers. The confocal image quality was sufficient to allow 100% counting of cell densities in the basal epithelium only. The basal cell layer was clearly demarcated with reflective cell borders to dark nonreflective cell bodies (Figure 3). Bowmans Layer appears as a transitional zone bordered by the basal epithelium and the anterior stroma, only distinguished by branching nerve fibres against an amorphous

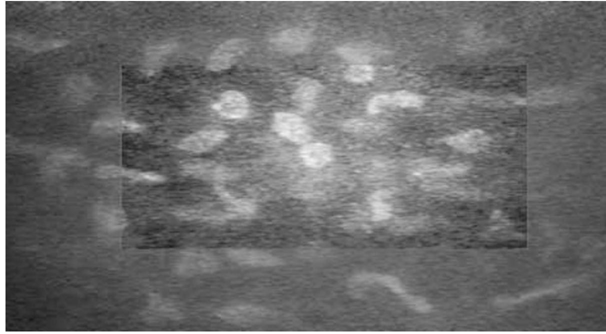

Figure 4 Anterior stromal keratocytes demonstrating oval/ellipsoid nuclei within a moderate keratoconus subject's stroma.

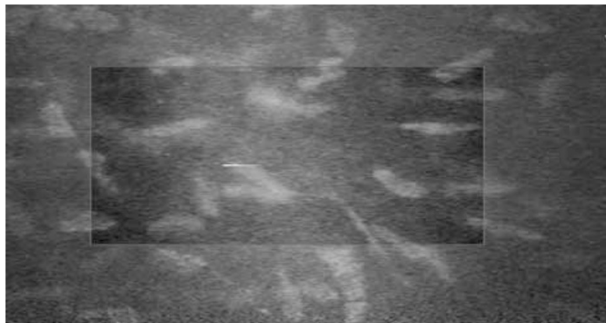

Figure 5 Posterior stromal keratocytes demonstrating elongated/spindle-shaped nuclei within a moderate keratoconus subject's stroma.

Figure 6 Anterior stromal scarring with corneal nerve innervation within a moderate keratoconus subject's stroma.

background. Corneal nerve fibres were highly reflective and were seen in both groups, innervating the area of Bowman's layer and into the anterior stroma.

In the corneal stroma the reflective keratocyte nuclei were seen against the darker ground substance; however, neither cytoplasm, cell boundaries, or collagen lamellae were visible. The keratocyte density was measured in two zones (1) just below Bowmans membrane (anterior stroma; Figure 4) and (2) just above the corneal endothelium (posterior stroma; Figure 5). In three cases within the moderate keratoconic group, stromal scarring prevented immediate visualisation of the anterior keratocytes directly under Bowmans (Figure 6).

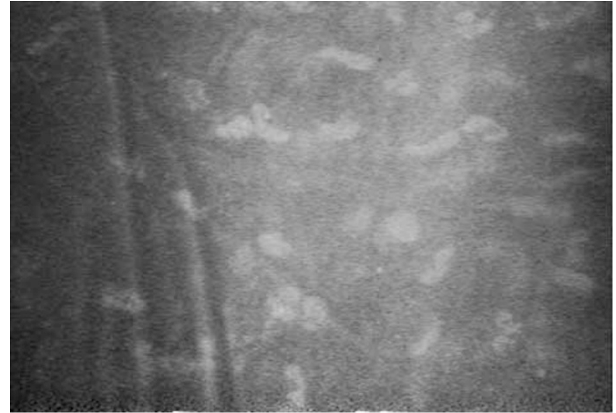

Figure 7 Stress lines within the posterior stroma of a moderate keratoconus subject. Vogts Striae are seen as dark lines within the stroma.

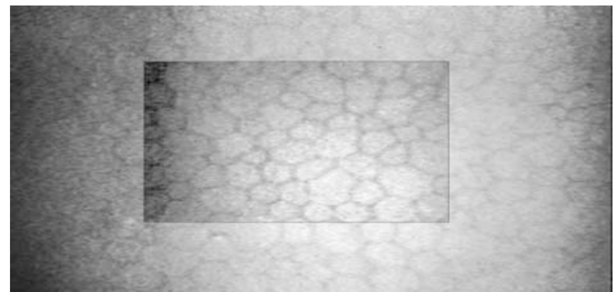

Figure 8 Endothelium mosaic of a mild keratoconus subject consisting of bright cell bodies and dark cell boundaries.

Although the immersion objective was normally positioned in front of the apparent corneal apex, in such cases the objective lens was repositioned until clarity was achieved.

The dark bands observed in the posterior stroma of keratoconic patients, (Figure 7) are similar to those observed by Somodi *et al*[7] and Hollingsworth and Efron[8] as being consistent with Vogt's striae. The banded appearance seen *in vivo* confocal microscopy was postulated to represent an irregular separation and spacing of individual collagen fibrils within the lamellae.[8] Descemet's membrane was not distinguishable in either group, however, the endothelial cell mosaic was easily visible with bright cell bodies and dark cell boundaries even in cases with dense stromal scarring (Figure 8).

Statistical methods

Analysis of variance (ANOVA) was performed to determine how much of the variability in corneal morphology could be explained by the presence and

severity of keratoconus. A contrast was performed to compare the groups with mild and moderate keratoconus against the controls and also to compare both these groups with each other. The age of the subject was included as a covariate to control for the expected effect of this variable. Levenes test for equality of variance was significant ($P=0.01$) for the ANOVA of basal epithelial cells. In order to avoid violating the assumption of equal variance, this data were transformed (squared transformation) before analysis. Levenes test was not significant for all other dependent variables. For each of the groups the mean density is quoted along with the standard deviation.

Results

Orbscan measurement and classification of disease severity

In the keratoconus group the mean corneal apex power, measured at the thinnest part of the cornea using the Orbscan system was 52.55±4.87 D (48–61.63 D) with a mean corneal thickness at this point of 429±72 μm (300–529 μm). The 'moderate' group ($n=7$) was defined by corneal apex powers which ranged between 48 and 55 D, with a mean corneal thickness of 473±43 (420–529) μm. The 'advanced' group ($n=12$) included corneal power greater than 55 D, with a mean corneal thickness of 348±30 (300–395) μm. The mean corneal thickness for the control Groups A and B were 508±80 and 495±77 μm, respectively

In vivo *confocal microscopy*

The principle of confocal microscopy has been described in detail elsewhere[9,10] and there were no adverse effects following assessment.

Basal epithelium

The mean basal epithelial density was 4912±434 cells/mm^2 for the noncontact lens wearing control group. There was a significantly lower basal epithelial density in those with moderate keratoconus (4592±414 cells/mm^2, $P<0.05$), and in those with advanced keratoconus (4530±596 cells/mm^2, $P=0.01$). The difference in basal epithelial cell density between those with moderate and advanced keratoconus was not significant. There was a nonsignificant tendency for increasing epithelial density with increasing age ($P=0.06$). In the keratoconic group, desquamated superficial cells were easily visible with bright cell boundaries. No differences were observed within Bowmans' layer between the control and keratoconic group.

Stroma

The mean anterior keratocyte density was 761±118 cells/mm^2 for the noncontact lens wearing control group. There was a significantly greater keratocyte density in those with moderate keratoconus group (883±111 cells/mm^2, $P=0.001$) and in those with advanced keratoconus (952±122 cells/mm^2, $P<0.001$). However, the trend for increasing keratocyte density with increasing severity of keratoconus did not reach significance ($P=0.1$). There was a nonsignificant tendency for decreasing keratocyte density with increasing age ($P=0.056$). In comparison to the contact lens wearing control group (609±66 cells/mm^2), both the keratoconic group ($P=0.000$) and the nonwearing group ($P=0.000$) had significantly higher density of keratocytes.

The mean posterior keratocyte density was 504±80 cells/mm^2 for the noncontact lens wearing control group and 550±54 cells/mm^2 for the moderate keratoconus group. There was a significantly greater keratocyte density in those with advanced keratoconus (599±97 cells/mm^2, $P<0.001$). The increase in keratocyte density with increasing keratoconus was significant ($P<0.05$). In comparison to the contact lens wearing control group (470±63 cells/mm^2), the keratoconic group ($P=0.000$) had a significantly higher density of keratocytes and the noncontact lens wearing control group demonstrated a nonsignificant tendency ($P=0.056$).

There was a significant tendency for decreasing posterior keratocyte density with increasing age ($P<0.05$). There was a significant reduction in keratocyte density from the anterior to the posterior stroma for all groups, however, the rate of decrease was significantly higher in both keratoconic groups ($P<0.05$) compared with the controls.

Endothelium

The mean endothelial density was 3043±264 cells/mm^2 for the noncontact lens wearing control group. The presence of keratoconus did not affect the endothelial cell density which was 2888±380 cells/mm^2, $P=0.16$ in those with moderate keratoconus and 2941±464 cells/mm^2, $P=0.54$ in those with advanced keratoconus. Age did, however, significantly affect the endothelial cell density for all groups, with a decrease in cell density with increasing age ($P=0.001$). The mean endothelial size (μm) was not significantly different ($P=0.18$) for any of the groups (328±32 for the control group and 348±23 for the keratoconus group). Age did, however, significantly affect the endothelial cell size, with an increase in cell size ($P<0.01$) with increasing age for all groups.

Discussion

Within the literature there are two main theories in relation to the role of keratocytes in the development in keratoconus: keratocyte apoptosis or the modification of substances synthesised or expressed by the keratocytes. Additionally, contact lens wear in itself may[11–14] influence the corneal keratocyte population. During corneal development keratocytes are responsible for the production of the corneal collagen fibrils and mucopolysaccharides that comprise the ground substance. The collagen fibrils are organised parallel to each other within each lamella and it is possible that keratocytes are responsible for tethering these lamellae in order to maintain the correct spatial anatomical relationship. Indeed, keratocyte cell thickness is influenced by the physical pressure exerted by adjacent stromal lamellae.[15] From biomechanical studies, interlacing collagen lamellae within the human cornea appear to be an important factor in the cohesive and tensile strength of the cornea[16] especially within the anterior stroma.[17]

Disappearance of anterior keratocytes following epithelial injury[18] is mediated by apoptosis (programmed cell death), which is induced by the interleukin-1 (IL-1) system. Such injury can be due to mechanical forces such as contact lens wear and/or eye rubbing, both of which have been cited as possible aetiological factors in keratoconus.[2] In our study there was a reduction in basal epithelium density within the keratoconic corneas. Hollingsworth *et al*[19,20] also demonstrated this reduction and found similar difficulties in obtaining consistent imaging of the superficial epithelial layers. These authors also reported evidence of epithelial elongation, although this was not seen in our study this could be due to differences in experimental methodology. Hollingsworth *et al*[19,20] imaged the central cornea while our study examined the corneal apex (except in three cases where stromal scarring prevented good visualisation at the apex).

Greiner *et al*,[21] however, demonstrated that in a rabbit model, eye rubbing caused alterations to the epithelium of the upper tarsal conjunctiva and not to the squamous epithelium of the cornea. Perhaps the greater epithelial fragility of keratoconics is the differentiating factor,[22] as the epithelium has been shown to be involved with keratoconus development.[23,24] Bureau *et al*[25] demonstrated that keratocytes from keratoconic corneas have a greater number of IL-1 receptors than normal. This increased expression of the IL-1 receptor may sensitise the keratocytes to IL-1 from the epithelium, thereby causing keratoctye apoptosis and a subsequent decrease in stromal mass over time.[26] Keratoconic corneal tissue examined histopathologically supports apoptosis as the mode of cell death.[27,28]

Although the contact lens association with stromal hypoxia, hypercarnia, and thinning has been well documented,[29–32] there is conflict within the reported *in vivo* confocal literature as to how contact lens wear affects the corneal keratocyte (see Table 1 [11–14, 33–35]). Variations in the reported number of keratocytes may be due to the contact lens type worn, the number of years wear and/or wearing schedule or even the type of *in vivo* confocal microscope used.

Jalbert and Stapleton[13] and Efron *et al*,[12] in assessing extended soft contact lens wear, both found reduction in keratocyte density in the posterior stroma within normal corneas. Jalbert and Stapleton[13] additionally demonstrated a reduction in the anterior stroma as well, as did Bansal *et al*[11] using a mix of contact lens types. Kallinikos and Efron[14] also reported significant decreases in anterior and posterior keratocyte densities as a result of soft and rigid contact lens wear in neophytes after 2 h within an anoxic environment. However, Efron reported differently in a third study, this time with Hollingsworth and Efron,[33] when they agreed with Patel *et al*[34] who found no changes in anterior or posterior keratocyte density with normal corneas. All authors used a scanning slit *in vivo* confocal microscope, except Patel *et al*[34]and Erie *et al*[35] who used a tandem scanning version.

Detecting the actual stromal depth of the confocal image is less accurate with the scanning slit than the tandem *in vivo* confocal[34] and therefore it is possible that images in the anterior 10% of the stroma (where there is the highest density of keratocytes) could be variably included within the groups. Additionally, the measurements recorded by the scanning slit authors cite the density in mm^2, whereas Patel *et al*[34] and Erie *et al*[35] report the volume in mm^3. This variation becomes vital in interpretation of results when the corneal thickness measurements are different.

As the apical ectasia with keratoconus progresses there is a decrease in corneal thickness.[2] In our study, corneal thickness was measured with the Orbscan which has been found to be noninvasive and reproducible,[36,37] although it measures normal corneas to be thicker than with ultrasound devices (in the order of 28 μm).[38,39] Within the literature, the definition of what constitutes early, mild, moderate, advanced, or severe keratoconus varies.[40–43] In our study an empirical combination of corneal apex power[44] and thickness was used.[45] Therefore, in this context the *in vivo* confocal microscopy data may not always be readily correlated with the small number of published studies on the topic.

Erie *et al*[35] observed that the decrease in the keratocyte density, in a study of mild to moderate keratoconus was only in association with contact lens wear. As, all our

Table 1 Effect of contact lens wear on stromal keratocyte densities

	Bansal *et al*[11]	Jalbert and Stapleton[13]	Efron *et al*[12]	Patel *et al*[34]	Erie *et al*[35]	Kallinikos and Efron[14]	Hollingsworth and Efron[33]	Weed *et al*
In vivo confocal microscope	Scanning slit	Scanning slit	Scanning slit	Tandem scanning	Tandem scanning	Tandem scanning	Tandem scanning	Scanning slit
Contact lens normals	Soft $n=18$ RGP $n=15$ PMMA $n=6$	EW soft $n=9$	EW soft $n=23$	Soft $n=11$ Mixed $n=6$ RGP $n=2$ PMMA $n=1$	Soft $n=9$ RGP $n=3$	Soft $n=20$ RGP $n=20$	RGP $n=22$	Soft $n=9$ RGP $n=6$
Non-CL wearers normals	$n=36$	$n=9$	N/A	$n=20$	$n=17$	$n=20$	$n=22$	$n=23$
keratoconus	N/A	N/A	N/A	N/A	$n=17$	N/A	N/A	N/A
Contact lens keratoconus	N/A	N/A	N/A	N/A	RGP $n=9$ soft $n=3$	N/A	N/A	RGP $n=19$
Age CL (years)	37 (23–57)	35 (25–43)	23	38 (25–54)	40 (20–70)	26	44	29 (18–41)
Age non-CL (years)	38 (20–73)	36 (31–40)	N/A	37 (26–50)	39 (20–72)	26	N/A	38 (20–58)
Number years wear normals		5.9 (5.3–6.5)	6 months	18.1 (10–33)	6 (2–18)	2 h anoxia	22 (6–30)	7 (1–15)
Number years wear keratoconus	N/A	N/A	N/A	N/A	7 (2–30)	N/A	N/A	11 (2–35)
Normals								
ASK CL	757 mm^2	544 mm^2	1112 mm^2	30.545 mm^3 32 056 mm^3	35 630 mm^3	See	958 mm^2	609 mm^2
ASK non-CL	925 mm^2	804 mm^2	N/A		31 168 mm^3	graph	1028 mm^2	761 mm^2
Keratoconus			—	N/A				
ASK CL	N/A	N/A	N/A	N/A	24 564 mm^3		N/A	883/952
ASK non-CL	N/A	N/A	N/A		32 724 mm^3		N/A	N/A
Normals								
PSK CL	N/A	514 mm^2	560 mm^2	21 812 mm^3	18 704 mm^3	See	580 mm^2	470 mm^2
PSK non-CL	N/A	628 mm^2	N/A	20 920 mm^3	18 129 mm^3	graph	557 mm^2	504 mm^2
Keratoconus								
PSK CL	N/A	N/A	N/A	N/A	11 118 mm^3		N/A	550/599
PSK non-CL	N/A	N/A	N/A	N/A			N/A	N/A
Conclusion	CL wear reduces ASK density only normal corneas	EW soft CL reduces A and PSK density normal corneas	CL wear reduces PSK density only normal corneas	Daily CL no effect on normal corneas A or PSK density	Decrease A and PSK density with CL wear only with Keratoconus	RGP and soft CL wear reduces A and PSK density normal corneas	RGP CL no effect on A or PSK density normal corneas	CL wear reduces ASK density normal corneas

ASK = anterior stromal keratocyte; PSK = posterior stromal keratocyte; CL = contact lens; EW = extended wear; RGP = rigid gas permeable.

subjects needed to wear contact lenses for visual rehabilitation and exhibited corneal signs of keratoconus, including corneal scarring, the mean corneal thickness was considerably less ($429 \pm 72\,\mu$m) in comparison to Erie *et al*[35] since the keratoconic populations are different in their severity. The difference in corneal thickness may account for the differences in the results. Within our study, as the corneal protrusion advanced the corneal thickness decreased compressing the keratocytes within the stroma at the corneal apex, the measured density will increase for the same confocal slice thickness.

However, between the control groups, contact lens wear *per se* was associated with a significantly reduced anterior keratocyte density and demonstrated a reduction trend toward reduced keratocyte density in the posterior stroma. These data support most of the existing data in 'normal' eyes;[11–14] however, it is at variance with the postulate of Erie *et al*[35] that only diseased eyes demonstrate keratocyte reduction with contact lens wear. Unfortunately, there were no moderate or advanced keratoconics who had not worn contact lenses to compare the effects of contact lens wear with keratoconus in our study.

In a very recent series of *in vivo* confocal microscopy studies, Hollingsworth *et al*[8,19,20,46] have provided novel data with which the current study can be compared. One important difference between the two studies previously commented on is the corneal location examined (central cornea[19,20] *vs* corneal apex). Keratocyte cell density may not be uniform across the keratoconic cornea and this may explain the higher anterior and posterior keratocyte densities reported in our study. Additionally, the keratoconic group examined by Hollingsworth *et al*[20] also included a mixture of contact lens wearers and noncontact lens wearers and was compared to a noncontact lens wearing control group while in our study, only keratoconic contact lens wearers were examined and the stromal keratocyte density was compared with two control groups (contact lens and non contact lens wearers) separately.

The keratocyte shape is specific for corneal location.[47] In the anterior stroma the keratocyte nuclei are more ellipsoid, whereas in the posterior stroma the nuclei appeared more elongated/spindle-shaped (Figures 4 and 5, respectively). This regional difference has been reported elsewhere,[8] although Poole *et al*[48] reported the exact opposite. The functional relevance of these regional keratocyte shapes is unknown, although it has been hypothesised that different keratocytes produce different substances.[49] This is supported by the nonuniform proteoglycan distribution within the stroma.[50]

The production of abnormal substances within the keratoconic stroma previously has been reported: with abnormal production of matrix metalloproteinase-2;[51] upregulation of the expression of MT1-MMP;[52] overexpression of keratocan;[53] reduction in expression of type X11 collagen[54] and modification of type V1 collagen synthesis[55] all being documented. Although keratocytes appear to provide a vital role in the stability of the corneal structure,[17] perhaps the corneal changes exhibited by keratoconics are due to substances synthesised or expressed by keratocytes.

In this study, the anterior and posterior stromal keratocytes in the control group are similar to that reported within the literature[56,57] and the reduction in keratocyte density from the anterior to the posterior stroma is widely recognised.[58–62] Keratoctye density, in addition, has been reported to decrease with increasing age,[60,62,64] however, other authors[57,63] have reported consistency in the corneal keratocyte populations throughout life. In this study, reduction in keratocyte density with age was found to be significant in the posterior stroma only, with a strong trend within the anterior stroma.

The corneal endothelium is a monolayer of polygonal cells, the density of which is highest at birth (more than 3000 cells/mm^2)[65] and steadily reduces slowly with age.[66] This reduction with age was significant in the keratoconic and control group in our study. Anecdotally, we observed polymegathism (variability in cell size) and pleomorphism (variability in cell shape) within the keratoconic group (control group were non contact lens wearers). Additionally, there was an apparent decrease in the frequency of hexagonal cell shapes within the endothelium, all of which has been previously reported elsewhere with keratoconics.[20,67–69] Similar observations have been documented within 'normal' corneal endothelium due to the presence of contact lens wear.[70] We found no difference in endothelial density between the keratoconic group and the control group (age was included as a covariate), which differs from Hollingsworth *et al*[20] who reported a higher endothelial density within the keratoconic group. This could be due to differing keratoconic populations in age and disease composition and with the experimental corneal location differences.

Limitations of this part of the study include (a) data being interpreted by an unmasked observer and (b) the problem of identifying where exactly the image is located in the X, Y and Z axes of the cornea. Using randomised images examined by a masked observer would eliminate the potential observer bias, however, this was not feasible. Anterior stromal data were taken from the region of tissue that comes into focus immediately posterior to Bowman's layer and similarly the posterior stroma data were taken from the video image immediately anterior to the endothelial layer, in order to minimise error in depth (Z axis) location.

Progression of subclinical keratoconus has been identified by serial corneal topography analysis.[71] Further *in vivo* confocal study is needed to follow patients with keratoconus from diagnosis to the end stage. Changes within the cornea can then be compared at varying stages of the disease especially the interplay between corneal thickness and keratocyte density. The impact of soft contact lenses on the cornea has been demonstrated by a monozygotic twin study.[72] Indeed, the majority of work on this subject (including this study) supports a degree of corneal thinning in association with contact lens within normal corneas.[73] To distinguish the effects of contact lens wear from the disease process of keratoconus, ideally, true unilateral keratoconics might be required to wear contact lenses in both eyes.

Additionally, in order to identify the effect of keratocyte populations before transplantation, *in vivo* confocal microscopy needs to be performed on advanced cones before trephination for corneal transplantation and then the data compared with the *ex vivo* confocal microscopy on the corneal button postoperatively. Conversely, *ex vivo* analysis of the corneal button before PKP followed by *in vivo* confocal microscopy of the transplant over time might provide interesting data in regard to changes in the donor keratocyte population postgraft.

The heterogeneity in keratoconus may explain the contradictory data that exist in the literature.[74] *In vivo* confocal microscopy can provide greater insight into the morphological changes that occur with this progressive noninflammatory corneal ectasia. By improving our understanding of keratoconus this may enable alternative therapies to be devised in order to retard its progression and therefore reduce the need for surgical intervention.

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