ANATOMICAL PATHOLOGY

An in depth analysis of histopathological characteristics found in keratoconus

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Summary
Aims: The aims of this study were to re-assess the histopathology of the disease by introducing more modern measuring techniques and to determine if axial stromal thinning, which is the most apparent change, is related to the other alterations observed.

Methods: Recipient keratoconic corneas from 36 patients following corneal transplantation were studied. Haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining were used to identify breaks in Bowman’s layer and Descemet’s membrane. Thickness of corneal layers was measured by Leica QWin software. Epithelial and stromal thickness were measured in each sample at the periphery of the corneal button and at the area of maximal stromal thinning. The presence of apoptotic cells in Bowman’s layer breaks was detected by terminal deoxynucleotidyl transferase mediated dUTP-X nick end labelling.

Results: In all 36 corneal samples the central stroma, at the apex of the cone, was thinnest than the peripheral. There was a negative correlation between central stromal and central epithelial thickness ($p = 0.009$). Bowman’s layer breaks were found in 92% of corneas. Apoptotic cells were detected at the level of Bowman’s breaks membrane. We found a positive correlation between epithelial thickness and the number of Bowman’s layer breaks ($p = 0.009$ for central epithelial thickness and $p = 0.003$ for peripheral epithelial thickness). Descemet’s membrane deformities were observed in 19% of corneas and central stromal thickness of these corneas was significantly less than corneas without breaks ($p = 0.006$).

Conclusions: There are various different histopathological features associated with keratoconus and some of them are very subtle and not very well studied. Accurate measurements also suggest some correlations between them. Stromal thinning is associated with the number of breaks in Descemet’s membrane, but it is the thickening of the epithelium which is associated with breaks in Bowman’s layer.

Key words: Cornea, ectasia, histopathology, keratoconus.

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INTRODUCTION
Keratoconus is a bilateral non-inflammatory corneal ectasia with a prevalence of 1 per 2000 in the general population. The corneal steepening presents clinically as irregular astigmatism and axial myopia. The subsequent reduced visual acuity can be corrected using spectacle correction or, in more severe cases, rigid gas permeable contact lenses. However, despite this, approximately 10–20% of keratoconics will require corneal transplantation for visual rehabilitation, as keratoconus remains one of the leading indications for corneal transplantation.2,3 Pathologically, keratoconus results from an undetermined abnormality in the mechanical properties of the cornea. Histological abnormalities have been described in all layers of the keratocoric cornea.4–12 The earliest detectable change in keratoconus occurs in the basal layer of the epithelium. At the apex of the cone, the usual 4–6 layers of epithelial cells can vary substantially and may increase to up to 10–15 layers of epithelial cells. In more severe cases, wing cells display large irregular shaped nuclei with epithelial cells assuming an elongated shape not otherwise seen in mild cases of keratoconus.13 Other associations include patchy disruption of Bowman’s layer, where focal defects are filled with collagen, epithelial cells or keratocytes from the anterior stroma.11,14,15

The strength of the cornea is in part dependent on an intact Bowman’s layer. Loss or damage to Bowman’s layer limits corneal rigidity and may play an important part in the pathogenesis of keratoconus. A consistent finding in keratoconus is thinning of the central stroma with a reduction in collagen fibres and lamellae as demonstrated by electron microscopy studies.16 Furthermore, reduction in the production of type IV collagen may hinder the matrix–matrix and matrix–cell interaction.17

In this study we have re-analysed the histopathology of keratoconic corneas using two widely used staining methods and by introducing more modern and accurate measuring techniques. The aim of the study was to attempt to correlate the well described histopathological characteristics of keratoconus, like central corneal thinning, with specific alterations in the various layers of the keratoconic cornea.

METHODS
Corneas
Thirty-six keratoconic corneal buttons embedded in paraffin blocks were retrieved from the Central Manchester University Hospitals NHS Foundation Trust Histopathology archive following ethics committee approval. All corneal buttons were resected from patients undergoing penetrating keratoplasty (PK) for keratoconus at the Manchester Eye Hospital between 1996 and 2003. All operations were performed by the same surgeon and the size of the corneal buttons was uniform at 8mm. All patients’ notes were available for clinical follow-up data, with at least a 5 year follow-up being available. Exclusion criteria included history of previous ocular trauma or surgery, cross linking therapy, corneal hydrops, systemic or genetic conditions affecting the cornea and contact lens wear for the last 12 months, as these factors may cause further histological changes in the keratoconic corneas.

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H&E and PAS staining
Paraffin blocks were trisected and each section was then cut into 5 μm sections, mounted on Thermo Shandon SuperFrost slides and rehydrated through xylene and a series of graded alcohols prior to histology staining. Haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stains were used to identify breaks in Bowman’s layer and Descemet’s membrane.

Detection of apoptosis by TUNEL
The presence of apoptotic cells were detected using a fluorescent TUNEL (TdT-mediated dUTP-X nick end labelling) kit (Roche Diagnostics, Switzerland) as per the manufacturer’s instructions. Briefly, the slides were incubated for 30 min at room temperature with proteinase K solution (20 g/mL in 10 mM Tris/HCl, pH 7.4–8.0) and rinsed twice with PBS. A 50 μL TUNEL reaction mixture was added to the samples while a 50 μL label solution was added to negative controls. Slides were incubated for 60 min at 37 °C in the dark. The slides were rinsed three times with PBS and mounted with a specific fluorescence mounting material. Slides were viewed on the Leica LEITZ DMRB microscope (Leica, Germany) with the excitation wavelength set in the range of 450–500 nm and detection in the range of 515–565 nm.

Recording and analysis of corneal histology
All slides were studied using the Leica LEITZ DMRB microscope, pictures were taken with a DeltaPix INFINITY-X digital camera (DeltaPix, Denmark). Epithelium, Bowman’s layer, stroma and Descemet’s membrane of the corneas were studied and measurements were performed using the Leica QWin software (Leica QWin pro; Leica Microsystems). Epithelial and stromal thickness was measured in each sample at the periphery of the corneal button and at the area of maximal stromal thinning corresponding to the apex of the cone. Both thicknesses were measured on a x200 magnification. A direct measurement of the epithelial thickness was used in contrast to a semi-quantitative analysis of the stromal lamellae. The measurement of stromal thickness was modified to exclude artefact gaps created in the corneal stroma during the histological processing. The Leica QWin software was set up to detect the pink coloured stromal tissue on PAS staining and differentiate this from clear artefact gaps, in any manually selected region (Fig. 1). The software was further able to provide data of the breadth and area of the region under study, including stromal tissue area. Knowing these data, the true length could be calculated. Bowman’s layer was further studied using the x20 objective lenses of the microscope and full thickness interruptions were considered breaks. Only the interruptions replaced by stromal or epithelial tissue were counted. These breaks were counted in six serial corneal sections at three different levels and the mode was used in each cornea. Descemet’s membrane was studied only from the PAS slides, as being a basement membrane it is better represented by this staining method.

Statistical analysis
All statistical analyses were performed with SPSS/PASW for Windows version 17.0 (PASW, USA). Mann Whitney U test, Spearman’s rank correlation and Fisher’s exact test were performed. All data are expressed as mean ± standard error of the mean (SEM). A level of p < 0.05 was accepted as statistically significant.

RESULTS
Qualitative changes
Changes in all levels of the keratoconic corneas were demonstrated. Irregularities of corneal thickness and irregular distribution of epithelial cells were observed in all samples. At the apex of the cone, epithelial cell layers varied in number, ranging from more than 10 layers in some cases, to just 2–3 cell layers in others. The well known variability of the epithelial cells18 was once more confirmed. More specifically, superficial epithelial cells appeared elongated or spindle-like shaped, wing cells displayed a large, irregularly spaced nucleus, while the basal epithelial cells appeared enlarged and, in some cases, showed possible cytoplasmic swelling. Additionally, in two samples, ingrowth of epithelium could be observed at the sites of the breaks, extending into the anterior stroma (Fig. 2).

Defects in Bowman’s layer, characterised as breaks, were observed in 33 (91.6%) of the corneas studied in this project (Fig. 2). The appearance of the breaks, and those of the underlying distorted stroma beneath, varied among corneal samples. Although these defects were usually filled with collagen, various nuclei could sometimes be observed at the...
level of Bowman’s layer, which were often very intensely stained, resembling an apoptotic or pre-apoptotic stage (Fig. 3). TUNEL analysis confirmed that these cells were apoptotic (Fig. 4). In other cases, aggregated nuclei appeared on the anterior stroma, lying adjacent to, or within the same plane as Bowman’s layer consistent with stromal scarring at the edge of the break (Fig. 3).

The architecture of the stroma and the arrangement of the collagen fibres were distorted in areas near to breaks in Bowman’s layer and it was observed that the structural deformities caused at the level of the break were not confined to Bowman’s membrane but extended also to the epithelium and the anterior stroma. Stromal thinning at the area of the apex of the cone was observed in all corneas studied. Also, the density of keratocytes was lower in the anterior stroma contrary to findings in normal corneas.

Descemet’s membrane was assessed on the PAS stained slides and ruptures as well as folds were identified. Depending on the age of the breaks, these defects were either filled with endothelium (recent break) or sealed by a newly formed Descemet’s membrane (older break) (Fig. 5).

Analysis of histological findings
In all 36 corneal samples the axial stroma was thinner than the peripheral stroma of the corneal button. The mean stromal thickness at the periphery was 477 ± 22 μm while at the region of maximal thinning it was 214 ± 11 μm. In contrast, the measurements of central and peripheral epithelium were variable with values ranging between 44.46 ± 3.52 μm and 50.08 ± 1.73 μm. It appeared that, axially, the epithelium was thinner in 23 (63.9%) corneas and thicker in 13 (36.1%) corneas. A statistically significant negative correlation between central stromal and central epithelial thickness (p = 0.009) was found. This was not confirmed in the peripheral cornea (Fig. 6).

Bowman’s layer breaks were found in 33 corneas (91.6%) with a range of 1–11 per slide and a mean of 3.4 ± 0.4. In the remaining three corneas there were no apparent breaks in at least three sequential histological sections. Most of the breaks were located at the central third of the corneal samples. Only the corneas that were observed to have four breaks or more (8; 22.2%) appeared to have breaks both centrally and
peripherally. There was a statistically significant positive correlation between epithelial thickness and the number of Bowman’s layer breaks with $p = 0.009$ for central epithelial thickness (Fig. 7A) and $p = 0.003$ for peripheral epithelial thickness (Fig. 7B). Correlation was not observed between stromal thickness and the number of Bowman’s layer breaks.

One or more breaks were observed in Descemet’s membrane in seven (19.4%) corneas. In addition, one also presented folds incorporated by posterior stroma. The axial stromal thickness of the corneas that presented breaks in Descemet’s membrane was significantly less than those that did not ($p = 0.006$). The mean epithelial thickness of those corneas with breaks...
in Descemet’s membrane was 181 ± 6 μm and those without breaks was 222.8 ± 12.8 μm.

It is worth mentioning that the mean age of our cohort was 33.28 ± 1.16 and ethnicity data were also available but there was no correlation of the measurements with these factors in our cohort.

**DISCUSSION**

Histological variations in all layers of the keratoconic cornea have been documented in several studies. Many of these studies have reported contrasting results for epithelial characteristics,9–11,13,19–21 highlighting the variable nature of the disease. The Manchester keratoconus study13,22,23 reported variability across the epithelium, with central epithelial thinning and peripheral epithelial thickening which was more obvious in the inferior cornea, but the number of samples used was small as only two corneas were studied under light microscopy. Scroggs and Proia10 used histology to study 95 corneas of patients who underwent penetrating keratoplasty for keratoconus and performed measurements with the use of an ocular micrometer to find significant central epithelial thinning in most of the corneas, a result that has also been reported by previous early reports.9,21 On the contrary, more recent reports11,22 have documented epithelia being severely thickened, while a study performed by Erie et al.19 in vivo by the use of confocal microscopy found no difference in thickness between control and keratoconic epithelium. In the Erie study epithelial thickness was measured as the distance between the first focused image of superficial epithelium and the sub-basal nerve plexus.19

In the case of the stroma, authors of all the above studies are in agreement that the most apparent change observed is central thinning, which is a consequence of a decrease in the number of collagen lamellae, as has been demonstrated by transmission electron microscopy.16 Additionally, many in vivo confocal microscopy studies have demonstrated reduced keratocyte densities,10,24 something which was also observed in our samples. We confirmed the variation in epithelial morphology since we found both patterns of epithelium, while stroma was found to be thinned centrally in all the 36 corneas studied. The difference in our study was that measurements of stromal thickness were slightly decreased compared to previous reports, as expected due to the semiquantitative method used. There was also an important difference in the measurements taken at the periphery. The mean peripheral stromal thickness in our study was 477 μm, in comparison to the study made by Scroggs and Proia10 where it was around 700 μm, which can be explained by the fact that the artefactual gaps that occur in the corneal stroma during histological processing are much wider in the periphery. This observation points to the fact that although there is a central thinning of the stroma in keratoconus, the actual proportion of the thinning is smaller than has been previously assumed. Furthermore, by having performed computer-assisted measurements on the epithelial and stromal thickness that evaluated tissue only, eliminating artefactual gaps, we were able to identify a statistically significant negative correlation between epithelium and stoma centrally. This is something that has not been documented previously and suggests that there may be a pattern in the structural deformities found in keratoconus. The semiquantiative method used in our study has not been applied before and whether it is more accurate than more conventional methods remains to be proven. The advantage of the specific method is that measurements are not affected by the wide artefactual gaps that occur during histological processing at the periphery of the corneal button, and the accuracy or not of our findings could be confirmed with in vivo studies. However, although central corneal measurements in keratoconus have been performed in vivo in various studies,24,25 to our knowledge there is no study to date measuring the thickness of the corneal stroma in keratoconic corneas mid-peripherally and this is something that could be studied in the future.

Ruptures and deformities of Bowman’s layer in keratoconus have also been reported by most of the aforementioned study groups with the use of light, electron or even confocal microscopy. The prevalence of corneas with breaks in our sample was 91.6%, while other studies have mentioned smaller rates of 80%10 and 71%.11 Many different patterns of deformities have been described, like Z-shaped interruptions23 or down-growth of epithelium into the layer,7 but there are no specific patterns found in individual corneas. Ruptured areas in Bowman’s layer, which were also identified in the majority of the corneas in our sample and were characterised as breaks, have been reported to be filled with either epithelium or proliferated collagenous tissue that is derived from the anterior stroma.8,20 Other studies6,11 documented stromal keratocytes occupying the breaks, while hyperreflective keratocyte nuclei were observed with the use of confocal microscopy at the level of Bowman’s layer in some of the patients studied by Hollingsworth et al.13,27 Isolated or aggregated nuclei were also identified at sites of Bowman’s breaks in our samples, but their origin could not be identified. These nuclei were often very intensely stained, and TUNEL showed they were apoptotic. This has also been documented in previous studies20,28 with the majority of apoptotic cells being localised at the level of Bowman’s layer and more specifically within areas of breaks. These cells have been labelled as apoptotic keratocytes20 but their role in the pathogenesis and progression of the condition is still unclear. As described by Hughes and Gobe,29 when using biochemical means for apoptotic cell identification one must be aware of a few potential pitfalls and this is why it is necessary for some morphological checks and balances. In our study, TUNEL positive nuclei were considered apoptotic only if their microscopic morphology was apoptotic as well (Fig. 3), in accordance with widely accepted practices.30

Regarding the involvement of Descemet’s membrane, which is considered an atypical finding by most of the studies and is mostly associated with cases of corneal hydrops, our findings were in accordance with previous groups, as our rate of 19.4% was similar to the 18% of Fernandes et al.,7 while the 12% in the early study by Chi et al.8 may be explained by the fact that only H&E staining was used to identify the breaks. Our opinion is that reproducibility of these results confirms its importance, while its possible role in stromal thinning may reveal a role of Descemet’s membrane breaks in the pathophysiology of the disease.

Finally, central stromal thinning, epithelial thickness variation and Bowman’s layer, as well as Descemet’s membrane breaks, are all typical findings in the keratoconic cornea. In an attempt to study possible relations between them, we have correlated our measurements of thickness with the number of breaks, in both Bowman’s layer and Descemet’s membrane, found in each of the corneas studied. Surprisingly, a very significant correlation was found between the thickness of
the epithelium and the number of the breaks in Bowman’s layer; however, the causative event between these is difficult to determine in a retrospective study using paraffin blocks of full thickness corneas transplanted following long standing keratoconic changes.

Once more these correlations suggest that there are some specific patterns in the structural changes observed in keratoconus and further study is needed to try and establish a model regarding the progression of changes in this ectatic dystrophy of the cornea.

Conflicts of interest and sources of funding: None to declare.

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References