



PII: S0275-5408(98)00003-9

## Prevalence of 'non-hexagonal' cells in the corneal endothelium of young Caucasian adults, and their inter-relationships

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### Summary

The corneal endothelium is often described as a mosaic of hexagonal cells, despite numerous reports that the relative number of 'hexagonal' cells is often only around 65%. Such estimates also cannot define the relative area contribution to the cell mosaic of either these cells, or those that are not 6-sided. Specular micrographs were therefore taken of 20 healthy young Caucasian adults aged 21 to 34 years and the apical surface areas of at least 105 contiguous cells from each central corneal endothelium measured by planimetry. The numbers of each cell type and their surface areas were assessed so that the summated area occupied by each cell type could be calculated. The results reveal that endothelia that show only modest variance in cell areas are usually composed of 4-, 5-, 6-, 7- and 8-sided cells. Assessments of relative contribution of each cell type to the mosaic indicates that the 6-sided cells can be expected to constitute just over 60% of the total cell area. Increases in the variance in the areas across all the cells can be strongly correlated with a reduction in the 6-sided cells and increases in the 5- and 7-sided cells. Each cell type (4-, 5-, 6-sided etc) has a preferred area that is proportional to other cells. The results firmly indicate that the mosaic is not random and follows a specific order that is presumably dictated by the physics of cell packing. These methods of comparing endothelia should allow distinctions to be made between endothelia that are different, versus being abnormal (i.e. with disease). © 1998 The College of Optometrists. Published by Elsevier Science Ltd. All rights reserved

### Introduction

Since the advent of photo slit-lamp photography and clinical specular microscopy, there have been numerous reports on the appearance of the human corneal endothelium (Doughty, 1989). Some of these reports have attempted to provide quantitative data on the actual cellular composition of the mosaic, rather than just presenting images for subjective interpretation. This morphometry is important since it would be very useful to be able to assign a certain type or level of function to a particular arrangement of the cells in the corneal endothelium.

For quantitative analyses of the corneal endothelium (morphometric assessments), it has become commonplace to assess the endothelial cell density (the ECD,

in cells/mm<sup>2</sup>), and/or the average cell area (usually presented in  $\mu\text{m}^2$ ) (Doughty, 1989). Studies using such assessment methods have clearly indicated that the human corneal endothelium can be expected to show an age-dependent decrease in ECD, and that this age-dependent change could be significantly altered by intra-ocular surgery, a range of acute-onset diseases of the anterior segment and by long-term contact lens wear, especially if of the PMMA type. A substantial correlation has not been found between central corneal thickness (CCT) and ECD or endothelial cell area (Doughty, 1989). It may be possible however to correlate the rates of change in CCT (following acute stress to the endothelium) with ECD (Erickson *et al.*, 1998), and some retrospective analyses indicate that the variance in cell area can be positively related to CCT in corneas that have been subjected to intra-ocular surgery (e.g. following recovery from cataract operations). Notwithstanding, these types of relationships are not

Received: 30 June 1997  
Revised form: 7 January 1998

obvious in the normal cornea or in the adapted contact lens wearer (Doughty, 1990). It has to be considered therefore whether assessments of ECD and cell areas are indeed adequate (Jacobi and Strobel, 1981).

Estimates of cell density or calculation of the average cell area can be easily made yet do not indicate, in themselves, that the endothelial cell mosaic is actually made up of polygonal cells with a range of areas and which normally have 4, 5, 6, 7 or even 8 sides (Doughty, 1989). Therefore, despite such extensive assessments of ECD and average cell areas, we still have only limited data on the actual composition of the cell mosaic and even fewer ideas on what determines the make up and organisation of this cell mosaic.

The rationale behind the present studies is that a better understanding of how this monolayer of cells is actually organised, and what determines this organisation, could provide the basis for then comparing morphology with function. One aspect of this organisation is the presence of cells that do not have 6 sides and what relationship might exist between them. As a first step towards its application in comparative studies, the relative contribution of each cell type to the endothelial cell mosaic was assessed from a set of normal human endothelia.

## Materials and methods

### Subjects

Twenty subjects were mainly recruited from graduate students and staff at Glasgow Caledonian University, with the protocol being approved by a departmental and University ethics committee. Based on responses to specific questions, all subjects were considered to be healthy and reported no major systemic disease. The subjects had no history of significant eye disease or surgery, and the only eye condition

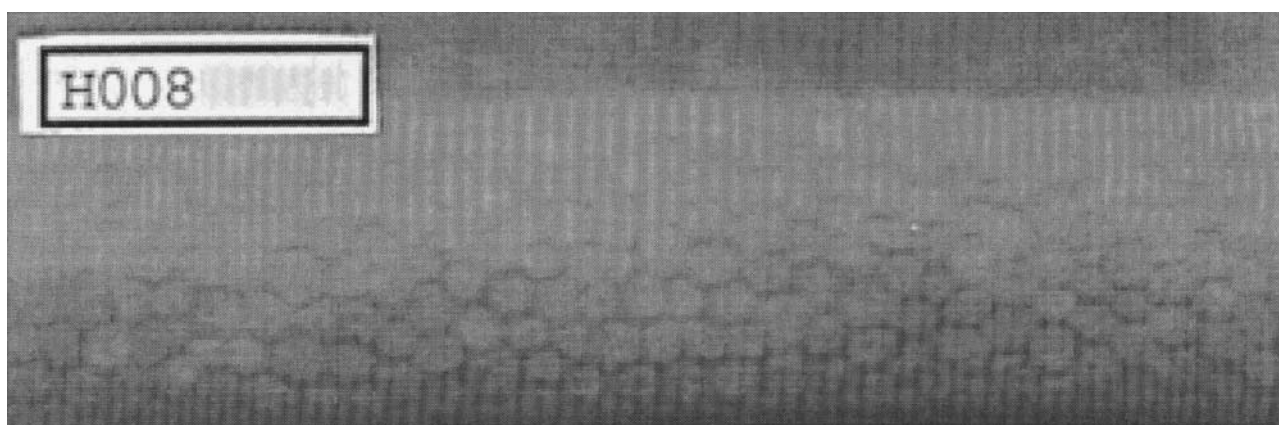
reported by some subjects was occasional mild discomfort or irritation. This was not considered important, and no specific tests were carried out to investigate this further. The subject group consisted of 9 females and 11 males with an overall average age of 27 years (range 21 to 34 years) and all were white Caucasians. The only medications that were reported were birth control pills by some of the female subjects. None of the subjects wore contact lenses. Half the subjects were habitual spectacle wearers for mild-to-moderate refractive errors only, i.e. there were no high myopes in the group.

### Corneal endothelial photography

A Topcon SP1000 non-contact specular microscope was used with a single micrograph of the central corneal endothelium being obtained, which has a nominal magnification of  $132\times$ . A scale bar was affixed to the resultant thermal paper image which was then scanned with an Apple Colour One scanned to generate a PICT file at a resolution of 53 lpc, in 35 mm slide format.

### Endothelial cell morphometry

The endothelial slide images were projected onto a white screen at a distance of 12 feet. From the projected image, a tracing overlay of the apical borders of the cells was made with the borders (sides) of as many cells that could be reliably identified being drawn. The overlay was generated only of cells that formed a contiguous array so that appropriate population statistics could be applied (Doughty *et al.*, 1993), but with the limit that the cells were not so close to the edge of the image that edge curvature effects could be a problem (Panzardi *et al.*, 1988). In *Figure 1* this is the zone just overlapping the scale bar/ID, i.e. where the cell-cell borders are less well defined. The overlay was drawn



**Figure 1.** Typical specular micrograph of the central corneal endothelium of a young Caucasian adult.

in pencil and then, after removal from the screen, a fine marker pen used to produce a final tracing. This overlay was then photo reduced to about 30% size and then the surface areas of the cells were then measured by manual planimetry to an estimated accuracy of  $\pm 3\%$  using the Bioquant IV image analysis system as previously detailed (Doughty, 1992). Each cell was numbered and the number of sides counted. This data, along with the cell area, was entered into a custom-written computer program written in CBasic operating on an IBM 386 computer and the resultant files read into a statistics program (Systat v. 3.0; Systat, Evanston, IL). The cells in individual files could be statistically analysed as a group, or cells with different numbers of sides selected as sub-groups and these analysed separately.

## Results

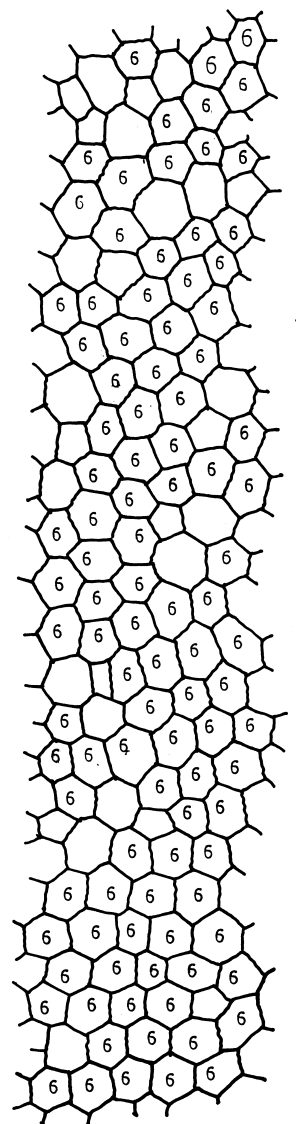
### *Overall characteristics of normal human corneal endothelia*

Based on evaluation of all 20 specular micrographs, one (H008) was subjectively selected as having a substantial apparent regularity and symmetry (Figure 1). A tracing overlay of this image is shown in Figure 2 to highlight this form; the 6-sided cells are identified. The image was taken from a 24-year-old female. The cell density was 3318 cells/mm<sup>2</sup> and the average cell area was 318  $\mu\text{m}^2$ .

Subjective assessments of all 20 endothelial micrographs indicated that they were qualitatively similar to this index example in terms of ECD and average cell area. None of the images showed any gross irregularities, blebs or guttae. The group-averaged ECD was 3442 cells/mm<sup>2</sup> (range 3212 to 3812), while the group averaged cell area was 322  $\mu\text{m}^2$  (range 301–344  $\mu\text{m}^2$ ). These morphometric criteria can be taken as indicators that these endothelia are those expected from young healthy adults.

### *Detailed analyses of the range and distribution of cell areas*

The data sets from each endothelial micrograph contained 105 to 135 contiguous cells. The distribution of the cell areas for H008 was essentially unimodal (not shown) with average cell area (318  $\mu\text{m}^2$ ) falling within the modal cell area (301 to 325  $\mu\text{m}^2$ ), indicating a normal distribution of the cell areas (range 146 to 536  $\mu\text{m}^2$ ). The calculated standard deviation for the distribution of the cell areas was consistent with the unimodal distribution in having a value of just 69  $\mu\text{m}^2$ . Alternatively, this standard deviation can be normalised as a percentage (the coefficient of variation, COV)



**Figure 2.** Tracing overlay of the cell-cell borders to show the mosaic arrangement of central corneal endothelial cells of a young Caucasian adult, and showing the 6-sided cells. Scale bar = 135  $\mu\text{m}$ .

which was 21.7% (see later). Other statistical measures can be applied to the set of contiguous cells and confirm that the distribution in the example was almost perfectly Gaussian as judged by the negligible coefficient of skewness (at 0.121) and very small kurtosis (0.378).

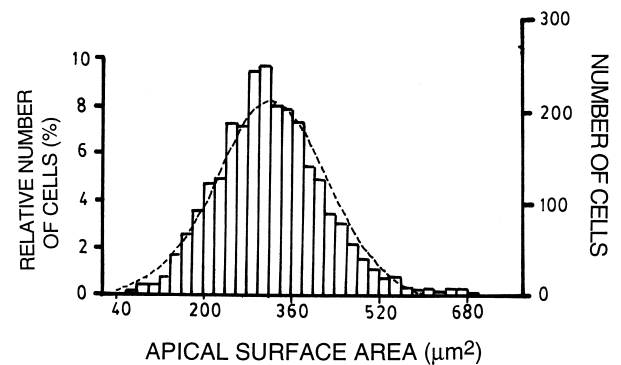
The distribution of cell areas in the other 19 endothelia were similarly unimodal although a few more of the cells were slightly smaller or larger, so removing some of the smoothness to the distribution. By taking all of the cell area measures for each endothelium image and pooling data from each set that was within successive discrete ranges of area (0 to

40  $\mu\text{m}^2$ , 41 to 80  $\mu\text{m}^2$ , etc), a pooled data distribution could be compiled as based on the total number of cells within each area range; this histogram is illustrated in *Figure 3*. This pooled profile for cell area data also conforms strongly to a Gaussian distribution (which has been superimposed on the profile in *Figure 3* as a dotted line). This indicates that relatively few cells fall outside of this distribution. This is confirmed from the calculations of skewness and kurtosis on each separate distribution. An average value for the coefficient of skewness was small at 0.544. The majority (17 of 20) of the data sets were within  $\pm 0.5$  of this value, although two endothelia had a modest positive skewness index of 1.201 and 1.358 and a single sample had a marginally negative skewness index ( $-0.031$ ). As indicated from the pooled distribution there are a significant number of slightly smaller cells that fall outside of the Gaussian distribution, and consistent with this is the finding that there was a slight positive kurtosis on the cell area distributions in just over half of the data sets (12 of 20). Overall, these slightly accentuated distributions had small-to-modest kurtosis values ranging from 0.271 to 2.086; the group-averaged kurtosis was positive at 1.084.

Notwithstanding these slight deviations from a normal distribution, the statistical analyses can be taken as indicators that these sets of cells are generally homogeneous.

#### *Analyses of the contribution of different cells to the endothelial cell mosaic*

Calculations of average cell area and the overall variation in cell area provides little information on the actual composition of the cell mosaic, other than perhaps indicating some heterogeneity. Further detail can however be obtained by assessments of the different types of cells based on the number of cell sides. The index endothelium (H008) was composed of a mixture of 4-, 5-, 6- and 7-sided cells, and the relative number of 6-sided cells was high at 79.2%. The rest of the cells were either 4-sided (0.77%), 5-sided (8.5%) or 7-sided (11.5%). Three of the endothelia were composed solely of 5-, 6- and 7-sided cells, but the rest of the endothelia also contained 4- or 8-sided cells or both. Statistical evaluation of all of the sets of endothelial cells



**Figure 3.** Histogram of the apical surface areas of all 2598 endothelial cells analysed from 20 different corneas to show its closeness to a Gaussian distribution (indicated by the dotted line).

revealed that the group-averaged percentage of 6-sided cells was 61.3%, with a range from 45.1% to 80%. The relative numbers of the other cells are summarised in *Table 1*.

Calculations of the relative numbers of each cell type however provide only limited information about the composition of the cell layer since even the relative numbers of different cells provides no information on the surface areas of these cells. The relative contribution of each cell type to the mosaic can however be assessed by measuring the cell areas and then calculating the total area occupied by each cell type. These total values can then be expressed in relative terms as a percentage of the area evaluated in the individual micrographs, i.e. the area-percent relationships. The average cell areas and the area-percent values are given in *Table 1*. It should be noted that the 4-sided cells tend to smaller than the 5-sided cells, and the 5-sided cells tend to be smaller than 6-sided cells, etc. In addition, it should be noted that with these areas now taken into consideration, the proportional contribution of smaller cells goes down (e.g. 5-sided cells number 19.6% yet contribute only 14.0% of the overall area) and that of the larger cells goes up (e.g. 7-sided cells number 16.4% yet contribute 21.3% of the overall area).

The relative contributions of each cell type, based on these area-percent values, can now be assessed by comparing the relative area contributions made by

**Table 1.** Morphometric data for 20 young human corneal endothelia

Cells	All	4-sided	5-sided	6-sided	7-sided	8-sided
Relative number (% of total, $\pm$ SD)	100	1.4 $\pm$ 1.2	19.6 $\pm$ 5.0	61.3 $\pm$ 9.9	16.4 $\pm$ 4.4	1.4 $\pm$ 1.5
Average area ( $\mu\text{m}^2$ )	322	126	230	324	420	513
SD on cell areas ( $\mu\text{m}^2$ )	12	33	18	10	29	95
Average area-percent	100	0.53	14.0	61.7	21.3	2.3

each cell type to the overall areas of the cells and the variance in cell areas. Since all of the endothelial data sets were essentially normally distributed, an acceptable normalised analysis of differences in cell area distributions can be made with calculations of the coefficient of variation (COV). An overall comparison of COV versus ECD revealed a statistically significant positive correlation (slope  $p < 0.05$ ) with COV increasing as ECD increased ( $F$  ratio 10.336,  $r = 0.365$ ). This relationship is not very substantial probably due to the scatter of data, which is perhaps not surprising because it includes all cell types. However, a comparison of the relative area occupied by 6-sided cells to the COV revealed a strong negative correlation (slope  $p = 0.000$ ,  $F$  ratio 41.866,  $r^2 = 0.703$ ), with a 10% change in the contribution of 6-sided cells being associated with a 5% change in COV (Figure 4). This result indicates that the variance in cell areas is the result of changes in cells other than the 6-sided ones and is confirmed by analyses of the relationship between the area occupied by 5- or 7-sided cells and the COV. In these cases, a statistically significant (slope  $p = 0.001$ ) positive relationship was obtained with the COV increasing as the relative area occupied by the 5- or 7-sided cells increased (Figure 5). As indicated by the relative slopes in Figure 5, the proportional increase in the area occupied by the 5-sided cells was only two-thirds of that for the 7-sided cells. This difference reflects the fact that the 5-sided cells are smaller than the 7-sided cells. While the slopes were still substantial and statistically significant, the correlation's ( $r^2$  values) were somewhat less at 0.495 and 0.566 respectively. Analyses of the relative numbers of 6-sided cells or their relative area

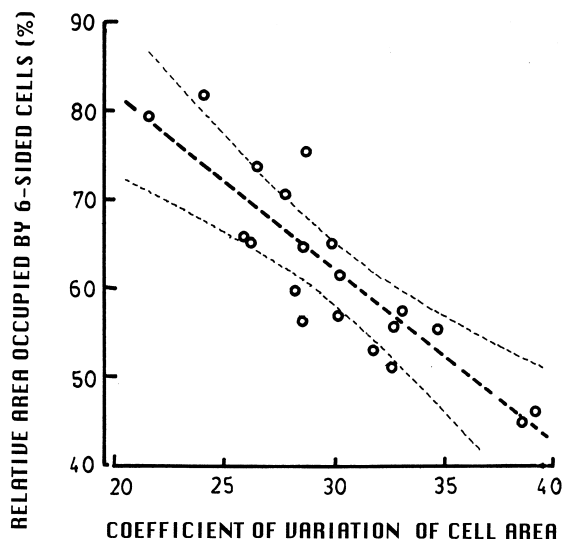


Figure 4. Relationship between the area percentage of 6-sided cells in a corneal endothelial cell mosaic and the overall variance in the area of all cells as estimated from calculations of the normalised standard deviation on the cell area distribution (the coefficient of variation in cell areas). Each data point is from a separate endothelium. The bold dotted line represents a linear regression of the data ( $r^2 = 0.703$ ) while the lesser dotted lines give the 95% confidence limits to this regression line.

contribution was strongly related to the contribution of the 5- and 7-sided cells (not shown). For example, the relative contribution made by the 5- and 7-sided cells was inversely and linearly related to the total area occupied by the 6-sided cells. These relationships were significant ( $p = 0.000$  that the slope was not zero) and

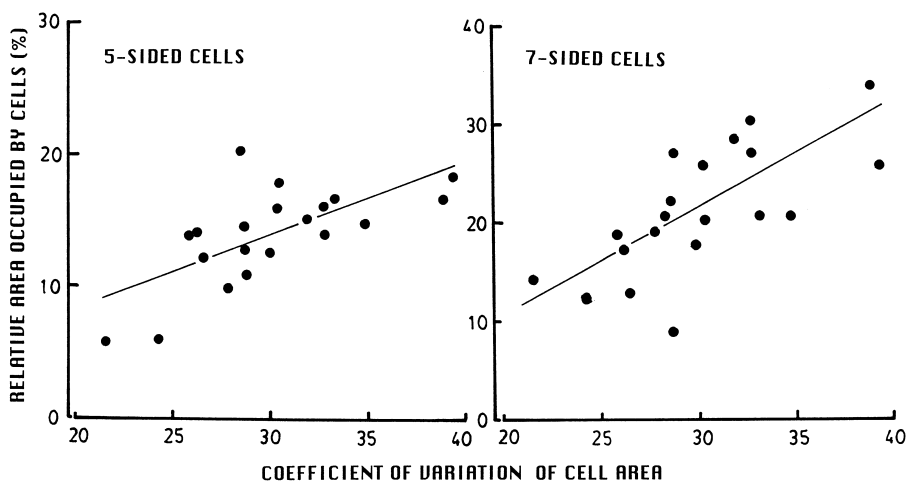


Figure 5. Relationships between the area percentage of 5- and 7-sided cells in a corneal endothelial mosaic and the overall variance in the area of all cells as estimated from calculations of the normalised standard deviation on the cell area distribution (the coefficient of variation in cell areas). Each data point is from a separate endothelium. In each plot the line represents that of a simple linear regression (see text for details).

had very high correlations ( $r^2=0.756$  and  $0.765$  respectively for the 5- and 7-sided cell area;  $F$  ratios of 55.859 and 58.449 respectively). However, there was a weaker correlation between either the actual percentages of 5- and 7-sided cells ( $p < 0.05$ ) or the relative areas occupied by the 5- and 7-sided cells ( $p < 0.05$ ;  $r^2=0.361$ ,  $F$  ratio = 10.181). This relationship was positive in that the contribution by both the 5- and 7-sided cells concurrently increased with the reduction in the total area occupied by the 6-sided cells.

## Discussion

The present studies indicate that there is some mild heterogeneity (i.e. mild polymegathism and pleomorphism) in the endothelial cell mosaic of young Caucasian adults but that, despite this, there are remarkable elements of organisation. The type of inter-relationships reported here for the endothelial cells have not been highlighted previously, but rather efforts have been made to find correlations based upon morphometry measures that do not make logical sense in terms of asking how the endothelial cell layer is organised, or whether there is some predetermined form to the cell layer. It is from this perspective that these analyses are presented to also emphasise that there is still much to be learned about the human corneal endothelial cell mosaic. There are a number of reasons why this sort of subtle heterogeneity has not been reported previously, and all relate to the methods used to analyse the mosaic.

Firstly, it is most unlikely that the presence of such subtle heterogeneity could be detected by assessments of ECD since this is an average of all cells, and may actually only be estimated from a sizing grid rather than repeatedly calculated from each cell area (Doughty, 1989; Landesz *et al.*, 1995). Similarly, while the often-used method to assess the variance in cell areas (the COV) may well be able to detect gross changes in the mosaic, even small numbers of very small or large cells (Doughty, 1990) can result in a very large calculated COV, despite the fact that most of the cells in the mosaic essentially appear normal. Furthermore, as previously pointed out (Doughty, 1990), any interpretation of changes in COV when applied to skewed distributions can be further complicated by the inherent ambiguous nature of the calculation which cannot distinguish between increases in COV that result from the addition of smaller versus larger cells. Furthermore, such standardised calculations are also prone to variance if the number of cells assessed is less than 50/endothelial sample (Doughty *et al.*, 1993); it is an inescapable fact that some determinations of COV have been carried out either on relatively small numbers of cells or even on

sets of non-contiguous cells (Doughty *et al.*, 1993). These issues of methodology also apply to other measures. The widespread reporting of the relative number of 'hexagonal' cells is also ambiguous and provides no information on other cells in the mosaic, especially when the percentage of these cells is low. The prime reason for the ambiguity is that the 6-sided cells do not necessarily conform to the very shape that the term hexagons implies, i.e. a 6-sided cell with sides of equal length and 3 axes of symmetry (Doughty, 1992). The once often-used calculations of the average shape factor for all the cells in a set of cells does absolutely nothing to remove this ambiguity (Doughty, 1989).

That neither ECD nor COV calculations could be expected to detect subtle differences in the endothelial cell mosaic is further underlined by the results of the present study that clearly reveal that the cells in the human corneal endothelium have different surface areas based on the number of cell sides. That this could be the case was indicated by preliminary photolamp studies on human endothelia (Doughty and Fonn, 1993) and detailed scanning electron microscopy studies on rabbit endothelia (Doughty *et al.*, 1997). So, as a consequence of this, smaller versus larger cells (compared to the 6-sided cell) will tend to be averaged out in ECD or COV calculations.

The present studies are designed to define what the actual composition and organisation of the endothelial cell layer is and are designed to address a number of general principles that are thought to dictate the packing of cells together in tissues (Thompson, 1961; Honda, 1983; Edelstein-Keshet and Ermentrou, 1990; Heckman, 1990; Whittle and Phillips, 1993). The rationale behind assessments of the area-side relationships, along with calculations of the relative total area occupied by each cell type, is to more fully describe the composition of the corneal endothelial cell layer but still in reasonably simple measures. These simple measures are however designed to go beyond previous assessments. For example, the reporting of the composition of the endothelial cell mosaic has commonly included a '% hexagons' (or '% SIX') measure, yet despite the widespread reporting of this measure this still does not tell us much about these cells. A numerical assessment of the percentage of these cells in a mosaic really does not provide an indication of their true contribution since the area of the cells is ignored. The method for reporting the '% hexagons' also tends to hide the variance in contribution of these cells. The value has been presented as a mean  $\pm$ SD (Good and Schoessler, 1988; Carlson *et al.*, 1988; Nieuwendal *et al.*, 1994; Jackson *et al.*, 1995). From this perspective, the SD value of 5.0 on the relative number of 6-sided cells in the endothelia assessed in this study is similar to that reported by others, and confirms that the pre-

sent set of endothelia are indeed similar to those considered to be suitable control subjects by others. The variance in the percentage of 6-sided cells can also be reported as a standard error of the mean (SEM) (Schultz *et al.*, 1984; Yee *et al.*, 1985; Erickson *et al.*, 1998) which needs to be carefully identified since the calculated SEM value will depend upon the number of samples averaged, even though it could be taken as a useful indication of minimal variance. For the present series of endothelia, the SEM calculated for the variance in the percentage of 6-sided cells would be just 0.7 in 61.3%.

The present studies are presented to illustrate that an assessment of '% hexagons' has its limitations, and that the relative contribution of all the 6-sided cells to the area analysed should be used, i.e. the area-percent measure. This is because the 6-sided cells may not be symmetrical hexagons, but also because their apical surface areas may not be randomly determined. The present studies of the area of the 6-sided cells in a set of young adult human endothelia indicate that there is a preferred range of areas for the 6-sided cells, although such a finding does not mean that there cannot be 6-sided cells that could have areas that were very substantially different. The present finding of there being an inverse relationship between the overall variance in the cell areas and the percentage of 6-sided cells in young adult endothelia is in accord with previous analyses on individuals over the entire human life span (Matsuda *et al.*, 1982; Yee *et al.*, 1985; Rao *et al.*, 1982). The correlation ( $r^2$ ) values were stronger in the present study probably because just a small age range was assessed. These relationships may be different at other ages, or may specifically change in certain corneal diseases; these issues are currently being studied.

If it is therefore accepted that the heterogeneity in the endothelial mosaic arises from predictable changes in the cells that are not 6-sided, then assessments of the area-side relationships is surely logical. It is this sort of analyses that need to be applied to the stressed or diseased corneal endothelium. A disproportionate shift in the contribution of smaller or larger cell types can now be identified and also be logically compared on an area basis (e.g. correlations between area-percent values and COV), rather than illogically based on a number versus area basis (e.g. correlations between % hexagons and COV). Similarly, a difference in the area-side relationship (e.g. its slope or variance) could provide a reasonable basis for distinguishing between endothelia that were simply slightly different as opposed to being abnormal. The same logic could also be applied to just one cell type (e.g. the 6-sided cells) to ascertain how they might change with age and/or various types of eye disease.

It is acknowledged that a number of previous investigators have reported on the relative number of each cell type in human corneal endothelia (Matsuda *et al.*, 1982, 1984, 1985; Karai *et al.*, 1984; Schultz *et al.*, 1984; Yee *et al.*, 1985; Matsuda and Bourne, 1985; Carlson and Bourne 1988; Carson *et al.*, 1988; Asano *et al.*, 1988; Bates and Cheng, 1988; Shetlar *et al.*, 1989; Corkidi *et al.*, 1994); it seems reasonable and possible to go one step further and define the relative contributions to the cell mosaic. Some current commercial systems do assess the areas of each cell and define the number of sides of each of the cells, and on this basis both the area-percent and area-side relationships should already be accessible. The first set of relationships is presented as a more meaningful assessment of the contribution of any one cell type (e.g. the 6-sided cells, and should replace the '% hexagons'). Combined with data on the area-percent contributions, the area-side assessments should provide a less ambiguous evaluation of the cells that make up the endothelial cell mosaic. Sets of endothelia can now be compared to see if their area-side relationship shows the same or a different slope to a standard. While the present study could be considered as only exploratory in that only 20 subjects were assessed, it is presented as the basis upon which more extensive studies on the organisation of human corneal endothelia can be undertaken, and such studies are currently underway.

This study was carried out with the intent of both better defining the actual composition of the normal human corneal endothelium. These results, on young adults, provide baseline data on a set of endothelia that could be considered as 'normal' in that they had no history of clinically significant eye disease, and were not contact lens wearers. The data obtained on the present set of corneas is not inconsistent with other reports for control subjects used in various studies. The average morphometric data obtained in the present study appears to be within that which could be expected from healthy young adults of Caucasian origin. While there was clearly some mild heterogeneity (mild polymegethism and pleomorphism) in the endothelial mosaic of these subjects, the overall statistical analyses of variance support the visual impression of 'normality'. The fact that such mild heterogeneity, as revealed in the present detailed assessments, can be present in young healthy adults surely indicates that more detailed studies need to be done rather than arguing that these individuals cannot be considered 'normal'.

#### Acknowledgements

This research was supported by funds from Glasgow

Caledonian University. My thanks go to the subjects who permitted their endothelial micrographs to be taken and to M. L. Zaman for technical assistance. The author has no proprietary interests in any equipment designed for corneal endothelial morphometric analyses.

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