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Assessment of the reliability of endothelial cell-density estimates in the presence of pseudoguttata

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Abstract
Purpose The purpose of this work is to assess the reliability of endothelial cell-density (ECD) estimates in corneas with different severity pseudoguttata.
Methods Specular microscopy was undertaken on grade 1, 2, or 3 pseudoguttata patients and age-matched controls aged 52–83 years. On high magnification prints of central cornea, areas of complete cells (all sides visible) and partial 'cells' (one or more sides obscured) were measured manually. Sets of 45 complete cells were selected, as well as 75 cells that were a mixture of complete and partial cells on guttate endothelia. ECD was calculated by a progressive averaging technique.
Results Each group comprised 12 patients with similar range of ECD values (1,230–4,587 cells/mm^2). Based on 40 complete cells, ECD could be estimated to within ±3.1% for grade 3 pseudoguttata versus ±2.0% for controls. If a mixture of complete and partial cells were measured, ECD could be estimated to within ±2.8% for grade 3 pseudoguttata images (n=70 cells) and ±1.1% for controls. The estimated variability increases to substantial levels of ±20% if only ten cells were measured. No statistical differences in ECD were noted between guttate and normal endothelia if only complete cells were measured, but could be different if partial 'cells' were included.
Conclusions Providing adequate numbers of complete cells are measured and in the absence of obvious polymegathism, ECD estimates can be made to within around ±3% in the presence of typical but significant pseudoguttata.

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Introduction

Assessments of the human corneal endothelium can be carried out in various ways. These include comparisons with grading panels or model schematic cell mosaic images [1–3], by the counting of the number of cells in a certain region of the endothelial image by a fixed or variable frame method [4–7], or by undertaking actual measures of the areas of the cells. Such area measures can be undertaken either by manual planimetry or software supplied with clinical specular microscopes or confocal microscopes [6–8]. From the cell counting or area measures, some type of estimate of endothelial cell density (ECD) can be made.

In undertaking morphometry (i.e., measures of cell areas which can then be used to calculate ECD), a natural question that arises relates to how reliable the cell measures are, and therefore how reliable the ECD estimates might be. Previous specific studies on this issue have not only led to the development of guidelines as to how many cells should be measured [6, 8, 9] but, far more importantly, show that there is a finite limit to the so-called repeatability or accuracy of such measures. One particular feature relates to whether the endothelium was uniform or non-uniform [8]; the latter condition is also widely known as endothelial polymegathism. Such studies have indicated that the variability in ECD estimates tends to decrease as the number of cells measured gets smaller. From such studies, recommendations have been made that some 75 to 100 cells would be a reasonable target number for cells measured in corneal endothelial morphometry [6, 8], and that that a

more robust outcome is likely if the set of cells forms a contiguous (tessellated) group [8, 9].

Such ideas should generally apply to endothelial cell mosaics that have a good quality (i.e., the cell–cell borders are reasonably well defined) and where the image is not otherwise compromised by features that either degrade or even obliterate cell–cell borders. There are however a number of conditions where the cell mosaic is of poor quality, or some or even most of the image is occluded by the presence of non-reflective areas. These dark (as opposed to light-reflective regions in specular microscopy or confocal microscopy) can be generally referred to as corneal (endothelial) guttata or pseudoguttata. True corneal guttata would generally be considered as being associated with a progressive endothelial dystrophy (e.g., Fuch's), while pseudoguttata can have similar appearances but be due to other reasons and have no obvious familial link [10–14]. Regardless of the reliability of being able to define the cause of endothelial guttate changes, there have been a variety of studies of the endothelial cell density in corneas affected with guttata or pseudoguttata [15–29]. The methodology used to generate ECD estimates in these various reports is however far from clear, and there appear to be few agreed guidelines on how the morphometry of such endothelia could or should be undertaken. As a result, the studies appear to show poor agreement with some studies reporting no statistically significant difference between normal and guttata-affected endothelia [12, 15, 17, 24–28] and others finding notable differences [16, 19, 21, 22]. A key feature of endothelia with guttata or pseudoguttata is that only part of a cell may be evident in the immediate vicinity of the darkened regions.

A broad interest prompting the present study was to further assess the endothelium with pseudoguttata, with the principal objective being to assess the impact of undertaking morphometry that either only considered measurements on complete cells or analyzed a mixture of complete and partial cells. As a secondary objective, the study was designed to assess the impact of only analyzing a relatively small number of cells (i.e., 45/image) in abnormal corneas. This is not an issue that appears to have been systematically studied (e.g., in endothelia substantially affected by guttata) which may only have a modest number of visible and complete cells suitable for analysis [19, 22, 29].

Methods

Patient selection

Following approval of the protocol by the university-based ethics committee, patients routinely attending the Glasgow-Caledonian University eye clinic were assessed by a special arrangement such that patients are asked if it was acceptable for their data to be used, anonymously, in clinical research studies. Approval was indicated by signing an informed consent form, that was then included in their records. Exclusion criteria, for the eye from which data was subsequently used, were any history of contact lens wear or ocular surgery (e.g., for cataracts). Most of the assessments were made by the staff optometrists as part of routine eye examinations wherein all patients had a single photograph taken of the central region of the corneal endothelium of each eye with a non-contact specular microscope (NCSM, Topcon SP-2000P). This was done prior to the administration of any eyedrops that might have been used as part of the examination (e.g., mydriatics or topical ocular anesthetics). The instrument has a chin and forehead rest and fixation lights to aid in correct positioning of the eye; the automatic mode was used throughout. In the majority of cases, a single image capture was of sufficient quality for use. The staff optometrists were simply requested print the images, write the patient ID number on the back of them and give them to the principal investigator (MJD). For cases where any endothelial abnormalities were present, the patient clinic file was later retrieved, and data extracted on a pre-prepared form; this included patient gender and age. The clinical record was also assessed for all recorded details of ocular health (cornea, lens, media, retinal vasculature, disc and macula assessments), and for any history of ocular surgery (e.g., cataract operation). Notes were also made of any systemic health conditions (high blood pressure, diabetes, thyroid, etc.) and any medications the patients were taking. Some patients with endothelial pseudoguttata were not further assessed for this study if, for example, they were found to have had ocular surgery. Similarly, if there was any indication of a familial history of corneal endothelial abnormalities, these were considered to be true guttata (and the patients referred).

Endothelial image analysis

The prints were examined and assigned one of four grades [30]; these were 0 (no pseudoguttata/normal), 1 ('several dark spots'), 2 ('many dark spots not in contact with each other') or 3 ('numerous dark spots that are in contact with each other'). The latter can also be referred to as semi-confluent or confluent pseudoguttata. The NCSM printed images (approx. 7×3 cm) were then scanned, a JPEG image file generated which was then printed at 1200 dpi to an enlarged A3 size (42.5×29.5 cm) with a final magnification of close to 750 X. On these prints, all cell–cell borders were carefully marked (to define the cell domains; see Results) as were the borders of the pseudoguttata (see Fig. 1). The sizes (areas) of the cell domains were then measured by planimetry using a DigiPro digitizer pad (Gridmaster™,

Elstree Computing Ltd., London) by tracing round the pre-marked cell–cell border (stream mode). With the magnification chosen, the accuracy of the tracing is to ±1% or better. With the known scale marker bar on the images, the absolute area values (in μm^2) were then calculated.

Data analysis

All data was entered into spreadsheets in Systat (v 11, Systat, Evanston, IL) for generation of global statistics and graphics. Data set normality for the distribution was checked using the Shapiro–Wilk test (where $p>0.05$ reflects a normal distribution). Statistical comparisons were made using two-sample Student's *t* tests or Kruskal–Wallis ANOVA as appropriate (and sometimes with both tests when one set was normal and the other was just outside normal limits). The measured area values were used to calculate average values from which an overall estimate of the ECD could be made based on 1000000/average area (to give a value in cells/mm^2). In addition, an estimate was made of the predicted variability and random error in such ECD estimates by a cumulative averaging technique essentially as outlined previously [8]. By this, an ECD value was calculated from each area measure and these were progressively summed and averaged (i.e., ECD1 + ECD2/2, ECD1 + ECD2 + ECD3/3, etc., to give a final mean value. Each of the averages obtained as the number of measured cells was increased, was then compared to the final mean value as a percentage. From the sets of percentage values, for cells 1 through 45 or 75 (see Results), a group mean percentage value ±1 SD was calculated.

Results

Patient profile

Forty-eight patients were examined, with 12 in each of the four groups. The overall average age was 67±9 years (range 52–83 years), with the average ages in each group being very similar at 67, 69, 65, and 67 years. The age range was largely selected based on those with grade 3 pseudoguttata, with other patients then essentially age-

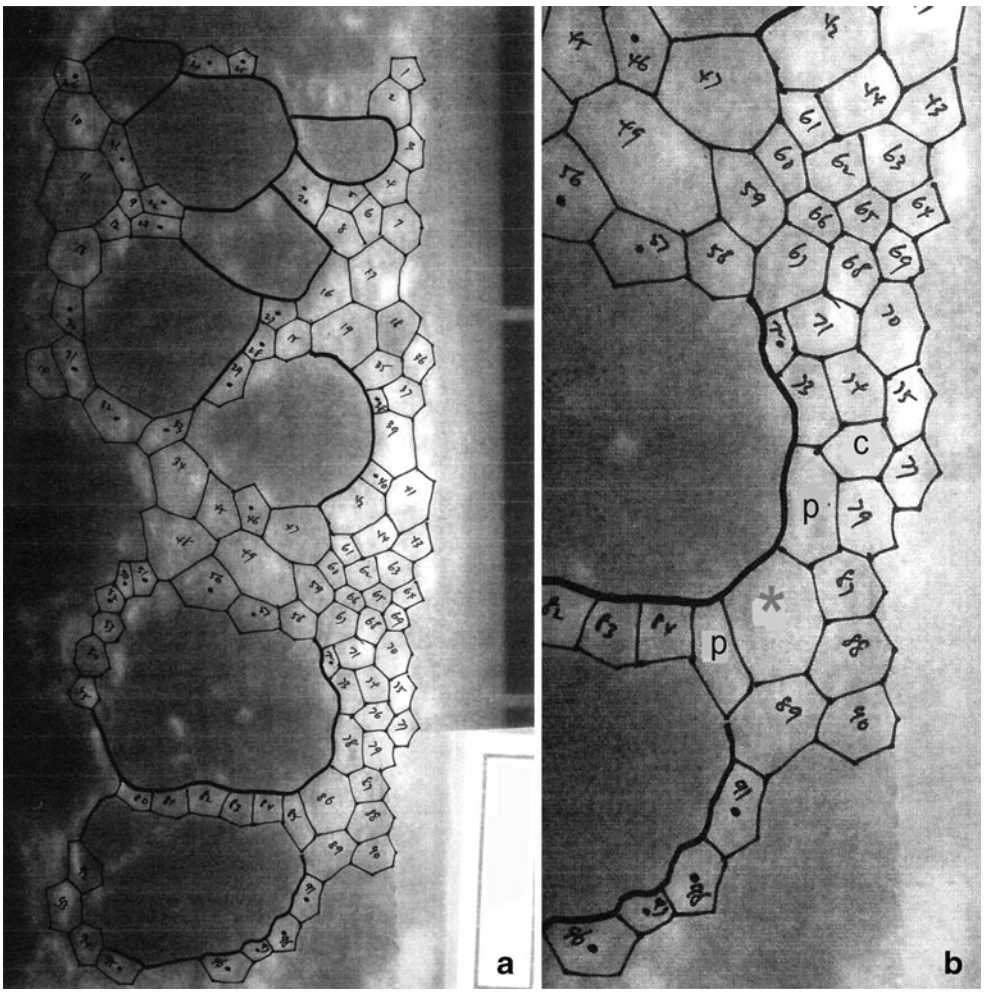

Fig. 1 Non-contact specular microscopy image of a patient with grade 3 confluent endothelial pseudoguttata. **a** Full image from central corneal region with cell–cell borders marked and the pseudoguttata outlined. **b** Part of the same image enlarged to highlight complete cells. **c** Partial 'cells' (p) adjacent to affected regions. For the cell marked with an *asterisk* (*), see text

matched to the most severely affected group. A number of the patients in all of the groups had systemic health problems such as high blood pressure (n=23), thyroid problems (3) or type 2 diabetes (3), but there were no obvious differences in the health characteristics when comparing the groups. Medication use, as reported by the patients, was consistent with their reported health conditions with 21 different cardiovascular medications reported (e.g., calcium channel blockers, beta-blockers, angiotensin converting enzyme inhibitors), with eight of these patients also taking diuretics. Overall, there was no obvious association between the use of cardiovascular medications and/or diuretics and whether the patients showed corneal endothelial pseudoguttata, regardless of grade. Half the control group were actually taking these medications and so, stated another way, multiple systemic medication use (polypharmacy) was not obviously associated with the occurrence of pseudoguttata. The average number of medications reported were 1.8/patient in the control group, then 1.3, 1.9, and 2.3 in the three guttata groups. Other notable medications included thyroxine (3), statins for cholesterol (8), analgesic NSAIDs presumably for arthritis-like conditions (6) with two of the osteoarthritis patients also reporting use of bisphosphonates (aledronic acid). All three diabetics were considered well controlled and were taking oral hypoglycemics (glicazide, etc.) and one was on intermittent insulin therapy. No neurological conditions were reported (e.g., Parkinson's, Alzheimer's. etc.) or medications for the same. Only one patient reported HRT use, and none of the patients reported regular use of eyedrops (e.g., for dry eye, glaucoma, etc.).

No patients had been diagnosed with glaucoma, but three did have early signs of diabetic retinopathy, and two were being evaluated for possible early stages of ARMD. The clinic records did not indicate any remarkable findings for corneal epithelium (e.g., epithelial edema) for any patients, although fluorescein staining and grading was not routinely undertaken. Five patients were noted to have corneal arcus and three had early conjunctival pingueculae. Quite a few patients (37.5%) had early signs of cataract, but none had undergone a cataract operation in the eye examined. For the four patients who had had a cataract operation on the fellow eye, it is assumed that standard post-operative medications were used on the operated eye but details were not obtained. No indications were given of the presence of any anterior chamber and aqueous humor changes (flare, cells, etc.), but a few patients (6) were noted as having a history of vitreous floaters. Optic nerve head (disc) assessments and those for the retinal vasculature (other than in the diabetic patients) were reported as unremarkable, except for three patients noted as having mild peripapillary atrophy. There were no substantial or obvious differences when comparing the four study groups.

The specular microscope images and the approach to the morphometry

In Fig. 1 is shown a case with semi-confluent grade 3 pseudoguttata to illustrate the main issues relevant to the endothelial morphometry and to explain the rationale behind the systematic approach adopted for these studies.

In the panel 1A, the guttata are outlined and, far more importantly, the cell–cell borders as evident on the A3 sized prints. Some of the cells are very distinct, are surrounded by at least four neighboring cells and are clearly separated from any of the darker regions where no cell–cell borders are evident. In many regions however, the cell borders appear to run up to the edge of the non-reflecting (dark) regions and then disappear. This is illustrated in more detail in the enlarged region in the right hand panel (Fig. 1b). Within this image, one can see some cells, clearly surrounded by other cells and these were designated as complete cells (and marked with a 'c'). In other locations, the 'cells' are not completely surrounded by other cells and one or more sides of the cell domain is formed by the edge of the pseudoguttata. These were designated as partial cells (marked 'p') simply because it was impossible to define the complete extent of the cell.

In this example, considered representative of a number of cases of more severe grade 3 pseudoguttata that have been observed (only some of which are included in this analysis), just 45 of the cell domains were considered to be complete. In all other images used for this study, more than 45 complete cells could be measured. Even for this image, however, it was easy to identify numerous partial cells. In previous studies on normal endothelia [8], sets of 75 contiguous cells were used to assess the variability in ECD estimates. Therefore, for all 48 endothelia examined for the present study, morphometry was undertaken on 45 complete cells (so that a complete and balanced set of analyses could be undertaken using the same lower value for the number of cells measured). The difference to previous studies [8] is that the 45 cells did not form a contiguous set (i.e., the cell domains were not all in contact with each other as part of the mosaic was interrupted by non-reflective pseudoguttata). For the sets of images including pseudoguttata, all contained at least 75 cell domains (i.e., a mixture of complete and partial cells) and so for all 48 images, analyses were undertaken of 75 'cells', again with the caveat that these did not form contiguous sets in the guttata endothelia. For the control set, the comparisons between using 45 and 75 cells were on contiguous sets of cells and are included to illustrate the effect of using less than 75 cells on the overall outcome of the morphometry.

Grade 3 pseudoguttata

In the example illustrated (Fig. 1), the cell domains constituted a mixture of both complete and partial 'cells'. This combination was also found for all 12 grade 3 pseudoguttata cases selected. All 12 of the grade 3 pseudoguttata examples did include at least 45 complete cells, although not contiguous with one another. If just these compete cells were selected (n=45 for each image), the ECD estimates ranged from 1,072–4,329 cells/mm^2, for a group mean of 2,621±862 cells/ mm^2. As illustrated in Fig. 2a, when 40 such complete cells were measured, the estimated variability in the ECD estimates was with±3.1% (SD). However, if the number of complete cells measured was reduced to much lower than this, then the variability in the ECD estimates increased quite obviously, e.g., the ±1 SD (as a percentage) progressively increased as the cell count was lowered (Fig. 2a). It was ±19.5% when n=10 cells, an option on some specular microscope software analysis systems (see Discussion). If the analyses of the images included both complete cells and the partial 'cells' (for a total of 75), the range of ECD estimates was between 1,230 and 4,587 cells/mm^2, for a group mean of 2,866±937 cells/mm^2. This apparent increase in ECD, when both complete and partial cells were used for the morphometry, was statistically significant (p=0.021, Friedman rank analysis) (see Discussion). When using a mixture of complete and partial cells, the estimated variability in ECD was small at ±2.8% for n=70 (see Fig. 2b), but increased to a very substantial ±25.0% for n=10 'cells'.

Grade 2 pseudoguttata

These images all had many dark spots (pseudoguttata) but were generally clearly separated from each other by at least a line of visible cell domains; the size of the dark spots was variable. As with the grade 3 images, the resultant sets of 'cells' were a mixture of complete and partial cells. All 12 examples included at least 45 complete cells, although not contiguous with one another. Analyses of these complete cells yielded ECD estimates between 2,075 and 3,011 cells/mm^2, for a group mean of 2,551±351 cells/mm^2. This set of ECDs calculated from 45 cells was not significantly different from the set of grade 3 pseudoguttata (p>0.1). However, the variability in ECD estimates was noticeably less (compare Figs. 3a and 2a). With 40 cells measured, this was ±2.5% and was only ±12.3% when just ten cells were measured. When a mixture of complete and partial cells were used for the morphometry (n=75) the ECD estimates ranged from 2,212 to 3,703 cells/mm^2 (group mean of 2,633±499 cells/mm^2) that were not different from those for when just 45 complete cells were measured (p=0.564). When using 75 of these complete and partial cells, the estimated variability in ECD was ±13.9% for n=10, and down to just ±1.7% for n=70 (see Fig. 3b).

Grade 1 pseudoguttata

There were only scattered and small-sized dark spots on these images, although all of them exceeded the dimensions of single cells, i.e., were not blebs. Since there were sizeable parts of these images where there were no pseudoguttata, the strategy adopted was to select a region that included at least one dark spot so that an analysis could again be made of the impact of assessing both complete and partial cells. In these cases however, the number of partial cells were relatively small as compared to the complete cells. Overall, if 45 complete cells were measured and analyzed, the ECD estimates ranged from 1,852 to 3,333 cells/mm^2 for a group mean of 2,412±492 cells/ mm^2. If 75 'cells' were analyzed, then the ECD estimates seemed little changed (group mean 2,494±477 cells/mm^2, range 1,905 to 3,436 cells/mm^2) and were statistically higher than for when just 45 complete cells were analyzed (p=0.021). For either of the sets of analyses of the grade 1 pseudoguttata, the variability in ECD (see Fig. 4) estimates was lower than for the grade 2 (Fig. 2) and grade 3 (Fig. 3) image sets. For analyses of just ten complete cells, the variability was ±11.9%, and was ±2.4% for 40 cells measured (Fig. 4a). A similar result was found for the sets of 75 complete and some partial cells, being ±13.9% for ten 'cells' and just ±1.2% for n=70 (Fig. 4b).

Control group (age-matched to pseudoguttata groups)

The last set of comparisons was made on corneal endothelia without any obvious pseudoguttata, although some individual cells in such older individuals were darker in appearance. None of these normal endothelia were considered to show notable polymegathism. None of the patients indicated any history of contact lens wear nor other conditions considered to be associated with corneal endothelial abnormalities, although two did have fairly pronounced corneal arcus. The comparisons between assessing 45 or 75 cells now simply reflect the possible difference when analyzing two sets of contiguous and complete cells (see Discussion). With 45 complete cells measured, the ECD estimates ranged from 2,012 to 2,881 cells/mm^2 (group mean 2,374±282 cells/mm^2). When 75 complete cells were measured, the range was from 2,049 to 2,667 cells/mm^2 (group mean 2,333±204 cells/mm^2) and these ECD estimates were no different from those obtained from 45 complete cells (p=0.241) nor from those from the pseudoguttata sets (p≥0.248). When just ten cells were measured in the small sample set (total of 45 cells), the inter-sample variability was±8.4% (Fig. 5a) and was just ±2.1% when

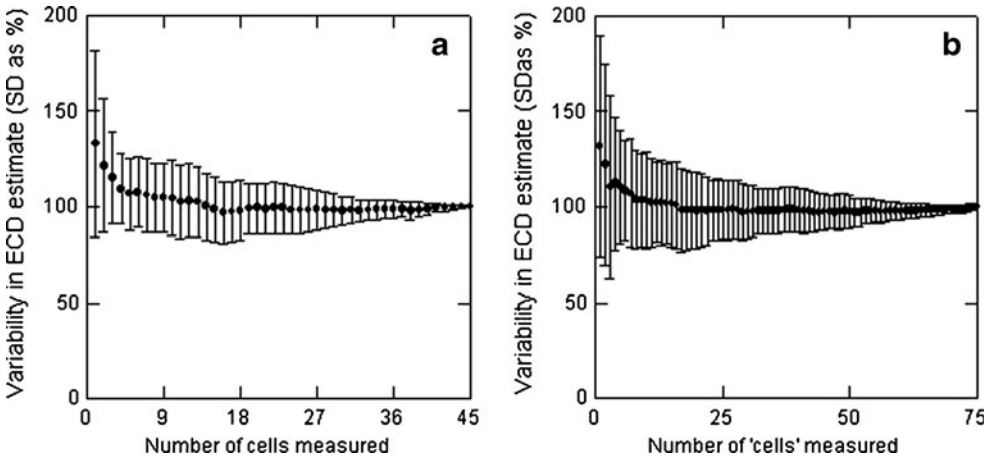

Fig. 2 Assessment of the variability in endothelial cell-density (ECD) estimates in relation to the number of complete cells measured (**a**) or in relation to the number of cells in a mixture of complete cells and partial cells (**b**) for 12 cases with grade 3 pseudoguttata

40 cells were measured (Fig. 5a). For such control non-polymegethous endothelia, when a set of 75 contiguous cells were analyzed, the ECD variability was just ±7.9% for n=10 cells and ±1.1% for 70 cells (Fig. 5b).

Overall comparisons between endothelia

For the present set of studies, three sets of endothelial images affected by pseudoguttata were essentially randomly selected, a selection process that was made subjectively based on whether the darkened regions met the grading criteria proposed by Nakashima (see Methods). Purely by chance, the age range and average age of the patients in these three groups were very similar (see earlier). A database was then examined to find 12 control patients whose ages fell within the same range as the guttata cases, and their average age was therefore very similar to that of the guttata cases.

Comparison were then made of the ECD estimates based on sets of 45 complete cells or from mixtures of complete and partial cells (n=75 'cells'/image). As can be seen in Table 1, the estimated ECD values in the control group, that would be to within ±2.1% or better, were lower than those of the pseudoguttata. This difference could not be shown to be statistically significant when 45 complete cells were measured (but note large SD value), but was statistically lower when 75 complete cells were measured and compared to the mixtures of 75 complete and partial cells measured for the pseudoguttata cases (p=0.047). Stated another way, the ECD estimates from 45 complete cells from images with grade 3 pseudoguttata, which would be to within±3.1% (rather than ±2.2%), were both statistically higher than the set of controls (p=0.047) as well as statistically higher than for if a mixture of 75 complete and partial cells were measured (p=0.021). As also detailed in Table 1, the ECD estimates showed a clear trend to increase as the grade of pseudoguttata increased regardless of whether 45 or 75 cells were measured. However, analysis of the median ECD values and the ±25% interquartile intervals (Fig. 6) clearly reveals the variability in the ECD estimates. When just 45 complete cells were measured, there is a lesser trend in the ECD in relation to the grade of the pseudoguttata (Fig. 6a), although this trend is evident when a mixture of complete and partial cells were

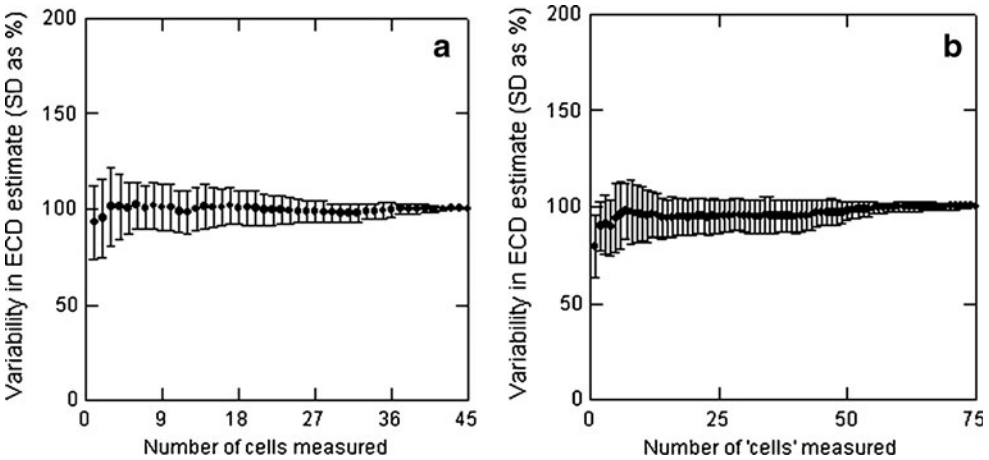

Fig. 3 Assessment of the variability in endothelial cell-density (ECD) estimates in relation to the number of complete cells measured (**a**) or in relation to the number of cells in a mixture of complete cells and partial 'cells' (**b**) for 12 cases with grade 2 pseudoguttata

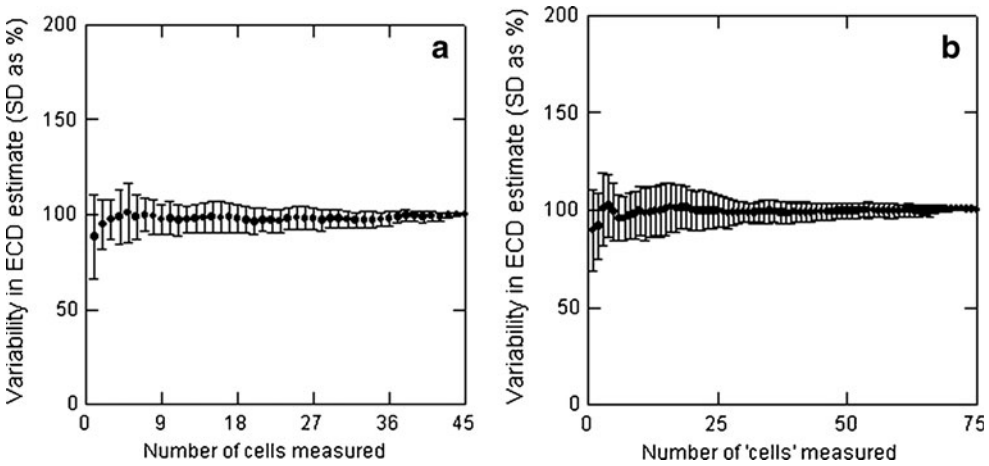

Fig. 4 Assessment of the variability in endothelial cell-density (ECD) estimates in relation to the number of complete cells measured (**a**) or in relation to the number of cells in a mixture of complete cells and partial 'cells' (**b**) for 12 cases with grade 1 pseudoguttata

used in the analyses (Fig. 6b). What is particularly notable is the extremely wide ECD range for the grade 3 pseudoguttata cases, i.e., these endothelia could clearly have a high or a low cell density. While the box plot shows no obvious outliers, a regression or correlation analysis indicated two of the pseudoguttata cases to be outliers (one low, one high). A linear regression model indicated the association between the estimated ECD and the pseudoguttata grade was significant if a mixture of 75 complete and partial cells was used for the analyses (p=0.024, r=0.324), but not if 45 complete cells were used (p=0.208, r=0.185). A least-squares analysis, however, indicated no statistically significant association ($p \geq 0.171$).

Discussion

This was an observational study related to assessment of corneal endothelial cell density (ECD) in guttate endothelia so that any patients were considered eligible for inclusion if their endothelia showed signs of (pseudo)guttata; history of anterior segment surgery and contact lens wear were the only exclusion criteria so that the effects of these would not be confounding variables in any secondary assessment of whether ECD was indeed different when (pseudo)guttata were observed. The study design was also balanced in that there were an equal number of cases in each group. Perhaps the outcome of primary interest from this study will be that, in the cohort studied, the mean ECD values are not obviously different when comparing controls to those with different grades of pseudoguttata (see later). Closer scrutiny of the data, however, shows very substantial inter-subject variability in the grade 3 severe cases and also that the absolute values obtained (for ECD) very much depend upon the method used to assess ECD. Therefore, the main and most important outcome of the present study is that the methods for estimates of endothelial cell density (ECD) need to be more carefully defined in corneas affected with guttata or pseudoguttata.

The present analyses, of the use of just 45 complete cells for ECD estimates in guttata-affected endothelia, indicate that the overall outcome was no worse than for normal endothelia. The cell morphological characteristics for guttata endothelia can be largely similar to normal endothelial in being of a relatively consistent size (as opposed to showing notable polymegathism). In terms of

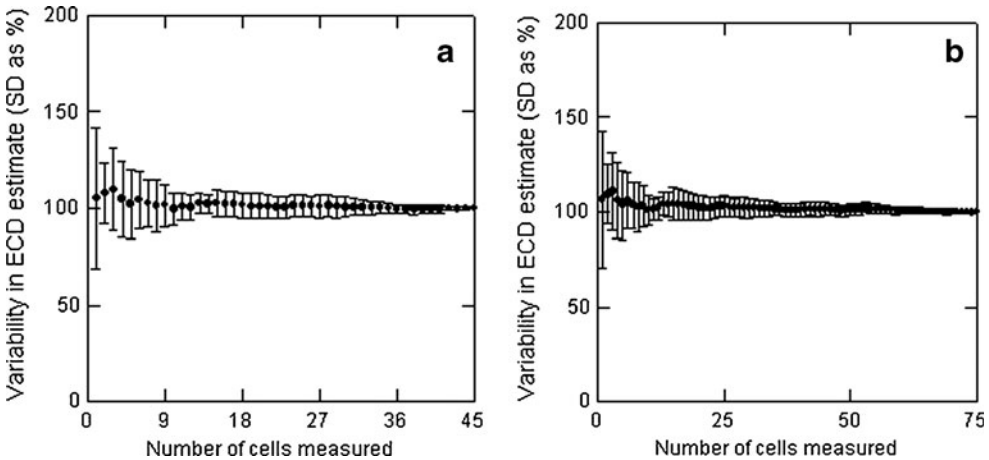

Fig. 5 Assessment of the variability in endothelial cell-density (ECD) estimates in relation to the number of complete cells measured up to 45 in total (**a**) or up to 75 in total (**b**) for 12 age-matched controls

Table 1 Endothelial cell-density estimates for different grades of endothelial pseudoguttata

Grade	ECD n=45	p vs. grade 0	ECD n=75[a]	p vs. GRADE 0	ECD 45 vs. 75
0	2,374±282	-	2,333±204	-	0.241
1	2,412±492	0.875	2,494±477	0.295	0.357
2	2,551±351	0.564	2,633±499	0.172	0.264
3	2,621±862	0.356	2,866±937	0.047	0.021

[a]Includes a mixture of complete and partial cells for pseudoguttata grades

the reliability of the actual ECD calculations, the variability associated with using just 45 cells was actually only modest; for grade 3 endothelia, the estimate of variability was just ±3.1% (i.e., based on that observed at n=40 cells). This overall outcome is consistent with previous studies that indicate that ECD (or the coefficient of variation, COV) can only be defined to a certain limit of 'accuracy'. In previous studies, it was demonstrated that assessments of the reliability of COV estimates very much depends on how uniform or non-uniform the cell mosaic might be [9] but, as yet, this has yet to be systematically assessed for estimates of ECD. In evaluation of 75 cells/endothelial image from older adults, a previous estimate was that the ECD value could be determined to within approximately ±3.4% (± SD as a%). This was determined on endothelia that showed little non-uniformity (mild polymegathism) with relatively few larger cells present. However, if just 40 cells were measured then the ECD could likely only be determined to within ±7% [8]. The present studies clearly indicate that ECD can be assessed to within ±2% when the endothelia are uniform in appearance. Further studies are needed to assess the impact of polymegathism on the reliability of ECD estimates. Notwithstanding, the non-uniformity assessed in the present studies was not the variation in cell size per se (i.e., polymegathism), but more that some cells are complete while others are only partial cells. The images included regions where part or all of the cells would not be seen by specular microscopy or confocal microscopy.

A related outcome from the present analysis is, perhaps not surprisingly, that ECD can be altered in patients affected with substantial (grade 3) endothelial pseudoguttata. However, only some cases will be expected to have a lower ECD and the overall result from the present cohort is that the mean ECD was actually marginally higher than that for a small set of age-matched controls or less-affected cases. Early studies [16] reported that most endothelia affected with 'prominent guttata' had larger cells but that some could have average areas within the range expected for aged-matched (older) non-affected corneas. Similarly, an almost 2-fold increase in average cell area (and so an even greater difference in ECD) was reported for another group of patients with guttata associated with Fuch's corneal endothelial dystrophy [21]. Another study on those with posterior polymorphous endothelial dystrophy noted that most had endothelial cell areas that were much larger than age-matched controls, but that the average area in affected corneas could also be smaller than such controls [19]. The current studies reveal a similar dichotomy in that some endothelia with grade 3 pseudoguttata have a rather lower ECD than age-matched controls while the others have similar or even higher apparent ECD values; the overall mean ECD may however not be different compared to age-matched controls without endothelial abnormalities. Beyond these studies, most other reports indicate that the ECD may only be slightly lower than age-matched controls or that no statistically significant difference could be detected [12, 15, 17, 24–28]. A confounding variable that

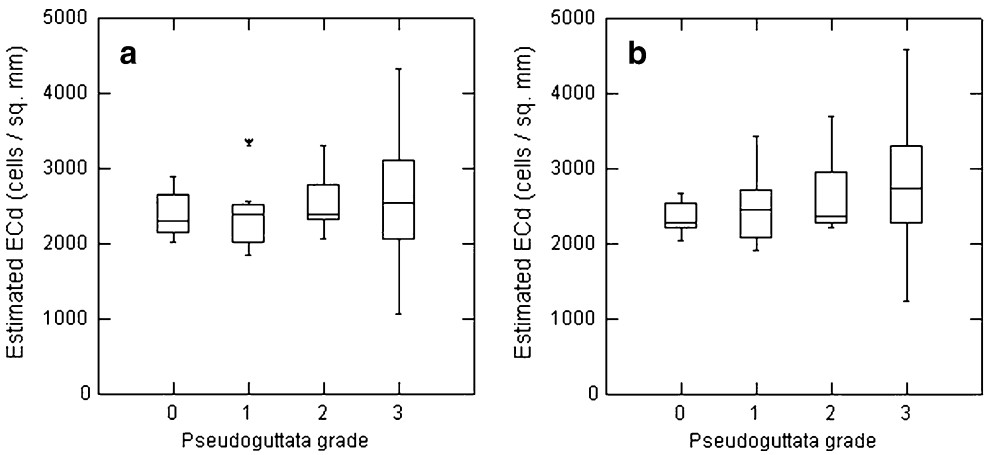

Fig. 6 Box plots to illustrate the trends in ECD measures in relation to the grade of pseudoguttata depending on whether just 45 complete cells only were measured from each image (**a**) or whether a mixture of complete cells and partial 'cells' were measured (**b**)

can substantially affect ECD in guttata cases is whether the patients had had intraocular surgery (e.g., for cataracts) [15, 28, 31], and this aspect of patient history is not clearly identified in some reports.

The present analyses are presented to illustrate that ECD estimates can be highly variable in cases with severe endothelial pseudoguttata. This variability in the ECD values is, as predicted, sensitive to or even determined by the absolute value of the cell count (i.e., how many cells were assessed to then obtain an ECD estimate). It is, however, also sensitive to whether only complete cells or a mixture of complete and partial cells were used in the ECD calculations. These two issues are inter-related but can be considered from slightly different perspectives in relation to previously published studies.

One of the goals in the present analyses was to further assess the impact of only using a modest number of cells for ECD estimates for cases where only this number of cells were easily available for routine analysis. This number, of 45 complete cells/image, is somewhat arbitrary but was selected after consideration of several factors. First, and foremost, single endothelial images affected by moderate-to-severe (pseudo)guttata are unlikely to include the 75 to 100 complete cells [10] that are often recommended for routine ECD assessments in non-guttate endothelia [6]. It could be argued that a simple solution to such a problem would be that more than one image should be taken, e.g., of the central corneal region, ensuring that there was no substantial overlap between the images. In reality, in really severe cases, several images may be required yet it still may be difficult to acquire 75–100 cells for measurements of the endothelial cell density at any particular location (e.g., central cornea or superior cornea, etc.) especially if that location was to be specified to be within a fairly narrow zone (e.g., at a certain distance from the centre of the cornea). Indeed, in a wide-field specular microscope study of endothelial guttata, an average of 12±3 micrographs were taken for each cornea (to include both the central region and all four quadrants) to allow for measurement of an average of just 47±23 cells/location [22]; data from the five locations were then pooled to able to have 100–200 cells available for analysis/endothelium. From other studies on guttata-affected endothelia, it can also be noted that it is not unusual to only obtain narrow-field cell images that include less than 40 cells suitable for measurement [19]. From these perspectives, the number of 45 complete cells is relevant to the types of endothelial images that can be expected for moderate-to-fairly severe cases of guttata. In less affected cases, there should be less of a problem in obtaining 75 or even 100 cells for analysis (assuming, of course, that the cell–cell borders were of sufficient clarity for measurement). For the current studies, the systematic assessment of the potential error in ECD estimates was undertaken on all the grades of pseudoguttata as well as an age-matched control so as to be able to draw a balanced conclusion on using just 45 cells in the more severely affected cases.

As indicated earlier, with just 45 complete cells for ECD estimates in guttata-affected endothelia, the overall outcome is really no worse than for normal endothelia with minimal polymegathism, i.e., the estimate of variability was just ±3.1% (i.e., based on that observed at $n=40$ cells). However, it has to be very strongly noted that as fewer and fewer cells (in such grade 3 guttata images) are used for the calculations, then this estimated uncertainty can become very substantial, e.g., to ±13.0% if 20 cells were measured and to ±19.5% if just ten cells were measured. This variability is greatly in excess of that expected for normal endothelia and can be considered as a characteristic feature of grade 3 pseudoguttata-affected endothelia and not a result obtained simply because just a few cells were used in the ECD analysis. In less-affected endothelia, the estimated uncertainty in ECD was closer to ±10.0% when just ten cells were measured. A minimum of ten cells in the vicinity of guttata was the criterion used in a study of uveitis-related endothelial changes [29], and only 10–20 cells have been used in other studies considering the reliability of the calibration of non-contact specular microscopes [2]. If the endothelia are even more substantially affected than those studied here, then it is unlikely that even 45 complete cells / image could be measured. If this were the case, then the present analyses indicate that the ECD estimates would therefore be of questionable value. If substantially fewer than 45 cells were to be available for measurement then it is suggested that any morphometry data be accompanied by a note on this uncertainty and the (n) given, e.g., as in a previous study on such endothelia [19].

An alternative approach to the morphometry might be to consider that any feature that could be considered to be a ‘cell’ would be measured, i.e., the resultant data set would include a mixture of complete cells and partial ‘cells’. In most papers on guttata-affected endothelia, insufficient details are provided for the morphometry to know if the measurements were undertaken on a mixture of complete and partial cells. Any ‘cell’ that can be seen to be in direct contact with guttata-affected (dark) regions needs to considered as an incomplete cell. In some instances it might be considered that the shape of the cell was so distinctive that it was likely to be that which it appeared to be, e.g., the slightly larger six-sided cell in the lower half of Fig. 1b (marked with an asterisk). Such an assignment also means that a decision has been made that all of the apical surface of the cell is indeed visible, and so this would be designated as the measurement of a complete cell. However, in considering the immediately adjacent cells, the same confidence in assignment of completeness of the

cell surely should not be considered appropriate, i.e., the cell above the asterisk-marked one (marked 'p') should not be considered to be a six-sided cell, and the cell to the left (also marked 'p') is not a five-sided cell, etc. These are considered to be partial cells, being partly obscured by the dark guttata. A comment has been made that in guttata-affected endothelia "cells can only be examined in areas where the guttae are few enough to allow a flat sheet of mosaic to be seen" [14]. Furthermore, in between the guttata (guttae), a distinct line of partial cells may be a distinctive feature, actually referred to as a 'chicken wire' visual effect [14], i.e., these can be distinctive 'cellular' features even if not necessarily of complete cells. Examples can be found where some cells included in morphometric analyses (e.g., as marked using a centre-dot analysis program) are clearly touching the guttata edges [21]. The same applies to application of an alternative 'automated contour' method [28], although the use of this type of software technique (also perhaps known as a 'polygonal' method) can be done such that cells in close contact with guttata are not included [4]. Such a selective approach appears to be very similar to that also used in assessing eye bank corneal endothelia where the 'contour' only included groups of cells with clearly resolved cell borders [5].

The intentional analyses of mixtures of complete and partial cell provides data confirming that the assignment of a partial cell feature was indeed correct. This is because the average cell area values from the sets of mixtures were lower in all the guttata cases. As a result, the ECD estimates were slightly higher, as were the overall mean ECD values when comparing measures on 75 'cells' versus 45 complete cells. For grade 3 pseudoguttata, this difference in mean ECD was close to 250 cells/mm^2 (Table 1). Such a systematic shift would not be the expected outcome from simply measuring more cells [8, 9, 32]. Indeed, the mean ECD in the control group (with no partial cells) was slightly lower if 75 cells were measured (rather than just 45 cells) (Table 1). A logical explanation is that some of the apical surface of the partial cells was obscured and so the measurable area was slightly less, with the net effect being summed over all of such cells to result in a slightly higher ECD. A case can be made that the inclusion of partial cells in morphometry of grade 3 pseudoguttata, perhaps in an attempt to get a reasonable cell count, does nothing useful. The estimated uncertainty in ECD is just as substantial when a set of 75 'cells' is chosen as compared to a realistically-obtainable number of 45 complete cells (Fig. 2b). The same generally applies to grade 2 pseudoguttata, but any such effect is likely to be insignificant for grade 1 pseudoguttata.

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